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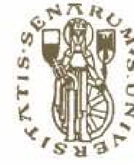

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EFFECT OF 7-KETOCHOLESTEROL AND 7β-HYDROXYCHOLESTEROL ON CELLULAR VIABILITY OF HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS.

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Oxidized low density lipoproteins (oxLDLs) are involved in atherogenesis (1) and their cytotoxicity has been linked to the formation of oxysterols (oxidized products of cholesterol) (2). The major oxysterols found in oxLDLs are 7β-hydroxycholesterol (7βOHC) and 7ketocholesterol (7-KC) and it has been reported that high concentrations (>40μg/mL) of these oxysterols induce apoptosis on endothelial cells (EC) (3). However it has been shown that oxLDLs have a dual effect on EC viability inducing apoptosis or proliferation depending on their concentration (4). Recently it has been reported that low concentrations of oxLDLs induce upregulation of endothelial NO synthase (eNOS) leading to EC survival, while high concentrations of oxLDLs induce downregulation of eNOS leading to endothelial damage (5). The objective of this study was to investigate the effect of low concentrations of oxysterols on human umbilical vein endothelial cells (HUVEC) and the pathways involved. HUVEC were isolated from human umbilical cord and used from passage two to six. Cell viability was determined by the measure of MTT reduction. Cell proliferation was measured by [^{3}H] thymidine incorporation assay. Apoptosis was determined by: a) flow cytometric analysis of annexin V and propidium iodide binding; b) caspase-3 activity of cell lysates. Long term treatment (24 h, 48 h, 96 h) of HUVEC with low concentrations of 7-KC or 7βOHC (1-10 μg/mL) increased MTT reduction in a concentration dependent manner. For both oxysterols maximal effect was obtained at 10 μg/ml at each time of treatment (+100% and +200% for 7KC and 7βOHC, respectively). This effect was not associated with an increase in cell proliferation as assessed by [^{3}H] thymidine incorporation assay. Low concentrations of 7βOH caused a 25% decrease in caspase-3 activity (after 6 h). 7-KC was not able to reduce caspase-3 activity after 6 and 10 h treatment. Antiapoptotic effect of 7βOH was confirmed by flow cytometric analysis after 24 h and 48 h of incubation. Treatment of HUVEC for 24 h with cholesterol (1-10 μg/mL) produced no effect on cell viability indicating that oxidation in C7 is necessary for the antiapoptotic activity. Higher concentrations of the two oxysterols induced cell death. As reported for oxLDL, 7βOHC and 7-KC show a biphasic effect on HUVEC viability depending on their concentration. The pathways involved in these effects and in particular the role of eNOS are the object of current investigations in our laboratory.

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NK-1 AND NK-3 TACHYKININ RECEPTORS IN PANCREATIC ACINAR CELLS AFTER ACUTE EXPERIMENTAL PANCREATITIS IN RATS

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Activation of neurokinin-1 (NK-1) receptors but not of NK-3 stimulates amylase release from isolated pancreatic acini of the rat. Immunofluorescence studies show that NK-1 receptors are more strongly expressed than NK-3 receptors on pancreatic acinar cells under basal conditions. No studies have examined the expression of the two NK receptor populations in pancreatic acini during pancreatitis in rats. We therefore investigated the relationships between expression of these two TK receptors and experimental acute pancreatitis induced by stimulating pancreatic amylase with caerulein (CK) in rats. Hyperstimulation of the pancreas by CK caused an increase in plasma amylase and pancreatic water content, and resulted in morphological evidence of cytoplasmic vacuolization. Immunofluorescence analysis revealed a similar percentage of NK-1 receptor antibody immunoreactive acinar cells in rats with pancreatitis and in normal rat tissue but a larger percentage of NK-3 receptor immunoreactive cells in acute pancreatitis than in normal pancreas. Western blot of NK-1 and NK-3 receptor protein levels after CK-induced pancreatitis showed no change in NK-1 receptors but a stronger increase in NK-3 receptor expression in pancreatic acini in comparison with normal rats thus confirming the immunofluorescence data. These new findings support previous evidence that SP-mediated functions within the pancreas go beyond sensory signal transduction contributing to neurogenic inflammation, and suggest that SP plays a role in regulating pancreatic exocrine secretion via acinar NK-1 receptors. The significant increase in NK-3 receptors during pancreatic stimulation suggests that NK-3 receptors also intervene in the pathogenesis of mild acute pancreatitis in rats.

FUNCTIONAL COUPLING OF ANGIOTENSIN II TYPE I RECEPTOR WITH INSULIN RESISTANCE AT GLUCOSE AND FATTY ACID UPTAKE IN IMMORTALISED CARDIOMYOCYTES (HL-1 CELLS)

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Heart insulin-resistance causes incorrect energetic substrates supply, an increasingly recognized feature of cardiac disease. So far, the role of angiotensin II in raising insulinresistance has been studied limitedly to glucose uptake. We investigated whether, in isolated cardiomyocytes, angiotensin II raised insulin-resistance not only at glucose but also at fatty acid uptake. HL-1 cells, immortalised cardiomyocytes, were chosen as experimental model. In these cells facilitated glucose and palmitic acid uptakes were measured radiochemically using [^{3}H]-2-deoxy-D-glucose and [^{14}C]-palmitic acid respectively. To mimic pathological microenvironments, cells were exposed to angiotensin II (100 nM) for 18 h in the absence or in the presence of irbesartan, PD123319, or PD98059, an inhibitor of ERK1/2 activation. Reactive oxygen species and angiotensin II receptor subtypes mRNA levels were visualised by dichlorofluorescein and by semi-quantitative RT-PCR respectively. HL-1 cells show basal glucose and palmitic acid uptake (3.7 ± 0.7 pmol/10^{4} cells/10 min and 53.9 ± 4.0 pmol/10^{4}cells/5 min respectively) that are stimulated further by insulin. Angiotensin II increased cell oxidative stress and mRNA for type I receptor. Exposure to angiotensin II increased the palmitic acid uptake (76.6 ± 3.7 pmol/10^{4} cells/5 min) but blunted the insulin stimulation of glucose and of palmitic acid uptake. Insulin effects at substrate uptakes were restored in the presence of irbesartan or PD98059. Our results show the potential role of angiotensin II in dysregulating heart energetic substrates supplementation and support the effectiveness of type I receptor blockers in the restoration of insulin regulatory role at fatty acid uptake.

5-FLUOROURACIL EXPOSURE IN ADJUVANT CHEMOTHERAPY REGIMENS IS SIGNIFICANTLY RELATED TO DISEASE FREE SURVIVAL IN COLORECTAL CANCER PATIENTS

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5-Fluorouracil is still the mainstay in adjuvant protocols for colorectal cancers, alone or in combination with other antineoplastic agents. Several studies have investigated the disease free survival (DFS) improvement after fluoropyrimidine-based adjuvant chemotherapy, despite data concerning the possible role of 5-FU exposure on DFS are not yet available. Therefore the aim of the present study was to evaluate any correlation between 5-FU pharmacokinetics, including that of the first inactive metabolite 5-fluoro-5,6-dihydrouracil (5-FDHU), and DFS in colorectal cancer patients candidate to receive 5-FU-based adjuvant chemotherapy. Through 3 years, 82 consecutive colorectal cancer patients, 53 men and 29 women (mean ± standard deviation age, 62.3 ± 9.2 and 58.8 ± 8.2 years, respectively), were enrolled. Planned treatment consisted of six cycles of L-leucovorin 100 mg/sqm and 5-FU 370 mg/sqm administered as i.v. boluses for 5 consecutive days every 4 weeks. Blood samples were withdrawn for up to 3 h after drug administration on day 1 of the first cycle, and 5-FU and 5-FDHU plasma levels were determined by using a validated UV-HPLC method. Individual plasma concentrations of drug and catabolite were fit according to a 2-compartments open model with the use of APO2PR computer software (Mediware, Groeningen) and the area under the time/concentration curve (AUC) value was calculated. Staging of tumors according to Astler and Coller's classification and their histological grade (WHO grading system) were recorded, as well as relevant clinical and laboratory informations during the entire course of planned chemotherapy. In this study, 39 patients (28 males and 11 females) experienced disease recurrence, and in 89.7% of them the disease recurred within 3 years of study entry. Tumor staging affected significantly DFS among patients ($p < 0.05$), whereas a trend was observed for age ($p = 0.056$) and histological grade ($p = 0.052$). Pharmacokinetic analysis demonstrated that 5-FU AUC and 5-FU/5-FDHU AUC ratio were significantly lower ($p < 0.05$) in patients who experienced a recurrence (7.34 ± 3.61 hxmg/l and 0.73 ± 0.34, respectively) compared to other subjects (9.19 ± 4.13 hxmg/l and 0.97 ± 0.60, respectively). However, multivariate analysis showed that 5-FU exposure was not an independent factor ($p = 0.07$), when staging and grading were also considered, even if the risk of disease relapse was halved in patients exposed to higher concentrations of 5-FU (AUC > 7.9 hxmg/l). Although the large interpatient variability observed in the present study, analysis performed on pharmacokinetic data demonstrate that reduced 5-FU AUC values are significantly associated with decreased DFS in colorectal cancer patients in adjuvant regimens based on i.v. boluses.

INHIBITION OF P-GP/ATPASE ACTIVITY OF RAT SMALL INTESTINE MEMBRANE VESICLES BY THE NOVEL, DIHYDROPYRIDINE MDR REVERTER DP7

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Permeability glycoprotein (P-gp) is an ATP-dependent active transporter that confers multidrug resistance (MDR) to cancer cells against many unrelated drugs. The energetic coupling of ATP hydrolysis with drugs transport allows to investigate P-gp function by assaying its ATPase activity. We have developed a test system for P-gp/ATPase activity in rat intestine membrane vesicles and analysed the effects of novel 3,5-dibenzoyl-1,4-dihydropyridines proven to inhibit P-gp of mouse lymphoma MDR-1 transfected cell line(1). Vesicles were prepared by following the method described by Kessler (2). ATPase activity was measured spectrophotometrically at 37°C using an ATP-regenerating system (pyruvate kinase and phosphoenolpyruvate) coupled to lactate dehydrogenase and NADH, by monitoring NADH absorbance decay with time at 340 nm (3). Sodium azide, EGTA, ouabain were added to inhibit, respectively, H^+-, Ca^{2+}- and Na/K-ATPase, residual ATPase activity being thus mainly attributable to P-gp and representing basal ATPase activity that could be stimulated by drugs known as transport substrates (4). NADH absorbance decay with time curve followed a polynomial equation. ATPase activity was calculated by the curve tangent at 1300 s. The modulation by verapamil, as a reference compound, of P-gp basal activity was analysed. Verapamil gave a typical bell shaped concentration-activation curve with 30 µM maximum effective concentration. The novel 3,5-dibenzoyl-1,4-dihydropyridine derivative, DP7, was dissolved in DMSO, and it was shown that the solvent alone, which never exceeded 2% v/v in the assay medium, had no effect on the P-gp ATPase activity. DP7 was tested at various concentrations (0.025-10 µM) either on basal or 30 µM verapamil-stimulated ATPase activity. Neither pyruvate kinase nor lactate dehydrogenase were inhibited by DP7 so we can conclude that DP7 inhibits in a concentration-dependent manner both P-gp ATPase activities with IC_{50} values of about 1 µM.

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EFFECTS OF A NOVEL SELECTIVE ADENOSINE DEAMINASE INHIBITOR ON TISSUE INFLAMMATORY INJURY IN AN EXPERIMENTAL MODEL OF COLITIS

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Inflammatory bowel disease (IBD) is characterized by altered immunological responses, and recent evidence indicates that adenosine modulates the immune system and inhibit inflammatory reactions. The use of drugs which increase tissue concentrations of endogenous adenosine, such as adenosine deaminase inhibitors, could represent an innovative strategy in the treatment of IBD. The present study investigates the effects of a novel adenosine deaminase inhibitor in an experimental model of colitis. Experimental colitis was induced in male Sprague-Dawley rats by intrarectal administration of 2,4-dinitrobenzenesulfonic acid (DNBS, 15 mg in 0.25 ml of 50% ethanol). Control rats received DNBS-vehicle. Animals were treated as follows: EHNA (reference adenosine deaminase inhibitor, 10 mg/kg), 3K (novel adenosine deaminase inhibitor, 5 mg/kg), dexamethasone (0.1 mg/kg). Drugs were administered intraperitoneally for 7 days, starting 1 day before colitis induction. Spleen weight, and serum levels of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) were assessed as systemic inflammatory indexes. Macroscopic and microscopic damage and myeloperoxidase (MPO) levels in colonic tissues were evaluated as local phlogistic indexes. Treatment with DNBS resulted in a significant increment of spleen weight (from 754±45 to 981±57 mg; +30.1%). Such an increase was reduced by treatment with EHNA (+10.7%) and mainly with 3K (+2.5%) or dexamethasone (+4.1%). In rats with colitis, serum TNF-α and IL6 were increased (TNF-α: from 5±1.3 to 41±9.1 pg/ml; IL-6: from 122.7±2.5 to 206.5±18 pg/ml). EHNA, 3K or dexamethasone decreased TNF-α and IL-6 levels towards basal values. Six days after DNBS treatment, the distal colon appeared thickened and ulcerated with areas of transmural inflammation; the macroscopic damage accounted for 7.5±2. 3K or dexamethasone reduced the macroscopic score (4.2±1.1 and 3.3±1.7, respectively), while EHNA was less effective (5.9±1.5). Histologically, colonic sections from DNBS-treated rats showed the most severe damage after 6 days (4.2±1.2). In tissues from rats treated with 3K or dexamethasone a decrease in microscopic score values was observed (2.3±0.6 and 1.6±0.7, respectively), while EHNA was without effect (3.7±1). MPO levels in colonic specimens from control rats were 3.2±1.8 ng/100 mg, and these values were increased in animals with colitis (17.1±4.5 ng/100 mg). 3K or dexamethasone significantly reduced MPO levels in DNBS-treated rats (7.5±1.8 ng/100 mg and 7.2±1.6 ng/100 mg, respectively), and EHNA was less effective (11.2±2.4 ng/100 mg). The novel adenosine deaminase inhibitor 3K exerts a significant antiinflammatory activity in animals with DNBS-induced colitis. Such an effect appears to be mediated by a reduction in plasma cytokine and tissue MPO levels.

ADENOSINE MODULATES VASCULAR ENDOTHELIAL GROWTH FACTOR EXPRESSION VIA HYPOXIA-INDUCIBLE FACTOR-1 IN HUMAN GLIOBLASTOMA CELLS

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Adenosine exerts its effects through four subtypes of G-protein-coupled receptors: A_1, A_{2A}, A_{2B} and A_3. Stimulation of the human A_3 receptor has been suggested to influence cell death and proliferation. Hypoxia appears to induce a program which shifts the cellular phenotype toward an increase in intracellular adenosine. Hypoxia-inducible factor-1 (HIF-1) is a key regulator of genes crucial to many aspects of cancer biology. Since in gliomas there is a strong correlation between HIF-1α expression, tumor grade and tumor vascularization, the aim of this study was to investigate whether adenosine may regulate HIF-1 in human glioblastoma cell lines. Human glioblastoma cell line A172 and U87MG; *Hypoxia treatment* in a modular incubator chamber and flushed with a gas mixture containing 1% O_2, 5% CO_2 and balance N_2; *Treatment of cells with siRNA* to silence A_3 receptor and HIF-1alpha protein expression; *Real-Time RT-PCR experiments:* total RNA was reserve transcribed and amplified by PCR using specific primers for A_3 receptor and HIF-1α; *Western blotting:* total cellular extracts were analysed using antibodies specific against HIF-1α and HIF-1β, phosphorylated (Thr183/Tyr185) or total p44/p42 MAPK, phosphorylated (Thr180/Tyr182) or total p38 MAPK and for A_{2A} or A_3 receptor; *Enzyme-Linked Immunosorbent Assay:* the levels of VEGF protein secreted by the cells in the medium were determined by a VEGF ELISA kit; *Transient Transfection and Luciferase Reporter Assays:* to quantify VEGF-promoter activity. The results indicate that in the human hypoxic A172 and U87MG glioblastoma cell lines adenosine upregulates HIF-1 α protein expression via the A_3 receptor subtype. In particular, we investigated the effect of A_3 receptor antagonists on HIF-1 and vascular endothelial growth factor (VEGF) expression. We found that A_3 antagonists inhibit adenosine-induced HIF-1α and VEGF protein accumulation in the hypoxic cells. Investigations in the molecular mechanism showed that A_3 receptor stimulation activates p44/p42 and p38 MAPKs that are required for A_3-induced increase of HIF-1α and VEGF. Our study on GBM cells, along with the earlier study on melanoma cells, suggests that a therapeutically feasible approach of targeting HIF is through the use of A_3 receptor antagonists.Additional studies are needed to determine whether these inhibitors are capable of blocking in vivo the HIF-1α-dependent invasiveness, survival, and angiogenesis of GBM.

EFFECT OF HEMODIAFILTRATION WITH ON-LINE REGENERATION OF ULTRAFILTRATE (HFR) ON OXIDATIVE STRESS IN DIALYSIS PATIENTS

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Increased Oxidative Stress (OxSt), inflammatory state and endothelial dysfunction are known “nontraditional” risk factors for cardiovascular events and determinant of overall uremic cardiovascular risk which remains the most common cause of excess morbidity and mortality for ESRD (End-stage renal disease) patients. These patients have, in fact, upregulation of both inflammation related proteins, such as IL-6 and CPR, and oxidative stress related proteins, such as NAD(P)H oxidase, which contribute to reduced NO availability and endothelial dysfunction. A novel dialytic method, HFR, has recently been reported to reduce IL-6 and CPR counteracting inflammatory state, while no data are available on the effect of HFR on OxSt. The present study evaluates in 14 hemodialysis patients the effect of HFR treatment on plasma level of oxidized LDL (oxLDL) determined by ELISA, marker of OxSt and, using a molecular biology approach (RT-PCR and Western Blot), on the mononuclear cell gene and protein expression of OxSt-related proteins such as $p22^{phox}$ (subunit of NAD(P)H oxidase, essential for superoxide production), PAI-1 (induced by OxSt and atherothrombogenetic) and Heme-oxygenase-1 (HO-1) (protective from oxidative stress). Two groups of seven patients were randomized in a cross-over protocol AB e BA and treated for six months with HFR (A) (SG8 Plus-Bellco, Mirandola, Italy) and compared with low flux bicarbonate dialysis (BD) (B) using a polysulphone 1.8 m^2 dialyser. Blood samples were collected at beginning of the study, after six months (cross over) and after twelve months. HFR reduced mRNA production and protein expression of $p22^{phox}$ and PAI-1 compared with BD (-0.09±0.05 Δ % vs. -0.02±0.06, p<0.0001 and -0.15±0.20 Δ % vs. 0.03±0.17, p<0.05 for p22phox; -0.186±0.06 Δ % vs. -0.052±0.05, p<0.0001 and -0.237±0.12 Δ % vs. 0.09±0.15, p<0.0001 for PAI-1). HO-1 was unchanged (-0.12±0.08 Δ % vs. -0.10±0.08 e -021±0.12 Δ % vs. -0.14±0,08) while plasma oxLDL were reduced (-0.14±0.19 Δ % vs. 0.01±0.14, p<0.01). These data indicate that HFR treatment is able to reduce the expression of proteins related to and the level of markers of OxSt. Given the relationships between OxSt and inflammation and the determinant role played by OxSt in inducing inflammation related mechanisms in ESRD patients, it could be that the HFR induced reduction of OxSt plays an important role in the reduced inflammation state observed after treatment with HFR. These important effects on OxSt and inflammation state might give to HFR treatment high potential clinical impact in counteracting progressive atherosclerotic cardiovascular disease in dialysis patients.

ANTI-INFLAMMATORY ACTIVITY OF *APIUM GRAVEOLENS* EXTRACT AND ITS MAIN CONSTITUENT, APIIN

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Apium graveolens L. var dulce (*Umbelliferae*) is an edible plant used as a flavouring and as a spice. The composition of the volatile oil has been largely studied (1,2). Its leaves contain large amount of antioxidant molecules (flavonoids and α-tocopherol) for which are used in folk medicine or usually as food in the mediterranean diet. The objective of this study was to assess the potential anti-inflammatory properties of a polar extract obtained from *Apium graveolens* leaves and its major component, apiin. Increasing concentrations of a dried polar extract (A) of *A. graveolens* leaves (0.01-0.1 mg/ml) and apiin (0.005-0.05 mg/ml) were evaluated *in vitro* on *Escherichia coli* lipopolysaccharide (LPS)-induced nitrite production (NO_2^-) production and on inducible nitric oxide synthase (iNOS) expression by Western blot analysis, in J774.A1 murine macrophage cell line (3). The cytotoxicity of increasing amount of A (0.01-0.1 mg/ml) and apiin (0.005-0.05 mg/ml) was evaluated on J774.A1 and HEK-293 cell viability by MTT assay (3). The *in vivo* topical antiinflammatory activity of A was investigated using the croton oil ear test in mice (4). Extract A and apiin showed *in vitro* a significant ($P<0.001$) inhibitory activity on nitrite production and iNOS expression in LPS-induced J774.A1 cells. *In vivo* anti-inflammatory evaluation showed for extract A also significant anti-inflammatory effect evaluated using indometacin as reference drug. Our results strongly support the anti-inflammatory properties of polar extract of *A. graveolens* leaves containing apiin as major constituent and other phenols. The presence of all these compounds may contribute to validate the use in folk medicine of *A. graveolens* plant extract against cutaneous inflammation (5-6).

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CYP2C9*3 (1075 A>C) POLYMORPHISM AND CYCLOPHOSPHAMIDE TOXICITY IN BREAST CANCER PATIENTS

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Cytochrome p450 (CYP) is a superfamily of phase I enzymes that may be implicated in the metabolism of several antineoplastic drugs. Several subfamilies of CYP are involved in the hepatic bioactivation, through the 4-hydroxylation, of cyclophosphamide (CPA). In particular, CYP2C9 shows a genetic polymorphism that has been associated with interindividual variations in the enzyme activity and in CPA metabolism. The SNP (Single Nucleotide Polymorphism) called CYP2C9*3 (1075A>C) leads to the aminoacidic substitution Ile359Leu in the exon 7 of the gene. This modification causes a decreased enzyme activity *in vivo* that could modulate the cytotoxic effect of CPA. The aim of this study is to define the role of the CYP2C9*3 polymorphism in developing toxicity in breast cancer patients after the CPA treatment. One hundred twenty-nine patients affected by breast cancer and homogeneously treated with cyclophosphamide, methotrexate and 5-fluorouracil (CMF adjuvant regimen) have been tested on genomic DNA from peripheral blood mononuclear cells. The genotype analysis was performed using the Pyrosequencing, a mini-sequencing technique. After the first cycle of chemotherapy, there is an association (p=0.0436, Chi-squared Test) between genotype and any grade of hepatic toxicity (see table 1a). This association is confirmed when comparing patients carrying at least one 1075C allele and grade 1-2-3 of hepatic toxicity: these patients show a 3.5-fold increased risk to develop hepatic toxicity (p=0.0039, OR 3.733, CI 95% 1.552-8.983) with respect to the patients with AA genotypes (see table 1b). A trend for a significant association (p=0.053) was observed when considering the hepatic toxicity over the entire course of chemotherapy but is not confirmed comparing grade 0 vs. grade 1-2-3 of hepatic toxicity (data not shown). No association was found between the CYP2C9*3 genotype and other toxicities (haematological and non-hematological).

Conclusion: Breast cancer patients treated with CMF carrying at least one allele of the CYP2C9*3 variant have a higher risk of developing hepatic toxicity (grade 1-2-3) after the 1st cycle of chemotherapy.

Table 1a

1st cycle	Grade 0	Grade 1	Grade 2	Grade 3
1075 AA (100)	80 (80.0%)	13 (13.0%)	5 (5.0%)	2 (2.0%)
1075 AC (28)	15 (53.6%)	10 (35.7%)	2 (7.1%)	1 (3.6%)
1075 CC (1)	0 (0%)	1 (100%)	0 (0%)	0 (0%)

p=0.0436, Chi Squared Test

* Fisher Exact Test, (1075AA) vs. (1075AC+1075CC)

Table 1 b

Grade 0	Grade 1-2-3	OR (95% CI) *
80 (80.0%)	20 (20.0%)	1
15 (53.6%)	13 (46.4%)	3.586 (1.69-7.596)#
0 (0%)	1 (100%)	

# p<0.05

DEVELOPMENT OF PRIMARY CULTURES OF RAT ILEUM LONGITUDINAL SMOOTH MUSCLE CELLS: SURFACE EXPRESSION OF CD73

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Abnormal motility of the gastrointestinal tract is frequently associated with intestinal inflammation. The mechanisms underlying this altered motor function are not fully understood but they may reflect alterations in smooth muscle and/or neuron activity (1). CD73/ecto-5 minnucleotidase, an enzyme located on the cellular surface of many cell types, participates in immune/inflammation reactions (2) and converts extracellular 5 min-AMP to adenosine, a mediator that controls intestinal motility and exhibits tissue-protective actions during inflammation or hypoxia (3). CD73 activity has previously been detected in human duodenal mucosa, intestinal epithelial cells, longitudinal muscle strips of guinea pig ileum and intact isolated rat ileum strips (4). The purpose of this work was to develop primary cultures of intestinal smooth muscle cells (ISMCs) from rat ileum and verify their surface expression of CD73. ISMCs were explanted from freshly isolated rat ileum. The longitudinal muscle was gentlystripped from the circular muscle and mucosa, and cut into 0.5 cm pieces, which were plated in culture dishes and allowed to adhere to the coverglass bottom. After 7 to 10 days, fragments were removed and isolated ISMCs were cultured in DMEM enriched with 10% FBS, 2 mM L-glutamine, 0.1 mM non-essential aminoacids and 20 μg/mL gentamicin. Immunohistochemistry (IHC) and immunocytochemistry (ICC) experiments were performed on formaldehyde-fixed tissue sections and cells, respectively. The cells grown from the explant became near confluent 12 to 15 days since plating. On reaching confluence, they formed local areas of high density with intervening zones of low density, giving a "hill and valley" appearance. For characterization, direct and indirect immunofluorescent stainings were performed using anti-α-SMC-FITC and anti-enteric-γ-SMC monoclonal antibodies (mAb), respectively. Primary cultures stained strongly with either antibody. Smooth muscle isoactin protein expression was detected in more than 98% of cells, indicating a homogeneous population of ISMCs. IHC with anti-CD73 mAb demonstrated the presence of the enzyme on rat ileum longitudinal muscle strips, while ICC confirmed CD73 staining only at the ISMC surface and particularly on intercellular junctions. These results provide the first evidence of CD73 expression on rat ISMCs, suggesting its possible role in the control of intestinal motility. This may be the initial step for future investigation of how CD73 enzyme expression is affectedby drugs as well as pathophysiological conditions.

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INCREASED TISSUE FACTOR-EXPRESSING PLATELETS AND LEUCOCYTEPLATELET AGGREGATES IN PATIENTS WITH ACUTE CORONARY SYNDROMES

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Platelets have a critical role in coronary artery thrombosis and their activation is pivotal in the pathogenesis of acute coronary syndromes. Also the expression within the arterial wall of the Tissue Factor (TF), the main initiator of coagulation, plays a central role in thrombus formation. Studies on patients with acute coronary syndrome (ACS) showed that TF plasma levels, monocyte-associated TF and the number of leukocyte-platelet aggregates are higher than in stable angina (SA) patients. Recent data indicate that TF is present in human platelets; these cells contain not only the protein but also TF mRNA, probably derived from megacaryocytes. Intraplatelet TF can be exposed on the membrane by platelet agonists such as ADP, Trap and tromboxane A_2 analogue and its expression can be modulated by commonly used antiplatelet drugs. In the present study we investigated TF expression in platelets and in leukocyte-platelet complexes of patients with ACS and SA.**.** We enrolled 25 consecutive patients with ACS and 25 patients with stable exertional angina. At the time of blood sampling patients were not receiving antiplatelet drugs but aspirin. Blood was drawn from an antecubital vein using a 19-gauge needle at rest and in the absence of chest pain (within 12 hours after the onset of symptoms for ACS patients). Platelet-associated TF was measured, both under resting condition and upon ADP stimulation (10 micromol/L, 15 min), in whole blood (WB) and in platelet rich plasma (PRP) by flow cytometry using a specific monoclonal anti human TF antibody. Under resting conditions ACS patients showed a significant two fold increase of TF-positive platelets as compared with SA (10.23±1.91 and 5.37±1.91 respectively, p=0.02). In vitro ADP stimulation increased the percentage of TF-positive platelets both in WB as well as in PRP but no difference was found in the absolute number between ACS and SA patients. Flow cytometric evaluation of leucocyte-platelet complexes showed that ACS patients have significantly higher levels of monocyte-platelet (+250%, p=0.005) and granulocyte-platelet aggregates (+200%, p=0.02) compared to SA patients. Moreover these aggregates express ten times more TF compared to those from SA patients. Our data indicate that in ACS patients expression of TF associated to platelets and to leucocyte-platelet complexes is higher than in SA patients and in control subjects. This evidence may further strengthen the link between platelet activation, blood coagulation and thrombus formation.

PHENOTHYPIC MARKERS DISCOVERY IN GROWTH FACTORS-STIMULATED VASCULAR SMOOTH MUSCLE CELLS (VSMC) BY PROTEOMIC ANALYSIS

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Vascular smooth muscle cells (VSMC) are the main component of *tunica media* and under physiological conditions they display a quiescent, contractile phenotype. These cells can also modulate their phenotype to become predominantly proliferative and this capacity plays a major role in the progression of such cardiovascular diseases as atherosclerosis, hypertension and restenosis (1). This study investigates, with proteomic techniques , the molecular changes that promote VSMC switching from quiescent to activated-proliferating phenotype, focusing in the first stages of the activation process. In particular, it analyses the modulation in tyrosinephosphorylation due to serum or single growth factors (Insulin-like Growth Factor 1, Platelet Derived Growth Factor) (2,3). Aim of this work was to identify markers which could be utilized for the selection and development of new diagnostic tools or new drugs. Proteins Tyr-Phosphorylation was observed for 10 min,30 min,60 min or 2-days after stimulation (time course exp.). Whole protein extracts from contractile phenotype (control) or stimulated cells were separated by 2D-electrophoresis PAGE and tyrosine-phosphorylated proteins were detected with specific antibodies. Several spots differentially phosphorylated were collected from a preparative gel, digested and subjected to mass spectrometry analysis by MALDI-TOF/TOF. Comparison of the 2D-PAGE profiles resulting from quiescent or activated-proliferating VSMC let us identify a number of differences in protein expression and phosphorylation. Several of these proteins were sequenced by mass spectrometry and classified in three principal groups: (a) cytoskeleton elements, (b) glucose metabolism enzymes, and (c) chaperones and enzymes involved in redox regulation. General decrease of the tyrosine-phosphorylation level was documented within the first minutes after stimulation followed by a recovery that is quick and dramatic for some chaperones and redox enzymes, otherwise it is not so significant for glucose metabolism enzymes. As far as the cytoskeleton components are concerned, no remarkable fluctuations were detected at the earliest time points except for those relative to α-actin that displays an impressive decrease. A comparison of the early stages of cell stimulation after serum or single growth factors administration enlightened important differences in the phosphorylation of chaperones suggesting their crucial role in VSMC activation. Each serum component, depending on its concentration, could control cellular metabolic pathways differently. This work opens new investigative perspectives exploiting mass spectrometry to reasearch new early markers implicated in cardiovascular diseases.

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CONSENSUS FOR SPORT IN ATHLETES WITH PRIMITIVE MYOPATHY

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This study investigated the effect of exercise on serum CK levels in athletes with persistent hyperckemia at rest compared with a control group of healthy athletes. We recruited 18 male Caucasian athletes playing different sports and high CK levels at rest (CK group). We also studied 25 male Caucasian athletes playing different sports and normal CK levels at rest, who were recruited as control group (C group). To assess CK and its isoenzymes activity, blood samples were taken at rest, 30 min, 6 h, 24 h, 48 h and 72 h after the test. The athletes who showed high serum CK values at rest or after exercise underwent careful clinical examination to evaluate localised hypotonic or hypotrophic muscles in the pelvic and upper limb girdle to ascertain whether signs of muscle weakness were present. They also underwent clinical and echocardiographic examination to identify genetic muscular pathology phenotypically expressed as a cardiomyopathy. If a mypoathy was diagnosed they received genetic counselling. In the control group, the CK values at rest were normal (48.18 ± 14.14 U/L). After exercise, they increased slightly, though they always remain lower than 80 U/L, decreasing to the rest level after 48 h (48 h 49.69 ± 21.90 U/L; 72 h 42.00 ± 14.14 U/L).The CK group always had CK levels at rest higher than normal (116.56 ± 33.30 U/L). After the test, CK showed a wide range of results, and did not return to the normal range (< 80U/l) neither after 48 h (130.11 ± 46.95 U/L) and 72 h (116.55 ± 24.84 U/L). The two groups had both, before and after the stress CK levels significantly different. Most of the athletes in control group referred muscular symptoms such as fatigue, pain and cramp after intensive training. . In the CK group, 7 athletes had clinical signs of Facioscapulohumeral muscular dystrophy (FSHD), 3 subjects with LGMD showed a prevalent myopathic involvement, and the serum CK level were higher. In one subject symptoms became suddenly worse after increasing the training workload and we suspected a CoQ10 deficiency myopathy, as he referred that a brother had died for cerebellar ataxia the year before. Other two subjects (M.C. and G.R.) showed hyperckemia at rest and after exercise and clear signs of progressive muscle involvement, but a conclusive diagnosis has not been reached yet. We demonstrated that, in athletes with sign of silent myopathy, CK levels can be also slightly elevated but had a different kinetics after exercise compared with healthy athletes: CK serum levels are almost always elvated even in preclinical stages of muscular pathology, therefore it may be safe to assess once, serum CK at rest, even in asymptomatic athletes and when time muscular symptoms are resistant to rest and therapy, or recur too frequently.

TUMOUR CELL DEPENDENT EFFECT OF NAMI-A ON CELL MALIGNANT BEHAVIOUR

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The progression of a benign tumour into a metastatic phenotype is the major cause of poor clinical outcome in cancer patients. Metastasis formation is a highly complex multistep process: tumour cells must modulate their adhesion ability, degrade the surrounding extracellular matrix (ECM), migrate and proliferate to a secondary site. NAMI-A is a ruthenium compound endowed with a peculiar anti-metastatic activity *in vivo* and it is devoid of cytotoxic effects on primary tumour. To study NAMI-A selectivity towards metastatic tumour cells, three cell lines with different metastatic and tumorigenic ability are used: HBL100, a non tumorigenic mammary epithelial cell line, MCF-7 poorly-invasive breast cancer cell line and MDA-MB-231 a highly-invasive breast cancer cell line. *In vitro* experiments that could mime the steps of metastatic process are done: for adhesion and resistance to detachment assay, a number of ECM substrates are used. To evaluate cell motility, migration and invasion assays are done. Morphological cell changes are detected by immunofluorescence: cells are stained with Alexa Fluor-488 conjugated phalloidin to detect actin filaments and with antipaxillin antibody to detect focal adhesion complexes and then they are observed at confocal microscope. To evaluate gelatinase activity, zymography of cell supernatants is performed. RhoA activity are measured by the affinity-precipitation assay on the basis of the specific interaction of activated RhoA with Mhotekin: protein expression and activation status are detected by electrophoresis and western blot techniques. NAMI-A interferes with every step of metastasis formation: it increases adhesion force of tumour cells to the growth substrates; it interferes with tumour cell adhesion to several substrates and to endothelial cells; NAMI-A reduces significantly cell invasion and migration, abolishing TGF-ß1 pro-invasive effect. NAMI-A's anti-migratory effect is stronger in haptotaxis than in chemotaxis, suggesting that NAMI-A activity involves tumour cell contacts with molecules of extracellular matrix; and finally it inhibits gelatinase production, without affecting TGF-ß1 pro-gelatinolytic effect, suggesting that NAMI-A's anti-metastatic effect is unrelated to TGF-ß machinery modulation. These effects are stronger on highly-invasive tumour cells than on poorly-invasive tumour cells or on not tumourigenic cells. On the basis of recent data describing NAMI-A-induced ß1integrin activation associated to actin remodelling, we also analysed the status of an important downstream molecule, RhoA, on the metastatic cells MDA-MB-231. NAMI-A increases RhoA activation on cells still adhered to the substrate, followed by the increase of actin polymerization and by a significant modulation of cell morphology. NAMI-A antagonizes TGF-ß1 pro-invasive effects without affecting its machinery. Besides, probably, NAMI-A interacts with integrins, leading to cell adhesion inhibition, when cells are in circulation, or blocking their movement if they are already attached to the substrate.

HYDROGEN SULPHIDE (H_2S): A PATHOPHYSIOLOGICAL MODULATOR IN VASCULAR HOMEOSTASIS

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Hydrogen sulphide (H25) has been considered for long time as a toxic gas, but recently its pharmacological effects have been revaluated. H_2S is produced in many tissues mainly by two enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS), and seems to be involved in several pathophysiological processes (1-4). It has been recently proposed that a reduction in plasma levels of H_2S could contribute to the development of spontaneous hypertension (5). Therefore we have investigated on the molecular mechanisms underlying H_2S-induced vasorelaxation in normal mice or in non-obese diabetic (NOD) mice. NOD mice show a progressive endothelial dysfunction that involves a reduced acetylcholine-induced vasorelaxation as well as an alteration of the adrenergic system (6). Mice were divided into three groups according to the different stages of pathology (NOD-I: diabetes onset; NOD-II: diabetic; NOD-III: severe diabetes. Aorta was explanted from NOR (wild type) and NOD mice at different stage of the disease and successively mounted on isolated organ baths, linked to isometric force transducers. We evaluated effects of administration of NaHS, a H_2S donor, and L-Cysteine (L-Cys), a H_2S metabolic precursor (1 μM-3 mM) on phenylephrine pre-contracted aortic rings. Both NaHS and L-Cys-induced a consistent vasorelaxation (max value: NaHS 100%, L-Cys 67%) that was not affected by endothelium removal. Conversely, administration of nitric oxide synthase inhibitor L-NAME, reduced both NaHS and L-Cys-induced vasodilatation (NaHS EC_{50}: 4.9×10^{-4} vs. 1.1×10^{-4}, $p<0.05$, n=5; L-Cys EC_{50}: 8×10^{-1} vs. 1.0×10^{-3}, $p<0.001$, n=5). Next we have investigated on the involvement of cAMP and cGMP in H_2Sinduced vasorelaxant effect, using SQ-22536 and ODQ as specific inhibitors of adenylate cyclase and guanylate cyclase respectively. The data obtained from these experiments suggest an important role for cGMP, particularly in L-Cys-induced relaxation (max rel: 23.5±2.1%vs.75.2±12.3%, $p<0.001$, n=5). In NOD-I, NOD-II e NOD-III mice, we observed a different response to NaHS or L-Cys. Indeed, while progression of the pathology from NOD-I to NOD-III correlates with an increase in NaHS induced vasorelaxation, L-Cys-induced vasodilatation progressively decreases (max relaxation: NOD-III 31.2±10.1% vs. NOD-I 73.4±10.7). This latter data is also supported by the finding that there is a reduced conversion of L-Cys to H_2S in NOD-III mice aortas (increase in production: 10% vs. 35%, n=3). In conclusion, our data show that NO contributes to H_2S-induced vasorelaxation in physiological conditions while in pathological conditions, where a reduced NO availability occurs, H_2S appears to play a compensatory role.

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THE ROLE OF SEROTONIN RECEPTOR FOR CARDIAC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

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During my PhD course I have studied the factors implicated in the expression of the functional properties of cardiac myocytes in order to better understand the mechanisms involved in cardiac development and pathology. In the first year I focused my attention on the effects of hypoxia during fetal life. In the second year I studied the effects of endogenous hypertrophic factors like endothelin on cultured HL1 cardiomyocytes. In this last year I studied possible factors regulating cardiac differentiation of embryonic stem cells (ES). Serotonin (5-HT) is known to play a crucial role during cardiac development, via type 2 receptor subtypes (5-HT2). Nevertheless the role of 5-HT2 during cardiac differentiation of ES is unknown. The aim of this work was to find out how 5-HT can affect cardiac differentiation of mouse ES (mES).mES were propagated in medium and differentiated via formation of embryoid body (EB). Since mES spontaneously differentiate toward a cardiac phenotype invitro, we first determined 5-HT content in the medium used to induce differentiation by HPLC. RT-PCR analysis is used to revealed if 5-HT2 receptor isoforms (2A, 2B and 2C) were selectively expressed in undifferentiated and differentiated cells. Functional coupling of 5-HT2 receptors was tested by measuring changes in intracellular Ca2+ content by fluorescence detection of FLUO-3 at the confocal microscope by exposure to either 5-HT (1 µM) or αMe-5-HT (100 nM, a 5-HT2 receptors selective agonist). T-type Ca2+ current was recorded by acute exposure to 5-HT of patch–clamped differentiated cells. The capacity of mES to differentiate into cardiomyocytes was also assessed in the presence of mianserine (1-10 µM, a selective 5HT2 antagonist) or SB215505 (1 µM, a selective 5-HT2B antagonist) by counting clusters of pulsating cardiomyocytes. In these condition, quantitative RT-PCR was used to quantify mRNA expression of cardiac transcription factor MEF2C and ventricular myosin light chain. 5-HT content in the medium resulted to be ≅5 µM. 5-HT2 receptor isoforms were selectively expressed during cardiac differentiation of mES, while they were absent in the undifferentiated population. In differentiated single myocytes, exposure to either 5-HT or αMe-5-HT resulted in a decrease of intracellular Ca^{2+}. This effect was accompanied by a reduction of T-type Ca^{2+} current. In control conditions, more than 20% of 3-dimensional EBs originated from mES exhibited spontaneously beating at day 7 of differentiation. In contrast, only 6% of EBs grown in the presence of 1 µM mianserine showed beating foci. No pulsating activity was detected if mianserine was raised to 10 µM. SB215505 reduced the appearance of beating foci to 16%. Accordingly, blockade of 5-HT2 receptors impaired expression of early cardiac transcription factor MEF2C, which was present in control condition (mRNA relative expression/GADPH 0.18%±0.033) and undetectable in the mianserine-treated EBs. Finally, structural cardiac genes expression was markedly affected, being ventricular myosin light chain present in control condition (mRNA relative expression/GADPH 1.2%±0.23) and undetectable in mianserine-treated EBs. These data show for the first time that 5-HT2 isoforms are selectively expressed in cardiomyocytes differentiated from mES and have a functional role in modulating intracellular calcium handling. Their pharmacological blockade during differentiation suppresses/reduces the occurrence of spontaneous contractile activity, the expression of cardiac specific transcription factors and sarcomeric proteins. Thus, 5-HT via 5-HT2 stimulation plays a key role in cardiomyocyte development.

GLUTAMATE INDUCES JURKAT T-LYMPHOCYTES ADHESION TO FIBRONECTIN: ROLE OF NMDA RECEPTOR

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L-glutamate (Glu), the main excitatory aminoacid neurotransmitter in the mammalian CNS, regulates response of T cells and Jurkat expressing both ionotropic (iGluR) and metabotropic (mGluR) Glu receptors. iGluR are ligand-gated ion channels, which, on the basis of their sequence homology and agonist preference, are classified into *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole propionic acid (AMPA) and kainate receptor types. mGluR are members of class G-protein couplet receptors. They are classified into three groups (I, II and III) and are linked to several effector systems (1,2,3).
The aim of this study was to investigate the ability of Glu to induce the adhesion to fibronectin (FN) of Jurkat T-lymphocytes. The iGluR or mGluR subtypes involved were also investigated. Jurkat cells were treated with Glu and seeded in 24 plates wells covered whit FN (4); the adhesion was quantitated by computerised microimaging analysis.
The proadhesive effects of Glu (range 10^{-11}-10^{-4}M) were illustrated by a bell-shaped concentration-response curve, whit a maximum at 10^{-6}M (231±23% adhesion vs control; P<0.01; n=5). iGluR specific agonist, NMDA, mimicked Glu effects (10^{-6}M NMDA % adhesion vs control: 216±19; P<0.01; n=5), while mGluR agonists were not able to reproduce the same adhesion effects.
D-(-)-2-Amino-5-phosphonopentanoic acid (D-AP5) and (+)-5methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine [(+)-MK 801], competitive and non-competitive NMDA receptor antagonists, completely inhibited Jurkat cells adhesion induced by Glu.
In conclusion the results demonstrated that Glu induces adhesion of Jurkat cells to FN and that NMDA receptors are involved in this process.

HOW DOES RAT ILEUM HANDLE EXOGENOUS ADENOSINE *IN VITRO*?

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Adenosine is well recognised as an important extracellular signalling molecule, acting via specific cell surface receptors to regulate several membrane and intracellular proteins. The nucleoside plays a key role in tissue protection under conditions of cellular stress, e.g. in ischemia and in inflammatory conditions. In the intestine, adenosine affects motility and secretion both directly and through the regulation of nervous discharge. The availability of adenosine to adenosine receptors depends on its degradation by adenosine deaminase and on the activity of nucleoside concentrative (CNT) and/or equilibrative (ENT) nucleoside transporters at the plasma membrane.The present study was meant to evaluate adenosine metabolism and transport in rat ileum strips incubated *in vitro*. The role of adenosine deaminase and of ENTs was specifically investigated. For this purpose male Wistar rats (320350 g b.w.), Harlan, Italy, were used. After sacrifice, distal ileum was removed and cut into 3 cm segments. Each segment was divided into four longitudinal strips, and these were placed into vials (2 for each vial) containing 2 ml of aerated (95%O_2, 5% CO_2) Tyrode solution. At the end of a 30 min preincubation at 36.5°C, they were exposed to exogenous adenosine (50 μM). The adenosine deaminase inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) and the ENT blocker, S-(4-nitrobenzyl)-6-thioinosine (NBTI) were added to the incubation medium 15 min before adenosine. At the indicated times medium aliquots (1 ml) were taken and the reaction was terminated by adding $HClO_4$ (1 M, final concentration), while the tissue was rapidly immersed in liquid N_2 and omogenized in 0.5 ml of $HClO_4$ 1 M. After centrifugation, the tissue residue was used to determine protein contents by the Lowry assay (1). The medium samples were analysed by a modified HPLC method (2) to quantify adenosine and its metabolites (inosine and hypoxantine). The reported data are means±SEM of the values obtained in 3 to 8 experiments. A time-course study was performed over 10 min showing that exogenous adenosine (50 μM) is rapidly cleared from the incubation medium of rat ileum, its half-life being approximately 0.5 min. The nucleoside concentration returns to basal levels (0.20±0.04 μM) within 5 min and its changes are quantitatively correlated with the increase of inosine and hypoxanthine. No degradation of adenosine occurs when samples are incubated in the absence of intestinal tissue. At 1 min, when 16.3±1.0 μM adenosine is still detectable, EHNA (1 μM) allows the recovery of 45.0±0.6 μM of the nucleoside and reduces inosine from 20.5±1.5 to 1.4±0.4 μM, not significantly different from baseline level (0.8±0.2 μM). Even after 60 min of incubation 9.0±0.9 μM adenosine can be measured in the presence of EHNA. By contrast NBTI (10 μM) does not affect adenosine and inosine concentrations in samples incubated for 1 min or 60 min. These results indicate that in rat ileum adenosine removal from the extracellular environment is a very rapid process and is primarily due to metabolic conversion by adenosine deaminase, while the nucleoside uptake through ENTs is irrelevant. A CNT-mediated transport can not be excluded and it might account for the decrease in the total pool of adenosine and its metabolites observed after 60 min of tissue incubation. Thus locally acting adenosine deaminase inhibitors can be expected to afford intestinal tissue protection in inflammatory and ischemic conditions.

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EFFECTS OF DELTA9-THC ON NOP RECEPTOR IN SH-SY5Y CELLS

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The neuropeptide nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for NOP. This receptor presents marked structural analogies with the three different opioid receptors, nevertheless it is not able to interact with the ligands for such receptors (1). Nociceptin has been shown to have a variety of functions such as nociceptive transmission, learning and memory, locomotory activity, food consumption, rewarding mechanism. The pharmacological characterization of this neuronal system allowed to suggest that nociceptin acts as a functional antagonist towards the endogenous opioid system (2). At this regard, previous studies reported the influence of acute and chronic morphine treatment upon proN/OFQ biosynthesis in the rat brain (3) and evidence has been provided that the endogenous opioid system is involved in the regulation of several effects elicited by cannabinoids, such as analgesia, reward, immunological responses or anxiety-like behaviour (4-6). With the aim to elucidate possible relationships between the N/OFQ-NOP and cannabinoid systems, in the present study the effects of delta9-THC exposure on NOP gene expression and receptor density have been investigated in the human neuroblastoma SH-SY5Y cell line. NOP receptor density has been determined by saturation and competition homologus binding studies. NOP mRNA levels have been determined by reverse transcription polymerase chain reaction (RT-PCR). The cells were exposed to 50-100-150-200 nM delta9-THC for 24 h. RT-PCR analysis showed a decrease of NOP receptor mRNA levels after delta9-THC exposure (95±4.5, 81.5±4.3, 71.0±5.7, 60.0±4.7*, vs. controls (100%,) for 50 nM, 100 nM, 150 nM and 200nM delta9-THC exposure, respectively, *p<0.05). A concentration-dependent decrease of NOP receptor B_{max} was observed after exposure to delta9-THC (95.0±2.7, 73.0±5.0*, 67.0±3.5**, and 62.0±5.4** versus controls (100%), for the concentrations of 50, 100, 150 and 200 nM , respectively, *p<0.05 and **p<0.01). These findings show that delta9-THC is able to affect NOP receptor, providing evidence for the existence of interactions between nociceptin/NOP and cannabinoid systems.

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COMPARATIVE STUDIES OF BRAIN METABOLISM IN ANIMAL AND HUMAN DISEASE MODELS

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Increasing interest in drugs active in the brain has suggested the relevance of brain metabolism in the modulation of efficacy and or toxicity. The study is focused on the metabolic differences between liver and brain in human healthy and tumoral tissues, in comparison with animal model. As preliminary a laboratory of Drug Profiling was set up and some assays for the investigation of metabolic stability were implemented. Metabolic stability was evaluated at 37°C, on 1µM compounds, in 125 incubation mix containing 30pmol/ml of cDNA expressed CYP3A4 (0.1÷ 0.2mg/ml protein) at a single time point (1h). Final concentration of organic solvent was 0.25%.
Intrinsic Clearance assay was evaluated by the substrate depletion approach [(1)]. Different concentrations of proteins and of compounds were investigated using verapamil as reference substrate. All depletion data were fitted to the monoexponential decay model, with a 1/y weighting, using Prism software. A Packard Multiprobe™ EX HT robot with WinPrep™ software was used for sample preparation. The analysis was performed by an UPLC/LCT-Premier TOF. Quantitative data were generated from the mass chromatograms measuring the peak area of the unchanged drug molecular ions using MassLynx 4.1 software. **.** A high throughput assay for quick ranking of large number of compound, up to 80 per session, was set up using cyp3A4 that is the most represented P450 in human brain. Intrinsic Clearance of different drugs was investigated. At odd of Omeprazole and nifedipine, Verapamil clearance showed to be strongly influenced by the amount of protein used: compound 2µM in rat liver microsomes (RLM) 0.5 and 0.2 mg/ml resulted in Clint of 92 µl/min/mg and 323 µl/min/mg respectively. Final experimental set up was realised with compounds at 1µM and 0.2mg/ml of microsomes. Results were in line with previous data reported (see Table 1). Moreover, the use of TOF instrument in accurate mass mode for Clint determination, allowed a preliminary evaluation of metabolites produced.

Table 1

Compounds	***HLM CL_{int} (µl/min/mg)***	***RLM CL_{int} (µl/min/mg)***
Verapamil	166	540
Propranolol	100[a]	
Omeprazole	35	
Diclofenac	193	181
Nicardipine	705	
Tolbutamide	14	

[a]All microsomial incubations were performed at 0.2 mg/ml of HLM (Human Liver Microsomes) with exception of propranolol (0.1 mg/ml).

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HYPER-HOMOCYSTEINEMIA ALTERS AMYLOID PEPTIDE-CLUSTERIN INTERACTIONS AND NEUROGLIAL NETWORK IN THE CAUDATE AFTER INTRASTRIATAL INJECTION OF AMYLOID PEPTIDES

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Amyloid peptides (Aβ) are fragments of the Amyloid Precursor Protein (APP), an integral membrane protein. They are continuously generated by neurons and non-neuronal cells via sequential cleavage of APP by secretases. Glial cells participate in the uptake of soluble extracellular Aβ and in the clearance of this material at localized sites where the Aβ are concentrated. It has been shown that clusterin (Apo J) exerts important additive effects in reducing Aβ deposition. In agreement with the fact that homocysteine (Hcy) potentiates Aβ peptide neurotoxicity, and Hcy brain levels increase with age, it has been demonstrated that high plasma levels of Hcy are a risk factor for AD. Accordingly, possible interactions between the β-fibrils, in Aβ intra-striatally injected animals, and ApoJ have been evaluated in an animal model of hyper-homocysteinemia. In the same animal model, the possible ability of Aβ peptides to activate astrocytes and to affect tyrosine-hydroxylase immunoreactivity (IR) in the basal ganglia, as well as the influence of Aβ on pain threshold, have been investigated. Male Sprague Dawley rats of 185-200 g (n=20/group) were used. Aβ1-40 and Aβ1-43 were dissolved in 35% acetonitrile, 0.1% TFA solution and injected into striatum (500 nl) by means of stereotaxic instrument. To induce hyper-homocysteinemia, animals were subjected to chronic intake of methionine (1 g/kg/day) in the drinking water for 35 days. The following parameters have been evaluated: pain threshold by plantar test, Hcy plasma and brain levels by HPLC, striatal tyrosine hydroxylase (TH) and glial fibrillary acidic protein (GFAP) IR, fibrillary amyloid visualization with thioflavine S, clusterin immunofluorescence and their colocalization. Our data show that methionine chronic consumption increases plasma and striatal Hcy levels by 30 and 100%, respectively. Moreover, Aβ injection decreases TH-IR while increases GFAP-IR in striata; no significant effects of hyper-homocysteinemia were observed. It has been observed that the colocalization between Aβ-fibrils and ApoJ-IR is increased in hyperhomocysteinemic animals. Furthermore, a reduction of the pain threshold has been found in animals intrastriatally injected with Aβ peptides. Our previous data have shown a pro-βfibril action of Hcy both *in vitro* and *in vivo* . The present finding demonstrating that Aβ fibrils-ApoJ high density deposits are increased in hyperhomocystinemic animals give further evidence to the ability of ApoJ in buffering toxic compounds, such as Hcy, and reducing Aβ deposition. Furthermore, our data demonstrate that Aβ peptides are not only signals capable of activating astrocytes but also capable of reducing TH-IR in the basal ganglia probably leading to a reduction in dopamine-mediated volume transmission. These alterations in the neuroglial network might explain, at least in part, the reduced pain threshold observed in the Aβ injected animals.

THE EFFECT OF HYPOXIA ON FREE IRON AND F2 ISOPROSTANES LEVELS IN RAT CORTICAL SYNAPTOSOMES

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Synaptosomes are particularly affected by oxidative stress and the release of free iron and F2-isoprostanes is a reliable method to assess oxidative state *in vivo* and *in vitro* In this paper we examined the effect of hypoxia (2% O_2) on rat cortical synaptosomes. We evaluated modifications of physiological behaviour and free iron and esterified F2-isoprostanes levels after different periods of hypoxia and after 30 min of re-oxygenation. Synaptosomes were isolated from adult male Sprague rats by discontinue Ficoll gradient centrifugation, re-suspended in Kreb's buffer (2mg/ml), incubated for 2 h at 37 °C in 2% O_2:73% N_2:5% CO_2 and re-oxygenated for 30 min. Controls were maintained in air:CO_2 atmosphere. Immediately after hypoxia and re-oxygenation we measured ATP production, membrane potential, Ca^{2+} concentration and levels of free iron and F2 isoprostanes. Oxygen consumption decreased significantly ($p<0.001$) during 120 min of hypoxia and was restored after re-oxygenation. At the same time, ATP production decreased and remained significantly lower even after re-oxygenation. This involved a depolarization of the synaptosomal mitochondrial membrane, although the $[Ca^{2+}]_I$ remained practically unchanged. Indeed iron and F2-isoprostanes levels, representing useful prediction markers for neurodevelopmental outcome, increased significantly after hypoxia from 0.22 ± 0.04 to 0.29 ± 0.08 nM/mg proteins and from 106 ± 25 to 126 ± 30 pg/mg proteins, respectively. Moreover there was a strong correlation between the two variables being r = 0.873. On the whole our results indicate that synaptosomal mitochondria undergo mitoptosis after two hours hypoxia.

ADOLESCENT EXPOSURE TO CANNABINOIDS AND LONG-TERM CONSEQUENCES ON THE ADULT BEHAVIOUR

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Despite the increasing use of cannabis among adolescents, there is little information about the neuropsychological long-term consequences of cannabinoid consumption in juveniles. Adolescence represents a critical phase for cerebral development and it is characterized by strong neuronal plasticity, with sprouting or pruning of synapses, myelinization of nerve fibers, variations of neurotransmitter concentration and their receptor levels. The endocannabinoid system plays an important role during early development stages of the brain as cannabinoids modulate the release and the action of different neurotransmitters. Therefore, early exposure to cannabinoids might lead to subtle but long-lasting neurobiological changes. To mimic the human juvenile exposure to cannabinoids, adolescent male and female rats (35-45 PND) have been treated intraperitoneally with increasing doses of THC twice a day for 11 days (2.5 mg/kg day 1-4; 5 mg/kg day 5-8; 10 mg/kg day 9-11) and left untreated until their adulthood (75 PND). During adult age (75-90 PND) rats have taken behavioural tests to evaluate locomotor activity, anxiety, depression and analgesia. The results demonstrate that chronic administration of THC during the adolescent period resulted in an altered behaviour in adulthood. The nature of these effects depended on the sex of the animals. THC-exposed female rats exhibited a trend to increase of anxiety in the elevated plus maze test, as demonstrated by the reduced number of entrances in the open arm and the time spent in that arm. Furthermore, female rats pretreated with THC exhibited in the forced swim test a reduction in the time spent in "swimming" and "climbing" and an increase in "immobility", alterations that reflect a state of despair. On the other hand, THC-exposed male rats showed an increased stress-induced analgesia (tail-flick test) compared to not preexposed. Since the endocannabinoid system partakes in the modulation of several physiopathological events (for example analgesia, motor behavior, memory, anxiety, stress…) and the adolescent exposure to THC could have altered the cannabinoid system homeostasis in adulthood, the evaluation of CB1 receptor functionality, in term of receptor density and receptor/G protein coupling was also investigated showing region-specific alterations. Our results suggest that cannabis consumption during adolescence may induce different longterm behavioural effects depending on animal sex. Alterations in CB1 receptor functionality in specific brain areas represent part of the molecular mechanisms underlying these effects.

POSTWEANING SOCIAL ISOLATION PRODUCES REGION-SPECIFIC EFFECTS ON BIOGENIC AMINE CONTENT IN POSTMORTEM ADULT BRAIN

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Recent development of typical and atypical neuroleptics for the treatment of schizophrenia has produced great improvement in the clinical symptoms of patients, but it is still inadequate, and the overall prognosis for schizophrenia is far from satisfactory. Recent advances in neuroscience have shown, indeed, that this pathology is a complex, multifactorial disease probably involving genetic, neurodevelopmental and environmental factors. Numerous efforts have been made to develop animal paradigms of schizophrenia in order to mimic characteristic neurobiological and behavioural features of the human disease (1). One of the so-called "environmental model" of schizophrenia is the isolation rearing of rats (2), which provides a non-pharmacological and developmental specific method of inducing schizophrenic-like behavioral deficits (3). The aim of this study was to describe changes in adult neurochemistry in the rat following postweaning social separation. In particular, we examined the effects of social isolation on biogenic amine content in postmortem adult brain. Our results have shown that socially isolated rats (ISO group) had decreased tissue levels of serotonin (5-HT) in the striatum, but not in the prefrontal cortex (PFC). The turnover of 5-HT, as determined by the ratio of its metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA) to 5-HT, was increased in the striatum of ISO animals. Moreover, dopamine (DA) and metabolites, 3,4-dihydroxyphenilacetic acid (DOPAC) and homovanillic acid (HVA), levels were not affected by housing conditions either in the striatum or in the PFC. The turnover of DA, calculated as DOPAC/DA, HVA/DA and (DOPAC+HVA)/DA, was not modified by social isolation. Noradrenaline levels were increased in the striatum but not in the PFC. These data provide evidence that isolation rearing in the rats results in striatal dysfunction. These effects may in part underlie the behavioural consequences of isolation relevant to human developmental disorders.

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IN VITRO AND _IN VIVO_ P2X7 SILENCING IN RAT HIPPOCAMPAL GLIAL CELLS BY RNA INTERFERENCE

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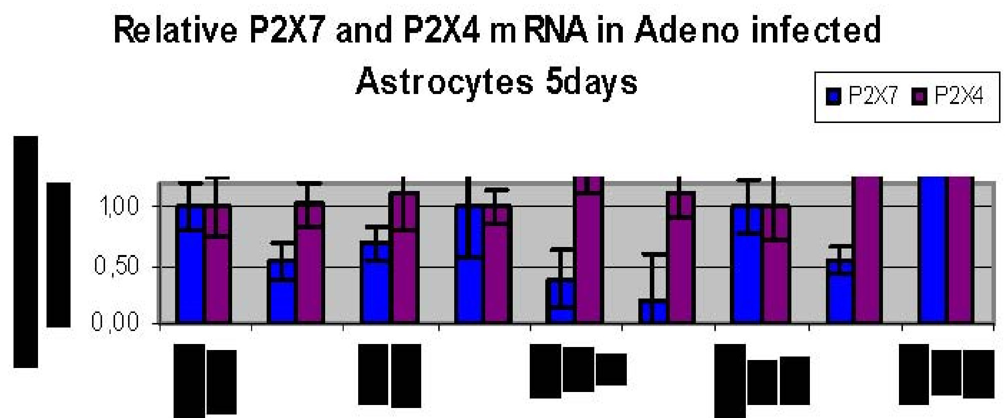
P2X7 is a member of the ATP-gated cation channel family, predominantly expressed in immune cells and glial cells. There is some evidence for a possible role of this gene in depression and anxiety, based on its pattern of expression, its potential role in neurotransmission modulation and on genetic linkage studies. To evaluate its involvement in psychiatric diseases, RNA interference was used to suppress P2X7 expression in rat hippocampal glial cells to assess any effect on anxiety and depression behaviour. Two AdV vectors were designed and produced to express a hU6 promoter-driven shRNA specific for P2X7. Hek-P2X7 recombinant cells and astrocytes [prepared as previously described (1)] were infected with different amount of virus. Each animal (male Wistar-Han rats, 240-260 g) was anesthetized with isofluorane and the virus was injected unilaterally in both dorsal and ventral hippocampus using a stereotaxic device and a Hamilton microsyringe. All the experiment was performed following the current national and international laws and policies. Total RNA was isolated from sample (cells or tissue), mRNA was converted in cDNA and P2X7 and P2X4 expression was quantified by TaqMan analysis using the Applied Biosystem method. Both AdV-shRNA vectors generated were able to suppress only P2X7 expression *in vitro* in recombinant cells and in primary rat astrocytes. The most active and specific viral vector was selected (max 90% of target mRNA suppression respect to the control after 5 days, tab. 1) and injected through unilateral infusion in rat hippocampus. An AdVshRNA suppressing P2X7 was identified as able to suppress only the target gene in rat astrocytes and was infused in rat hippocampus.

Tab.1



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ANTIPROLIFERATIVE EFFECTS OF POLYACETYLENES FROM *E. PALLIDA* ON HUMAN PANCREATIC CANCER CELL LINE MIA PACA-2

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Three main classes of active constituents have been identified in medicinal Echinacea species, angustifolia, pallida and purpurea: a) caffeic acid derivatives b) alkylamides and polyacetylenes c) polysaccharides. They are responsible for the most important pharmacological activities of this plant, the antiinflammatory, immunostimulatory, antioxidant and cicatrising ones (1). Previous studies in our laboratory showed that medicinal Echinacea root hexanic extracts were cytotoxic on human pancreatic adenocarcinoma cancer cell line MIA PaCa-2 and E. pallida was the most cytotoxic species. This pharmacological evidence was in line with the different phytochemical profile of the E. pallida hexanic extract, rich of polyacetylenes, which are almost absent in the other two species (containing mainly alkylamides) (2). Different polyacetylenes have been already reported to be cytotoxic against a number of solid and leukemic cancer cell lines (3,4) and potentiate the cytotoxicity of other anticancer drugs (3,5). In the present work, we investigated the effects of five polyacetylenes isolated from hexanic extract of E. pallida on MIA PaCa-2 cell line. Cell viability has been evaluated by the colorimetric WST-1 assay and expressed as % of control (cell viability under no treatment). Moreover, apoptotic cell death was assessed by an immunoenzimatic assay which evaluated the cytosolic internucleosomal DNA enrichment (ELISA test) and by the evaluation of caspase activity (Caspase 3/7 activity test). Our results indicated a concentrationdependent antiproliferative effect in the range 1-100 µM for most of the compounds under study and an apoptotic cell death at 50 µM for two polyacetylenes after 24 h exposure. On the basis of these data, we propose to evaluate the antiproliferative effect on other human cancer cell lines, the potential effects of combination with drugs used in the clinical chemotherapy, and to better characterize the mechanisms of cytotoxicity of these compounds.

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EXPRESSION AND REGULATION OF CYTOCHROME P450 AND PHASE II ENZYMES IN THE LUNG OF RAT, RABBIT AND PIG

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Lung is a prominent target organ for numerous types of chemically induced damage as a result of exposure to xenobiotics after inhalation or following systemic administration. Many of the compounds associated with lung diseases, such as cancer and chronic obstructive pulmonary disease (COPD), require an enzymatic activation, mostly catalysed by cytochrome P450 (CYP) enzymes (1). The lung is a very heterogeneous organ and may contain over 40 different cell types. There are marked species differences in the number and morphology of the pulmonary cells and this could explains, for example, a different metabolic capacity of mouse lung and a higher susceptibility of this species to toxicity by some compounds with respect to rat and human lung. Knowledge about the presence and regulation of CYPs in laboratory animals (mostly rodents) and human lung is rather limited. Therefore, it is important to deepen the studies on the laboratory animals and to develop new models in larger animals such as pig, since it is anatomically and physiologically similar to man. In this study we treated rats, rabbits and domestic pigs with typical CYP inducers to examine the presence and inducibility of specific CYPs in the lung through RT-PCR, specific enzymatic activities and immunoblot analysis. These analysis were also made in the liver for comparison.Rats were treated for 4 days with phenobarbital (80 mg/kg i.p.), clotrimazole (90 mg/kg by gavage) or a mixture of dexametasone plus pregnenolone 16 carbonitrile (50 mg/kg each i.p.), whereas rabbits were treated with phenobarbital (50 mg/kg) or rifampicin (50 mg/kg), known as specific CYP2B and 3A inducers through the constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Pigs were treated for 4 days with β-naphtoflavone (30 mg/kg i.p.), an inducer of enzymes belonging to the aryl hydrocarbon receptor (AhR) gene battery (CYP1A, 1B, 2S1, UDPGT, NQO1 and GST) (2). Phenobarbital treatment did not induce the CYP2B isoforms, unlike the liver, in either rat and rabbit lung. Also the treatment with the CYP3A inducers did not affect, unlike the liver, the expression of the four CYP3A isoforms (3A2, 3A9, 3A18 and 3A23) in the rat lung and the CYP3A6 in the rabbit lung. A low, but not inducible, expression of CAR mRNA was detected in the lung of rat but not in the lung of rabbit, whereas a low PXR mRNA expression was demonstrated in rabbit but not in rat lungs. These results indicate that the role of CAR and PXR in the lung, when present, is different to that observed in the liver of rat and rabbit. In pig the study was conducted, in addition to lung, in trachea and bronchi and the results showed a higher expression in the latter two tissues. In pig, for the first time, we identified (by RT-PCR with degenerate primers) and sequenced totally or a portion of CYP1A2, 1B1, 2S1 and AhR genes. The sequences of these porcine genes showed a high homology with the human orthologoues. Unlike AhR, the expression of CYP1A1, 1A2, 1B1 at mRNA, activity and/or immunoprotein level was induced by βNF although at different extent both in liver and lung tissues. CYP2S1 mRNA, a new isoform, was detected only in pulmonary tissues and more markedly after the βNF treatment. Among all phase II examined, βNF induced the activity of UDP-GT in the liver but not in the lung, demonstrating a tissue-specific regulation of this important enzymes by AhR.

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EFFECTS OF STATIN CHRONIC TREATMENTS ON RESTING CHLORIDE CONDUCTANCE AND CALCIUM HOMEOSTASIS OF RAT SKELETAL MUSCLE

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The use of statins is restricted by side effects they can produce on skeletal muscle. We showed that resting chloride conductance (gCl) and the Ca^{2+} handling structures, are potential targets of statin-induced muscle damage. The effects of 2-month chronic treatment with fluvastatin (5 mg/kg and 20 mg/kg) and atorvastatin (10 mg/kg) on the above mentioned functional parameters are reported here. Resting gCl was measured by means of two intracellular microelectrodes technique (1) and $[Ca^{2+}]_i$ by FURA-2 cytofluorimetric technique (2), in extensor digitorum longus muscle of control and treated rats. Rats treated with 5 mg/kg (10 rats) and 20 mg/kg (3 rats) fluvastatin showed a significant 25% gCl reduction. Also atorvastatin (10 rats) decreased gCl by 20%. Resting $[Ca^{2+}]_i$ was increased by 15% in muscle fibers of rats treated with 5 mg/kg fluvastatin and 10 mg/kg atorvastatin and by 40% in fibers of rats treated with 20 mg/kg fluvastatin as compared with control animals. No increase in the sarcolemmal permeability to cations (SpCa) was found in all treated rats. Statins treatment produced a significant alteration of the response to caffeine, activator of ryanodine receptor. Particularly, a drastic reduction of the caffeine responsiveness was observed in 20 mg/kg fluvastatin treated rats, showing a 80% reduction of amplitude of the 40 mM Caffeine-induced Ca^{2+} transient with respect to control rats. To investigate on the mechanism responsible for statins-induced $[Ca^{2+}]_i$ increase, we studied the effects of *in vitro* application of fluvastatin. on skeletal muscle. Fluvastatin (200 µM) enhanced basal $[Ca^{2+}]_i$ by 105±21 nM. and resulted still effective in the absence of extracellular Ca^{2+}, suggesting that the drug-induced $[Ca^{2+}]_i$ increase is not sustained by extracellular Ca^{2+} influx. In the presence of 5µM ruthenium red, inhibitor of ryanodine receptor, fluvastatin produced a $[Ca^{2+}]_i$ increase of 22±1 nM, indicating an involvement of sarcoplasmic reticulum (SR). Interestingly, pre-incubation with 2 µM cyclosporine A, an inhibitor of the mitochondrial permeability transition pore, completely abolished the fluvastatin-induced $[Ca^{2+}]$ increase suggesting that fluvastatin promoting a Ca^{2+} mitochondria efflux triggers a conspicuous Ca^{2+} release by SR. Our data suggest that the reduction of gCl, the importance of which is well known for mantainance of excitability and contraction in working muscle, and the disregulation of Ca^{2+} homeostasis, initiated by mitochondria, is related to muscle toxicity.

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GLIAL CELL DETECTION AND C-FOS IMMUNOREACTIVITY IN A TRANSGENIC MOUSE MODEL OF HUNTINGTON'S DISEASE

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Huntington disease (HD) is an autosomal, dominantly inherited neurodegenerative disorder characterized by progressive motor and cognitive disturbances caused by an expansion in CAG repeats in the IT15 gene which encodes the huntingtin protein. A pathogenetic role for excitotoxic cell death mediated by increased glutamatergic excitoxicity in the striatum has been proposed. Drugs able to modulate striatal levels of glutamate are thus candidates to protect striatal neurons from neurodegeneration. We have recently demonstrated that the extracellular concentration of adenosine increases in the striatum of HD transgenic (R6/2) mice and that the selective antagonist of adenosine A_{2A} receptors, SCH 58261, directly administered in the striatum, significantly reduces glutamate outflow (1). Furthermore we demonstrated that the p38 mitogen-activated protein kinase (MAPK) pathway is activated in the neurons of the striatum of HD transgenic mice (1). The p38 MAPK is known to be a factor activated and inducing the expression of pro- and inflammatory mediators and involved in the early gene c-Fos phosphorylation. Products of Fos family are players in inducing inflammatory gene expression in glial cells. In HD patient brain microglial activation in the striatum and in the cortex (2) and reactive astrogliosis in the striatum during the late stage of pathology (3)were reported. A first aim was therefore to investigate glial cells and c-Fos activation in heterozygous transgenic R6/2 male and wild-type mice at different ages, 10-11 (presymptomatic phase) and 14 week old (symptomatic phase), and to evaluate the effect of selective adenosine A_{2A} receptor antagonist SCH 58261. SCH 58261 was subchronically administered (0.01 mg/kg i.p.) at time -20, -17 and -2 h from sacrifice. Mice were transcardically perfused with paraformaldheyde and brains were cut by cryostat into 30 µm thick slices. Microglial cells immunostained by CD11-b antibody and astrocytes immunostained by GFAP were not activated in the striatum of 10-11 (n=5) and 14 week old R6/2 mice (n=3). Astroglial cells were detected only in the cingolate cortex of 14 week old mice (n=3). In the brain of 10-11 week old R6/2 treated mice (n=5) specific c-Fos immunostaining was not changed in comparison to wild-type mice (n=6). On the other hand, specific c-Fos immunostaining was increased in the piriform cortex, but not in the striatum, of 14 week old mice (n=3) in comparison to wild-type mice (n=6). The selective antagonist of adenosine A_{2A} receptors decreased c-Fos immunoreactivity in the piriform cortex of 14 week old mice (n=3). The results demonstrate that in transgenic R6/2 mice in the terminal phase, there is a modest activation of glial cells and activation of c-Fos early gene in the cortex, and that adenosine A_{2A} antagonism reduces c-Fos activation in the cortex.

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EFFECTS OF SOCIAL ISOLATION ON SEROTONERGIC SYSTEM IN PREFRONTAL CORTEX OF RATS: AN *IN VIVO* STUDY

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Schizophrenia is a chronic and severe psychiatric disorder which typically begins in late adolescence or early adulthood and it is characterized by psychotic periods with positive symptoms separated by periods with negative symptoms. Despite the extensive investigations on its clinical, psychological, biological and genetic aspects, schizophrenia neurobiology is only partially understood. Animal models are important tools in research of the physiopathological mechanisms underlying human diseases and offer an useful approach to test novel hypotheses that, in turn, may lead to innovative therapies. One of the so-called "environmental model" of schizophrenia is the isolation rearing of rats (1), which provides a non-pharmacological and developmental specific method of inducing schizophrenic-like behavioral deficits (2). The prefrontal cortex (PFC) plays a major role in the pathophysiology of schizophrenia. Thus, a neurochemical study on the activity of serotonergic system in the PFC was carried out on socially isolated (ISO) or group-housed (GRP) male Wistar rats. *In vivo* microdialysis with morphine (5 mg/kg s.c.) stimulation was performed in ISO animals and their controls, examining the PFC for serotonin (5-HT) and its metabolite, 5-hydroxyindolacetic acid (5-HIAA). While basal levels of 5-HT did not differ between housing conditions, the time course of 5-HT efflux produced by morphine was delayed in GRP rats. Moreover, basal levels of 5-HIAA in ISO group were significantly higher than GRP animals. Morphine injection significantly reduced 5-HIAA over the observation period. Additionally, to characterize further the generality of the effects of social rearing on serotonergic function, the effects of a locally applied depolarising pulse of potassium ions (100 mM for 20 min) was examined in ISO rats and their controls. Results showed that high K^+-induced increase in extracellular 5-HT levels was potentiated in isolation-reared rats. There was no effects of high K^+ perfusion on 5-HIAA levels. Our experiments demonstrate 5-HT changes in PFC subsequent to social isolation of rats. More broadly, they support the involvement of 5-HT system in chronic stressor (isolation rearing).

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REPEATED TREATMENT WITH A *CANNABIS SATIVA* EXTRACT EVOKES PAIN RELIEF IN A RAT MODEL OF NEUROPATHIC PAIN: MECHANISMS INVOLVED

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In recent years, some reports suggested that plant extracts consisting in combinations of cannabinoids may provide benefits that surpass treatment with single ones and may be potentially useful in the treatment of many diseases, such as chronic pain. On these bases, the aim of this research was to develop a novel analgesic regimen using low dose combinations of cannabinoids to treat neuropathic pain, a debilitating chronic pain refractory to actual drugs. Previously, we demonstrated the antihyperalgesic properties of cannabidiol (CBD) when orally given to neuropathic rats. In the present study, the antinociceptive properties of an high CBD extract (H-CBD) were tested in a rat model of chronic constriction injury of sciatic nerve (CCI) to investigate whether H-CBD may be powerful than pure CBD in counteract neuropathic pain. Rats were orally treated daily for a week, starting from the 7th day following the injury. A group of animal was treated with CBD 10 mg/kg (a dose that did not reach a maximal effect), a second group received H-CBD containing the same dose of CBD and a third group was administered with Δ^9THC at the corresponding mixture dose (0.42 mg/kg). As expected, CBD treatment partially relieved thermal hyperalgesia assessed by Plantar test (Ugo Basile, Varese, Italy). When CBD was administered through H-CBD, we observed a total relief of thermal hyperalgesia, in spite of Δ^9THC chronic treatment did not reduce thermal hyperalgesia. These data suggest that the chronic treatment with H-CBD evokes pain relief in CCI rats improving the effects of single cannabinoids. In order to investigate whether this synergism could occur during pharmacodynamic phase, different antagonists were tested to study the involvement of cannabinoid and/or vanilloid receptors. Alteration in pharmacokinetic phase could be also responsible for H-CBD synergic effect observed. Hepatic cytochrome P450-mediated metabolism and intestinal P-glycoprotein-mediated transport, which cannabinoids are able to modulate and whose are substrates, were studied in CCI rats chronically treated with H-CBD extract and CBD, to evaluate whether they contribute to an increased bioavailability of CBD and/or other compounds contained in the plant extract tested. Data showed a marked decrease of total hepatic cytochrome P450 and an inhibition of P-glycoprotein activity. All together these findings suggest that natural extracts, containing compounds such as terpenes and flavonoids that might increase cannabinoid bioavailability, could represent a new pharmacological tool for the treatment of neuropathic pain, leading to the use of lower doses avoiding side effects. Moreover, both terpenes and flavonoids possess antioxidant and antinflammatory properties which could significantly contribute to the therapeutical effects.

GEMCITABINE AND NIMESULIDE MODULATE BCL2 EXPRESSION IN HUMAN PANCREATIC TUMOR CELL LINES

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Gemcitabine (Gtb) is an inhibitor of ribonucleotide reductase and DNA synthesis and is an effective antitumoral agent used for the treatment of pancreas cancer. The majority of human primary pancreatic carcinoma overexpress the prostaglandin endoperoxide synthase Cox-2 (inducible form) in contrast to the benign pancreatic tumor. Cox-2 activity produces prostaglandin E2 and induces the production of angiogenic factors, which can favour tumorigenesis through neoangiogenesis. The present study analysed the effects of gemcitabine and nimesulide (Nms), a selective cox-2 inhibitor, on the expression of the anti-apoptotic Bcl-2 protein in two pancreatic tumor cell lines BxPc3 and MiaPaCa, characterized by high and low cox-2 expression respectively. We defined 2 pharmacological doses, for each drug, able to induce a nontoxic stimuli. We assayed the mitochondrial activity of 30x10^4 living cell treating with different concentractions (Nms 15-30-60-120 µM and Gtb 15-30-60-120 nM) for 24-48-72h. The cytotoxicity test shows that Bxpc3 are more sensitive than MiaPaca to both drugs and the lowest toxicity effect corresponds to Nms 30µM and Gtb 15nM. Cell lines were also exposed to parallel treatments for 24,48,72h: Gtb 15nM; Nms 30µM, Gtb 15nM+Nms 30 µM. Total proteins were extracted and the Bcl-2 expression level was assayed by westhern-blot.BXPC3 showed: at 24h an increase of Bcl-2 upon Gtb and Gtb+Nms treatment, while Nms alone did not modify the Bcl-2 level; at 48h Nms induced an increase of Bcl-2 higher than that induced by Gtb or Gtb+Nms; at 72h a similar high level of Bcl-2 was observed for each treatment. The Bcl-2 levels in Miapaca-2 at 24h and 48h were increased only upon Gtb+Nms treatment. On the contrary at 72h showed a decrease in Bcl-2 level upon each treatment. The increase of the antiapoptotic Bcl-2 protein upon chronic treatment of BxPC3 and Miapaca2 with gemcitabine 15nM may be part of a resistance mechanism of cancer cell to this chemotherapeutic agent. The decrease of Bcl-2 observed in MiaPaca (low cox2 expression) upon Nms 30µM may be explained by inhibition of antiapoptotic cox-2-dependent pathway. High Cox-2 expression in BxPc3 may be responsible for the lack of inhibition of the antiapoptotic pathway by nimesulide.

QUINACRINE ANTAGONISES THE U 87-MG CELL PROLIFERATION INDUCED BY POLYAMINES

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Variations in polyamine concentration are observed in case of neurodegenerative illness(1). Quinacrine is an alkylating agent characterised by the presence of an aliphatic chain similar to that of putrescine and spermine, two natural polyamines whose activation is closely associated with cell proliferation. In a previous study we observed that MCF-7 cell proliferation induced by polyamines was antagonised by quinacrine. In the present study, we investigated the effect of quinacrine and natural polyamines, alone and in combination, on glioblastomas cell cultures. Human glioblastomas cells (U87-MG) were cultured in EMEM medium, maintained at 37ºC in a fully humidified atmosphere of 5% CO_2 in air and treated with increasing concentrations of quinacrine (from 3.3 µM to 100 µM), putrescine (from 0.05 µM to 0.5 µM), spermine (from

1. 0.5 µM to 10 µm) and quinacrine plus putrescine or spermine. Results from MTT test showed that putrescine and spermine 0.5 µM significantly stimulate U87-MG cell growth (+53.86% and +28% respectively), whereas quinacrine confirmed to be a potent cell inhibitor. The combination of sub-inhibitory concentrations of quinacrine (34 µM) plus putrescine (0.5 µM) resulted in a significant inhibition (-63%) of cell replication, but the same inhibitory effect (26%) was reached using a lower concentration of quinacrine (17µM). The same results were obtained by the combination quinacrine (34 µM)/spermine (0.5 µM): -65% cell growth inhibition and quinacrine (17µM)/ spermine (0.5 µM): -28% cell growth inhibition. Preliminary results from the western blotting test performed on U87-MG cells indicate that bcl2 expression seems to be reduced in the cultures treated with the combinations polyamines/quinacrine (34 µM). Natural polyamines putrescine and spermine added to U87MG cell cultures significantly stimulate cell growth. Quinacrine at low concentration (17µM), antagonises cell proliferation induced by the polyamines. Previous studies performed on MCF7 cells showed that quinacrine blocks cell replication on the G1 phase. We suppose that the combination quinacrine/polyamines could result in the same effect on the U87-MG cells, moreover the presence of the aliphatic chain both in quinacrine and in polyamines could explain this interaction. If our hypothesis will be confirmed, it will be possible to postulate the use of low concentrations of quinacrine in the treatment of neurodegenerative illness or malignancies induced by variations in polyamine levels in human brain.

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ROLE OF P2 PURINERGIC RECEPTORS ON SYNAPTIC TRANSMISSION: ELECTROPHYSIOLOGICAL STUDIES IN HIPPOCAMPAL AND STRIATAL BRAIN SLICES

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Purinergic P2 receptors have been extensively investigated in recent years and growing evidence indicates their involvement in numerous cell-to-cell communication systems. In particular, in the central nervous system (CNS), their role in neuron-to-glia communication (1) and in synaptic transmission (2-4) has been described. P2 receptors are widely distributed in the central nervous system and comprise seven different subunits of P2X receptors, ligandgated non-specific cation channels (5), and eight subtypes of P2Y receptors, G-protein coupled receptors (6). The role of P2 receptors in the CA1 region of rat hippocamapl slices has been investigated under normoxic conditions and during *in vitro* oxygen and glucose deprivation (OGD). Field excitatory postsynaptic potentials (fEPSPs) from the CA1 dendritic layer or the population spike (PS) from the soma in the same brain area were extracellularly recorded. Under normoxic conditions, exogenous application of ATP or its stable analogue ATPγS significantly decreases the amplitude of evoked fEPSPs and PSs in the CA1 region of rat hippocampal slices. These effects are blocked by the unselective (PPADS) and the $P2Y_1$ selective (MRS 2179) P2 antagonists. After prolonged (7 min) OGD, slices do not recover their electrical activity (fEPSPs disappearance) when reperfused with normally oxygenated and glucose containing artificial cerebro spinal fluid (ACSF). The block of P2 receptors, 10 min before, during and after the 7 min OGD insult, prevents the irreversible loss of neurotransmission in the CA1 hippocampal region. This effect was always accompained by the block or delay of the anoxic depolarization (AD), and was observed in the presence of all the P2 antagonists tested (PPADS, suramin, MRS 2179, and brilliant blue). These results are suggestive that P2 receptors exert a deleterious role on synaptic transmission after prolonged *in vitro* OGD. The role of P2 receptors in rat striatal slices was investigated by whole-cell patch clamp technique in morphologically identified medium spiny neurons. Under normoxic conditions, exogenous application (8 min) of the selective $P2Y_1$ agonist 2MeS-ADP activates an iberiotoxin and apamin-sensitive potassium conductance elicited by a voltage-ramp depolarization (from -100 mV to +40 mV, 1800 ms). This effect was blocked by the selective $P2Y_1$ antagonist MRS 2179. Application of 2MeS-ADP also decreases the action potential firing rate of medium spiny neurons during suprathreshold current injections under current clamp conditions. These results indicate a role of $P2Y_1$ receptors in output striatal GABAergic pathways.

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INFLAMMATORY PATHWAYS AND DYSTROPHIC PROGRESSION: EFFECT OF *IN VIVO* TREATMENT WITH TARGET-SPECIFIC DRUGS ON FUNCTIONAL, BIOCHEMICAL AND HISTOLOGICAL MARKERS OF MUSCLE DAMAGE IN EXERCISED MDX MICE

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A chronic inflammatory state may act as a secondary mechanism strongly contributing to progression of Duchenne muscular dystrophy. Accordingly, wide anti-inflammatory glucocorticoids are the sole clinically useful drugs. Cyclosporine A, another wide acting immunosuppressant, contrasts some of the pathological signs in the model of exercised adult mdx mice (1). To gain insight in the pathways involved, we performed a 4-8 week treatment of adult exercised mdx mice with either etanercept (0.5 mg/kg s.c. twice a week), contrasting tumour necrosis factor (TNF)-alpha, or meloxicam (0.2 mg/kg/day, i.p.), which blunts inflammatory eicosanoids by inhibiting COX-2 enzyme. A multidisciplinary approach, involving *in-vivo* evaluation of mouse strength, and *ex-vivo* electrophysiological, biochemical and histological analyses, was used. Etanercept contrasted the exercise-induced mouse weakness. In fact the values of normalized strength increment after 4 week of exercise (strength/body weight time 4 – strength/body weight time 0) were 0.47 ± 0.04 (n = 6) and 0.76 ± 0.13 (n = 8) in untreated and etanercept-treated mdx mice, respectively ($p < 0.02$). *Ex vivo*, the etanercept treatment significantly counteracted the impairment of chloride channel function, a cellular functional index of myofiber damage, in both EDL and diaphragm muscle. Also it reduced the level of plasmatic creatine phosphokinase from 11405 ± 332 U/l to 3645 ± 988 U/l ($p <0.05$) and of muscular pro-fibrotic cytokine TGF-beta1, although the histological profile was not ameliorated. On the other hand, meloxicam was modestly effective and only on fewer parameters, supporting a marginal role of COX-2 derived eicosanoids in dystrophic progression. Similarly to cyclosporine A, none of the drugs ameliorated calcium homeostasis, which may explain the poor amelioration of histology. Thus, the present results confirm the role of inflammation, and in particular of the pro-inflammatory cytokine TNF-alpha, in the progression of dystrophic disease opening interesting therapeutic perspectives.

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EXPRESSION OF P-GLYCOPROTEIN AND GLUTATHIONE-S-TRANSFERASE: THEIR ROLE IN THE RESISTANCE OF COLORECTAL TUMORS AND IN THE RESPONSE TO CHEMOTHERAPY

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Colon cancer is the second leading cause of cancer death in most western countires. 40 to 50 percent of patients who undergo potentially curative surgery alone ultimately relapse and die of metastatic disease; chemotherapy is used as first-line treatment for metastatic colorectal cancer to destroy cancer cells that have spread. The new agents incorporated into frontline therapies, irinotecan (a semisynthetic derivative of camptothecin) and oxaliplatin, have improved the prognosis, but still, drug resistance is the major cause of chemotherapy failure. The expression of P-glycoprotein (PGP), the multidrug resistance-related protein (MRP) and gluthatione-S-transferase (GST) has been shown to be associated with the prognosis and response to therapy. PGP and MRP are transmembrane proteins that are involved in the extrusion of many substances, including some antineoplastic drugs, from cells. GSTs are enzymes that metabolize drugs, by conjugation of electrophylic molecules with reduced glutathione and protect cells from oxidative damage induced by antineoplastic drug, reducing their efficacy. GSTP1, a member of the GST family, highly expressed in the colon carcinoma cells, inhibits the kinase activity of JNK, a member of the MAPK family, which leads to the inhibition of apoptosis induced by oxidative stress and by antineoplastic drugs. Flavonoids show strong antioxidant activities and inhibitory effect on the growth of some cancer cells and therefore are of interest for increasing the efficacy of antitumor agents. Aims of the research will be: 1) to evaluate the role of PGP, MRP and GST proteins and of the proteins of the MAPK pathway in the resistance of colon carcinoma cells, *in vitro*, to irinotecan, camptothecin and oxaliplatin; 2) to test the effect of a treatment with anthocyanidins, polyphenolic ring-based flavonoids, on the citoxicity induced by these antitumor drugs; 3) to study the expression of PGP, MRP and of the MAPK pathway proteins after a treatment with the antineoplastic agents alone, or in combination with anthocyanidins. LoVo cells, derived from human colon carcinoma, expressing PGP, MRP and GSTP, were used. Cytotoxicity assay using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT test) was performed to determine IC_{50} of anticancer agents and anthocyanidins and to evaluate the effect of their combination. IC_{50} for camptothecin and oxaliplatin was respectively 37.0 nm± 5.3 (camptothecin) and 876.2 nm± 245.5 (oxaliplatin). IC_{50} for anthocyanidins was: 37.6 µm± 3.3 (delphinidin), 46.9 µm±1.8 (cyanidin), 80.5µm±9.8 (malvidin). The effect of the combination of anthocyanidins and antineoplastic drugs was also studied. Delfinidin (25µM) significantly increased the cytotoxic effect of both camptothecin 0.01µM ($p<0.001$) and oxaliplatin 0.1µM ($p<0.001$). The same concentration of cyanidin had similar effect in combination with camptothecin ($p<0.001$) but 50 µM cyanidin significantly increased cytotoxicity of both camptothecin ($p<0.001$) and oxaliplatin ($p<0.001$). Our results show that anthocyanidins, in particular delphinidin and cyanidin, exert an important cytotoxic action in the LoVo colon cancer cell line and increase the cytotoxicity of oxaliplatin and camptothecin. Studies are in progress to clarify the mechanisms of this cytotoxic action (detection of apoptosis, caspase-3 activity, GSH level measurements, expression of transport proteins and proteins of the MAPK pathway in cells treated with different concentrations of the antineoplastic drugs and flavonoids).

INTERACTIONS BETWEEN DP7, A NEW POWERFUL P-GP INHIBITOR, AND LIVER MICROSOMAL CYP FROM RAT AND HUMAN

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Multidrug resistance (MDR) is often associated with failure of cancer chemotherapy in many tumour cells. Several compounds inhibit P-glycoprotein (P-gp) and other membrane transporters that carry out of tumour cells antineoplastic agents. Blocking these transporters is a good strategy to overcome MDR. Many cytotoxic agents and MDR modulators that are substrates for P-gp, are also substrates for the cytochrome P450 (CYP) isoform 3A4. The competition between cytotoxic agents and P-gp modulators for CYP3A4 activity has resulted in unpredictable pharmacokinetic interactions. In principle, the ideal MDR-reverter should inhibit P-gp leaving unaltered CYP (1). In recent studies, a new dibenzoyl-1,4-dihydropiridine compound (DP7) has been shown to be a powerful P-gp inhibitor, almost avoid of cardiovascular effects (2). The aim of this study was to test the effects of DP7 on CYPcatalysed reactions, focusing the attention on human CYP3A4 isoform. Experiments were performed on rat and human liver microsome preparations. When rat microsomes were incubated with DP7, concentration-inhibition curves were obtained with use of selective markers of CYP activities. Ethoxy-resorufin (ETR) was marker of CYP1A1, penthoxyresorufin (PTR) of 2B, methoxy-resorufin (MTR) of 1A2 and benzyloxy-resorufin (BZR) of 1A1/2, 2B, 3A. Furthermore, kinetic analysis of the enzymes, using different substrate concentrations, were performed to obtain information on the mechanism by which each isoform was inhibited. IC_{50} values were 3.8 µM for PTR, 3.8 µM for ETR and 10.4 µM for BZR. All enzymes activities were inhibited non competitively. Moreover, DP7 inhibition of CYP3A4 family, in rat and human microsomes, was assessed fluorimetrically using a selective probe, 7-benzyloxy-quinoline (BQ) (3). With this substrate, however, the concentrationinhibition relationship on rat liver microsomes gave an IC_{50} value of 4.17 µM, while in the case of human liver microsomes it was not possible to achieve a 50% inhibition even at 75 µM concentration. In conclusion, the moderate inhibition of CYP isoforms in rat liver microsomes and the minimal interaction with human CYP3A4 family suggest that DP7 may be considered a lead compound for the development of novel MDR reverter dihydropyridines of therapeutics interest.

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A MODEL OF INSULIN-RESISTANCE INDUCED BY A HYPERCALORIC DIET ASSOCIATED WITH LONG-TERM TREATMENT WITH OLANZAPINE IN RATS

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Type 2 diabetes mellitus (DM2) is one of the most common endocrine diseases worldwide. Many studies have indicated a genetic predisposition for the development of DM2 according to the 'thrifty gene' hypothesis. Moreover, some forms of psychopathology such as the bipolar disorder, depression, and schizophrenia show a DM2 prevalence significantly higher compared to that of general population. However, the increased risk of developing DM2 for psychotic patients became a health-emergency only with widespread use of new generation antipsychotic drugs, such as olanzapine (1). Clinical studies show an association between the therapeutic response and the metabolic alterations during antipsychotic therapy. The aim of this study was to verify whether in rats a long-term treatment with olanzapine could potentiate the diabetogenic effect of a hypercaloric diet (high fat and carbohydrate content, HF). Moreover, we examined whether olanzapine would more easily induce metabolic alterations in rats that already showed initial signs of metabolic derangement. Thus, two different experimental protocols were used. In the first, rats fed the HF diet were divided into two groups, one receiving the vehicle and the other olanzapine (0.25 mg/Kg/die) for 8 weeks. That is, olanzapine treatment and exposure to the HF diet began simultaneously. In the second experiment rats were divided into two groups, one fed the standard diet and the other the HF diet. After 4 weeks of exposure to the standard or HF diet, each group was divided into two subgroups, one treated with vehicle and the other with olanzapine (2 mg/kg/die) for 4 weeks, while continuing the respective diet. At the end of the first experiment the body weights and weight of white adipose tissue showed no significant differences between the vehicle and olanzapine treated groups. Blood glucose levels in basal conditions were decreased in rats treated with olanzapine, while plasma insulin levels were increased. After an oral glucose load the time-curves of glycemic values and plasma insulin values were similar in the two groups. However, insulin sensitivity was significantly decreased in the olanzapine group. At the end of the second experiment the body weights and weight of white adipose tissue showed significant differences between rats fed the standard versus the HF diet, irrespective of the treatment. Blood glucose levels in basal conditions were lower in olanzapine treated rats compared to the respective vehicle treated group, while plasma insulin levels were higher in the rats fed the HF diet. After an oral glucose load the time-curves of glycemic values and plasma insulin values showed some statistical differences between groups. However, insulin sensitivity was significantly decreased in the rats fed the HF diet compared to their standard diet fed reference group. During olanzapine treatment we observed a transitory condition of hypothermia of about -2°C compared to the vehicle treated groups lasting for several h after each treatment. Thus, we examined by western blot the expression of UCP-1, a protein implicated in nonshivering thermoregulation, in brown adipose tissue. UCP-1 expression was increased in olanzapine treated rats, fed the standard or the HF diet.

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HADSC (HUMAN ADIPOSE DERIVED STEM CELLS) IN SKELETAL TISSUES REGENERATION

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Human mesenchymal stem cells (MSCs), found in bone marrow, adipose tissues, dermis, muscles and peripheral blood, have the potential to differentiate into cells of connective tissue lineages, including bone, cartilage, fat, muscle and neuron. This differentiation potential makes MSCs excellent candidates for cell-based tissue engineering. In this study, we have examined the profile of the human adipose tissue-derived stem cells (hADSC) in the undifferentiated states, and then we have differentiated these cells towards osteogenic and chondrogenic lineages. hADSCs were enzymatically isolated from adipose tissues obtained by liposuction from 18 adult human donors (1-3). They were expanded in monolayer with serial passages until confluence in presence of non inductive control medium. All the preparations of hADSC were assayed for the presence of MSCs-related cell surface antigens by flow cytometric assays (2,4,5). Under appropriate culture conditions, ADSCs were then induced to differentiate towards the osteogenic, chondrogenic and adipogenic lineages (6,7). Differentiation was assessed using histochemistry, immunoistochemistry and enzymatic assays. We also analyzed the interactions between cells and specific biomaterial for tissue engineering. These cells can be maintained *in vitro* for many generation with stable population doubling and low levels of senescence**.** Immunofluorescence and flow cytometry showed that the majority of hADSCs have a marker expression very similar to that of MSC; they infact expressed CD 13, CD29, CD44, CD54, CD90, CD105 and were absent for CD117 (c-kit), CD71, CD 45 and CD34 expression. (8). The osteogenic cells were strongly positive for Alizarine Red S staining and the levels of osteopontin are sensibly higher than in the cells cultured with control medium. The alkaline phosphatase activity reached the highest value between the second and the third week. The cell induced towards the chondrogenic lineage showed an high positiveness to the Alcian Blue staining, while the adipogenesis of the hADSCs cells were assayed by Oil red O staining. The cells seeded on biomaterials shoved high adhesive properties and a good differentiation potential is confirmed. No cytotoxicity effect were present. The data support the hypothesis that a human lipoaspirate contains multipotent cells and may represents an alternative stem cell source to bone marrow-derived MSCs. Our preliminary results on the interactions between hADSCs and scaffolds are really encouraging for a hADS Cs -based skeletal tissue regeneration.

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ROLE OF PROSTAGLANDINS IN GHRELIN GASTROPROTECTION AGAINST ETHANOL-INDUCED MUCOSAL DAMAGE IN RAT

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In our previous studies we have shown that ghrelin, a gastric-derived peptide considered the endogenous ligand for GH secretagogue receptor (GHS-R), exerts a central protective activity against ethanol (EtOH)-induced gastric lesions in rats. It is known that one of the main gastric systems involved in the maintenance of mucosal integrity and protection against EtOH injury is the prostaglandin (PG) system. PGs are synthetized by cycloxygenase (COX) which have constitutive (COX1) and inducible (COX2) isoforms. To assess the contribution of endogenous PGs in the gastroprotective activity of ghrelin, we examined the effects of central ghrelin (4000 ng/rat/i.c.v) on EtOH (50%, 1 ml, rat/os)-induced gastric ulcers in rats pretreated (30 min before) with celecoxib, a selective COX2 inhibitor, or indomethacin, a non selective COX inhibitor. Furthermore, the gastric mucosal content of prostaglandins was determined at the end of the experiments.Indomethacin (10 mg/kg/os), but not celecoxib (3.5 mg/kg/os), prevented the significant reduction (-57%; $p<0.01$ vs. EtOH) elicited by ghrelin on EtOH-induced gastric ulcers.Mucosal PGE2 measured one hour after EtOH was significantly increased (280%; $p<0.001$ vs. control) by EtOH. Ghrelin was able to prevent the increase of PGE2 production induced by EtOH (-67% $p<0.01$ vs. EtOH). The same reduction of mucosal PGE2 was obtained with celecoxib (-69% vs. EtOH) while indomethacin induced a suppression of PGE2 production more marked than that observed in ghrelin-treated rats (-84% vs. EtOH). The present results indicate that prostaglandins are involved in the gastroprotective activity of ghrelin.The fact that indomethacin but not celecoxib prevented ghrelin induced gastroprotection indicates that the maintenance of COX1-derived prostaglandins is relevant for the protective action of the peptide against EtOH-induced gastric ulcers.

ASSESSMENT OF POSSIBLE NEPHROTOXICITY FROM IODINATED CONTRAST MEDIUM IN PATIENTS UNDERWENT CORONARY MSCT

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Use of iodinated contrast media for diagnostic and interventional procedures is increasing as computed tomography (MSCT) and percutaneous coronary intervention (PCI) technologies provide increasing patient benefit. Although some complications associated with contrast media are mild and transient, contrast-induced nephropathy (CIN) can negatively affect longterm patient morbidity and mortality. The purpose of our study was to investigate the risk of contrast-induced nephropathy in patient underwent coronary MSCT The study population consisted of 236 patients (201 men and 35 women; mean age 54 years, range 38-79) with serum creatinine concentrations of 1.0 to 1.5 mg/dl who underwent electrocardiography (ECG)–gated multi–detector row CT. Patients were monitored in the hospital for 2 days and followed-up at 7 days. The primary end point was the peak increase from base line in the creatinine concentration during the two days after MSCT. Other end points were an increase in the creatinine concentration of 0.5 mg/dl or more, an increase of 1.0 mg per deciliter or more, and a change in the creatinine concentration from day 0 to day 7. Resource utilization data for 236 patients were analyzed. Increase of at least 0.5 mg/dl of the creatinine concentration 48 h after the procedure occurred in 3/236 patients but none of them required any treatment because there were no significant changes in serum creatinine after 7 days. About 140 ml contrast media and a moderate flow rate of about 5 ml/s ensure good results in coronary MSCT with no significant risk of CIN in the daily routine.

LAMOTRIGINE INHIBITS BASAL AND NA^{+}-STIMULATED, BUT NOT Ca^{2+}-STIMULATED, RELEASE OF CORTICOTROPHIN-RELEASING HORMONE FROM THE RAT HYPOTALAMUS

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Corticotrophin releasing hormone (CRH) is currently envisioned as a peptide neurotransmitter also involved in the aetiology and pathophysiology of various endocrine, neurologic, and psychiatric diseases, particularly affective disorders, anxiety and depressive disorders (1); in depressed patients an altered regulation of the HPA is often observed, which reflects CRH hyper-secretion from PVN hypothalamic neurons. The antiepileptic lamotrigine (LTG) is currently used as a mood stabilizing agent in patients with bipolar disorders (2); however, its mechanism of action for the latter indication is still poorly characterised. We investigated whether LTG can modulate CRH production and release by using the incubation of rat hypothalamic explants as an *in vitro* model. Furthermore we studied LTG interference with CRH release was dependent on a Na^{+} and/or Ca^{2+}-related mechanism. The experimental model has been described in detail elsewhere (3). Briefly, rat hypothalamic explants were incubated in a 24-well plate (1 hypotalamus/well) at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% O_2 in 300 µl incubation medium; explants were treated with medium alone (controls) or medium containing test substances namely LTG, KCl, veratridine. CRH released into the medium was measured by radioimmunoassay (RIA), whereas CRH mRNA was measured by RNase protection assay. We found that LTG reduces in a time- and concentration-dependent manner basal CRH release. LTG also inhibits veratridine-stimulated, but not K^{+}-stimulated, CRH release in 1-h experiments. Moreover, LTG tended to reduce CRH mRNA expression in 1-h experiments, regardless of whether the drug was given alone or in combination with veratridine or high K^{+} concentrations; such reduction achieved statistical significance after 3 h of incubation. In 1-h experiments, LTG reduces CRH release from hypothalamus neurons by interfering with Na^{+} -driven secretion mechanisms in accordance with observation that LTG is able to inhibit veratridine but not KCl stimulated CRH release. After prolonged (3h) incubations, the reduction of CRH release is also accounted for by LTG–induced decrease in CRH gene expression.

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DE NOVO SYNTHESIS OF CYCLOOXYGENASE-1 COUNTERACTS THE SUPPRESSION OF PLATELET THROMBOXANE BIOSYNTHESIS BY ASPIRIN

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Aspirin affords cardioprotection through the acetylation of serine^{529} in human cyclooxygenase (COX)-1 of anucleated platelets inducing a permanent defect in thromboxane (TX)A_2-dependent platelet function. However, heterogeneity of COX-1 suppression by aspirin has been detected in cardiovascular disease and may contribute to failure to prevent clinical events. The recent recognized capacity of platelets to make proteins de novo, paves the way to identify new mechanisms involved in the variable response to aspirin. We found that in washed human platelets the complete suppression of TXA_2 biosynthesis by aspirin (300 µmol/L added to Platelet Rich Plasma) recovered in response to thrombin (1 U/mL) and fibrinogen (0.38 mg/mL) in a time-dependent fashion [at 0.5 and 24 h, TXB_2 averaged 0.1+0.03 and 3+0.8 ng/mL; in the presence of arachidonic acid (10 µmol/L) it was 2+0.7 and 25+7 ng/mL, respectively, mean+SEM, n=9-22]. These concentrations of TXA_2 are biologically active and in the presence of other platelet agonists may further boost platelet responses. A similar effect was detected with other platelet agonists [ADP (20 µmol/L), collagen(5µg/mL) or thrombin receptor-activating peptide (50 µmol/L]. Recovery of TXA_2 biosynthesis in aspirin-treated platelets was blocked by translational inhibitors, by rapamycin, and by inhibitors of phosphatidylinositol-3-kinase. The results that COX-1 mRNA was readily detected in resting platelets and that [^{35}S]-methionine was incorporated into COX-1 protein after stimulation, strongly supports the occurrence of *de novo* COX-1 synthesis in platelets. In conclusion, signal-dependent *de novo* synthesis of COX-1 occurring in aspirin-treated platelets following activation may represent a mechanism involved in the interference of complete and persistent suppression of TXA_2 biosynthesis by aspirin necessary for cardioprotection. The occurrence of this phenomenon *in vivo* in patients with cardiovascular disease treated with low-dose aspirin remains to be verified. Our results may contribute to shed some light on the rather nebulous concept of aspirin resistance.

ISOLATED AND PERFUSED BRAIN: A METHOD TO STUDY THE WHOLE CEREBROVASCULAR SYSTEM

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Microvessels are targeted in cerebral diseases, including focal ischemia, hyperglycemia hypertensive angiopathy, and inflammatory disorders. The technical difficulties involved in accessing brain vasculature in mammals under *in vivo* conditions have stimulated the development of alternative *in vitro* techniques to tackle biological problems under more favorable experimental conditions than *in vivo* . In recent years a new technique has been developed to isolate and maintain *in vitro* a guinea pig brain via arterial perfusion with an artificial blood solution and allowing the study of a complex functional cerebrovascular system where the microcirculation plays a pivotal role. With this tecnique we studied the effect of intravascular activation of neutrophils on the synthesis of cysLT and the formation of cerebral edema. We showed that challenge (fMLP 0.1 µM) of neutrophil-perfused guinea pig brain *in vitro*, resulted in edema (brain wet weight increase control: 17±1% n=8, challenged: 51±9% n=10, $p<0.01$) associated with increase of cysLT (pg/ml of perfusate: control: 14.50±4.45 n=8, challenged: 40.73±6.69 n=10, $p<0.01$). Neutrophil pretreatment with a 5-lipoxygenase inhibitor, MK886 (1 µM), prevented both edema and cysLT formation. Moreover, the dual cysLT1-cysLT2 receptor antagonist BAYu9773 resulted more potent and effective than a selective cysLT1 antagonist (Iralukast) in preventing the brain permeability alteration induced by neutrophil activation. Unfortunately a major limitation of this preparation is represented by the difficulty to find pharmacological and biological tools tailored for the guinea pig, such as specific antibodies or DNA or RNA sequences. In order to overcome these limitations, we have set-up a new method of isolated and perfused brain of the rat. We have characterized the pharmacological profile of its vascular bed founding that rat brain vasculature is sensitive to the tromboxane A_2-receptor agonist (U46619), while 5HT is a very weak vasoconstrictor, and NA is fully ineffective. The intact brain vasculature of the rat retains a functional endothelium, and histamine causes full relaxation of U46619precontracted vessels via histamine H_2 receptor activation. Also in rat brain the perfusion with challenged neutrophil resulted in edema formation (brain wet weight increase control: 27.7±4.05% n=5, challenged: 65.04±7.57% n=5, $p<0.01$) associated with increase of cysLT formation. In the meantime, we have studied the cerebrovascular system in streptozotocin-diabetic rats showing that the vascular responsiveness to vasocontracting agonists does not change, while the vasorelaxant effect of histamine decreased (EC_{50} value: non diabetic 0.32 µM, diabetic 1 µM, $p<0.01$). In conclusion these results show that the method of isolated and perfused brain can be used to study the whole cerebrovascular system and the interaction between blood cells and vascular wall, and with rat brain it is possible also to study this in experimental model of cerebral deseases as diabetes and stroke.

STATE-DEPENDENT BINDING OF PILSICAINIDE TO THE LOCAL ANESTHETIC RECEPTOR WITHIN THE PORE OF HUMAN SKELETAL MUSCLE VOLTAGEGATED SODIUM CHANNEL

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Pilsicainide is a class IC antiarrhythmic drug widely used in the treatment of ventricular tachyarrhythmias in Japan. Electrophysiological studies performed on myocardial cells suggested that pilsicainide is a pure sodium channel blocker. In this study we compared the effects of pilsicainide on human wild-type (WT) and mutant (F1586C and Y1593C) skeletal muscle sodium (hNa_v 1.4) channels. The F1586C and Y1593C mutations were engineered into the wild-type $hNa_v1.4$ template. These two aromatic residues are part of the local anesthetic (LA) binding site within the sodium channel pore (1). Transient or permanent transfection of HEK-293 cells with these channels were performed using the calcium-phosphate coprecipitation method (2). In transient transfection the cells were cotransfected with plasmid DNA encoding CD8 receptors, to allow recognition of transfected cells with CD8 antibodies. For permanent transfection, the clone selection was carried out with Geneticin (G418). Sodium currents I_{Na} were recorded in the whole-cell patch clamp configuration. We evaluated the block of sodium channels by pilsicainide by measuring the reduction of I_{Na} elicited from the holding potential (HP) of -120 mV to -30 mV at 0.1 (tonic block) and 10 Hz (use-dependent block) frequency stimulation. The concentration-response curves were fitted with a first-order binding function, given the half-maximum inhibitory concentration (IC_{50}) at 0.1 Hz of 113 ± 4 µM for WT, 835 ± 27 µM for Y1593C mutants, and 4084 ± 1086 µM for F1586C mutants. At 10 Hz, the IC_{50} values were 31 ± 2 µM, 533 ± 9 µM and 3297 ± 666 µM, respectively. Through specific protocols, we measured affinity of pilsicainide for resting channels (K_R) and inactivated channels (K_I) of WT channels at HP = -180 mV and -70 mV, respectively. The K_R value on WT channels was 2,9 mM and K_I value was 300 µM. This study demonstrates that pilsicainide blocks sodium channel by interacting with the LA binding site. This drug has a use-dependent behavior, suggesting a higher binding affinity to open and/or inactivated channels with respect to closed channels. Indeed, we show that the drug is a weak blocker of closed and inactivated sodium channels, thereby needed channel opening and/or interacting with open channels to exert its inhibitory action.

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ROSIGLITAZONE REDUCES INFLAMMATORY RESPONSE AND PLATELET AGGREGATION IN AN EXPERIMENTAL MODEL OF RAT CAROTID INJURY

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Rosiglitazone, agonist of peroxisome proliferator-activated receptor-gamma, appear to regulate cellular proliferation and inflammation, modulating the production of inflammatory mediators and inhibiting hyperplasia and restenosis after balloon-injury in rats. The aim of our study was to investigate the anti-inflammatory activity of rosiglitazone in a rat experimental model of carotid surgical injury (1), specifically analysing the time course of the Nuclear Factor-kB (NF-kB) activation, and the expression of inflammatory factors such as cyclooxygenase 2 (COX-2) and phosphorilated p38 MAP kinase (pp38 MAPK) and the effect of rosiglitazone on platelet aggregation. Rats were divided in two groups: injured treated group (TI) with rosiglitazone (10 mg/kg/die), administered by gavage for 7 days before carotid injury and continued for 21 days after injury, and injured untreated group (CI). Animals were sacrificed at different times after injury (4 h, 48 h, 7 days, 14 days and 21 days) and in both groups the expression of COX-2 and pp38 MAPK was evaluated by western blot, while the time course of activation of NF-kB was evaluated by electrophoretic mobility shift assay. Blood samples from the groups were drawn from the abdominal aorta and platelet aggregation, induced by 10 μM ADP, was measured by aggregometer. After 14 days, rosiglitazone treatment resulted in a significant reduction of p38 MAPK and COX-2 expression in the injured carotid compared to CI group ($p<0.0001$ and $p<0.001$, respectively). Platelet aggregation, induced by ADP 10 μM, was reduced by 30% in TI compared to CI ($p<0.0005$). Moreover, NF-kB activation was significant decreased in TI vs. CI group ($p<0.0005$). Our preliminary data demonstrated that after 14 days rosiglitazone has a protective role in arterial injury by reducing antinflammatory processes and inhibiting platelet aggregation.

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MACROMOLECULAR COMPLEXES IN ALZHEIMER'S DISEASE PATHOGENESIS: PARTNERS OF ACETYLCHOLINESTERASE

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Acetylcholinesterase (AChE) catalyses the rapid hydrolysis of acetylcholine (ACh) to acetate and choline, resulting in termination of neurotrasmission at brain cholinergic synapses as well as at neuromuscular junctions. In addition, AChE is believed to play non-catalytic roles in both physiological processes, as synaptogenesis and neuritogenesis, and pathological conditions, as Alzheimer's disease (AD). The cholinergic hypothesis of AD suggests that the degeneration of cholinergic nerve terminals and the concomitant decrease in ACh levels in the brain regions involved in cognition, together lead to the symptoms of AD. Moreover, high level of AChE have been found inside senile plaques and neurofibrillary tangles. Biochemical analyses show a selective loss of specific AChE molecular variants in AD. Therefore, detailed understanding of AChE's alternative splicing in AD is of great potential interest. The human AChE gene contains 4 alternative first exons, of which only one encodes an in-frame extended N-terminus (hN-AChE). hN-AChE includes a 66 amino acids extension which precedes the signal peptide and prevents its cleavage. As a consequence, the amino terminal region plus signal peptide becomes a transmembrane domain of the hN-AChE protein. To challenge this hypothesis, a biochemical fractionation approach was taken to determine the distribution of AChE among the different subcellular compartments of murine hippocampal neurons. Immunoblot analysis of the biochemical fractions using anti-AChE antibodies revealed the presence of the predictable heavier N-AChE protein in purified synaptosomes as well as in the Triton-insoluble synaptic fraction. Confocal microscopy confirmed the existence of AChE in these fractions by immunofluorescence labeling. In view of this information, and because the extended hN-AChE primary sequence includes an atypical proline-rich domain, we searched for putative interactions between N-AChE and the SH3 domain of candidate proteins. Co-immunoprecipitation assays demonstrated a specific interaction between AChE and Src, which, in addition, shows a similar subcellular distribution. We are now trying to demonstrate whether this interaction is direct or not through a pull down assay, using a fusion protein of GST with the Src SH3 domain.

MUCIN DEPLETED FOCI ARE MODULATED BY DIETARY TREATMENTS AND SHOW DEREGULATION OF PROLIFERATIVE ACTIVITY IN CARCINOGEN TREATED-RATS

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Colon carcinogenesis occurs through consecutive steps, starting from the transformation of normal crypts into preneoplastic lesions and their eventual change into adenomas and carcinomas. Recently, mucin depleted foci (MDF), characterized by absent or scarce mucous production, have been described by Caderni et al. (1), in the colon of rats treated with the colon-specific carcinogen azoxymethane. MDF are histologically dysplastic, increase in rats treated with known promoters of colon carcinogenesis and decrease with chemopreventive agents (1, 2). In addition, they show marked alteration of the Wnt signalling, a pathway involved in colon carcinogenesis (3). On this basis, we hypothesized that MDF represent preneoplastic lesions in the process of colon carcinogenesis. To give more support to these data MDF were determined in carcinogen-treated rats fed with a low or high risk diet for developing colon cancer; moreover, since altered proliferation has been involved in colon carcinogenesis, the mitotic index (MI) and the expression of p27 and p16, two cyclin-dependent kinase inhibitors of the cell cycle progression, were determined in MDF and in the normal mucosa. Materials and Methods: Male F344 rats (n=20) were treated with two subcutaneous injection, one week apart, of the colon carcinogen 1,2-dimethylhydrazine (DMH). After one week from the last injection rats were randomly divided in two groups fed the following two diets: a control low- fat diet based on AIN-76 diet (5% corn oil) or a high fat diet (23% corn oil). Sixteen weeks after the last DMH dose, rats were sacrificed and colon excised and processed for MDF determination (1). p27 and p16 expression were analysed by immunohistochemistry in MDF and in their normal mucosa. Results were expressed as percentage of labelled cells over the total cells scored; MI was determined by morphological observation on haematoxylin and eosin stained slides and results expressed as number of mitoses/number of cells scored x100. Differences between groups were analysed using unpaired *t-test.* Paired t-test was used for p27 and p16 expression data. MDF determination showed that high-fat fed rats had significantly ($p<0.05$) more MDF than controls (5.7 ± 0.5 vs. 4.2 ± 0.3; mean ± S.E). On the contrary, multiplicity, i.e the number of crypts forming each MDF, was not varied (data not shown). Nuclear p27 expression was significantly reduced in MDF compared to normal mucosa (36 ± 3 vs. 11 ± 2, $p<0.01$). The expression of p16 was also reduced in MDF compared to normal mucosa, but to a lesser extent than for p27 (35 ± 3 vs. 24 ± 2; $p<0.05$). Determination of the MI showed that MDF had a higher proliferative activity than normal mucosa (1.71 ± 0.23 vs. 0.85 ± 0.10, $p<0.01$). The data reported show that MDF have a deregulation in the proliferative activity, a phenomenon observed in colon carcinogenesis. Moreover, MDF increase in rats fed a high risk diet (high fat) compared to a low risk diet (low fat). These results suggest that MDF might be a good biomarker for experimental colon carcinogenesis.

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3,5-DIBENZOYL-4-(3-PHENOXYPHENYL)-1,4-DIHYDRO-2,6 DIMETHYLPYRIDINE (DP7) AS A NEW MDR REVERTER DEVOID OF CARDIAC EFFECTS

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Several compounds have been studied as reverters of multidrug resistence (MDR), one of the main reasons of failure in cancer chemotherapy. Potent P-glycoprotein (P-gp) inhibitors have been tested in clinical trials so far, including Ca^{2+} channel blockers such as verapamil and dihydropyridines, first-generation-MDR reverters (1). However, clinical application of these agents has not been extensively pursued to date, mostly owing, to their unwanted cardiovascular side effects. This has promoted the search for congeners of these first-generation-MDR reverters characterized by a reduced cardiovascular toxicity. In a previous study, a novel dihydropyridine, namely 3,5-dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7), at concentrations two orders of magnitude higher than IC_{50} as a P-gp inhibitor, showed neither antispasmodic nor spasmolytic effects and inhibited L-type Ca^{2+} current [$I_{Ca(L)}$) by a mere 20%, thus outlining the clear divergence of its P-gp inhibition and the vascular activity (2). The aim of this study was to investigate the effects of DP7 on rat isolated hearts, perfused according to the method described by Langendorff (constant flow) (3). Left ventricular pressure (LVP), coronary perfusion pressure (CPP) and surface electrocardiogram (ECG) were measured in paced hearts (6 Hz, 0.6 msec, 8 V). The ECG parameters measured were: QRS interval, i.e. the intraventricular conduction time and QT interval, i.e. the duration of ventricular repolarization. Nifedipine, a well known dihydropyridine Ca^{2+} channel blocker, was used as the reference compound. CPP was not affected by nifedipine at any of the concentrations tested in the range 1 nM - 3 μM, whereas LVP was reduced in a concentration-dependent manner (pIC_{50} = 7.07 ± 0.1). In the presence of nifedipine, both QT and QRS intervals did not change appreciably (QT = 70.8 ± 2.0 msec, control n = 6; 65.1 ± 2.1 msec, 3 μM nifedipine n = 4; QRS = 13.5 ± 0.8 msec, control; 14.9 ± 0.1 msec, 3 μM nifedipine, respectively). DP7 did not modify both QT and QRS (QT = 72.5 ± 1.3 msec, control n = 7; 72.1 ± 1.9 msec, 3 μM DP7 n = 3; QRS = 16.5 ± 0.1 control; 16.2 ± 0.2 msec, 3 μM DP7, respectively) as well as LVP. In conclusion, these results suggest that DP7 may represent a novel lead compound for the development of potent dihydropyridine MDR chemosensitizers devoid of cardiovascular effects.

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EP2 PROSTANOID RECEPTOR PROMOTES TUMOR CELL GROWTH AND INVASION BY INOS AND ERK1-2 PATHWAYS

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In several type of tumor the levels of nitric oxide (NO) derived from inducible NO synthase (iNOS), and prostaglandin E2 (PGE2) derived from cyclooxygenase-2 (COX-2) originated from tumor cells or tumor associated inflammatory cells has been reported to correlate with tumor growth, metastasis and angiogenesis. The present study examined the role of iNOS signaling pathway in PGE2-mediated tumor invasiveness and proliferation in A431 cells, a model of squamous carcinoma cell line. Cell invasion and proliferation promoted by PGE2 were blocked by iNOS silencing RNA (si-iNOS) or iNOS/guanylate cyclase (GC) pharmacological inhibition. Consistently, iNOS-GC pathway inhibitors blocked mitogen activated protein kinase-ERK1-2 (MAPK-ERK1-2) phosphorylation, which was required to mediate PGE2 functions. PGE2 effects were confined to the selective stimulation of the EP2 receptor subtype, leading to epidermal growth factor receptor (EGFR) transactivation via protein kinase A (PKA) and c-src activation. EP2-mediated ERK1-2 activation and cell invasion and proliferation, were abolished by inhibition of PKA, c-Src, and EGFR, as well as by silencing iNOS, or by pharmacological inhibiting iNOS-GC pathway. Silencing of iNOS also impaired the EGFR-induced ERK1-2 phosphorylation. Together, these results indicate that in A431 tumor cell line, PGE2 mediates ERK1-2 phosphorylation, cell growth and invasion through EGFR transactivation via PKA activity and activation of iNOS/GC pathway.

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ANTIANGIOGENIC AND ANTITUMOUR EFFECTS OF METRONOMIC IRINOTECAN (CPT-11)

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Low-dose metronomic chemotherapy is the chronic administration of chemotherapy at relatively low, non-toxic doses on a frequent schedule of administration (e.g. daily) continuously or with no prolonged drug-free breaks. Indeed, some results highly suggest the presence of a specific "antiangiogenic window" when low-dose chemoterapy is used on endothelial cells, which becomes apparent after protracted exposure time[(1]. 1) *In vitro* assessment of the anti-endothelial effect of continuous low concentrations of SN-38, the active metabolite of CPT-11, for prolonged periods of time (144 h); 2) investigation of the relationship between expression, synthesis and secretion of pro- and anti-angiogenic factors (e.g. VEGF and TSP-1) in human normal and tumor cell lines after metronomic administration of SN-38 and the anti-endothelial activity; 3) *in vivo* assessment of the antitumour efficacy of metronomic CPT-11 alone and in combination with the antiangiogenic drug SU5416. *In vitro* human endothelial cells (HMVEC-d and HUVEC) and colorectal cancer cell line (SW620) and (HT-29) were treated both at low and high concentrations using a continuous (144 h) drug exposure protocol[(1)] to evaluate the antiproliferative effects (expressed by experimental IC_{50}s), the inhibition of modulation of gene expression of pro- (VEGF) and anti-angiogenic (TSP-1) factors by real time-PCR. Nude mice with s.c. HT-29 xenografts were administered with metronomic CPT-11 alone or in combination with SU5416, or with CPT-11 at the maximum tolerated dose (MTD). Results. *In vitro* SN-38 inhibited selectively the proliferation of HMVEC-d and HUVEC (IC_{50}=0.014±0.0024 and 0.21±0.029 nM, respectively; mean±SD). The IC_{50} of the colorectal cancer cell line SW620 and HT-29 were 0.64±0.014 and 1.5±0.05 nM, respectively. The expression of TSP-1 gene was greatly increased in endothelial cells treated at the IC_{50} (143.4% vs. 100% of control for HUVEC and 249.8% vs. 100% of control for HMVEC-d), whereas the expression of VEGF was different between the two cell lines (62.6% vs. 100% of control for HUVEC and 133.7% vs. 100% of control for HMVEC-d). The *in vivo* therapeutic effect of CPT-11 metronomic regimen (4 mg/kg/day) after 47 days was significant (24.7% vs. 100% of controls, P<0.05), and similar to the metronomic CPT-11 plus SU5416 (10 mg/kg twice weekly) (24.1% vs. 100% of controls, P<0.05). MTD CPT-11 (150 mg/kg once weekly), as expected, inhibited the tumour growth (9.7% vs. 100% of controls, P<0.05); moreover, a single high dose of CPT-11 followed by metronomic CPT-11 plus SU5416 obtained an almost complete regression of tumours (5.54% *vs*. 100% of controls, P<0.05). The toxicity profile was extremely favorable for metronomic/antiangiogenic regimes, whereas the MTD CPT-11 treated animals showed a significant weight loss. *In vitro* results shows the antiangiogenic properties of low doses SN-38, suggesting the possible role of TSP-1 in this effect. *In vivo* the CPT-11 metronomic schedule alone and in combination with antiangiogenic drugs is effective on colorectal cancer without toxic effect on mice.

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bv8, THE AMPHIBIAN ANALOGUE OF PROKINETICIN-1/ EG-VEGF DECREASES th2 CYTOKINES AND INDUCES A th1 PROFILE IN MICE

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The small 77 aminoacid protein Bv8, isolated from amphibian skin, belongs to a novel family of secreted proteins also present in rodents and human (Prokineticin-1 or EG-VEGF). Two Gprotein coupled receptors, PKR-1 and PKR-2, have been identified for this family of proteins. Several biological activities associated with Bv8/Prokineticin protein have been described: we demonstrated a PKR-1 mediated proinflammatory action of Bv8 on mouse macrophage; in particular we showed its capability to increase IL-12, the critical factor that drives the development of T-helper cells towards a Th1 differentiation, and to decrease IL-10 macrophage production. On this basis we could hypothesise an impact of Bv8 on Th1/Th2 cytokine balance; so we checked the effect of the protein *(in vitro/ in vivo)* on mouse splenocyte cytokines both in presence of the policlonal mitogen Concanavalin-A and after mouse immunization with the protein antigen keyhole-limpet hemocyanin (KLH). We also investigated the involvement of PKR-1 receptor with the use of KO mice. BALB/C male mice, PKR-1 KO mice and their wild type controls were used in this study. Spleen cells ($4x10^6$ cells/ml) were incubated for a period of 24 h with or without Con-A (10 µg/ml) for IL-2, IFNγ and for 48 h for IL-10 and IL-4 in presence/absence of different concentrations of Bv8. In *in vivo* experiments Bv8 was administrated s.c. at the dose of 250 pmoles/Kg, spleens were obteined 4h later. In the studies aimed to evaluate an antigen-specific answer, two weeks after mouse immunization with KLH (100 µg) spleens were removed and spleen cells ($7x10^6$ cells/ml) were incubated for a period of 48 h with or without KLH (80 µg/ml) for IL-2 and IFNγ evaluation and for 72 h for IL-10 and IL-4 in presence/absence of Bv8 10^{-11} M. In *in vivo* experiments Bv8 was administrated s.c. at the dose of 250 pmoles/Kg at the moment of the immunization with KLH or 4h before spleens collection. Our results shown that the *in vitro and in vivo* presence of Bv8 significantly affected the production of Th1/Th2 cytokines after stimulation with Con-A; in fact we observed a significant and relevant decreament of the Th2 cytokines IL-4 and IL-10, while the Th1 cytokines IL-2 and IFNγ were not modified. These effects were completely lost in PKR-1 KO mice. Similar data were observed after KLH immunization in fact, also in this case, we observed a significant decrement of the Th2 cytokine IL-10 and no modification in the levels of Th1 cytokines. In conclusion, we demonstrated that Bv8 is a potent modulatory factor of the immune responses driving to a Th1 inflammatory profile.

THE ROLE OF REACTIVE OXYGEN SPECIES ON GLUTAMATE-DEPENDENT NEURONAL APOPTOSIS IN THE NEUROPATHIC PAIN DEVELOPMENT

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We combined behavioural, morphological and molecular approaches to assess the role of mGlu1 and mGlu5 receptors and the involvement of reactive oxygen species (ROS) in neuropathic pain-associated neuron apoptosis induced by sciatic nerve chronic constrictive injury (CCI) in the mouse. It has been shown that glutamate-mediated excitotoxicity is a major contributor to cell death in several nervous system pathological conditions (1). ROS also play a key role in several forms of cell death occurring in ischemia and stroke (2), and in many types of apoptosis (3). We have shown that glutamate-dependent apoptosis increased the pro-apoptotic bax and bcl-xS mRNA in the spinal cord of rats, 1-3 days post-CCI (4, 5). Behaviour analysis, obtained through plantar test apparatus and Basile Analgesymeter , showed the presence of the hyperalgesia and allodynia 2-3 days after surgery in neuropathic mouse. RT-PCR analysis showed increased expression of the bax/bcl-2 ratio (77±28%), apoptotic protease-activating factor-1 (apaf-1) (117±21%) and caspase-9 (48±16%) in the spinal cord by 3 days post-CCI. Consistent with biomolecular data, sciatic nerve CCI induced a marked increase in the incidence of apoptotic-positive cell features by 3 days post-CCI on the side ipsilateral to the nerve injury (percentage mean ± SE of TUNEL-positive cells: 16±2% in mice with CCI of the sciatic nerve, compared to the sham mice: 7±1%; percentage mean ± SE of Hoechst apoptoticpositive cells: 18±1% in mice with CCI of the sciatic nerve, compared to the sham mice: 6±2%). PBN treatment (100 mg/kg i.p. twice, daily) prevented thermal hyperalgesia and mechanical allodynia and normalized the mRNA levels of bax, apaf-1 and caspase-9 genes 3 days post-CCI. PBN also caused a significant decrease in the number of TUNEL- (10±1%) and Hoechst-apoptotic (11±1%) positive profiles in the dorsal horn spinal cord 3 days after CCI. These findings suggest that reactive oxygen species and metabotropic glutamate group I receptors participates in increasing the expression of some apoptotic genes which might play a role in the onset of neuropathic pain development. The administration of molecules acting as ROS scavengers could pave the way to their use as anti-neuropathic agents.

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SELECTIVE TARGETING OF ANTITUMORAL DRUGS MEDIATED BY MACROMOLUCULES SPECIFICALLY DIRECTED TOWARDS MEMBRANE RECEPTORS

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Treatment of malignant neoplasias is based on cytotoxic drugs which are not able to distinguish between the malignant cell and the normal one. In order to allow to treat exclusively the tumoral cells in a selective way, it's been studied hyaluronan acid (HA) as a vehicle: it's a biological compound of the extracellular matrix and it binds to CD44, a receptor overexpressed on tumoral cells. HA binds also to another receptor, RHAMM, which mediates cell motility. Several bioconjugates (BCs), characterized by an antineoplastic agent (FA) linked to HA and by different degrees of substitution, have been tested both *in vitro* an *in vivo*. The research has been based on HA molecules conjugated to an antitumoral drug which we'll call FA, because of a secrecy agreement we have with the pharmaceutical industry we're working with. First we tested CD44 expression and the compounds' cytotoxicity on several human cell lines. Later we checked bionconjugates' tolerability and efficacy on mice bearing TLX5 lymphoma, B16-F10 murine melanoma and on nude mice bearing human breast cancer MDA-MB-231. Finally, it was tested the effect of the treatment with HAs characterized by different molecular weight (HA150kDa and HA16kDa) and doses (from1 to 0,062mg/ml), on RHAMM expression on human multiple myeloma cell line RPMI8226. All the cell lines taken into consideration express CD44. The *in vitro* results show that the BCs don't increase the drug's cytotoxicity while *in vivo* results show that they are more toxic respect to FA alone. The treatment of mice bearing TXL5 lymphoma with HA-FA cause a statistically significant reduction of tumour growth and an increase of life-time expectancy. HA-FA is also effective on B16F10 melanoma where it causes the complete disappearance of liver metastases while on xenotransplant the treatment significantly reduces the primary tumour growth and it increases the life-time expectancy. These datas are statistically significant compared to the effect checked by the only FA. Finally, the treatment on RMPI8226 with HAs doesn't cause any variation on RHAMM expression and it doesn't cause apoptosis. HA-FA BCs present interesting antitumoral characteristics: in fact, even if they have a lower activity *in vitro*, in *in vivo* systems they show a therapeutic efficacy higher if compared to the antitumoral drug alone. On the other hand, the treatment with the only HA doesn't cause any variation on RHAMM expression and function.

ARTERIAL HYPERTENSION INDUCES CIRCULATING BETA AMYLOID DEPOSITION IN BRAIN TISSUES

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Although Alzheimer Disease (AD) has been considered for long to be of non-vascular origin, a growing body of recent studies has indicated the possibility that cardiovascular risk factors could be involved in the pathophysiology of sporadic AD. In particolar, arterial hypertension is frequently associated but its role in the pathogenesis of AD appears difficult to elucidate. Typical features of AD are senile plaques mainly composed of amyloid β peptides, (AβP) generated from the amyloid precursor protein (APP), a ubiquitously expressed transmembrane glycoprotein. Aim of our study was to investigate the role of high blood pressure levels in the deposition of AβP into cerebral tissues. In this study we used two independent mouse models of high blood pressure: one in which we performed trans aortic coarctation (TAC) and another in which we infused angiotensin II subcutaneously. In both models the analysis of the impact of hypertension on the blood brain barrier (BBB) revealed an increased permeability in some cerebral areas such as cortex and hippocampus, which are particularly involved in the control of cognitive functions such as learning and memory. More interestingly, the hypertensive mice showed by immunohystochemistry a marked positivity to anti AβP antibodies as compared to normotensive control mice. Moreover, the positivity to anti AβP antibodies was particularly relevant on vessels wall and in the surrounding tissues suggesting a vascular rather than a neuronal source of such amyloid deposits. To investigate this issue we analyzed TAC-mice after passive immunotherapy with IgG against AβP, which hampers AβP migration from blood to brain. In particular, a group of TAC mice, and the relative sham control mice, received weekly intraperitoneal injection of a dose of 10 mg/kg of anti AβP antibodies for one month. Another group of TAC and sham mice received four injections monthly of only PBS. We observed that hypertensive mice treated with passive immunotherapy showed a markedly reduced AβP immunopositivity in both cortex and hippocampus as compared to hypertensive mice treated with vehicle alone. Thus, our study demonstrates for the first time that chronic hypertension determines in brain an impairment of the blood brarrier permeability with deposition of AβP. Moreover, the analysis of the effect of the administration of anti-AβP IgG revealed that passive immunotherapy is able to rescue hypertensive brains from AβP deposition and suggests a circulating vascular source of this latter. In the light of our results we can infer that arterial hypertension is not only a major risk factor for cerebrovascular accidents, such as stroke and cerebral hemorrhage, but could be an important event that, associated with other risk factors, triggers the pathophysiological cascade that finds its final expression in the AD onset. On this issue, the pharmacological treatment and the prevention of hypertension could be important not only for the protection of blood vessels, heart and brain from serious and immediate damages but also to prevent the later onset of as much as seroius pathologies such as AD.

SEROTONIN TRANSPORTER 5HTTLPR POLYMORPHISM IN PATIENTS WITH IRRITABLE BOWEL SYNDROME: RELATIONSHIPS WITH CLINICAL VARIANTS AND SYMPTOM SEVERITY

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It has been suggested that serotonin transporter (SERT) plays a role in the pathophysiology of irritable bowel syndrome (IBS). The expression of SERT gene is modulated by a promoter polymorphism (5HTTLPR), which gives rise to long (L) and short (S) alleles. The S allele causes a decreased expression of SERT protein, with reduced efficiency of cellular serotonin reuptake, a condition which seems to affect the response of depressive disorders to drugs acting as SERT blockers. Since SERT inhibitors have been recently proposed for treatment of IBS, this study was designed to evaluate possible associations of 5HTTLPR polymorphism with different clinical forms and symptom severity in patients with IBS. IBS patients were selected according to Rome II criteria, and subdivided into diarrhoea predominant (D-IBS), constipation predominant (C-IBS), and alternating bowel (A-IBS) groups. Symptom severity was estimated by means of a standard questionnaire. Healthy volunteers, matched for sex and age, were also enrolled. Genomic DNA was extracted from whole blood. The SERT gene region containing the 5HTTPLR polymorphism was amplified by PCR using primers designed on the basis of GeneBank nucleotide sequence. Expected sizes of PCR products were 484 bp and 528 bp, for S and L alleles, respectively. DNA bands were visualized by electrophoresis with 2% agarose gel in the presence of ethidium bromide.

119 IBS patients (30 M, 89 F; mean age 39.7 years; range 19-71 years) and 95 healthy volunteers (35 M, 60 F, mean age 46.6 years; range 24-84 years) were genotyped. All subjects were Italians of Caucasian origin. Frequencies in IBS patients (L/L 35.3%, L/S 54.6%, S/S 10.1%) differed significantly from healthy volunteers (L/L 25.3%, L/S 53.7%, S/S 21.1%; Pearson Chi-Square: P=0.05), with a lower prevalence of S/S genotype in the former group. When stratifying patients by clinical variants, the genotype distribution was: D-IBS (n=45), L/L 35.6%, L/S 51.1%, S/S 13.3%; C-IBS (n=46), L/L 41.3%, L/S 52.2%, S/S 6.5%; A-IBS (n=28), L/L 25%, L/S 64.3%, S/S 10.7%. Comparison of genotype frequencies in bowel habit subgroups versus healthy volunteers indicated a significant difference for C-IBS (P=0.036), but not for D-IBS or A-IBS patients. Mean symptom severity score values in IBS patients with L/L (253.9±66.4), L/S (276±66.4) and S/S (259.5±60) genotypes did not differ significantly (ANOVA: F=1.513, P=0.224). The present results show a reduced prevalence of S/S genotype in an Italian cohort of IBS patients, and suggest an association between the C-IBS clinical variant and 5HTTPLR polymorphism. No relationship appears to exist between 5HTTLPR genotypes and symptom severity in IBS patients.

USE OF CONTRAST AGENTS IN MRI TO IDENTIFY INFLAMMATORY CELLS IN THE CEREBRAL ISCHEMIC TISSUE

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Recently, contrast agents based on particles of dextran-coated iron oxide have been developed and represent a powerful diagnostic tools. These nanoparticles superparamagnetic iron oxide (SPIO) and ultrasmall superparamagnetic iron oxide (USPIO) create an excellent Magnetic Resonance Imaging (MRI) contrast, due to the large magnetic susceptibility produced by iron. Previous studies, in models of different pathologies, showed that sufficient amounts of USPIO particles can accumulate in cells with phagocytic function and that such labelled cells can be detected by MRI. Aim of this study is to validate the use of USPIO as a non-invasive tool to follow by MRI the inflammatory processes that occur in a model of focal ischemia. Male Sprague-Dawley rats underwent permanent middle cerebral artery occlusion (MCAO) and were allocated to two groups: control animals, that received an i.v. bolus of vehicle and another group, that received an i.v. bolus of USPIO 5 h after the MCAO. MRI was performed 24 h after USPIO/vehicle injection and then every 24 h, until animals were sacrificed, to monitor the distribution of the contrast agent. After 24 h from USPIO administration, MR images showed hypointense signals that appeared in the same regions of brain damage. To verify the correspondence between hypointense regions and presence of iron nanoparticels, a Pearl's Prussian blue staining was applied to the histologic sections. We found a correlation between the iron staining and MRI signal, which confirmed the role of the iron particles in generating hypontense regions in the injured tissue of animal treated with USPIO. Subsequent histologic and immunoistochemical analyses have been performed to detect the different typology of cellular infiltrates involved in the phagocytosis of USPIO particles. Our results show a direct correspondence between staining for USPIO and staining for microglia and monocytes/macrophages cells infiltrated in the tissues. USPIO appears to be a valuable tool to identify cellular infiltration and to monitor inflammatory process *in vivo,* by using a noninvasive imaging methodology. In view of these results we will perform other experiments to further understand which cells are involved in the USPIO uptake.

NIFLUMIC ACID PRODUCES A MITOCHONDRIA-DEPENDENT CALCIUM INCREASE IN NATIVE RAT SKELETAL MUSCLE

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Niflumic Acid (NFA), a non steroidal anti-inflammatory drug (NSAID) belonging to the class of fenamates, is able to induce changes of intracellular calcium levels in smooth muscle (1), neurons and gastric mucosal cells, but very little is known about the effect of these drugs on skeletal muscle. On the other hand, it modulates the activity of several ion channels; in particular it is a well known blocker of chloride channels, and in our laboratory was found that NFA inhibits CLC-1 muscular channel both through a direct block of the protein and indirectly by Ca^{2+}-dependent PKC. So, we considered the hypothesis that NFA could act on calcium homeostasis of skeletal muscle tissue. We used Fura-2 technique to investigate calcium movements in native skeletal muscle fibers (2): small bundles of rat EDL (*Extensor Digitorum Longus*) were mechanically microdissected tendon to tendon and loaded with Fura-2 AM fluorescent probe. Cytosolic calcium concentration, $(Ca^{2+})_i$, was calculated with the equation of Grynkiewicz (3). NFA enhanced *in vitro* the resting $[Ca^{2+}]_i$ of EDL fibers in a dose-dependent and reversible manner. Particularly, NFA 100 µM increased $[Ca^{2+}]_i$ from 23.7 ± 3.9 to 121 ± 14 nM (*n* fibers 21; *N* animals 6). Investigating the origin of this $[Ca^{2+}]_i$ raise, we found it was reproduced in absence of extracellular calcium (*n* fibers 15; *N* animals 3), and when the sarcoplasmic reticulum was depleted by a pretreatment with caffeine and thapsigargin (*n* fibers 21; *N* animals 4) or when RyR channels were blocked by Ruthenium Red (*n* fibers 25; *N* animals 4). Whereas the pre-incubation of cyclosporin A, an inhibitor of PTP, Permeability Transition Pore (*n* fibers 21; *N* animals 4), as well as oligomycin, that inhibits ATP synthase (*n* fibers 18; *N* animals 3), completely blocked calcium transient triggered by 100 µM NFA, suggesting that it was caused by the efflux of the ion from the mitochondrial pool. Furthermore, NFA's action on $[Ca^{2+}]_i$ in skeletal muscle seems to be not related with the cyclooxygenase-prostaglandin patway, because indomethacin, meloxicam, mefenamic and flufenamic acid, other NSAIDs tested, didn't produce the same effect on resting calcium. This study shows for the first time the capacity of NFA of acting on calcium homeostasis in myofibers and it may be a starting point in elucidating molecular mechanisms of NSAID-induced muscle toxicity and other side effects.

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ROLE OF mGlu1 RECEPTORS IN NEURONAL APOPTOSIS AND GLIOSIS IN A RAT MODEL OF NEUROPATHIC PAIN

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In this study, by using a combination of behavioural and biomolecular approaches, we investigated the involvement of mGlu1 receptors for the induction of apoptosis in the spinal cord of neuropathic (CCI) rats.
Behavioural testing was performed to verify thermal hyperalgesia and mechanical allodynia. The mRNA levels of the genes under analysis were measured by RT-PCR amplification. Thermal hyperalgesia and mechanical allodynia became apparent at 3 and 7 days after sciatic nerve injury. RT-PCR analysis showed increased expression of the bcl2 gene (263 ± 72 % vs. 100 ± 21%, in CCI/vehicle-rats and sham/vehicle rats, respectively), bax gene (451 ± 66% vs. 100 ± 8%, in CCI/vehicle-rats and sham/vehicle rats, respectively) and GFAP (glial fibrillary acidic protein) gene (337 ± 53% vs. 100 ± 7%, in CCI/vehicle-rats and sham/vehicle rats, respectively) 3 days post CCI.
Administration of JNJ16259685 (5 mg/kg s.c., daily), a selective mGlu1 receptor antagonist, reverted the thermal hyperalgesia and mechanical allodynia 3 days post-CCI. This treatament only reverted thermal hyperalgesia, at 7 days post-CCI but not mechanical allodynia. JNJ16259685 treatment increased expression of the bcl2 gene (933 ± 95% vs. 263 ± 72%, in CCI/treated-rats and CCI/vehicle rats, respectively), and GFAP gene (595 ± 48% vs. 337 ± 53%, in CCI/treated-rats and CCI/vehicle rats, respectively) 3 days post CCI, while did not affect the levels of mRNA of bax gene. Finally, this treatment did not modify the expression of all genes studied at 7 day CCI.
These results suggest that mGlu1 receptor antagonists may represent a novel therapeutic strategy for the treatment of the early phase for the onset of thermal hyperalgesia in neuropathic pain conditions.

PHARMACOGENETICS AND DRUG COMBINATIONS STUDIES IN PANCREAS AND NON-SMALL CELL LUNG CANCER (NSCLC)

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Pancreas and lung cancer are two of the most lethal tumours and efforts are focusing on both pharmacogenetic analysis, which may allow the identification of responding patients, and the use of drug combinations with new cytotoxic compounds, as pemetrexed, or with targetspecific agents, as the EGFR tyrosine kinase inhibitor erlotinib. The aims of this study were to define markers influencing clinical response and investigate the combinations of gemcitabine, pemetrexed and erlotinib in pancreas and lung cancer. Clinical data were available from 105 and 82 patients affected by pancreas cancer and NSCLC, respectively. Expression profile of microdissected neoplastic cells was studied by quantitative PCR, while genotyping of cytidine deaminase was performed by allelic discrimination. Association between clinical outcome and expression levels or genotype of drug determinants, was estimated using χ^2 test, Kaplan-Meier curves and Cox's regression. *In vitro* studies were performed on pancreas and NSCLC cells characterized by different levels of EGFR, as well as by heterogeneous patterns of activity and expression of gemcitabine and pemetrexed determinants, such as deoxycytidine kinase (dCK) and thymidylate synthase (TS). Cells were also selected for several mutations, i.e. the H1650 cell line contains EGFR deletion (DelE746-A750), while A549, SW1573, H460, MIA PaCa-2, PANC-1 and Capan-1 cells have K-Ras mutations. Pharmacologic interaction was studied using the combination index method, while effects on EGFR and Akt phosphorylation, cell cycle and apoptosis induction were studied with western blot, ELISA, flow cytometry and fluorescence microscopy, respectively. Finally, expression and activity analyses were performed to assess whether pemetrexed modulated the gemcitabine transporter hENT1 as well as the activating enzyme dCK, or whether erlotinib influenced TS levels.

In vitro studies confirmed the key role of dCK, CDA and hENT1 on gemcitabine sensitivity and their expression analysis in patients' samples suggested a stratification to create groups with different likelihood to respond to treatment. Indeed, a striking relationship was found between hENT1 expression and overall survival in 83 gemcitabine-treated pancreas cancer patients (P<0.001), as well as with clinical response in 21 NSCLC patients. In addition, the CDA A79A genotype was predictive for response in 61 NSCLC patients treated with gemcitabine and cisplatin. Synergistic cytotoxicity was showed, mostly with pemetrexed→gemcitabine sequence, and pemetrexed increased hENT1 and dCK expression. Similarly, pemetrexed and erlotinib had a synergistic interaction on NSCLC cells, associated with favourable modulation of cell cycle, induction of apoptosis and reduction of TS gene expression. This study demonstrates the rationale of the choice of molecular determinants of drug activity to be examined in cancer samples to predict clinical outcome and, by characterization of the genetic and molecular context in which synergistic interaction occurs, provides new strategies for combined treatment optimization with conventional anticancer drugs and targeted agents.

PHARMACOLOGICAL REVERSION OF THE PHENOTYPE OF HUMAN TSC2$^{-/-}$ SMOOTH-MUSCLE CELLS

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Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome resulting from the loss of function of the TSC1/2 complex. The TSC1 and TSC2 gene products, hamartin and tuberin, associate as a functional complex regulating the mammalian target of rapamycin (mTOR). The loss of tuberin function leads to constitutive S6K1 activation and phosphorylation of its ribosomal substrate S6 (1). We have recently reported the isolation and characterization of angiomyolipoma smooth muscle (A^+) cells showing loss of heterozigosity (LOH) for the TSC2 gene. EGF (epidermal growth factor) is necessary to promote the proliferation and maintenance of the A^+ cells, and its proliferative action cannot be replaced by IGF-1. Exposure to antibodies against EGF receptor (anti-EGFR) is lethal causing the total loss of A^+ cells in two weeks (2). The EGF-dependent A^+ growth is tuberin-dependent as rapamycin-induced blockade of mTOR causes the normalization of A^+ cells proliferation rate. A^+ cells are strongly positive to HMB-45 antibody, a marker of TSC and lymphangioleiomyomatosis (LAM) cells, that binds gp100 protein. When A^+ cells are exposed at plating time to anti-EGFR (5 μg/ml) or rapamycin (5 ng/ml) the labelling with HMB-45 is progressively down-regulated. The efficacy expressed by anti-EGFR is superior to that of rapamycin in suppressing HMB-45 labelling. When anti-EGFR or rapamycin are added to the culture medium 3 h after A^+ cells plating, the efficacy in reverting HMB-45 labelling is reduced, although anti-EGFR is still more effective than rapamycin. The activation of S6 and Erk was also evaluated as parameter of cell proliferation after exposure to rapamycin or anti-EGFR. Incubation at plating time with rapamycin (1 and 5ng/ml) for 24 and 48 h decreased phosphorylation of S6 in a dose- and time-dependent manner. Anti-EGFR strongly inhibited S6 phosphorylation in 48 h, while PD98059 (PD), an Erk inhibitor, had only a partial effect. Anti-EGFR decreased Erk phosphorylation mainly in 48 h, while PD inhibited Erk activation after 24 and 48 h of incubation in a similar way. Consistently with the previous data, Erk phosphorylation was more effectively inhibited by anti-EGFR than PD. Also rapamycin decreased Erk activation in 48 h and the effect was higher with the concentration of 5ng/ml. Rapamycin blocked the excessive proliferation rate when added at plating time (i.e. the rate of proliferation of A^+ cells was comparable to that of normal human aorta smooth muscle cells). However, the addition of rapamycin 3 h after plating failed to affect A^+ cell proliferation. On the contrary, the addition of anti-EGFR to the growth medium either at plating time or 3 h after plating initially blocked cell proliferation, then after 7 days caused the progressive loss of A^+ cells with a comparable efficacy. In conclusion, EGF pathway is strongly involved in A^+ cell growth and phenotype. Anti-EGFR exposure reverses the constitutive S6 and Erk phosphorylation, while PD (Erk inhibitor) has a partial effect. These data suggest that EGF might activate an alternative signaling cascade besides to mitogen activated protein kinase (MAPK) pathway.

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NF1 GENE ANALYSIS BASED ON DHPLC

Grassia Carolina

Neurofibromatosis type 1 (NF1; MIM# 162200) is a common autosomal dominant disorder, characterised by cafe-au-lait spots, peripheral neurofibromas, Lisch nodules and freckling. Other features found in a minority of patients include scoliosis, macrocephaly, pseudarthrosis, short stature, malignancies and learning disabilities. Spontaneous mutation rate is very high and ~50% of the affected cases represents *de novo* mutations. The NF1 gene spans 350 kb of genomic DNA, encodes a transcript of 12 kb, comprising at least 60 exons. The gene product, neurofibromin, is a 250 kD hydrophilic protein comprising 2,818 aminoacids that exhibits homology to a number of GTPase activating proteins (GAP), and is involved in the negative control of RAS-mediated signal transduction. Mutational analysis of NF1 patients is difficult because of the size of the gene, the presence of several pseudogenes and the wide variety of mutations, mostly unique to one family. Denaturing high-performance liquid chromatography (DHPLC), a fast and highly sensitive technique based on the detection of heteroduplexes in PCR products by ion pair reverse-phase HPLC under partially denaturing conditions, is in many ways ideally suited to mutation detection in these conditions. In the present study we attempted to delineate the NF1 mutational spectrum in the Italian population reporting our experience with the direct analysis of the whole NF1 coding region in related and unrelated subjects affected by NF1 by denaturing high performance liquid chromatography (DHPLC). We investigated 39 NF1 unrelated patients (19 males and 20 females) aged 1-18 years. Informed consent was obtained from all patients. In each case, the NF1 diagnosis was confirmed according to the NIH criteria (National Institute of Health Development Conference, 1988). Considering the large size of NF1 gene the DHPLC analysis was carried out on the cDNA, reverse transcripted from the total RNA extracted from lymphocytes. For each patient, a 2 ml blood sample was directly collected in Paxgene RNA blood tubes (PreAnalytix) and stored at -80°C. The PAXgene tube contains a proprietary reagent that immediately stabilizes intracellular RNA for days at room temperature and for weeks at 4° C. The ability to eliminate freezing, batch samples, and minimize processing urgency greatly enhances lab efficiency. The total RNA was extracted using the Paxgene RNA system kit (Qiagen). The purification process was designed to provide high quality RNA from samples that have been stabilized in Paxgene RNA blood tubes. The 8457 bp long NF1 coding sequence was amplified by RT-PCR in 21 partially overlapping fragments, using specific primers pairs. Oligonucleotide pairs have accurately been designed, paying particular attention to the GC content of the PCR product. The Primer3 software was normally used for oligonucleotide design in our laboratory. We have optimized the PCR conditions using a thermalcycler with gradient block. When possible, we utilized the same annealing temperature for all the exons. This condition allows us to amplify different exons on the same 96/384-well plate. The DHPLC analysis was optimized using the Transgenomic Navigator 1.5.4 software, utilizing at least three different column temperatures: we observed increased sensitivity with this approach, mainly with GC rich sequences. The PCR products were then run on DHPLC using the optimized conditions. Each variation observed in patients was further characterized by direct sequencing on ABI3130xl automated sequencer and its pathogenic role validated by analyzing at least 500 normal alleles. We have identified mutations in 18 patients so far. Six mutations were found to be novel. We observed 3 missense mutations, 5 nonsense mutations, 5 frameshift mutations and 5 exon deletions. As many as 10 mutations caused, directly or indirectly, a premature termination codon. Our study demonstrates that combined approach (RNA extraction and DHPLC analysis) is simple and inexpensive. Due to its simplicity and effectiveness, DHPLC may be considered as a screening step, especially for large genes such as the NF1 gene with its 60 constitutive exons. One limitation of this method is the lack of sensitivity for large deletions.

CHOLINERGIC DYSFUNCTION, NEURONAL DAMAGE AND AXONAL LOSS IN TGCRND8 MICE

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized pathologically by the presence of senile plaques, neurofibrillary tangles and marked neuronal death in the brain of affected patients. Cholinergic neurons originating in basal forebrain nuclei are the most vulnerable targets of degeneration in AD brain. Several lines of transgenic mice have been created to investigate the underlying neurodegenerative mechanisms in AD and to test new therapeutic approaches. Transgenic TgCRND8 mice expressing a double mutant form of amyloid precursor protein (APP) have been produced. In 7-month-old TgCRND8 mice, we studied the Aß deposits-associated neuroinflammation and the number and morphology of the forebrain cholinergic neurons, by means of histochemistry and single and double labelling immunohistochemistry, the extracellular cortical acetylcholine levels *in vivo*, by means of the microdyalisis technique, and the ability to acquire an inhibitory avoidance response in the "step-down" test. Neuronal shrinkage, particularly in correspondence to Aβ(1-42)-immunopositive deposits, and white matter demyelination were widespread in the transgenic mouse brain. In the brain areas where a strong Aβ(1-42) deposition and numerous plaques were detected, reactive microglia and hyperthrophyc astrocytes were found infiltrating and surrounding Aβ(1-42)-immunopositive deposits, respectively. A significant reduction in the number of choline acetyltransferase (ChAT)-positive neurons in the nucleus basalis magnocellularis and a decrease of both basal and K^+-stimulated extracellular acetylcholine (ACh) levels in the frontal cortex of TgCRND8 mice, respect to non transgenic controls, were found. M2 muscarinic receptor-immunoreactivity was significantly reduced in the primary motor cortex of TgCRND8 mice and no increase in the extracellular ACh levels was brought about by the administration of 0.5 mg/kg i.p. of the muscarinic antagonist scopolamine. A significant cognitive impairment was demonstrated in the "step-down" test. These findings demonstrate that neuronal damage and cholinergic dysfunction *in vivo* may underlie the impairment in learning and memory functions in this mouse model of Alzheimer's disease.

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PRESYNAPTIC $P2X_7$ RECEPTORS ON CEREBROCORTICAL GLUTAMATERGIC NERVE TERMINALS: MULTIPLE MODES FOR NEUROTRANSMITTER EXIT

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In the present study we investigated on the way of exit for glutamate from cerebrocortical nerve terminals following activation of presynaptic $P2X_7$ heteroreceptors. To this aim, we used the following experimental models: purified synaptosomes as neuronal models to study $[^3H]$D-aspartate and endogenous glutamate release in superfusion; HEK293 cells transfected with $P2X_7$ receptors to study the possibility that $[^3H]$D-aspartate can exit the cells through the $P2X_7$ channel/pore itself. Adult male Sprague-Dawley rats were sacrificed and cerebral cortex removed. Purified synaptosomes were prepared. The synaptosomal pellet was incubated with $[^3H]$D-aspartate, transferred in superfusion system and exposed to 2',3'-O-(4-benzoylbenzoyl)-adenosine-5'-triphosphate (BzATP). The effect of voltage-operated calcium channel (VOCC) toxins, DLTBOA or niflumic acid was evaluated. Ca^{2+}-free, EGTA (0.5 mM)-containing medium, was added 18 min before agonist. $[^3H]$D-aspartate efflux was determined by liquid scintillation counting and endogenous glutamate by HPLC; endogenous glutamate release was expressed as picomoles per milligram of synaptosomal protein. HEK293 cells stably expressing rat GFPtagged $P2X_7$ and native HEK293 were incubated with $[^3H]$D-aspartate. Specificity of the uptake through glutamate transporters was assessed be carrying out parallel uptake procedure in Na^+-free HEPES and with DL-TBOA. The effects of BzATP, of DL-TBOA, niflumic acid and oxATP on $[^3H]$D-aspartate efflux were evaluated. The BzATP (100 μM)-evoked $[^3H]$D-aspartate and endogenous glutamate efflux from synaptosomes were reduced when extracellular calcium was removed (42 and 55% respectively n=3-7) or when synaptosomes were exposed to BAPTA-AM (50 μM, about 50% inhibition n=3). These effects were not addictive. Blockade of VOOC did not affect the BzATP-evoked $[^3H]$D-aspartate or endogenous glutamate efflux. DL-TBOA (30 μM) or the niflumic acid (300 μM) did not affect the calcium-independent BzATP-evoked endogenous glutamate or $[^3H]$D-aspartate efflux. BzATP increased $[^3H]$D-aspartate efflux in HEK293 cells transfected with the $P2X_7$ receptor (about 800%, n=8), while it was ineffective in control native cells. Preincubation with oxATP (300 μM) prevented the BzATP-evoked $[^3H]$D-aspartate efflux. The BzATP-evoked efflux was unaffected by DL-TBOA (10 μM) or niflumic acid (300 μM). It is concluded that activation of the $P2X_7$ receptor appears likely to excite glutamate efflux from rat cerebral cortex nerve endings by multiple modes. Activation of $P2X_7$ receptor can evoke vesicular exocytosis of glutamate, dependent on extracellular calcium entry. We therefore propose that presynaptic $P2X_7$ receptor can play a role as action potentialindependent Ca^{2+}-channels linked to neurotransmitter exocytosis. Inefficiency of blockade of either neuronal glutamate transporter or anion channels to affect the calcium-independent fraction of the neurotransmitter efflux evoked by the $P2X_7$ receptor activation indicate that these modes of exit are not responsible for the receptor-evoked glutamate efflux. On the other hand, activation and opening of the $P2X_7$ receptor in HEK293 cells allowed $[^3H]$D-aspartate passage. We therefore suggest that glutamate might exit the nerve terminals through the receptor itself, which can therefore represent a new mode of exit for glutamate.

MATERNAL ADAPTATIONS TO PREGNANCY AND LEPTIN AND GHRELIN EVALUATION: EXPERIMENTAL STUDY ON SPONTANEOUSLY HYPERTENSIVE RATS

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Pregnancy is a hypermetabolic state associated with numerous neuroendocrine changes. During pregnancy in the rat, food intake is increased, whereas thermogeneis is inhibited. This produces a positive energy balance required for growing and development of fetal and maternal tissues besides building up a fat store for subsequent lactation. Leptin (Ob) is thought to play a critical role in the regulation of food intake and adipose tissue mass, and may directly or indirectly influences reproductive function. It has been shown that a dysregulation of autocrine and paracrine function of Ob in fetal-maternal interface can be implicated in pregnancy-induced hypertension (1). The aim of the present study was to evaluate the mutual effect between pregnancy and hypertension and its relationship with leptin and leptin receptor (Ob-R) modulation. Normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive female rats (SHR), were divided into four groups: normotensive non pregnant (WKY-NP); WKY pregnant (WKY-P); SHR non pregnant (SHR-NP) and SHR pregnant (SHR-P). Body weight, food intake, frequence and systolic blood pressure were evaluated on days 0 (before breeding), 6, 14 and 20 of pregnancy. On day 20 bioelectrical impedance analysis was applied for body composition assessment, then all rats were sacrificed and blood was collected for metabolic and hormonal parameters determination. Subcutaneous adipose tissue, hypothalamus and placenta were excised for Ob and Ob-R expression by Western blot analysis. Pregnancy in WKY as well as in SHR rats induced a significant increase ($p<0.001$) in body weight gain, food intake and fat mass. No differences in systolic blood pressure and heart rate were observed in WKY-P compared to WKY-NP rats. In SHR-P rats systolic blood pressure and frequence were significantly reduced at 14 days of pregnancy compared to SHR-NP ($p<0.05$) and the fall was more marked at 20 days ($p<0.001$), where blood pressure and heart rate value became similar to that of normotensive pregnant rats. SHR strain animals presented a significant reduction of plasma leptin concentration than that of the respective WKY controls ($p<0.001$). Conversely, a clear increase of plasma ghrelin was observed in SHR animals compared to that of WKY controls ($p<0.001$). In normotensive animals, pregnancy induced a significant decrease of total cholesterol ($p<0.001$), LDL cholesterol ($p<0.001$) and glucose ($p<0.05$), and an increase of plasma tryglicerides ($p<0.05$). In SHR-P all these parameters were similarly modified; in particular a very strong increase of tryglicerides was observed in comparison to WKY-P ($p<0.001$). In subcutaneous adipose tissue pregnancy induced in normotensive as well as in hypertensive rats a significant increase ($p<0.01$ and $p<0.05$, respectively) in Ob expression vs their respective not pregnant controls. A significant increase ($p<0.01$ *vs* WKY-P) of Ob expression in placenta from SHR-P rats was revealed. The expression of Ob-Rb in adipose tissue was reduced in both strain by pregnant status ($p<0.05$). A form of leptin resistance, due to a Ob-Rb down regulation, was found at the hypothalamic level in SHR rat that may explain the hyperphagia observed in this rat strain.These data provide new insights into the adaptive mechanisms that take place during pregnancy in SHR rats.

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ANALYSIS OF THE MECHANISMS UNDERLYING THE VASORELAXANT ACTION OF TWO NATURAL RELATED FLAVONOIDS: QUERCETIN AND (+/-)NARINGENIN

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Epidemiological studies, indicating that high dietary intake of flavonoids-plant-derived polyphenolic compounds -reduces the risk of mortality due to cardiovascular diseases (1), have promoted the interest in the mechanism(s) of their possible cardioprotective effect. Although the *in vivo* as well as the *in vitro* vasodilatatory activity of flavonoids has been well documented (2), the mechanisms responsible for this effect are not yet elucidated. Therefore, the aim of this study was to investigate the mechanical and/or electrophysiological effects of two structurally related flavonoids, quercetin and (+/-)- naringenin, in vascular smooth muscle preparations. Rat tail artery rings and single myocytes were employed for functional and patchclamp experiments, respectively. In rat tail artery myocytes, both (+/-)-naringenin and quercetin increased BK_{Ca} currents in a concentration-dependent manner; these stimulatory effects were fully reversible and iberiotoxin-sensitive. (+/-)-Naringenin increased the activation kinetics of BK_{Ca} current and shifted in the midpotential of the curve the voltage dependence of the activation to more negative potentials by 22.1 mV, as well as decreased the slope of activation. Preliminary results show that 30 µM quercetin markedly suppressed both frequency and amplitude of spontaneous transient outward currents (STOCs). In endothelium-denuded rat tail artery rings pre-contracted with serotonin, quercetin caused a concentration-dependent vasorelaxation (pIC_{50}= 5.09), which was antagonised by iberiotoxin or KCl 60 mM. Quercetin-induced vasorelaxation was accompanied by a decrease of both cytosolic and stored Ca^{2+} which was not EGTA-like. Application of 30 µM 2-APB, a blocker of store-operated Ca^{2+} influx, did not reproduce this effect of quercetin at all, whereas 30 µM CPA, a blocker of the sarcoplasmic reticulum Ca^{2+}-ATPase, mimicked those on stored Ca^{2+}. Furthermore, quercetin appeared to sensitize the contractile apparatus to Ca^{2+}. Quercetininduced relaxation was reduced significantly in the presence of 10 µM Rp-8-Br-PET-cGMPS, a specific PKG-inhibitor (pIC_{50}= 4.75); pre-treatment of vessels either with 1 µM ODQ, a specific inhibitor of guanyl cyclase, or with 300 µM Rp-8-CPT-cAMPS, a specific PKAinhibitor, however, did not alter the mechanical effect of quercetin. In conclusion, these results demonstrate that the two flavonoids hit various targets involved in Ca^{2+} homeostasis of vascular smooth muscle cells. Furthermore, findings indicate the role of BK_{Ca} channel in the vasorelaxation sustained by quercetin needs to be clarified.

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GEFFECTS OF 3,5-DI-*T*-BUTYLCATECHOL (DTCAT) AND 3,5-IISOPROPYLCATHECOL (DIICAT) ON CONTRACTION–RELAXATION CYCLE OF RAT PHRENIC NERVE-DIAPHRAGM PREPARATION

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A recent study has demonstrated that 3,5-di-*t*-butylcatechol (DTCAT) and 3,5-diisopropylcatechol (DIICAT) are capable to trigger Ca^{2+} release from rat skeletal sarcoplasmic reticulum (SR) vesicles (1); this effect is expected to modify the skeletal muscle contractionrelaxation cycle. The aim of the present study was to investigate the effects of DTCAT and DIICAT on the contractile response to electrical stimulation of rat nerve phrenicdiaphragm preparation *in vitro.* Male Sprague-Dawley rats (250-350 g) were anaesthetized and killed by decapitations. Phrenic nerve and diaphragm were immediately removed and suspended in a Krebs-bicarbonate solution. Diaphragm, with its intact phrenic nerve, was dissected out of the right hemidiaphragm in a Petri dish containing Krebs-bicarbonate solution. A triangular muscle piece was prepared, transferred into an organ bath and attached by a thread to a tension transducer, while the phrenic nerve was pulled through a stimulating electrode. The muscle was electrically stimulated either in the presence or absence of 10 μM d-tubocurarine, added to block nerve response. The baseline tension, muscle tension (RelAfit), rise time from 10% to 90% of the leading edge of the peak [$T_{rise(10-90)}$] and fall time from 10% to 90% of the trailing edge of the peak [$T_{fall\ (10-90)}$], were measured with MacLab/Chart Vs 4.2. In the presence of d-tubocurarine, both DTCAT and DIICAT increased, in a concentrationdependent manner, baseline tension but not RelAfit. 2,6-Di-*t*-butyl-4-methoxyphenol (DTBHA), a stimulator of the SR Ca^{2+}-ATPase (2), was able to antagonise the effects of DTCAT but not those of DIICAT. Ruthenium red, a blocker of the ryanodine receptor Ca^{2+} channel, was ineffective on both DTCAT and DIICAT. DIICAT increased in a concentration-dependent manner $T_{fall\ (10-90)}$; ruthenium red, but not DTBHA was able to antagonise this effect. Preliminary experiments indicate that in the absence of d-tubocurarine, DTCAT decreased, in a concentration-dependent manner, RelAfit of the nervous component when the muscle was stimulated indirectly. Similar effect on RelAfit were obtained when the muscle was stimulated directly under the same experimental conditions. In conclusion, these findings indicate that neither DTCAT nor DIICAT behave as skeletal muscle positive inotropic agents, but rather they stimulate the release of stored Ca^{2+} from SR under control conditions. Furthemore, DTCAT seems to impair the nervous transmission of the impulse to the muscle.

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POLYMORPHISMS OF THE ABCB1 GENE PREDICT DRUG RESISTANCE IN EPILEPTIC PATIENTS

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Mdr1 gene is a member of the ABC (ATP-binding cassette) transporter superfamily. It reduces cytoplasmic drug accumulation as a result of transmembrane drug transport, and contributes to protect human brain from drug exposure through the blood-brain barrier (1). Genetic polymorphisms were first identified from *in vitro* studies with cancer cells (2), and subsequently, the entire Mdr1 coding region has been extensively investigated (3). Preliminary reports documented that high or low expression of Mdr1 gene is associated to the C or T allele at position 3435 of exon 26, respectively (4). Two additional SNPs (G2677 T/A in exon 21 and C1236T in exon 12) which are in linkage disequilibrium with Mdr1 C3435T also seem to influence Pgp function (5). To evaluate the correlation among C3435T, G2677T and C1236T polymorphisms and the efficacy of antiepileptic treatment in patients affected by partial epilepsy. Genomic DNA was extracted from blood samples of 70 patients, C3435T and G2677T polymorphisms were analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Incubation of PCR products with Sau3AI and Ban I enzyme yielded to the complete digestion of the C allele (39-bp and 158-bp fragments) and G allele (198 and 26) but not the T allele. C1236T polymorphism was detected by the TaqMan probe-based 5 min nuclease assay. By using two probes, one specific to each allele of the SNP and labeled with distinct fluorogenic tags, both alleles are specifically detected in a single tube. Frequencies of CC, CT and TT genotype for the SNPs C3435T in exon 26 and C1236T in exon 12 were 25.5%, 62.7% and 11.8%, and 37.2%, 51% and 11.8%, respectively. GG, GT and TT genotypes of G2677T SNP displayed a frequency of 43.1%, 41.2%, and 15.7%, respectively. The analysis of ABCB1 haplotypes based on exons 12 and 26 revealed that the AED resistant phenotype was related to the CC3435 and to the CC or CT1236 genotypes, whereas other haplotypes were associated to drug responsiveness. The pharmacogenetic analysis of DNA from epileptic patients showed the existence of a correlation between the Mdr1 genotype and clinical response to AEDs. Since the Mdr1 gene may influence the bioavailability and distribution to the central nervous system of therapeutic agents, genotyping for Mdr1 polymorphisms may support the identification of individuals at risk for drug resistance and it should become a reliable assay in clinical settings.

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THE ISOTHIOCYANATE SULFORAPHANE INDUCES CYCLIN D3- AND P53- MEDIATED CELL-CYCLE ARREST AND APOPTOSIS OF NON-TRANSFORMED HUMAN T LYMPHOCYTES

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The isothiocyanate sulforaphane has been shown to function as cancer chemopreventive agent (1). It is a strong inducer of phase 2 enzymes and exerts cytostatic and cytotoxic effects on different tumor cell lines (2) and particularly on Jurkat T- leukaemia cells (3). Whether these effects are specific for neoplastic cells has yet to be established. Since selective targeting and low toxicity for normal host tissues are fundamental requisites for a good chemopreventive agent, we tested sulforaphane on non-transformed human cells. We investigated proliferation and apoptosis induction by sulforaphane in freshly isolated non-transformed phytohemagglutinin-stimulated human T lymphocytes. Moreover, we analyzed some of the molecular pathways that are triggered by sulforaphane. Human peripheral blood was obtained from normal healthy volunteers of AVIS (Italian Association of Voluntary Blood Donors). Human mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque. After depletion of adherent cells on plastic dishes, T lymphocytes were isolated by erythrocyte rosetting. T cells were cultured in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5 µg/ml phytohemagglutinin and 30 µM bromodeoxiuridine. Cells were treated with different concentrations of sulforaphane (3-10-30 µM) and the effects on cell-cycle progression, apoptosis induction and expression of p53, bax, bcl-2 proteins and cyclins were analyzed by flow cytometry. We demonstrated that sulforaphane arrested cell-cycle progression in G1-phase, particularly after treatment with concentration 30 µM for 72 h. The analysis of cell-cycle-regulatory molecules operative in G1-phase indicated that treatment with sulforaphane resulted in a significant down-modulation of cyclin D3, whereas the expression of cyclin D2, CDK4 and CDK6 was more mildly attenuated. In particular, treatment with sulforaphane greatly decreased the expression of cyclin D3 at 24, 48 and 72 h. The maximum effect was registered at 24 h, where the expression of cyclin D3 was decreased by 60%. Moreover, a progressive time- and concentration - related increase in the fraction of apoptotic cells (i.e., annexin V^{pos}-propidium iodide^{neg} cells) was observed after treatment with sulforaphane. In fact, after 72 h of treatment at 30 µM the number of apoptotic cells increased to 50% with respect to the control. It is important to remark that the highest concentration of sulforaphane also induced necrosis (i.e., annexin V^{pos}- propidium iodide^{pos} cells) in a time-dependent manner. Finally, to examine the effect of sulforaphane on levels of p53, bcl2 and bax, cells were treated with sulforaphane 30 µM for 72 h. p53 expression was found to be increased in treated cells relative to untreated cells. Taken together, these findings suggest that sulforaphane is active not only in transformed lymphocytes, but also in their normal counterpart. Although *in vitro* studies do not necessarily predict *in vivo* outcomes, this study raises important questions regarding the suitability of sulforaphane for cancer chemoprevention.

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LIPID MICRODOMAINS IN NEUROSECRETION

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A number of proteins and signaling molecules are known to modulate $Ca_v2.1$ (P/Q type) calcium channel activity and neurosecretion. Because recent findings have indicated the presence of $Ca_v2.1$ and SNAREs in cholesterol-enriched microdomains of neuroendocrine and neuronal cells, we have investigated whether key molecules involved in calcium channel gating, such as heterotrimeric G proteins and Neuronal Calcium Sensor 1 (NCS-1), were also recruited in these microdomains. Analysis of flotation gradients from Triton X-100 treated synaptosomal membranes, revealed the presence of two populations of detergent resistant membrane (DRMs): one containing proteins of the exocytic machinery ($Ca_v2.1$ channels and SNAREs) and the second containing NCS-1. Interestingly, heterotrimeric G ptotein subunits were localized in both DRM subtypes. Direct immunoisolation confirmed the colocalization of NCS-1 with heterotrimeric G proteins in DRMs but these microdomains are different from those containing the exocytic machinery and G-protein subunit as well. Furthermore, PIP2, a molecule known to regulate different cellular processes including neurosecretion and calcium channel gating, is present in both population of lipid microdomains. In our recent studies we have also used hippocampal neurons in culture as experimental model in order to analyze the role of cholesterol enriched domains in the transport, recruitment and stabilization of exocytic protein complexes and $Ca_v2.1$ channel in intact cells. We have used drugs (such as mevastatin and/or fumonisin) to alter the cellular level of cholesterol and sphingolipids. In this contest we have also developed a capillary gradient system which enables us to separate the DRMs using the little amount of proteins derived from cultured neurons. Our results demonstate, biochemically and immunocytochemically, that SNAREs and $Ca_v2.1$ localize in lipid microdomains isolated from cultured neurons. Preliminary data have also shown that cholesterol/sphingolipids depleting drugs impaired the distribution of lipid microdomains markers, GM1 and flotilin. On the other hand the distribution of protein of exocytic machinery is partially altered. In the next future, I will investigate the effects of cholesterol/sphingolipids depletion on: the exo-endocytosis of synaptic vescicles; transport of $Ca_v2.1$ at the level of presynaptic membrane; the stabilization and gating properties of $Ca_v2.1$

MORPHINE WITHDRAWAL-INDUCED INCREASE IN EXCITABILITY OF MEDIUM SPINY NEURONS IN THE NUCLEUS ACCUMBENS

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Among the various neurotransmitter systems influenced by morphine administration the dopaminergic (DAergic) system is well established to play a pivotal role. Indeed, it is well documented how this system, especially its mesolimbic subdivision, is involved in druginduced reward (1), and in opiates addiction (2). Morphine withdrawal produces a hypofunction of mesencephalic dopamine neurons that impinge upon medium spiny neurons (MSN) of the forebrain. These neurons are the principal post-synaptic target of DAergic afferents from the VTA (3). The present work will permit to study in detail the physiology of MSNs in the various phases of morphine dependence to fill an extremely important gap in the understanding the functionality of DAergic transmission in its entirety (pre and post-synaptic elements). The present study was designed to investigate the modification of MSNs at various times during withdrawal after chronic administration. Male albino rats were used and subdivided in two groups: chronically treated (n=26)(see 4) with morphine and relative controls (chronic vehicle) (n=25). In addition chronically treated rats were withdrawn from chronic treatment and furter subdivided in spontaneously and pharmacologically whithdrawal (naltrexone 5 mg/kg) and studied at various times (1, 3, 7, 14 days). Further the effect of an acute morphine challenge was evaluated through the use of the cells/track method, to conduct "population studies" (5). Briefly, by passing the recording electrode different times through the Nacc (tracks), identified cells can be counted to obtain the cells/track index. All data were analyzed utilizing one-way ANOVA and sucessively F-test for repeated measures when appropriate. Further statistical analysis utilized two-way analysis of variance (ANOVA; treatment × naltrexone and treatment x time x morphine). Results show that cells/track was increased (100%) in the spontaneously withdrawn group as compared with chronic saline controls; this effect persist beyond 14 days. A one-way ANOVA revealed a significant morphine/controls treatment effect [F(1.51)=3.34; P=0.07]. The same analysis show a significant interaction time x treatment effect [F(4.51)=2.59; P=0.04]. A qualitativly similar but larger increment (542%) was observed after naltrexone administration in the chronic morphine (1 h) group. A two-way ANOVA showed a significant interaction treatment x naltrexone [F(1.8)=6.81; P=0.031]. In addition, a decrease in these cells/track was detected (274%) after acute morphine (i.v.). A two-way ANOVA revealed a significant main effect of acute morphine [F(1.23)=52.55; $P<10^{-6}$].results show that excitability of MSN is increased during morphine withdrawal as revealed by the cells/track index. In addition, the increased index observed upon withdrawal persits over time. Coherently acute treatment with morphine (4mg/kg) decreased excitability by they same means. The results suggest that MSN of Nacc show an increased excitability during morphine withdrawal which persist after somatic signs of withdrawal vanished, thereby supporting a major role played by these neurons in opiate addiction mechanisms.

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ACUTE BUT NOT CHRONIC TREATMENT WITH OLANZAPINE REDUCES EXPERIMENTAL ANXIETY IN RATS

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Olanzapine is an atypical antipsychotic whose particular action is due to the blockade of both dopaminergic (namely D_2/D_4 type) and serotonergic receptors (namely $5HT_2$ type). Furthermore, previous studies from our laboratory demonstrated that the pharmacological effects of olanzapine could also involve the glutamatergic system (1), whose neurotransmitter glutamate, besides in neurological and psychiatric diseases (Parkinson's disease and schizophrenia), seems to be implicated in the genesis of anxiety (2). Several clinical studies have recently reported that olanzapine is effective in treatment – resistent depression (TRD) and in panic disorder (PD), showing antidepressant and anxiolytic properties (3). The aim of this study is to confirm the anxiolytic and antidepressant effects of olanzapine in animals and to clarify the underlying mechanisms. To examine the hypothesis, we used the elevated plus-maze test (EPM) either in basal condition or after restrain stress and the forced swim test (FST). These methods are considered valid animal models of anxiety and depression. Rats were acutely or chronically (21 days) treated with a dose of olanzapine (0.5 mg/Kg i.p.) which didn't affect their spontaneous motility and examined in EPM and FST. The results showed an anxiolytic effect of the acute ($n=30$, 1-way ANOVA, main effect $p<0.0036$) but not of the chronic treatment ($n=40$, 1-way ANOVA $p>0.05$) with olanzapine since Tukey multiple comparison post-hoc test revealed that 45 min restrain stress induced a significant reduction in time spent in open arm ($p<0.01$) and this effect was counteracted by an acute treatment with olanzapine. Furthermore, no antidepressant effect could be observed after either an acute or chronic treatment with olanzapine ($n=30$, 1-way ANOVA $p>0.05$). These preliminary data suggest that a chronic treatment with the antipsychotic olanzapine could induce drug tolerance. Since previous results have shown that olanzapine acts on the serotonergic system (4), radioreceptorial binding and second messanger transduction studies on the $5HT_1$ and $5HT_{2A}$ receptors will be performed to better elucidate the biochemical mechanisms involved.

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DETECTION AND QUANTIFICATION OF ENDOGENOUS N/OFQ IN THE CEREBROSPINAL FLUID BY SPINAL PERFUSION IN THE RAT

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The neuropeptide nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the opioidlike receptor NOP. This peptidergic system presents marked structural analogies with the three classic opioid receptors end their related peptides, nevertheless there is no cross-interaction between the two systems. N/OFQ has been shown to be involved in a variety of functions including the modulation of nociceptive transmission, with different actions on the nociceptive threshold at the spinal and the supraspinal level respectively. The intracerebral administration of exogenous N/OFQ produces an apparent hyperalgesic effect, whereas the spinal administration seems to induce an antinociceptive effect. To better understand the role of this peptidergic system, in the nociceptive transmission at the spinal level, we investigated the presence of endogenous N/OFQ in the cerebrospinal fluid and the possible modifications of its levels by pharmacological and non-pharmacological manipulations, by means of a spinal perfusion method in anesthetized rats coupled with a sensitive radioimmunoassay. Sprague-Dawley rats were anesthetized with sodium pentobarbital/chloral hydrate (30/130 mg/kg i.p.) and a tracheotomy was performed. Rats were then fixed in a stereotaxic apparatus and a first intrathecal (IT) catheter was inserted into the subarachnoid space through the atlanto-occipital membrane, carefully lowered down 8 cm toward the lumbar enlargement and connected with the inlet of the perfusion pump. A second catheter was lowered down 2 mm under the atlanto-occipital membrane and connected to the outlet of the perfusion pump. Perfusion flow-rate was set at 100 µl/min and fractions of 15 min were collected, purified by sep-pak C18 columns, liophilized and stored at –80 °C until the radioimmuno assay. The endogenous N/OFQ resulted detectable in the perfusion samples, and it was also possible to progressively increase its recovery by using precautions to avoid proteolysis such as the addition of specific proteases inhibitors to the artificial cerebrospinal fluid (CSF), the use of an high albumin concentration in the CSF and the collection the perfusion fluid in an acid ambient. By means of these measures, a sixfold higher concentration of N/OFQ (3.05 ± 2.07 fmol/tube vs. 18.60 ± 2.01 fmol/tube, $P < 0.001$) was achieved. The perfusion with K^+-enriched CSF (60 mM) induced an about twofold increase of N/OFQ levels (+ 187.77 % ± 15.05 %, $P< 0.001$). The application of two different noxious stimuli to rats, represented by the subplantar administration of carrageenan (3%, 100 µl) or formalin (5%, 50 µl) caused different effects. Carrageenan did not induce any significant change in N/OFQ levels within 4 h from the administration, while formalin produced a 42% increase in release, 30 min after administration. In conclusion, the spinal perfusion procedure allowed us to detect and quantify the endogenous N/OFQ levels in basal conditions and also its alterations caused by the perfusion with K^+-enriched CSF, thus suggesting a neuronal origin of the peptide. The different results obtained after carrageenan or formalin administration suggest the possibility that the delayed increase of N/OFQ observed after formalin could be more related to central sensitisation phenomena rather than to nociceptive stimuli themselves.

CO-EXISTENCE OF GLUTAMATERGIC NMDA, AMPA AND METABOTROPIC PRESYNAPTIC RECEPTORS ON HUMAN AND RAT NORADRENERGIC NERVE TERMINALS

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Interactions between postsynaptic group I metabotropic glutamate receptors (mGluRs) and ionotropic NMDA receptors (NMDARs) have been described (1). We here report functional interactions between presynaptic mGluRs and NMDAs mediating noradrenaline (NA) release in hippocampus and human neocortex. The experiments of release were carried out with superfused synaptosomes, a technique best suited to study receptor-receptor interactions occurring presynaptically on a particular family of nerve endings (2). Isolated hippocampal and neocortical nerve endings (synaptosomes) were labelled with $[^3H]$NA (final concentration 50 nM) in the presence of the transporter blockers 6nitroquipazine (0.1 µM) and GBR12909(0.1 µM), to avoid false labelling with $[^3H]$NA of serotoninergic and dopaminergic nerve terminals, respectively. In rat hippocampus, the mGluR1/5 agonist 3,5-DHPG, inactive on its own (+ 2,23 ± 3.21 % increase over basal $[^3H]$NA release, n= 5, but see also 3), gained activity when releasemediating NMDARs were activated (100 µM NMDA = + 36.82 ± 3,45 %; + 100 µM 3,5-DHPG = + 62.13 ± 5,45 %, P<0.01, n=7). When NMDARs were made functional through activation of co-localized AMPARs in presence of Mg^{2+} ions (100 µM AMPA = +40.50 ± 3.4%; +100 µM NMDA = 58.72 ± 6.34%, P<0.001, n= 5, but see also 2) the mGluR-mediated component of release (100 µM AMPA + 100 µM NMDA + 100 µM 3,5 DHPG = 98.86 ± 4.6%, P<0.001, n=6)was insensitive to the mGluR1 antagonists CPCCOEt (+ 5 µM CPCCOEt = 89.5 ± 2.64%; P<0.001, n=6), but it was blocked by the mGluR5 antagonist MPEP (+ 1µM MPEP= 58.72 ± 2.35%, P<0.001, n=5). When NMDARs were activated by Mg^{2+} removal (100 µM NMDA + 100µM 3,5-DHPG = 60.15 ± 3.89%, P<0.05, n=7), contemporary activation of mGluR5 and mGluR1 contributed to the evoked release, the mGluR-mediated component of release being blocked by 4-CGP, a mixed mGluR1/5 antagonist or by a mixture of CPCCOEt and MPEP (+ 100µM 4-CGP = 35.26 ± 2.9%, P<0.01, n=6), but apparently being insensitive to the presence of the respective mGluR1 or 5 antagonists alone (+ 5µM CPCCOEt = 51.25 ± 7.12%; + 1µM LY367385 = 59.91 ± 3.1%; 1µM MPEP = 49.95 ± 3.4%, n=6) . Interestingly, the HIV-1 protein Tat, recently proposed to selectively activate mGluR1, mimicked 3,5-DHPG in Mg^{2+}-free conditions (100µM NMDA + 1µM glycine = 72.76 ± 3.44%; 100µM NMDA + 1µM glycine + 1nM Tat = 124.88 ± 5.7%, P<0.001, n=6), but not in presence of Mg^{2+}(100µM NMDA + 100µM AMPA + 1µM glycine = 112.67 ± 4,89; 100µM NMDA + 100µM AMPA + 1µM glycine +1nM Tat = 89.75 ± 8.7%, n=5) Experiments with human neocortex synaptosomes show mGluR-NMDAR interactions similar to those observed in rodents. mGluR1 and mGluR5 co-localize with NMDARs on noradrenergic terminals and can be differentially recruited to contribute to transmitter release depending on the mode of NMDAR activation.

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INFLAMMATION AND NEURODEGENERATION IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS, EFFECT OF MINOCYCLINE AND ATORVASTATINE IN COMBINED TREATMENT

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The hallmarks of Multiple Sclerosis include multifocal perivascular mononuclear cell infiltrates in the CNS, oligodendrocytes loss, demyelination and axonal damage. Experimental Autoimmune Encephalomyelitis (EAE), the available animal model for Multiple Sclerosis, is a T cell-mediated autoimmune disease. Immunomodulators combined with neuroprotective drugs may represent a useful treatment in Multiple Sclerosis. We tested the effect of combined treatment of Minocycline, that exhibits multiple anti-inflammatory effects with neuroprotective properties, and Atorvastatine, an immunomodulator. C57Bl6/j female mice were immunized with myelin oligodendrocyte protein (MOG) 35-55 peptide emulsified in complete Freund's adjuvant. Pertussis toxin was administered i.p. Controls were injected with saline. Immunized mice were divided into four groups treated with: Minocycline (50 mg/kg, i.p.), Atorvastatine (1 mg/kg, os), Minoccycline (50 mg/kg, i.p.) plus Atorvastatine (1 mg/kg, os), and saline. The mice were sacrificed 16, 26 and 60 days after immunization.. Weight loss and clinical signs were examined daily. Anti-MOG antibody response was assessed by solid-phase ELISA (sera obtained from three animals in each group at 26th day p.i.). Histo- and immunohistochemical studies were carried out on paraffin-embedded coronal brain and spinal cord sections. Inflammation was assessed by the presence of inflammatory infiltrates stained with hematoxylin and eosin an by the glial fibrillary acidic protein (GFAP) immunoreactivity. Axonal and neuronal pathology were assessed by Bielshowsky staining and by the antibody anti NeuN, respectively. At 16th and 26th day post-immunization (p.i.) infiltrates were 3.62±1,4 ($P<0.001$) and 3,77±1,2 ($P<0.001$), respectively. Few or no infiltrates were observed at 60th day p.i.. At 16th day p.i. an intense GFAP immunoreactivity (+170%, $P<0.05$) which became even more intense at 26th day (+193%, $P<0.001$) was observed. Neuronal and axonal damage was found in spinal cord 16th (-59%, $P<0.05$) and 26th (-61%, $P<0.001$) day p.i. The loss in the number and staining of NeuN-positive neurons (-50%, $P<0.05$) and Bielshowsky staining lasted up to 60 day p.i.. Luxol Fast Blue staining revealed demyelination at all three times of sacrifice but with some differences in the degree of myelin degradation. Demyelinated plaques were associated with inflammatory infiltrates at 16th and 26th day post-immunization. All treated groups, sacrificed at 26th and 50th day p.i., resulted in a better clinical score as compared to saline group. Moreover combined treatment resulted in a significative reduction of MOGantibody response (one way Anova and Tukey post comparison test, $P<0.001$). These findings demonstrate a fundamental role of inflammation in the development and progression of the disease. The axonal damage observed during the chronic phase, which may depend on a lack of trophic support, may significantly contributes to the clinical progression of the disease. In conclusion these data demonstrate the usefulness of the combined treatment in the therapy of Multiple Sclerosis.

PRECOCIOUS CARDIOVASCULAR ABNORMALITIES IN CHILDREN WITH MILD-MODERATE CHRONIC RENAL FAILURE (CRF)

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Survival in children with CRF has increased over the last 20 years, but gross mortality rate remains high. Data from US Renal Data System and from the Dutch registry for renal transplantation showed a cardiac death rate significantly higher than of the general pediatric population. In adults recent studies demonstrated an increased cardiovascular risk even in mild CRF independently from other classic risk factors. To recognize early signs of cardiovascular involvement, new echocardiographic parameters were obtained from 13 pediatric patients (pts) with CRF. Myocardial function could be characterized by 2 regional deformation parameters: the regional systolic Strain (S) and the peak systolic Strain Rate (SR). S represents the magnitude of myocardial deformation from end diastole to end systole; SR the maximal velocity of deformation during mechanical systole. The cohort comprised 5 M and 8 F, mean age 13.3±7.8 years, affected by mild-moderate CRF without clinical heart disease and echocardiographic signs of left ventricular hypertrophy. Pts were selected to have blood pressure values under the 95th centile for sex and age. Radial and longitudinal systolic function showed significant reduction when compared with healty pediatric subjects. Body Mass Index, levels of creatinine, haemoglobin, cholesterol, calcium, and phosphorus did not correlate with cardiac abnormalities. A correlation was evident between parathyroid hormone (iPTH) and SR ($p<0.01$). We could not make any hypotesis about the genesis of these findings, but earlier detection of cardiovascular abnormalities in these patients might allow earlier interventions to reduce morbidity and mortality.

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TRANSPORT OF FLAVONOIDS IN ENDOTHELIAL CELLS: COMPARISON BETWEEN EA.HY 926 CELL LINE AND PRIMARY CELLS FROM RAT AORTA

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Endothelial cells play a key role in the maintenance of the cardiovascular homeostasis. They have multiple functions, such as the preservation of integrity of the vascular wall; moreover, they represent a functional barrier between the circulating blood and the surrounding tissues, through which the transport of several substances is regulated. A great deal of epidemiological studies investigated the correlation between a flavonoid-enriched dietary intake and a reduced risk factor in the development of cardiovascular diseases, such as atherosclerosis (1). However, the actual mechanism of transport of flavonoids through the endothelium remains unclear. It was demonstrated that flavonoids can interact with a number of membrane transport proteins. One of them is bilitranslocase (BTL), a membrane carrier of diverse organic anions such as bilirubin and anthocyanins, which was recently shown to mediate the uptake of some dietary flavonoids in liver cells (2). In addition, some ABC transporters acting as multispecific efflux pumps by an ATP-mediated mechanism may cause a decrease in the cytoplasmic levels of flavonoids (3). The aim of this research was to investigate the expression of these proteins in endothelial cells and to study their role in the transport of flavonoids. The permanent human cell line Ea.hy 926, exhibiting an endothelial phenotype, was used for the purpose. Some selected comparative studies were also carried out in primary endothelial cells from rat aorta. To detect the above mentioned carriers, specific antibodies were used in immunohistochemical, immunocytochemical and Western blot analyses. Transport studies were carried out using quercetin as a substrate, alone or with specific inhibitors of the membrane carriers: this enabled an evaluation of the contribution of these carriers to the specific permeability of endothelial cells to flavonoids. The data obtained indicated that BTL, MRP1, MRP2 and BCRP were expressed and took part in quercetin uptake in both cell lines. Thus, these results suggest that the expression and function of these carriers in the vascular endothelium could be responsible for the transport of flavonoids and could be involved in the protection of blood vessels from atherosclerosis as well as in the modulation of the vascular tone. Further studies will be addressed to investigate those transport mechanisms in human primary endothelial cells.

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EXPRESSION OF MUTANT NICOTINIC RECEPTOR BETA2 SUBUNIT IN TRANSGENIC MICE CAUSES ABNORMAL EEG AND SPONTANEOUS SEIZURES

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Nocturnal Frontal Lobe Epilepsy (ADNFLE) is a paediatric form of epilepsy characterised by clustered attacks of epileptic episodes occurring during light sleep and originating from the frontal lobe. This autosomal dominant focal epilepsy is caused by mutations in either �4 or �2 subunits of the nicotinic acetylcholine receptor (nAChR). We have shown that patients affected by type 3 ADNFLE linked to chromosome 1p21 carry a missense mutation (V287L) in the �2 subunit of neuronal cholinergic nicotinic receptor (*CHRNB2*). Moreover, we demonstrated that this single amino acid substitution dramatically alters the electrophysiological properties of the receptor complex by retarding its desensitisation (1). This delay may determine a gain of function of the receptor. The aim of the project is to study the role of mutant nicotinic receptor in the pathogenesis of ADNFLE by developing and characterizing a type 3 ADNFLE mouse model. A transgenic mouse, carrying the ��V287L mutation was generated by use of the TET-OFF conditional gene expression system. *Chrnb2* mouse mutant cDNA has been cloned under the control of the tetracycline-responsive promoter Ptet, whose activation is dependent on binding of the activating protein tTA. Administration of doxycycline (an analogous of tetracycline) prevents tTA binding therefore silencing the expression of the transgene. Responder pTetO-Chrnb2VL mutant mice were crossed with a transgenic strain carrying the gene coding for tTA under a prion protein (PrP) promoter to produce double transgenics where the transgene is specifically expressed in Central Nervous System (Tg(tTA:Chrnb2VL) mice). Three lines of double transgenic mice have been developed. Transgene's stability and site of insertion were verified through molecular assays. Expression of the transgene has been evaluated in several brain areas (cortex, thalamus, hypothalamaus, caudate-putamen, hippocampus, cerebellum and brain stem). Brain electrical activity of transgenic mice was evaluated by electroencephalographic (EEG) recordings. Screw electrodes were inserted bilaterally through the skull over primary motor cortex and hippocampus (anteroposterior, +2.0 –3.0 mm; left-right 2.0 mm from bregma) in mice aged 2-8 months; a further electrode was placed into the nasal bone as ground. One week after surgery, freely moving mice were recorded for 24 h. EEG data have been analyzed by use of computerized algorithms (2): transgenic mice (n=8) showed frequent spikes (rapid events of high amplitude) that were absent in wild-type mice ($p < 0.01$). One transgenic animal showed spontaneous seizures. We are currently evaluating the effect of chronic oral administration of doxycycline on the EEG behaviour of transgenic mice to assess the possibility to revert the epileptic phenotype of mice by switching off the expression of mutated beta2 subunit. Further investigation of electrophysiological and functional properties of mutant receptors will gain new insights on the pathogenetic mechanisms leading to ADNFLE.

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NO-DONORS AND PLATELET AGGREGATION: MECHANISMS OF ACTION IN RAT

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Platelet aggregation is a direct index of platelet function. High levels of platelet aggregation seem to be correlated with an elevated risk of cardiovascular disease development. Inhibition of platelet activation is an important function of endothelium-derived nitric oxide (NO) and is supported by NO synthesized in the platelets themselves. NO-donors are molecules that release NO and exert many biological functions by activating guanylate cyclase (cGMP-dependent pathway) and/or other complex mechanisms (cGMPindependent pathway). There is conflicting data in the literature regarding the mechanism of action of the various donors. For example, S-nitrosoglutathione (GSNO) seemed to inhibit platelet aggregation by a cGMP-dependent mechanism (1); however, Sogo et al. (2) have shown GSNO to be partly cGMP-independent. In this study, we analyzed the antiaggregant effect exerted by different NO-donors and we also evaluated their mechanism of action. We compared the entity of inhibition of platelet aggregation exerted by sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), and 3-morpholino-sydnonimine (SIN-1) in *in vitro* test using rat platelet rich plasma (PRP). All three drugs were used in a range of concentrations from 5 μM to 2 mM. Platelet aggregation was performed according to Born's method (3) and induced by ADP (10-15 μM); each aggregation rate was evaluated as an increase in light transmission. In order to evaluate which of these donors inhibits platelet aggregation by a cGMP-dependent mechanism, we also incubated each of them with PRP in the presence of 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a selective inhibitor of soluble guanylate cyclase. All of these NO donors inhibited aggregation and the maximum effect was evidenced at higher doses. GSNO was more effective than SNP and SIN-1. However, treatment of platelets with ODQ (100 μM), indicated that only SNP-mediated inhibition was exclusively cGMP-dependent. These results seem to confirm the hypothesis that not all NO-donors have a cGMP-dependent mechanism of action. Further measurements of intraplatelet cGMP levels in rat PRP treated with SNP, GSNO, and SIN-1 alone and in the presence of ODQ should be necessary.

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ROLE OF CYCLOOXYGENASE ISOFORMS AND NITRIC OXIDE SYNTHASE IN THE MODULATION OF TRACHEAL MOTOR RESPONSIVENESS IN NORMAL AND ANTIGEN-SENSITIZED GUINEA-PIGS

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Both nitric oxide (NO) and prostaglandins (PGs) are relevant mediators in airway physiology and pathology, and their production is regulated by different enzyme isoforms It is generally believed that the production of NO and PGs by constitutive enzyme isoforms is important for physiological functions, while their formation via the inducible isoforms is involved mainly in inflammatory and other pathological responses. Increasing evidence suggests a link between NOS and COX pathways, but there is no consensus on the functional consequences resulting from their mutual interactions. NO has been reported to regulate the activity of COX enzymes both by enhancing and inhibiting their expression or modulating their activity; on the other hand, a possible influence of PGs on NOS activity has been proposed and current evidence suggests either inhibition, stimulation or no interaction. The effects of selective cyclooxygenase isoform (COX-1, COX-2) inhibition, alone or in combination with NOS blockade, on tracheal muscle responsiveness to histamine were investigated in healthy and ovalbumin (OA)-sensitized guinea-pigs. Cumulative concentration-response curves to histamine (10^{-7}-10^{-4} M) were carried out on isolated tracheae from three different groups of animals: 1) Naïve 2) OA-sensitised and 3) OA-challenged guinea-pigs. OA-sensitisation was performed with 100 mg/kg i.p. and 100 mg/kg s.c. of the antigenic protein, 14-19 days before the experiments. OA-challenge was carried out by 10 min animal exposure to an aerosol of OA solution (1 %) in saline on the days 7,14,15 and 16 from the sensitisation, experimenting 6 h after the last aerosol.The effects of SC-560 and DFU, COX-1 and COX-2 selective inhibitors respectively, both at 10^{-6} M, were evaluated. L-NAME (10^{-4} M) was used as a non-selective NOS inhibitory agent. Responses were expressed as % of KCl 40 mM-induced contraction. Immunohistochemical analysis showed that COX-1 and COX-2 are constitutively present in normal guinea-pig trachea, particularly in the epithelial layer, and that COX-2 expression is enhanced in OA-sensitized animals both in epithelial and subepithelial tissues. In normal guinea-pigs, SC-560 or DFU significantly increased the contractile response to histamine, these effects being not additive. NOS inhibition by L-NAME did not affect histamine-induced contraction, but reversed the increase caused by COX-1 blockade, while not modifying the enhancement associated with COX-2 inibition. In guinea-pigs subjected to OVA-sensitization and challenge, COX-2, but not COX-1, inhibition enhanced the motor response to histamine, without any influence by L-NAME. In normal, but not in sensitized animals, the removal of epithelial layer from tracheal preparations abolished the enhancing action of DFU on histamine-mediated contraction. Finally, the release of PGE2 from tracheal tissue of normal and sensitized animals, was inhibited only by SC-560. In conclusion, the present findings suggest that: a) both COX-1 and COX-2 are constitutive in guinea-pig trachea, and COX-2 expression in enhanced by antigen sensitization; b) in normal animals epithelial COX-2 mediates an inhibitory action on tracheal contractility not PGE2-, but probably PGI2dependent, and this enzyme is subjected to upstream regulation by epithelial COX-1 and NOS, through a complex interplay where COX-1 inhibits NOS, preventing the inhibitory action of NOS on COX-2; c) following antigen sensitization, the modulation function of the epithelial COX-1/NOS/COX-2 network is impaired, and the inhibitory control on tracheal contractility is maintained by COX-2 induced at level of subepithelial cell sites.

GHRELIN AND ITS NATURAL UNACYLATED FORM INHIBIT SERUM STARVATION-AND CYTOKINE-INDUCED APOPTOSIS OF PANCREATIC β CELLS STIMULATING camp/pka, ERK1/2 AND PI3K/AKT SIGNALING

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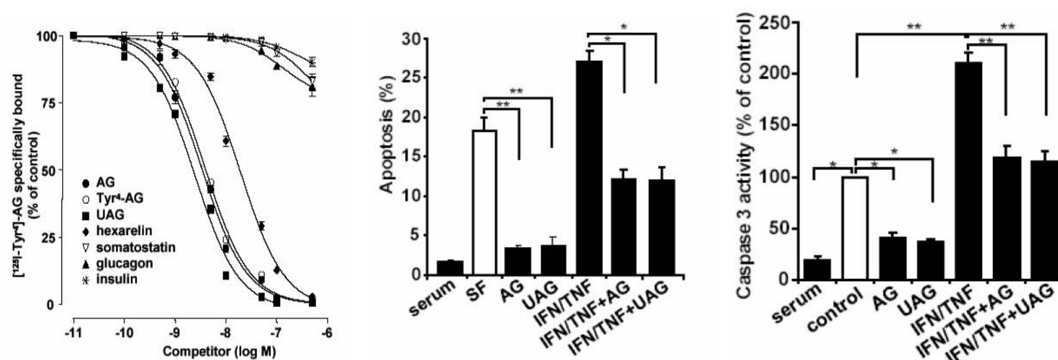
Ghrelin (GRLN) is expressed in many tissues, including the human pancreas, and a GRLNproducing ε cell population has been demonstrated in rodent islet (1). Moreover, GRLN promotes β cells proliferation (2) and modulates insulin secretion, suggesting a role in endocrine pancreas function (3). Among its pleiotropic actions, which include modulation of glucose metabolism, ghrelin regulates inflammation and the immune system (4). We investigated the role of GRLN in pancreatic β cell apoptosis induced by serum starvation or interferon (IFN)-γ/tumor necrosis (TNF)-α; indeed, IFN-γ/TNF-α synergism is one of the major responsible of β cell destruction in type I diabetes. By using RT-PCR, western blot, ELISA, EIA, Hoechst staining and radioreceptor assays we have obtained the following data. Hamster HIT-T15 pancreatic β cells expressed GRLN but not the type 1a GRLN/GH Secretagogue receptor (GHS-R1a), that binds GRLN in its acylated form (AG) only. However, both AG and unacylated GRLN (UAG) recognized common high affinity binding sites on these cells (Fig. A). Either AG or UAG prevented serum starvation- and IFN-γ/TNF-α-induced apoptosis (Fig. B) and caspase-3 activity (Fig. C), an important pro-apoptotic marker. Further, anti-GRLN antibody enhanced apoptosis of either cells cultured with or without serum but not with IFN-γ/TNF-α. AG and UAG even up-regulated intracellular cAMP. Notably, blockade of adenylyl cyclase/cAMP/protein kinase A signaling with MDL12330A and KT5720 inhibitors prevented GRLN cytoprotective effect. Finally, AG and UAG activated PI3K/Akt and ERK1/2 and GRLN antiapoptotic effect was blocked by PI3K and MAPK inhibitors (wortmannin and PD98059).

A B C



In all, GRLN, either acylated or not, prevents apoptosis of pancreatic β cells via a non type 1a GRLN/GHS-R receptor that signals through cAMP/PKA, ERK1/2 and PI3K/Akt. These findings suggest that the GRLN system plays a significant role in regulating β cell fate.

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SHHF/Mcc-fa/fa RAT, A GENETIC MODEL OF SYNDROME X: CHARACTERIZATION OF THE MODEL AT PRE-HEART FAILING STAGE

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Cardiovascular complications represent the major causes of morbidity and mortality in the diabetic population. It is also well established that obesity and diabetes increase cardiovascular complications in humans. The SHHF/Mcc-fa/fa rat, a strain characterized by dyslipidemic obesity, insulin resistance and hypertension, developing decompensated heart failure, seems an good Syndrome X model. The aim of the study is a comprehensive exploration of vascular reactivity in this strain in comparison with Wistar-Kyoto, as paradigm of normal rat. Fiveteen rats (age 6 months) of each strain were randomized. To establish the metabolic state of animals, blood samples were obtained to determine plasma levels of glucose (Glu), triglycerides (Tri), cholesterol (Cho), insulin (Ins) by commercial kits. To determine nociceptive reflex integrity all rats were tested on tail-flick apparatus. Next, blood pressure (BP), heart rate (HR), aortic blood flow (ABF), total peripheral resistance (TPR) and peripheral blood perfusion (PP) were monitored and recorded in anesthetized, ganglion-blocked (Pentolinium 0.01 mg/kg/min) animals, during bolus administration of vehicle or endothelin-1, in escalating doses (0.03, 0.1, 0.3, 0.5, 1, and 1.5 nmol/kg). Finally, organs were weighed with weight expressed as organ weight/brain weight ratio (OW/BW). Preliminary data confirm dysmetabolic state of SHHF, in fact, found in fasted animals, in the typical hematochemical picture of Syndrome X were: very high ($p<0.0001$) plasma levels of Tri (1052±353 mg/dl), Cho (240±46 mg/dl) and Ins (59±17 ng/ml) in presence of normoglycemia. Moreover, the tail-flick test showed reduction of nociceptive sensibility as demonstrated by latency increase: SHHF 6.8 (5-11) s vs. WKY 2.9 (2.5-3.3) s ($p<0.01$). SBP (137±6.0 mmHg) and TPR (90±18 AU) of anesthetized SHHF were significantly higher before ($p<0.04$ and $p<0.03$, respectively) and after ganglion-blocking agent administration ($p<0.001$ and $p<0.04$, reps.) than those of WKY. Consequently, ABF showed specular behavior with respect to TPR. PP was tendentially lower in SHHF with respect to WKY whereas ganglionblock increased it at level similar to WKY. The biphasic effect on hemodynamics, induced by Et-1 administration, seemed substantially similar in both strains, although the maximal hypertensive response induced in SHHF was tendentially lower than in WKY (21±19 vs. 48±24 mmHg, $p>0.42$). On the contrary, in SHHF PP was significantly ($p<0.001$) increased by Et-1 administration. Finally, Body weight, liver W/BW, kidney W/BW, LVW/BW of SHHF were increased in statistically significant fashion ($p<0.0001$). The preliminary data summarized above extend knowledge of this interesting SHHFMcc-fa/fa rat strain, confirming it as a useful model of Syndrome X and associated cardiovascular complications, which will result in latter stage (10-12 months) heart failure, currently under investigation.

INVOLVEMENT OF IL-1 AND IL-6 IN A MOUSE MODEL OF NEUROPATHY: MODULATION WITH THE PURINERGIC RECEPTOR ANTAGONIST PPADS

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Neuropathic pain consequent to peripheral injury constitutes a considerable problem in medical practice. Recent animal studies led to the hypothesis that neuropathic pain may be associated with local inflammation and overexpression of inflammatory cytokines in locally recruited macrophages and Schwann cells and microglia. Recent studies suggest that activation of purinergic receptors, ion channels P2X and G-protein-coupled P2Y, is involved in painful perception. In the present work we investigated the temporal quantitative expression of inflammatory cytokines at different anatomic steps of pain transmission at the peripheral nervous system in a mouse model of neuropathic pain. Moreover we determined the effects of a non-selective P2 purinergic receptor-antagonist, PPADS, on pain behaviour and cytokine system. A model of mononeuropathy was induced in right sciatic nerve of male C57BL/65 mice (2530g) by chronic constriction injury (CCI). Thermal hyperalgesia was evaluated by using a Plantar Test Apparatus, mechanical allodynia by the von Frey test. The latency of nociceptive reaction and the mechanical stimulus intensity threshold were measured at 3, 7 and 14 days after injury and after daily administration of PPADS (20 mg/kg) for eleven days, starting from day 3 after the nerve injury. In the same days the level of IL-1 and IL-6 expression was evaluated by real time RT-PCR in ipsilateral dorsal root ganglia (DRG) and lesioned sciatic nerve (SN). Hyperalgesia and allodynia were already maximal three days after CCI, and they remained similarly elevated up to 14 days. At 3, 7 and 14 days after injury IL-1 and IL-6 significantly increased in SN. In DRG, a precocious increase of IL-6 (3 days) was observed, while IL-1 expression level was elevated only at later time (7, 14 days). PPADS administration attenuated both the mechanical allodynia and hyperalgesia in CCI mice. PPADS treatments significantly decreased IL-1 level at 7 and 14 days after CCI in SN and DRG. IL-6 expression was significantly decreased by PPADS in SN 14 days after injury. This study shows that in a mouse model of neuropathy proinflammatory cytokines may contribute to the development and maintenance of pain hypersensitivity. Moreover the purinergic receptor antagonist PPADS was able to attenuate painful neuropathy condition possibly normalizing cytokine levels.

PRECISION-CUT LIVER SLICES APPROACH TO STUDY XENOBIOTIC METABOLISM

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Several *in vitro* systems are available for studing in the liver the metabolism or toxicity of novel compounds. Although subcellular fractions, such as microsomes, and isolated hepatocytes have been most extensively used, precision-cut liver slices could be an useful tool and it has some typical advantages: no proteolitic enzymes are necessary for preparation, normal polarity of the cells remain intact and there is the maintenance of cell heterogeneity and cell-cell interactions (1). The use of liver slices is not new but it has become more widespread after the introduction of apparatus that minimally traumatized the tissue with slices very uniform for shape and dimension (2). The aim of the present study is to set up the preparation of liver slices in order to study the xenobiotic metabolism. Precision-cut liver slices were prepared from tissue core (8 mm) using a Krumdieck tissue slicer filled with oxygenated, ice- cold Krebs-Henseleit buffer. After a pre-incubation period of 30 min, slices were individually incubated in RPMI1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 wells plates with a continuous gentle shaking (3). Incubation medium were supplemented with 7-ethoxycoumarin or Ldeprenyl for the corresponding metabolism studies. After various time (30 min, 2 h, 4 h, 6 h, 24 h) medium samples were taken and slices homogenizated. Total glutathione (GSH) content and lactate dehydrogenase (LDH) leakage were measured. LDH leakage was evaluated both in pre-incubation and incubation medium and in whole slice homogenates. 7-ethoxycoumarin metabolism was determined in the medium samples by means a fluorimetric method. The formation of L-deprenyl metabolites was determined by GLC in the incubation medium. The microscopy measured indicated that the slices were 250-300 μm thickness and weight about 25 mg. The LDH leakage increased with time up to 24 h incubation (60% of the total). On the contrary GSH content remained constant up to 24 h of incubation. Furthermore the time-dependent formation of amphetamine, methamphetamine and nordeprenyl as well as hydroxylate, glucuronidate and sulphatate derivatives of cumarine when slices were incubated in presence of deprenyl and, respectively, cumarine indicated that the slices fully maintained their metabolic capacity. In conclusion the results of this study show that precision-cut liver slices prepared in our laboratory remain viable and metabolic competent to 24 h.

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MULTIDIRECTIONAL ROLES OF CAMKII PHOSPHORYLATION IN GOVERNING SAP97 FUNCTIONS IN EXCITATORY NEURONS

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The correct delivery of synaptic proteins is a key process in the regulation of physiological neuronal response. Membrane-associated guanylate kinase (MAGUK) protein family has been addressed as organising elements in excitatory neurons. Synapse-associated protein 97 (SAP97) is a member of MAGUK protein family and shares with other MAGUK members a multimodular structure made of one L27, three PDZ, an SH3 and a guanylate kinase domains. SAP97 has been involved in the correct targeting and clustering of glutamate ionotropic receptors subunits at postsynaptic sites. We have previously shown that Calcium/Calmodulin kinase II phosphorylates *in vivo* SAP97 on two major sites; one located in the first PDZ domain –Ser232- and the other in the N-terminal domain –Ser39-. CaMKII-dependent phosphorylation of SAP97-Ser232 disrupts SAP97 interaction with NR2A subunit thereby regulating synaptic targeting of this NMDA receptor subunit. Interestingly, CaMKII-mediated phosphorylation of the other site -Ser39- is necessary and sufficient to drive SAP97 to the postsynaptic compartment in cultured hippocampal neurons. In addition, SAP97-Ser39 phosphorylation represents a key step governing GluR1 containing AMPA receptor delivery to the postsynaptic complex. Nevertheless, the division in time and space of these CaMKII dependent phosphorylation sites of SAP97 is still unknown, as is the dissection of their physiological functions. To that, we studied the effects on NR2A trafficking of pharmacological tools stimulating or inhibiting calcium release from the endoplasmic reticulum which is required for SAP97 trafficking toward spines. Moreover, we transfected hippocampal neurons with NR2A and different phosphorylation mutants of SAP97. Further, using phospho-specific antibodies, we dissect the different roles of these two phosphorylation sites since they suggest that SAP97 is a multimodular element where distinct domains play differential roles in organizing the glutamatergic synapse.

NEUROTENSIN RECEPTOR INVOLVEMENT IN THE RISE OF EXTRACELLULAR GLUTAMATE LEVELS AND APOPTOTIC NERVE CELL DEATH IN PRIMARY CORTICAL COLTURES AFTER OXYGEN AND GLUCOSE DEPRIVATION

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It has been shown that Neurotensin (NT) enhances, both *in vivo* and *in vitro* (1), glutamatergic signalling. The aim of the present study was to investigate, under normotic conditions, the role of NT receptor1 (NTR1) in nerve cell death and extracellular glutamate levels, after oxygen and glucose deprivation (OGD) in cerebral cortex cell cultures. Primary cultures of rat cortical neurons have been prepared from 1 day-old SD rats(2). For OGD, the culture medium was replaced with a glucose-free Krebs-Ringer bicarbonate buffer, and multiwell or dishes were put into a hypoxic incubator (95% N_2/5% CO_2) at 37°C for 60 min; 24 h after OGD, lactate Dehydrogenase (LDH) levels**,** determination of endogenous extracellular Glutamate levels and nuclear staining with Hoechst 33258 were performed. The effects of NT and NTR1 antagonist SR48692 were evaluated during OGD. Exposure of cortical cell cultures to OGD, induced a significant increase of extracellular glutamate levels (n=15, p<0,05), LDH efflux (n=28, p<0.05) and number of the apoptotic nuclei (n=18, p<0.001) as measured 24 h later the ischemic insult, with respect to sister cell cultures not exposed to OGD (n=9, n=23, n=15 respectively). The addition of NT (100 nM) to the cells was associated with an enhancement of the OGD-induced increase of glutamate levels (n=10, p<0.001), LDH efflux (n=23, p<0.001) and number of apoptotic nuclei (n=9, p<0.001). The pre-exposure of the cells to the NTR1 antagonist SR48692 (100 nM) prevented these effect of the neuropeptide. Interestingly, SR48692 100nM, by itself, reduced the ODG-induced increase of extracellular glutamate levels, LDH efflux and number of apoptotic nuclei. This results lead to hypothesize that, under normothermic conditions in this *in vitro* model of ischemia (3), cortical NTR1 activation may contribute to apoptotic nerve cell death, with reduction of neuronal survival, by the ability of NT to modulate the glutamatergic transmission, opening up a therapeutic potential for NTR1 antagonists in the treatment of cortical ischemia.

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PRENATAL EXPOSURE TO A CANNABINOID RECEPTOR AGONIST(WIN 55,212-2) PRODUCES ALTERATIONS ON HIPPOCAMPAL AND CORTICAL GLUTAMATERGIC SYSTEM

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Fetal development is a period sensitive to environmental influences such as maternal drug use. The most commonly used illicit drug by pregnant women is marijuana. Human studies have shown motor, social, and cognitive disturbances in offspring exposed to cannabis prenatally. Cannabinoids can be transferred from the mother to the offspring through the placental blood during the gestation and interferes with the ontogenic processes. The aim of the present study was to investigate whether prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 (WIN), influences cortical and hippocampal glutamtergic system in rats offspring. For this purpose Wistar female rats weighing 250-280g were treated daily with WIN (0.5 mg/kg) or WIN vehicle from GD 5 through GD20**.** *In vivo(*microdialysis*)* and *in vitro* (primary cortical cell cultures) experiments were performed in the rat offspring of WIN-treated and vehicle-treated dams. Basal and K^+-evoked extracellular glutamate levels were significantly lower in cortical cell cultures obtained from pups exposed to WIN during gestation with respect to those measured in cultures obtained from neonates born from vehicle treated dams. The addition of NMDA (0.01–10 μM, 10 min) to cortical cell cultures from neonates born from vehicle-treated dams induced a concentration dependent increase of glutamate levels, and this was absent in cell cultures obtained from WIN-exposed pups (1). Furthermore, prenatal exposure to WIN induced a reduction in the number of cortical neuronal population. *In vivo* microdialysis showed a significant decrease in basal extracellular glutamate levels in the cortex (2) and hippocampus (3) of adult rats (90PDN) born from WIN-treated dams and an impaired responsivity of hippocampal CB1 receptors to challenge of WIN. Prenatal WIN exposure, induced: i) an alteration of the mRNA levels of the NMDA (NR2A and NR2B) and metabotropic (mGlur1, mGlur5) receptors, both in cortex and hippocampus in adult rats (90PDN) ii) a decrease of CB1 mRNA expression in hippocampus of adult rats (90PDN). Finally we found that prenatal exposure to WIN 55,212-2 significantly reduced the phosphorylated levels of ERK1/2 in hippocampal tissue respect to control. The present *in vivo* and vitro studies demonstrate that the prenatal exposure to the cannabinoid receptor agonist WIN induces a significant and persistent alteration of hippocampal and cortical glutamatergic and cannabinoidergic systems.

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SPONTANEOUS REPORTING SYSTEM IN VENETO: THE EXPERIENCE OF THE HOSPITALS IN VERONA

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Pharmacovigilance is defined as the detection, assessment and prevention of adverse drug reactions (ADR) of medicines in humans. Before a product is marketed, the experience of its safety and efficacy is limited to its use in clinical trials which are not reflective of practice conditions since they are limited by the numbers and types of patients as well as duration of the trial. Hence derives the relevance of pharmacovigilance. WHO (World Health Organization) defines an ADR as "A response to a drug which is noxious and unintended, and which occurs at doses normally used in man for the prophylaxis, diagnosis, or therapy of disease, or for the modification of physiological function". The spontaneous reporting system is the main tool in order to define the safety of commercialized medicines. In Italy the spontaneous reporting system and the estimation of ADR are unfortunately limited by a gross under-reporting, specially deriving from hospital physicians.

Our goal was to increase the number and the quality of the adverse drug reaction reports coming from the hospitals of Verona (Borgo Trento Hospital "Ospedale Civile Maggiore" and Borgo Roma Hospital "Ospedale Policlinico G.B. Rossi) combining different approaches: individuate in each ward a reference physician responsible for the collection of reports periodic visit by an internist to stimulate the recognising ADR cases and to help in the compilation of reports educational ward meetings with discussion of recent alarms on drug ADRs During the first six months of 2006 we carried out some of these interventions in medical wards of Borgo Roma Hospital.

The number and the quality of reports sent in the first semester 2005 were compared with those sent in the same period of 2006, giving the following results: Fifty three reports in 2006 compared with 19 in the same period in 2005 More than half of reports came from medical wards, but other departments even using drugs with many side effects did not improve their reporting. The more involved drugs were cardiovascular drugs, antibacterial drugs and central nervous system drugs

Spontaneous reporting is the primary method to detect important ADRs and physicians are in a position to play a key role in reporting programs but underreporting is very common. A targeted programme in the hospital wards is needed to improve ADR reporting: Among the various interventions, the periodic visit by a physician directly stimulating the compilation of reports seems to give best results, but the involvement of hospital doctors is still very low.

PROTECTIVE EFFECTS OF GUANOSINE AGAINST β−AMYLOID−INDUCED OXIDATIVE DAMAGE IN SH−SY5Y CELLS

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Neurodegeneration in Alzheimer's disease (AD) is associated with abnormal accumulation of neurotoxic β-amyloid (βA) protein, which causes neuronal death (1). Several distinct mechanisms have been implicated in βA-induced neuronal death, including the generation of free radicals, mitochondrial damage, and inflammation (2). The attenuation of deleterious effects of free radical by new molecules is proposed as a potential therapeutic intervention in AD (3). Guanosine and other nonadenine-based purines have a number of neurotrophic and neuroprotective roles such as stimulation of astrocyte proliferation, promotion of neurite outgrowth and increased synthesis and release of NGF (4). However, the neuroprotective efficacy of guanosine in AD is still not well established. In this study, we investigated the neuroprotective effects of guanosine against βA protein-induced oxidative damage in a human neuronal cell line (SH-SY5Y). The oxidative neuronal damage was induced by 3 h treatment with fragments of βA_{25-35} (1 μM), a neurotoxic core of βA protein. An experimental approach using tetrazolium salts and a fluorescent probe 2,7-dichlorofluorescein diacetate has been used to determine the loss of mitochondrial activity and free radical formation induced by exposure of SH-SY5Y cells to βA_{25-35}. Pre-treatment and cotreatment of SH-SY5Y cells with various concentrations of guanosine (12.5-100 μM) were found to display a dose-dependent inibitory effect on loss of mitochondrial activity induced by βA_{25-35}. The results also demonstrated that similar concentrations of guanosine inhibits the free radical formation induced by βA_{25-35} in a concentration-dependent manner. The maximum inhibition of mitochondrial damage (66%) and free radical formation (30%) was observed with 100 μM of guanosine. These preliminary results support further research aimed at identifying guanosine as novel lead structure that affect neurodegenerative disorders.

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CLONING OF CYP1A2 FROM PIG LIVER AND KINETIC PROPERTIES OF PURIFIED PORCINE 1A2 VS HUMAN RECOMBINANT CYP1A2

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The CYP enzymes belong to a superfamily of hemeproteins that are involved in the metabolism of exogenous and endogenous compounds. Many factors appear to affect the expression and activity of CYPs. CYP1A2 mediates the metabolic activation of aromatic amines and other aromatic hydrocarbons. In the latest years, the pig model has become of interest for the possibility of liver transplantation to man and for this reason it is important to characterize the expression and activity of the main porcine CYPs including CYP1A2. In the present study, we described the cloning of a cDNA from pig liver encoding CYP1A2. In addition, we compared the kinetic parameters of porcine CYP1A2, previously purified in this lab, from β-naphtoflavone (βNF) treated pigs, to those of human recombinant CYP1A2 in a reconstitution system (1). Five control pigs (of about 25-30 kg) were used for the analysis. Total RNA was extracted from the liver of each pig, treated with DNase, modified by Gene Racer Kit (Invitrogen), retrotranscripted and amplified by PCR using unspecific and specific human 1A2 primers. The PCR products were sequenced and analysed by CLUSTAL and BLAST programs. Liver microsomes were prepared in the standard way and ethoxyresorufine-O-deethylase (EROD) activity was determined as previous described (2). The reconstitution system contained 50 pmol of purified porcine CYP1A2 or recombinant human CYP1A2, 100 pmol of NADPH-cytochrome P450 reductase, 30 μgr of lipids (DLPC) and NADPH regenerating system. It was cloned a cDNA of 1930 bp containing an open reading frame of 1551 bp encoding a CYP1A2 protein of 516 aminoacids, which had an identity of 78% and 84% with rat and human, respectively. The analysis of five pigs showed differences in five nucleotides, which did not result in any difference in the encoded protein. Porcine microsomes oxidised ethoxyresorufine with a K_m of 0.6μM very similar to that of 0.2 μM obtained with the purified pig CYP1A2 and to that reported for human recombinant CYP1A2 (0.09 μM). It was also found that α-naphtoflavone inibited the porcine 1A2 with a IC_{50} of 0.25 μM similar to that reported for the human 1A2. The cloned CYP1A2 of pig had a sequence highly homologous to the human counterpart. The Kinetic properties of porcine 1A2 appeared to reflect closely those of human CYP1A2, suggesting that the xenobiotics substrates of this enzyme might be metabolised in pig as in human.

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CYTOTOXIC ACTIVITY OF GEMCITABINE IN COMBINATION WITH THE VEGF AND EGF RECEPTOR TYROSINE KINASE INHIBITOR ZD6474 AGAINST PANCREATIC AND BLADDER CANCER CELLS

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The deoxycytidine analog gemcitabine is an active agent against a variety of solid tumours, including pancreas and genito-urinary malignancies. However, a great deal of interest has been focused on research into new drugs and new drug combinations for chemotherapy of these diseases. In particular, several studies are investigating the emerging role of biological agents targeting molecular pathways involved in tumor growth and angiogenesis, such as ZD6474, a new inhibitor of the kinase domain-containing of VEGFR2 and EGFR. Therefore, the present study was performed in pancreatic and bladder cell lines to analyze the ability of ZD6474 to synergistically interact with gemcitabine, and to study cellular and genetic mechanisms underlying these effects. Cells were treated with gemcitabine, and ZD6474, alone or in sequence, and the cytotoxicity was assessed by the CellTiter 96 Non-radioactive cell proliferation kit. Pharmacologic interaction was studied using the combination index (CI) method. The effects of drugs on cell cycle, Akt (S473) and EGFR (Y992 and Y1173) phosphorylation and apoptosis were investigated by flow cytometry, ELISA and fluorescence microscopy, respectively. Finally, quantitative PCR was used to study target gene expression profile and its modulation by single drugs. A concentration-dependent inhibition of cell growth was observed with gemcitabine and ZD6474, with IC_{50} values of 0.08±0.01 and 0.49±0.06 μM (MIA PaCa-2), 0.18±0.04 and 0.54±0.07 μM (PANC-1), 0.10±0.02 and 1.19±0.32 μM (Capan-1), 0.92±0.05 and 1.35±0.16 μM (T24), and 4.37±0.83 and 1.68±0.29 μM (J82), respectively. The combination index analysis showed synergism for both drug sequences (CI<1). Flow cytometric studies demonstrated that gemcitabine enhanced cellular population in the S phase with respect to control (from 27.52 to 42.31%, from 30.21 to 39.11%, and from 22.49 to 34.50% in MIA PaCa-2, PANC-1 and Capan-1 cells, respectively). ZD6474 significantly decreased the amount of activated Akt and cells exposed to both drugs presented typical apoptotic morphology. In particular, drug combinations significantly increased apoptotic cell death with respect to control cells ($P<0.05$). Furthermore, gemcitabine significantly enhanced the content of pY1173 EGFR levels, while ZD6474 decreased EGFR phosphorilation at both tyrosine residues. PCR analysis showed that ZD6474 increased the gene expression ratio between the gemcitabine activating enzyme deoxycytidine kinase and the gemcitabine target ribonucleotide reductase in pancreatic cell lines. Several factors, including modulation of Akt and EGFR phosphorylation, induction of apoptosis and expression of genes involved in drug activity, may contribute to the synergistic effect between gemcitabine and ZD6474, and these data provide experimental basis for the rational development of this combination against pancreatic and bladder cancer.

HIGH FREQUENCY OF CONGENITAL POLYCYTHEMIA DEPENDENT ON VON HIPPEL-LINDAU GENE MUTATIONS IN CAMPANIA REGION

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Chuvash form of familial polycythemia (MIM 263400) is a non-benign autosomal recessive disorder characterized by high hemoglobin level, relatively high serum erythopoietin content and premature mortality mostly due to cerebral vascular events and peripheral thrombosis. The disease is due to Von Hippel-Lindau (VHL) gene (C598T>R200W) mutation which leads to an increased HIF-1α activity and, in turn, to an abnormal erythrocyte production in response to physiological O_2 blood level. This polycythemia is endemic in Chuvashia with a frequency of

about 0.057, while the estimated worldwide frequency is around 0.00137. The identification of an identical haplotype in all patients allowed the hypothesis that the disease originates from a single ancient event occurred from 15000 to 60000 years ago. In this study, we investigated for the first time the VHL gene *598C>T* transition in the italian polycytemic patients. In particular, we analyzed the frequency of mutation in Campania, a region of the South Italy. Twenty-two patients, belonging to 13 families with a presumed congenital Chuvash-like polycythemia were included in the study. We identified five families (corresponding to 14 patients) affected by this form of congenital polycythemia. Twelve live on the island of Ischia (Bay of Naples). The haplotype of all polycythemic patients was identical to that observed in Chuvashia, thus confirming the occurrence of a single founder. We also demonstrated that heterozygotes show an increased HIF-1α activity which might represent the selective biochemical advantage for the maintenace of mutation. In conclusion, we identify a high incidence of Chuvash erythrocytosis not localized in Chuvashia. This suggests that this form of familial polycythemia might be endemic in other areas of world allowing us to propose a more general "VHL-dependent polycythemia" definition for classifying such congenital red cell disorder.

HMGB1-EVOKED RELEASE OF GLUTAMATE FROM ADULT MOUSE BRAIN GLIA SUB-PARTICLES

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The High Mobility Group Box 1 protein (HMGB1) is a chromatin-binding factor highly conserved in mammals. Although this multifunctional HMGB1 is widely expressed in developing central nervous system, the adult brain maintains high levels of HMGB1 only in hippocampus and cerebellum (1). In developing rat brain, HMGB1 co-localizes with the Receptor for Advanced Glycation End Products (RAGE) (2). Our aim was to determine whether extracellular HMGB1 affects glutamatergic transmission in the CNS of adult rodent. Therefore, we monitored glutamate release from purified axon terminals (synaptosomes) and glial plasmalemmal vesicles, here referred as to gliosomes. Adult male mice were used. Sample preparation: purified glial re-sealed particles (gliosomes) and synaptosomes were prepared essentially according to Nakamura (3). Confocal microscopy: samples were prepared by labelling synaptosomes and gliosomes with the appropriate primary and fluorescent secondary antibodies. Images were collected by an inverted microscope endowed with 100x/NA1.4 objective. Glutamate release: synaptosomes and gliosomes were labelled with $[^3H]$D-Aspartate and exposed in superfusion (4) to HMGB1 in presence or absence of external Ca^{2+}, dl-TBOA or DHK. Endogenous glutamate was measured by HPLC. Immunoblotting: proteins were separated by SDS-10%PAGE and transferred onto nitrocellulose membrane; immunoreactivities were evaluated by peroxidase-conjugated secondary antibodies followed by development with ECL® Plus. HMGB1 increased the spontaneous efflux of $[^3H]$D-Asp and endogenous glutamate from gliosomes; the maximal potentiation was obtained around 3 nM HMGB1 and EC_{50} amounted to 0.57±0.04 nM and 0.32±0.04 nM for hippocampal and cerebellar gliosomes respectively (means ± SEM of 5-15 experiments in triplicate). The efflux of $[^3H]$D-Asp from gliosomes was strongly reduced (about 70%) in the absence of Ca^{2+} external, but it was indipendent on intracellular Ca^{2+} chelators. The HMGB1 evoketed-release of glutamate was abolished in the presence of the non-selective glutamate uptake inibitor dl-TBOA (10 µM), but not of dihydrokainate (50 µM), an inibitor of GLT1 (means ± SEM of 7 experiments, in triplicate). HMGB1 bound to gliosomes but not to synaptosomes and its binding depended upon the presence of Ca^{2+}. In gliosomes, HMGB1 co-immmunoprecipited with GLAST or RAGE, but not with GLT1. Only the interaction between RAGE-HMGB1 was promoted by Ca^{2+}. Nanomolar concentration of HMGB1 selectively increases the basal efflux of glutamate from gliosomes. The results suggest that the HMGB1-induced glutamate release is mediated by reversal of transportes and that the transporter involved is GLAST. The apparent Ca^{2+} dependency of the HMGB1-induced glutamate release is linked to the Ca^{2+} need for its binding to gliosomes. Moleculary, the effect of HMGB1 is promoted by Ca^{2+}-dependent interaction with RAGE. Taken together these findings indicate that HMGB1 cytokine could act as a modulator of glutamate homeostasis in adult mammal brain.

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A NEW PYRAZOLO-PYRIMIDINE-DERIVED C-SRC INHIBITOR REDUCES TUMOR ANGIOGENESIS

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Src tyrosine kinase family (SFKs) is involved in growth factor-induced cell proliferation, invasion and metastasis. Vascular endothelial growth factor (VEGF) is the most important factor involved in tumor angiogenesis and progression and c-Src is essential for these VEGFmediated processes. We investigated the influence of a new pyrazolo-pyrimidine-derived cSrc inhibitor,1l [1], on the angiogenic output of human epidermoid A431 tumor cells. The effect of 1l was assessed on growth and microvessel density in A431 xenografts implanted in immunodeficient mice (2). The effects of c-Src inhibition were investigated on VEGF induced angiogenesis *in vivo* (rabbit cornea assay)(3) and *in vitro* (proliferation and invasion test performed in endothelial cells)(3). A431 cells when implanted in nude mice were able to rapidly induce a palpable tumor mass. The peritumor administration of nanomolar concentration of 1l decreased tumor burden and tumor microvessel density though the inhibition of VEGF expression. VEGF-induced neovessel growth was also significantly decreased in the rabbit cornea assay. The antiangiogenic activity of 1l was confirmed *in vitro* where it reduced proliferation and invasion of endothelial cells promoted by VEGF. As a reference cSrc-inhibitor the commercially available PP1 was used. Notably, PP1 (500 nM) displayed inhibitory effects on VEGF-induced endothelial cell functions and the effect was comparable to the one elicited by 50 nM 1l. In conclusion this study demonstrates that inhibition of c-Src kinase activity with namolar concentration of the novel c-Src inhibitor 1l, reduces VEGF induced-angiogenesis both in tumor and endothelial cells and indicate that c-Src inhibitors might be potential therapeutic agents for angiogenesis associated diseases.

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NATURAL AND SEMISYNTHETIC PHENOLICS AND NUCLEAR MAGNETIC RESONANCE IN HUMAN OVARIAN CANCER CELL LINES

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Increasing epidemiological and experimental evidences indicate that natural polyphenols protect against cancer. Phenols have well known antioxidant properties, but it has also been documented that they modulate protein kinases and proteasesand are ligand for transcription factors (1, 2). The first aim of this work was to study the effects of many different natural and synthetic phenols on cell lines derived from human ovarian carcinoma: wt (2008) and cis-platin resistant (C13), in the absence or in the presence of cis-Pt. Semisynthetic phenols (P_{1-20}) were derived by in silico approach on the basis of a hightroughput consensus docking strategy of large class of natural flavones, flavonols, naphtoquinones, coumarins and protein kinase ATPbinding pocket. Compounds were tested at (0.1µM-0.1mM) on cell vitality, on the complexity of cell cycle progression and on ROS generation. The second aim was to possibly define by ^{1}H-NMR, a rapid, accurate and noninvasive technique (3), the metabolically profile of live intact wt and cis-platinum resistant cancer cells, being resistance still a the rate limiting step of platinum chemotherapy. Cell vitality was measured after 24 h of incubation with drugs by MTT test. DNA content was determined after 24 h incubation with drugs on propidium iodide stained cells by flow cytometry using FACScan instrument (Coulter Epics XL, Beckman). ROS generation was measured after 2 h incubation with drugs, on H_2DCFA probed cells by flow cytometry. ^{1}HNMR experiments were determined from samples of $2x10^{7}$ cells and spectra were collected on Brucker Avance DMX600 spectrometer. Results demonstrated differences between wt and cis-pt resistant ovarian cells in cell cycle phases, ROS generation and mainly in saturated lipid, lactate and glutathione. Quercetin, curcumin and plumbagin and P_6 and P_{20} at (0.1µM-1µM) concentrations significantly inhibited proliferation, but they differently affected cell cycle progression and ROS generation in both wt and cis-pt resistant cancer cells. The results indicate that natural and semisynthetic phenols may selectively inhibit at different phases cell cycle, suggesting different molecular targets in their mechanism of action. Only some natural phenols were able to increase the cytotoxicity induced by cis-pt on the two carcinoma cell lines, in comparison with single treatment. Chemoprevention is a new approach to develop efficient strategies of controlling cancer, (4). Our data indicate that natural occurring and may be chemosensitizing molecules as well as cell targeted cytotoxic molecules and they may constitute the basis for design more selective kinase inhibitors compounds.

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POSSIBLE ACETYLTRANSFERASIC ACTIVITY ENCODED BY A GENE CASSETTE WIDELY DIFFUSED IN *ACINETOBACTER BAUMANNII*

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Acinetobacter baumannii are Gram negative bacteria that during recent decades have been responsible for numerous hospital outbreaks. They are frequently resistant to antibiotics of different classes, i.e. wide spectrum β-lactams, aminoglycosides and quinolones. Very often resistance is determined by gene cassettes that can be found within elements known as integrons. These structures include a gene that encodes a site-specific recombinase, intI integrase, and a recombination site, attI, located adjacent to the intI gene. IntI integrase is responsible for both integration and excision of gene cassettes at the attI site. Integrons have been described in many Gram-negative species and in Acinetobacter of both environmental and clinical origin. In particular, epidemic strains of *A. baumannii* were found to carry these elements with high frequency and presence of integrons correlates with a multidrug resistance profile. Two integrons are most frequently observed in Europe in epidemiologically unrelated clinical isolates of *A. baumannii*. They have the same cassette array but one has two copies of a single cassette, named orfX, whose function is still unknown. The aim of this study is to identify the role of the product of this gene. Clinical isolates of *A. baumannii* from different European countries and positive for the presence of integrons were submitted to macrorestriction analysis in order to investigate their relationship. Most isolates could be included into one of two epidemic clones, while only 5 isolates were considered sporadic. All epidemic isolates carried one of the two integrons, while sporadic strains did not. The nucleotide sequence of the orfX cassette was obtained independently from the two integrons and resulted to be well defined and conserved. Using softwares that predict secondary structure of theoretical proteins it has been found that the product of orfX might be a member of the GNAT superfamily of acetyltransferases. This group includes also aminoglycoside-acetyl transferases, enzymes that inactivate aminoglycosides and make them ineffective in the treatment of clinical infections. To test the possibility that product of orfX has an acetyltransferasic activity, we decided to clone the gene in an expression vector. To this purpose, orfX was amplified by PCR and cloned in pBAD/Myc-His (Invitrogen), that allows control of expression levels by modulation of arabinose concentrations in bacterial medium. In an induction test we established that a final concentration of arabinose of 0.002% is sufficient to get good levels of expression. Under this conditions, a Kirby-Bauer assay was performed with antibiotic-susceptible bacteria that were transformed with the vector carrying orfX. The aminoglycosides amikacin, gentamicin, netilmicin and tobramycin were tested. Bacteria were susceptible to all of them, suggesting that the product of orfX do not inactivate these compounds. Other aminoglycosides and antibiotics must be tested.

EFFECTS OF CANNABINOIDS ON L-DOPA-INDUCED DYSKINESIAS: ROLE OF CB1 AND TRPV1 RECEPTORS

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L-dopa represents the most effective treatment for the motor symptoms of Parkinson's disease (PD). Although L-dopa causes a pronounced symptomatic improvement in the initial stages of PD, its long-term use results in disabling side-effects, such as dyskinesias, consisting of abnormal involuntary movements (AIMs). The unilateral (6-OHDA)-lesioned rat represents a well established animal model of PD that allows to investigate behavioral functions regulated by the basal ganglia and to examine the brain neurochemical changes after DA depletion. Stimulation of cannabinoid receptors, the pharmacological targets of the active ingredient of marijuana, is emerging as a promising therapy to alleviate L-dopa-induced dyskinesia (1). To investigate how the endocannabinoid system is involved in attenuating AIMs, 6-OHDA-lesioned rats were treated chronically with L-dopa (6 mg/kg, i.p., per day) in association with an inhibitor of aromatic aminoacid decarboxilase (carbidopa, 12 mg/kg, i.p.) for 12 days. This treatment causes severe AIMs increasing over time and that can be classified as axial, limb, locomotor and orofacial dyskinesias. Co-administration of the cannabinoid agonist WIN 55,212-2 (WIN, 1 mg/kg, i.p.) with L-dopa (day 9 to 12) significantly attenuated all axial, limb and oral AIMs (n=6, $p<0.05$, $p<0.01$, Kruskal-Wallis test followed by Dunn's multiple comparison test). This effects was reverted by the CB1 antagonist AM251 (1 mg/kg, i.p.) (n=6, $p<0.05$, Friedman test followed by Dunn's multiple comparison test). By contrast, systemic administration of URB597 (n=8,
1. 0.1 and 0.3mg/kg, i.p.), a potent FAAH inhibitor that increases the levels of the endocannabinoid anandamide by blocking its degradation, had no effect on AIMs scoring. Unlike WIN, anandamide is known to directly bind and activate TRPV1 receptors, which have been implicated in the regulation of basal ganglia functions by promoting dopamine neuron firing in the substantia nigra. (2,3). Interestingly, subchronic co-administration of URB597 and capsazepine (10 mg/kg), a selective TRPV1 antagonist, significantly decreased all AIMs subtypes (n=6, $p<0.05$, $p<0.01$, Kruskal-Wallis test followed by Dunn's multiple comparison test), whereas capsazepine (n=6) had no effect when administered alone. In support to this evidence co-administration of capsazepine and the anandamide analog, R(+) methanandamide (10 mg/kg), also able to activate TRPV1 receptor, showed a trend of decrease of all AIMs, whereas co-administration of AM251 and R(+) methanandamide was uneffective. Our data suggest that CB1 and TRPV1 receptors play opposite roles in L-dopa-induced dyskinesias.

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SULFORAPHANE PROTECTS SH-SY5Y CELLS AGAINST 6-HYDROXY-DOPAMINE-INDUCED OXIDATIVE STRESS

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Parkinson's disease (PD) is associated with a progressive loss of nigrostriatal dopaminergic neurons. Recent findings suggest that oxidative stress caused by dopamine could be closely involved in the pathogenesis of PD. Sulforaphane (SF), a glucosinolate-derived isothiocyanate found in cruciferous vegetables, is a known cancer chemopreventive agent (1) and a strong inducer of phase II detoxification enzymes (2). In this study we investigated the neuroprotective effects of SF against 6-hydroxydopamine (6-OHDA)-induced cell death using SH-SY5Y cells, a dopaminergic neuronal cell line. 6-OHDA is one of the most commonly used neurotoxins in *in vitro* and *in vivo* experimental models of PD. An experimental approach using a pulse/chase treatment with 6-OHDA (100 µM) to determine the neuronal apoptosis and necrosis has been applied. In particular, we detected early apoptotic events and necrosis, with Annexin-V/propidium iodide (PI) double-stain system. We also measured another apoptotic event, DNA fragmentation, with enzyme-immunoassay. Moreover, to determine the indirect antioxidant activity of SF, we measured the Total Antioxidant Activity (TAA) of cytosolic fraction (3), the levels of glutathione (GSH) in neurons (4) and the activity of phase II enzyme, such as Glutathione-S-Tranferase (GST) (5), Glutathione Reductase (GR), NADPH-quinone reductase (NAD(P)H-QR) (6) and Glutathione Peroxidase (GPx). Treatment of SH-SY5Y cells with SF (0.6-5 µM) for 24 h before 6-OHDA treatment showed a dose-dependent inhibitory effects on 6-OHDA induced early apoptosis and necrosis. In particular, the maximum inhibition of apoptosis (80%) and necrosis (50%) was observed with 5 µM of SF. The results also demonstrated that similar concentration of SF inhibited DNA fragmentation induced by 6OHDA (82%). Furthermore, SF led to an increase of TAA and GSH levels, in a dosedependent manner, with a maximum increase of 2 and 3 fold, respectively. Moreover, SF increased the activity of phase II detoxification enzymes, such as GST (60%), GR (30%) and NAD(P)H-QR (40%). Taken togheter, these results demonstrate that *in vitro* neuroprotective effects of SF could be ascribed to increase cellular antioxidant defences. Therefore, these results should encourage further research in PD animal models to explore the potential profile of SF as novel neuroprotective agent.

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CALCITONIN GENE RELATED PEPTIDE AFFECTS OSTEOCLASTOGENESIS DIRECTLY AND INDIRECTLY BY MODULATING THE EXPRESSION OF OSTEOPROTEGERIN IN HUMAN OSTEOBLAST-LIKE CELLS THROUGH THE INVOLVEMENT OF CAMP PATHWAY

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An increasing number of studies suggest that nerve-derived signals play an important role in the regulation of bone remodelling. Functional receptors for signalling molecules of skeletal nerve fibers are expressed in bone cells and the activation of these receptors leads to the modulation of osteoblasts and osteoclasts activities. Calcitonin gene related peptide (CGRP) is a neuropeptide which is present in the bone nerve endings and which has been denonstrated to play a role in bone metabolism. Recently the OPG/RANKL/RANK system has been recognized to be the main mechanism involved in the communication between osteoblasts and osteoclasts. OPG, a member of the tumour necrosis factor receptor superfamily, is an endogenous inhibitor of osteoclastogenesis produced by cells of the osteoblast lineage. RANKL is preferentially produced by osteoblasts, and its specific receptor RANK, is expressed on osteoclasts. RANKL binds either to RANK, leading to osteoclast activation or to OPG, thereby preventing osteoclastogenesis. In this study, we evaluated whether or not CGRP anabolic activity on bone could be mediated by the OPG/RANKL/RANK system. We studied the effect of CGRP on OPG expression and secretion in primary cultures of human osteoblast-like cells (hOB) and the signalling pathway involved in this effect. We also evaluated if CGRP modulation of OPG secretion in hOB could affect osteoclastogenesis from human peripheral blood monocitic cells (PBMCs) either through hOB or directly. Our results show that hOB express a functional CGRP receptor composed by calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1). CGRP treatment of hOB ($1x10^{-11}$ $1x10^{-7}$M) dose-dependently inhibited OPG expression and secretion with an EC50 of $1x10^{-8}$M. This effects was prevented by treating the cells with the peptide antagonist, CGRP $_{8\text{-}37}$ ($1x10^{-8}$M). Forskolin, a stimulator of cAMP production, and dibutyryl cAMP similarly reduced the production of OPG. CGRP ($1x10^{-8}$M) enhanced protein kinase A (PKA) activity in hOB, and the preteatment of hOB with the PKA inhibitor, H89 ($2x10^{-6}$M), abolished the inhibitory effect of CGRP on OPG secretion. Treatment of PBMC with the conditioned media derived from CGRP treated hOB induced a significant increase in the number of multinucleated tartrate resistant acid phosphatase positive cells (TRAP +) and in the secretion of cathepsin K compared to the PBMC treated with the conditioned media of untreated hOB. Conversely treatment of PBMC with CGRP ($1x10^{-8}$M) induced a significant inhibition of the number of multinucleated TRAP + cells. These results show that CGRP is able to exert both a facilitatory and inhibitory action on osteoclastogenesis. It can be suggested that CGRP by acting on both cellular component of bone remodelling, contributes to the fine adjustment of this process, favouring the gain or loss of bone mass depending on the local demand.

FUNCTIONAL SYNAPTOPROTEOMICS OF HIPPOCAMPUS IN A RAT MODEL OF DEPRESSION WITH GENE-ENVIRONMENT INTERACTION

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Many evidences showed an important influence of stress factors in depression. Recent studies suggest an involvement of glutamatergic transmission in the pathophysiology of mood disorder and in mechanism of action of antidepressant drugs (AD) and AD have been shown to induce various effects beyond neurotransmitters receptors, such as adaptative changes in neuroplasticity. Aim of this study (that takes part in the European project GENDEP) is to examine the effects of stress and antidepressant treatment on molecular correlates of synaptic plasticity in an animal model of depression. The Flinders Sensitive Line (FSL) rats are a validated animal model of depression carrying genetic vulnerability associated to distinct features of pathology (1). Moreover, to reproduce early life stress events the FSL rats and their control, the Flinders Resistant Line (FRL) rats, were subjected to a standard maternal separation protocol (2). *Maternal separation and drug treatments.* FSL/FSL rats were subjected to maternal separation from postnatal day 2 to 14. Treatment with ESC (25 mg/kg/day) was carried out at weeks 6-10, by using dietary chow (2). *Preparation of synaptosomes*. Synaptic terminal (synaptosomes) were prepared from rat hippocampi by purification on Percoll gradient and differential centrifugation and ultracentrifugation (3). *Western Blot and Immunoprecipitation analysis.* Phosphorylation and protein expression levels were measured by SDS-PAGE and immunostaning. Using specific antibody, αCaMKII was immunoprecipitated and the amount of co-immunoprecipitated proteins were measured by SDS-PAGE and immunostainig (3). Groups of 8 FSL/FRL rats were subjected to maternal separation (MS) and chronic treatment with escitalopram (ESC). We used Western Blot analysis to assess NMDA-NR1-NR2A/2B expression levels, αCaMKII/NMDA receptor interaction, ERKs MAP kinases and synapsin-1 expression levels and activation in purified hippocampal synaptic terminal (synaptosomes). Data were obtained with at least six replicates per determination. Basal expression level of NMDA-NR1 subunit in synaptosomes of FSL was significantly lower (about 30%) compared with FRL and MS upregulated NR1 levels. Interaction between αCaMKII and NMDA-NR2A/B subunit was reduced (about 30%) in FSL rats compared with FRL, consistent with a lower synaptic NMDA receptor content. Furthermore, we found a marked increase (about 60%) between FSL and FRL in basal phosphorylation level of synapsin-1 (αCaMKII site), a protein involved in vesicle trafficking. ERK1/2 are stress-sensitive kinases and synapsin-1 is one of their substrate. Basal phosphorylation levels of ERK1/2 in synaptosomes of FSL were higher (almost 100% of augmentation) and MS failed to further increase kinases activation, contrary to the results obtained in FRL. Our results combined suggest a dysfunction of glutamate neurotransmission in FSL.

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EXPRESSION OF ARYL HYDROCARBON RECEPTOR (AHR)-REGULATED GENES IN THE BRAIN FROM CONTROL AND βNF-TREATED PIGS.

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In the last years, the finding that many anatomical, physiological and biochemical properties of domestic and miniature pigs are close to those of humans has made these animals an alternative to traditional large species in pharmacological and toxicological studies. However, our knowledge of metabolic functions in pig organs, especially brain, is limited. Therefore, we began a cytochrome P450 (CYP) characterization in various porcine cerebral regions (cortex, cerebellum, midbrain, hippocampus) and in blood-brain interfaces (meninges and brain capillaries). Herein, we report the expression of AhR, CYP 1A1, 1A2, 2S1 and ethoxyresorufin O-deethylase (EROD) and methoxyresorufin O-demethylase (MEROD) activities (markers of CYP 1A1/2) in the above mentioned regions and in livers obtained from 3 control and 3 β-naphtoflavone (βNF) i.p. treated pigs (30 mg/Kg βNF for 4 days). Brain regions and meninges were excised; then microsomes and mitochondria were immediately prepared according to Novi et al. (1) and Sims (2) respectively. Brain capillaries were prepared from frontal cortex according to Mrsulja et al. (3). mRNA was extracted from each tissue, treated with DNase, retrotranscribed and amplified by PCR. βNF treatment enhanced the expression of CYP 1A1 mRNA both in blood-brain interfaces and in the other cerebral regions examined. At variance, CYP 1A2 transcript was detected at low level only in brain capillaries and in the cortex after βNF treatment. The expression of 2S1 was never observed. As expected, AhR was expressed in every studied cerebral area and not modulated by βNF administration. At the activity level, βNF treatment enhanced EROD (about 5-10 times) in microsomes (not in mitochondria) of both capillaries and meninges but not of cortex, cerebellum, midbrain and hippocampus. Microsomal and mitochondrial MEROD activities were not modulated by βNF in any of the studied brain and blood-brain barrier regions. In the liver, βNF treatment strongly activated the expression of CYP 1A1, 1A2 and 1B1 mRNA and increased microsomal EROD and MEROD activities about 13-15 times with respect to control animals. In conclusion, we showed for the first time the enhancement by βNF of CYP 1A1 and/or CYP 1A2 expression and the induction of microsomal EROD activity in the brain capillaries and meninges of a mammal. The observed expression of CYP 1A1, 1A2 in some cerebral regions after βNF treatment is possibly too low to give rise to induction of marker activities such as MEROD and EROD.

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ROLE OF APOPTOSIS AND GENE EXPRESSION MODULATION IN THE SYNERGISTIC CYTOTOXICITY OF OXALIPLATIN AND PEMETREXED AGAINST COLON CANCER CELLS

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Oxaliplatin is a third generation platinum compound used in the treatment of colorectal cancer. The effect of oxaliplatin appears to be improved when it is combined with thymidylate synthase (TS) inhibitors. Pemetrexed is a new multitargeted antifolate that blocks folate metabolism and DNA synthesis, mostly by inhibiting TS. The aim of the present study was to investigate the molecular mechanisms underlying the cytotoxicity of oxaliplatin and pemetrexed combinations against human colon cancer cell lines characterized by different genotypes. HT29, WiDr, LS174T and SW620 cell lines were treated with oxaliplatin and pemetrexed, alone or in combination; pharmacologic interaction was studied using the combination index (CI) method, while cell cycle was investigated with flow cytometry. Moreover, the effects of drugs on Akt phosphorylation and apoptosis were studied with ELISA and fluorescence microscopy. RT-PCR analysis was performed to assess whether these cytotoxic agents modulated the gene expression of Nucleotide Excision Repair proteins (i.e. excision repair cross complementig group 1, ERCC1 and group 2, ERCC2) and pemetrexed target enzymes, including TS. Finally, genotyping of the Single Nucleotide Polymorphisms (SNPs) ERCC2 A751C and G312A and ERCC1 C118T was carried out using TaqMan® primers and probes designed with File Builder. Oxaliplatin was cytotoxic against HT29, WiDr, LS174T and SW620 cells, with IC_{50}s of 0.33±0.02, 0.13±0.01, 0.19±0.01 and 1.13±0.35 µg/ml, respectively. A dose-dependent inhibition of cell growth was also observed after pemetrexed treatment, with IC_{50}s of 5.10±0.42 (HT29), 1.14±0.15 (WiDr), 1.05±0.36 (LS174T) and 0.87±0.23 (SW620) µg/ml. The CI analysis showed synergism for simultaneous and sequential combinations. Flow cytometric studies demonstrated that oxaliplatin significantly enhanced cellular population in the S phase in HT29, LS174T and SW620 cells. Fluorescence microscopy revealed that drug combinations increased apoptotic cell death with respect to single agents; furthermore, cell exposure to these agents resulted in Akt phosphorylation inhibition in all cell lines ($P<0.05$). RT-PCR showed that pemetrexed reduced the expression of ERCC1 in HT29 and LS174T cells and strongly down-regulated ERCC2 in HT29, LS174T and SW620 cells ($P<0.05$), whereas oxaliplatin decreased the mRNA levels of pemetrexed target enzymes; in particular, TS expression was reduced in LS174T and SW620 cells ($P<0.05$). Finally, SNPs genotyping of cell lines suggested a correlation between ERCC1 and ERCC2 genotypes and chemoresistance to oxaliplatin. These data demonstrate that oxaliplatin and pemetrexed synergistically interact against colon cancer cells, through cell cycle modulation, inhibition of Akt phosphorylation, apoptosis induction and reduction of mRNA levels of crucial enzymes. Furthermore, our results underline the role of SNPs of select genes in sensitivity/resistance to chemotherapeutic agents.

EXPRESSION OF BETA-ADRENOCEPTORS IN VENTRICULAR MYOCYTES ISOLATED FROM HEARTS OF DIABETIC AND NORMOGLYCEMIC RATS: EFFECT OF A TREATMENT WITH LOSARTAN

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Diabetes is known to be associated with cardiac dysfunction. Alterations of cardiac adrenoceptors-mediated responses in diabetic cardiomyopathy is still a matter of debate. Diabetes may affect both β-AR mediated responsiveness (1) and β-AR density and/or affinity in cardiac tissue (2). Recent evidence (3) suggests that the blockade of the renin-angiotensin system ameliorates diabetes-induced cardiac dysfunction: we investigated the effect of a treatment with losartan on the expression of β-AR in ventricular cardiomyocytes (VM). We measured the density of β_1- and β_2-AR in VM isolated from hearts of normoglycemic and diabetic rats treated and untreated with the AT1 antagonist losartan. Diabetes was induced by a single injection of streptozotocin (STZ) in the tail vein of male Wistar rats. Losartan was dissolved into the drinking water and its concentration adjusted to maintain a daily dosage of 20 mg/Kg for three weeks. Rats were divided into four groups: untreated and treated normoglycemic rats (CTR and CTR_{LOS}), untreated and treated diabetic rats (DIA and DIA_{LOS}). Binding assays were performed on membranes from VM isolated from hearts using a protocol based on previously described procedures (4). In homologous competition curves, β-AR were labelled with the non specific β-AR antagonist $[^3H]$-CGP12177. In heterologous competition curves, fixed concentrations of the tracer (0.3 nM) were displaced by increasing concentrations of CGP-26505, a selective β_1-AR antagonist. After three weeks, DIA rats showed all the typical metabolic parameters of the hyperglycemic condition, and the treatment with losartan did not modify any of these parameters. The number of β-AR (fmol/mg protein) was reduced from CTR to DIA rats and the treatment with losartan tends to restore the number of β-AR to normal values. Competition for $[^3H]$-CGP 12177 binding from CGP 26505 resulted in a biphasic curve, demonstrating two classes of β-AR binding sites. The proportion $\beta_1:\beta_2$ did not change sensitively in myocytes derived from treated and untreated rats Our results demonstrate that the β-AR density is reduced in diabetic myocytes and losartan tends to restore the number of β-AR to the normoglycemic levels. These findings are in agreement with our previous data (5) that suggest that the blockade of angiotensine II receptors may contribute to improve the cardiac β adrenergic pathway, which is typically depressed in diabetes.

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CHARACTERIZATION OF THE PHATOLOGICAL EVENTS OCCURRING SPONTANEOUSLY IN THE KIDNEY OF STROKE-PRONE RATS

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Spontaneously hypertensive stroke-prone rats (SHR-SP) represent a unique animal model that spontaneously develops severe hypertension, with subsequent renal and brain disorders and early death. In SHRSP, besides elevated blood pressure, genetic factors, abnormalities in the renin-angiotensin system, and an elevated salt intake have also been recognized as important factors in the pathogenesis of renal lesions. In fact, Spontaneously Hypertensive Rats (SHR), a closely related strain, develops similar degree of hypertension without presenting renal and cerebral damage. In SHRSP the pathological events occurs spontaneously in one year but the lag time is reduced if rats are exposed to a specific permissive diet, low in protein and high in sodium (Japanese diet, JPD). This strain provides an opportunity to disentangle the complexity of the process that occurs during the development of renal failure and, eventually, to test the potential of renal protective therapy. The aim of the present study was to characterize the chain of events occurring in the kidneys of SHRSP to identify possible target for pharmacological treatment. At six weeks of age the rats were placed on JPD, and weekly monitored for body weight and blood pressure. Serum and 24-h urine were recorded before the onset of JPD and every seventh day afterwards. The animals were sacrificed at different stage of proteinuria (40, 100 and 270 mg/day) and the kidney collected for the molecular and immuno-histological analyses. Some animals sacrificed at the start of dietary treatment represent the basal group. The SHRSP subjected to dietary treatment develop hypertension and proteinuria characterized by the accumulation in the urine of acute-phase proteins. Kidneys from animals sacrificed at different times during JPDtreatment exhibited progressive fibrocellular proliferative lesions, particularly in the glomeruli, and a disorganisation of the renal parenchyma. The arteries showed marked hyperplasia/proliferation, a so-called onion-skin lesion. The immuno-histochemical analysis showed a massive inflammatory cells infiltration around the arteries and the tubules. Most of these infiltrating inflammatory calls were positive for ED-1, a marker of macrophages derived from circulating monocytes. These rats develop also a massive fibrosis caractherized by collagen and fibrinogen deposition and by the accumulation of alpha-smooth muscle-positive myofibroblasts, the main source of the interstitial matrix proteins. Expression of MCP-1, TGFbeta1 and IL-1beta mRNA, as evaluated by RT-PCR accumulated drammatically in the kidneys of rats subjected to dietary treatment. These data indicate as the SHRSP represent an animal model in which the inflammation, both a systemic than local level, play a pivotal role in the pathological events occurring in different organs and in particular at renal level. This rat strain provides a useful tool to answer the question of whether or not anti-inflammatory strategies may affect the genesis, progression, and outcome of tissue damage.

IN VITRO AND *IN VIVO* ANTIANGIOGENIC AND ANTITUMOUR EFFECTS OF METRONOMIC CERAMIDE ANALOGS

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Low-dose metronomic chemotherapy is a novel approach to the control of cancer, working primarily through antiangiogenic mechanisms and significantly reducing toxic side effects.
Among promising anticancer drugs, ceramide analogs have been investigated as apoptotic inducers and proliferation inhibitors of tumor cell lines[1], but at concentrations too high to justify their development as antiproliferative agents. To evaluate the antiangiogenic/antitumour activity of low-dose metronomic ceramide analogs (e.g. AL6 and C2) and to investigate the modulation of pro- and anti-angiogenic factor expression due to the low-dose schedules in the preclinical setting. Human endothelial cells (HMVEC-d and HUVEC) and pancreas cancer cells (MIA PaCa-2) were treated at low concentrations (0.01-100 nM) using a continuous (144 h) drug exposure protocol[2] to evaluate the antiproliferative effects (expressed by experimental IC_{50}s), the inhibition of migration, the modulation of gene expression of pro- (VEGF) and antiangiogenic (TSP-1) factors by real time-PCR and their secretion with analysis of conditioned media by ELISA kits. *In vivo* experiments were conducted on s.c. MIA PaCa-2 xenografts in nu/nu mice that were treated for 42 days with i.p. 0.5 mg/kg/day C2 or AL6, and gemcitabine at the maximum tolerated dose (MTD), to evaluate the inhibition of tumour growth. AL6 inhibited selectively the proliferation of HMVEC-d and HUVEC (IC_{50}=4.85±3.14 and 4.37±1.30 nM, respectively; mean±SD) whereas MIA PaCa-2 growth was inhibited at concentrations higher than 100 nM; similarly, a superimposable antiproliferative profile was obtained with C2 (HUVEC IC_{50}=6.21±3.05 nM; HMVEC-d IC_{50}=4.89±3.6 nM and MIA PaCa-2 IC_{50}>100 nM). Moreover, both low concentrations of AL6 and C2 showed a significant inhibition of HUVEC cell migration (24.33±1.45% and 32.67±2.03% of migrated cell/image area vs. controls, respectively; $P<0.05$). HUVECs highly expressed TSP-1 gene after protracted administration of an AL6 concentration equal to the previously obtained IC_{50} (170% vs. 100% of controls); interestingly, also the VEGF expression was increased after IC_{50} administration (236% vs. 100% of controls). TSP-1 was secreted into the media at higher amounts in treated endothelial vs. control cells (198.3% vs. 100% of controls with AL6 100 pM). In contrast, VEGF secretion did not change in conditioned media. At the end of the *in vivo* experiment (42 days), metronomic ceramide analogs C2 and AL6 determined a significant inhibition of tumour growth of 49.9% and 31.0% ($P<0.05$) compared to controls, respectively, whereas MTD gemcitabine an inhibition of 21.4%. No signs of toxicity were recorded in ceramide analog treated animals, whereas gemcitabine determined a significant weight loss. Metronomic low dose ceramide analogs showed selective anti-endothelial effects and induced significant differences in the modulation of TSP-1 and VEGF expression. Moreover, these results seem to confirm the key role of TSP-1 in the selective antiproliferative activity of metronomic schedules. Furthermore, *in vivo* studies have demonstrated the antitumour activity of metronomic C2 and AL6 in absence of toxicity.

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$P2X_7$ RECEPTORS IN PURIFIED RAT CEREBRAL CORTEX SYNAPTOSOMES: PHARMACOLOGICAL CHARACTERISATION

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To investigate on localization, function and pharmacological characterization of purinergic $P2X_7$ receptor on rat cerebrocortical nerve terminals, in the present study glutamate release, calcium accumulation in single synaptosomes, immunohistochemistry and western blotting analysis were carried out in purified synaptosomes. Purified synaptosomes were prepared from cerebral cortex of adult male Sprague-Dawley rats, incubated with $[^3H]$D-aspartate (0.03 μM) and transferred in superfusion system. Synaptosomes were exposed to ATP or 2'-3'-O-(benzylbenzyl)-ATP (BzATP), and the effects of the $P2X_7$ antagonists oxidized ATP (oATP) or Brilliant Blue G, of the adenosine antagonists CGS 15943, DPCPX or SCH58261 or of extracellular Mg^{2+} on the release of $[^3H]$D-aspartate and endogenous glutamate were evaluated. Western blotting analysis was carried out on lysated synaptosomes or native and stably expressing GFP-tagged $P2X_7$ HEK293 cells with an anti rat $P2X_7$ polyclonal antibody. Confocal microscopy was carried out on synaptosomal preparation. The following antibodies were used: mouse anti-syntaxin-1, guinea pig anti-VGLUT1, rabbit anti-$P2X_7$. For calcium imaging, synaptosomes were loaded with FURA-2-AM and the fluorescence ratio F340/F380 via microfluorimetric technique was used to monitor $[Ca^{2+}]_i$ changes. The presence of $P2X_7$ receptors in cerebrocortical synaptosomes and in HEK293 was verified by Western blot. Confocal analysis shown that a subpopulation of synaptosomes was glutamatergic (63.3±3.4% of syntaxin-1 positive synaptosomes) and expressed $P2X_7$ receptors (36.3±3.2% of GLUT-1 positive synaptosomes) (mean±S.E.M; n=6 different images). The glutamatergic nerve endings represented 55.4 ±2.5% of the total number of $P2X_7$ positive synaptosomes, indicating that $P2X_7$ receptor was also localised in non-glutamatergic cerebrocortical nerve endings. Collectively, these data indicate that $P2X_7$ receptors are expressed in a neuronal subpopulation estimated in about 42%. Activation of $P2X_7$ receptors by Bz-ATP evoked intracellular calcium accumulation in about 45% of single synaptosomes. The values of the maximal increase from the basal level of F340/F380 ratio were 0.150±0.025 and 0.027±0.009 in presence of KCl 40 mM and BzATP 100 μM, respectively (n=155 determinations from 8 independent experiments). ATP or BzATP increased $[^3H]$D-aspartate efflux from synaptosomes in a concentration-dependent and extracellular Mg^{2+}-sensitive manner. oATP (300 μM) abolished the effects of Bz-ATP (100μM, ≅ 90% inhibition) or ATP (1-3 mM, ≅ 80% inhibition); Brilliant Blue G (1 μM) reduced effects of Bz-ATP (100 μM) or ATP (1-3 mM) (n=3 to 9 experiments). DPCPX (1 μM) SCH58261 (0.1 μM) or CGS15943 (1 μM) were ineffective on the ATP-evoked $[^3H]$D-aspartate or endogenous glutamate efflux (n=3 independent experiments). In conclusion, we obtained direct evidence for the presence of functional $P2X_7$ receptors on rat cerebrocortical glutamatergic nerve terminals, their activation evoking calcium influx and glutamate efflux. In particular, the main results of our work are: $P2X_7$ receptors linked to intracellular calcium elevation are present on subpopulation(s) of adult rat cerebrocortical nerve terminals; immunofluorescence quantification of the nerve terminals expressing the $P2X_7$ receptor fits in well quantification of the nerve terminal subpopulation carrying functional $P2X_7$ receptors linked to intracellular calcium accumulation; $P2X_7$ receptors linked to glutamate release excitation are expressed in a subpopulation of glutamatergic nerve terminals.

EXOCYTOTIC GLUTAMATE RELEASE EVOKED BY MILD DEPOLARIZING STIMULI IN GLIA PARTICLES FRESHLY PREPARED FROM ADULT RAT BRAIN AND IN ADULT ASTROCYTES CULTURES

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There is now a general agreement that glial cells respond to receptor-mediated increase of intracellular Ca^{2+} by releasing gliotransmitters which can mediate communication between glial cells and between glial cells and neurons. We have recently shown that glia particles acutely purified from the adult rat brain (gliosomes) can exocitate glutamate when subjected to non-depolarizing stimuli able to increase intracellular Ca^{2+} (1). We present here evidence for mild depolarization-evoked glutamate release from gliosomes. Gliosomes and synaptosomes were purified from cerebral cortex of adult rats on discontinuous Percoll® gradient (2). Release experiments were performed either measuring the radioactivity released by gliosomes labelled with [^{3}H]D-aspartate ([3H]-D-ASP) or the endogenous glutamate release. Confocal microscopy images were collected using Leica DM IRE inverted microscopes endowed with 100x/NA1.4 objective. Western blots were performed according to Laemmli (3). Primary cultures of cortical rat astrocytes were prepared both from 2-day-old pups or adult (60-80 day-old) rats. Western blot and confocal mycroscopy experiments with selective glial and neuronal markers indicated that gliosomes were poorly contaminated by synaptosomes and vice versa. Depolarization with KCl, 4-aminopyridine or veratrine increased endogenous glutamate or [^{3}H]D-ASP release from gliosomes. The release was Ca^{2+}-dependent, although part of the KCl-and veratrine-evoked overflow occurred by reversal of glutamate transporters. KCl increased membrane potential and cytosolic Ca^{2+} in gliosomes dependently on external Ca^{2+}. Both intracellular Ca^{2+} increase and glutamate release were sensitive to KBR7943, an inhibitor of the Na^{+}/Ca^{2+} exchanger but not to voltage sensitive Ca^{2+} channel blockade. Western blot analysis revealed that gliosomes contain proteins of the exocytotic machinery. KCl increased cytosolic Ca^{2+} and glutamate release from adult, but not neonatal, astrocytes in a Ca^{2+}-dependent and KBR7943-sensitive manner. Conditioning adult astrocytes with neurons enhanced their sensitivity to depolarization. It appears that gliosomes can release glutamate by a process resembling depolarization-linked neuronal exocytosis, triggered by Ca^{2+} entry through the Na^{+}/Ca^{2+} exchanger working the reverse mode. This characteristic seems to be related to their derivation from adult, *in situ* matured, astrocytes.

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EFFECTS OF DEFECTIVE HERPES SIMPLEX VECTORS EXPRESSING FGF-2 AND BDNF ON NEUROGENESIS IN VITRO AND ON PILOCARPINE EPILEPTOGENESIS IN VIVO

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Neurotrophic factors (NTF) are involved in the survival, as well as in the proliferation and differentiation of neurons from their precursors. Modulating NTF expression in lesion areas may thus represent a new approach for the therapy of seizure-induced damage (1, 2). We examined the effects of recombinant herpesvirus-based vectors (3) capable of expressing combinations of NTFs on survival, proliferation and differentiation of freshly isolated neural progenitors. Homotypical aggregates of neural progenitors were obtained from postnatal day 13 rat brains using density gradients. We named these aggregates “proneurospheres” because they can generate clones (spheres) from single precursors. Initially, we proved that, by using vectors expressing FGF-2 and CNTF together, it is possible to prompt substantial proliferation of nestin- and BrdU-positive progenitors, but most of the new cells generated by infection with these vectors display an astrocytic phenotype (4). We then generated and tested a second HSV vector, expressing a combination of FGF-2 and BDNF. Vectors expressing these NTFs together increase survival and proliferation of proneurospheres, and most of the new cells differentiate into neurons, based on positive labelling for MAP-2 and β-tubulin III. These new vectors are currently tested *in vivo* , in the pilocarpine model of status epilepticusinduced neurodegeneration and epileptogenesis. In this animal model we hope to augment the regenerative capacity of endogenous neural stem cells and/or progenitors in the hippocampus following seizure-induced damage by employing NTFs. For this reason we infuse our vectors in hippocampal area with a mini-invasive technique. We use, under a stereotassic control, patch-clamp glass needles, connected to an infusion pump to reduce the mechanical lesion. Preliminary data suggest that, when injected in the hippocampus 3 days after status epilepticus, FGF-2/BDNF expressing vectors reduce cell loss and partially prevent the occurrence of spontaneous seizures (5).

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DIFFERENTIAL EFFECT OF CHRONIC RENAL FAILURE ON THE PHARMACOKINETICS OF LIDOCAINE IN PATIENTS RECEIVING AND NOT RECEIVING HEMODIALYSIS

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The effect of chronic renal failure (CRF) on the pharmacokinetics of lidocaine, a drug cleared almost exclusively by hepatic metabolism, has been thus far evaluated only in patients on regular hemodialysis. This study had two objectives: 1) to investigate the effect of CRF on the pharmacokinetics of lidocaine in patients both undergoing and not undergoing hemodialysis; 2) to test the effects of plasma from the patients examined and of lidocaine metabolites possibly accumulated *in vivo* on lidocaine biotransformation *in vitro*. Methods: In a clinical investigation, we studied the kinetics of lidocaine and its metabolites, monoethylglycinexylidide (MEGX) and glycinexylidide (GX), after i.v. injection of 1 mg/kg lidocaine in 15 healthy volunteers (creatinine clearance per 1.73 m^2 [CL_{cr}] > 80 mL/min), 10 subjects with moderate renal insufficiency ($30 \leq CL_{cr} \leq 60$ mL/min), 10 subjects with severe renal insufficiency ($CL_{cr} < 30$ mL/min), and 10 functionally anephric patients on chronic hemodialysis. In experiments *in vitro*, we determined the effects of plasma and GX on the formation rate of the primary lidocaine metabolite, MEGX, by human liver microsomes. Results: In patients not undergoing hemodialysis, lidocaine kinetic parameters were altered in proportion to the degree of renal function impairment, but only in patients with severe renal insufficiency were differences clinically relevant: clearance was about halved with respect to controls (mean±SD 6.01±2.54 vs. 11.87±2.97 mL/min·kg; $P < 0.001$) and half-life was approximately doubled (4.55±1.71 vs. 2.24±0.55 h; $P < 0.001$). No such alterations were observed in patients on regular hemodialysis, whose values were similar to those of the control group. Steady-state volume of distribution and MEGX levels were independent of renal function, whereas GX levels were more than doubled with respect to controls ($P < 0.05$) in all CRF groups. No inhibitory effect of plasma, from any of the subjects examined, was observed on lidocaine biotransformation *in vitro*. GX was found to be a competitive inhibitor, but its K_i value (52 ± 4 μmol/L) was two orders of magnitude higher than its concentrations *in vivo* . Our *in vivo* findings have both clinical and methodological implications: 1) lidocaine dose adjustment may be required in patients with severe renal insufficiency not receiving hemodialysis; 2) results of studies evaluating the effect of CRF on metabolic drug disposition are not of general validity, unless patients both undergoing and not undergoing hemodialysis have been examined. Our *in vitro* observations exclude that impairment of lidocaine disposition is the result of direct inhibition of metabolizing enzymes by accumulated metabolites or uremic toxins. Inhibition of hepatic cytochrome P450 expression by dialyzable circulating factor(s) is a possible explanation consistent with the results of recent *in vitro* studies.

ENDOCANNABINOID AND ENDOVANILLOID SYSTEM IN CEREBRAL ISCHEMIA

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Endocannabinoids exert neuroprotective functions in several models of neurotoxicity via cannabinoid CB1 and vanilloid VR1 receptors (1-4). As soon as anandamide is released, the diffusion process is accelerated by a rapid and selective carrier system (5). The development of a series of anandamide transport inhibitors, to slow its elimination and to magnify its beneficial effects can provide a new tool to investigate the role of endocannabinoids (6). To further investigate the role of endocannabinoid and endovanilloid system against neuronal injury *in vivo*, we injected exogenous compounds such as the cannabinoid agonist Δ^9-tetrahydrocannabinol (THC) (0.05-2 mg/kg/i.p.), and the vanilloid agonist capsaicin (0.01-0.6 mg/kg/s.c.). The role of CB1 and VR1 receptors was investigated after treatment with rimonabant (0.05-3 mg/kg/i.p.) capsazepine (0.01-10 mg/kg/s.c.) respectively. Finally, the effect of the anandamide transport inhibitor AM404 (0.03-2 mg/kg/i.p.) was studied. To quantify the ischemic damage we measured from 1 h to 7 days after recirculation, electroencephalographic (EEG) mean total spectral power, spontaneous motor activity, cognitive function, rectal temperature and hippocampal neuronal count. These compounds showed a protective effect (versus vehicle treated group) against hyperlocomotion on day 1, evaluated in an “activity cage” ($p<0.01$), memory impairment (passive avoidance test) on day 3 ($p<0.05$) and EEG flattening ($p<0.01$) and neuronal loss ($p<0.01$) on day 7. THC showed a maximal neuroprotective effect at a dose of 1 mg/kg. Rimonabant and capsazepine partially antagonised this effect. The protective effect obtained with capsaicin (0.2 mg/kg) was fully reversed by capsazepine. Surprisingly, at high dose, rimonabant and capsazepine *per se* showed a protective effect. Finally, AM404 protected against ischemic damage (2 mg/kg) with a mechanism involving VR1 receptor. Taken together, these results showed that endocannabinoid and endovanilloid systems are closely connected suggesting that the modulation of AEA levels plays an important role in mechanisms underlying neuroprotection.

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POSSIBLE MECHANISMS OF ACTION ON DNA OF MEGAZOL, A TRYPANOCIDAL NITROIMIDAZOLE.

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The nitroimidazole-tiadiazole derivative CL 64855 (Megazol; MZ) could represent a promising alternative to drugs currently used for Chagas disease treatment, also taking into account the resistance development in *Trypanosoma cruzi*. A better understanding of drug action is necessary in order to minimise damage in host cells. In this context, MZ genotoxicity was assessed by different Comet assay protocols to investigate possible action mechanisms for DNA damaging. Fresh human leukocytes, previously treated for 5 or 60 min, at 5 or 37 °C with MZ (0, 1, 2, 4, 8 µg/ml), were analysed by the Comet assay (1), performed at pH=12.1, to measure single and double DNA strand breaks (SB) and at pH>13, to measure also alkalo-labile sites (ALS). DNA specific oxidative damage was detected by using Endo III (2). Each experimental point represents the mean (±sd) of three independent treatments. Regression analysis and Dunnet's C test were applied to the experimental data. The slope of DNA migration increase in dependence of MZ concentration is reported in Table 1 in relation with the cell treatment type and the different Comet assay condition. For the 1h, 37 °C treatment, the increase is greater at pH>13 than pH=12.1 ($p<0.05$): MZ is able to produce DNA SB lesions detectable at both pH levels, as well as ALS, but only detectable at the pH >13. The presence of oxidized pyrimidines was better detected ($p<0.05$) by using ENDOIII at pH=12.1 than at pH>13. Moreover, DNA oxidative stress could be partially detected in terms of alkalo-labile sites, at pH>13 without enzyme. The treatment was also performed on cells maintained on ice (5 min or 1h) to minimise the cellular mechanisms of DNA damage. In cells treated at low temperature (0 °C), no evident genotoxic effects induced by short-time MZ exposure (5 min) were detected (pH>13 or pH=12.1), whereas a significant increase was observed when increasing the incubation time (1h).

Table 1 *Cell pH=12.1 pH>13 pH=12.1 pH>13 Treatment +ENDOIII +ENDOIII*

Cell Treatment	pH=12.1	pH>13	pH=12.1 +ENDOIII	pH>13 +ENDOIII
1h, 37°C	0.65 (r^2=0.82)	0.85 (r^2=0.85)	1.41 (r^2=0.93)	0.80 (r^2=0.73)
1h, 0°C	0.45	(r^2=0.59)	1.66	(r^2=0.89)
5', 0°C	0.01 (r^2=0.00)	0.04 (r^2=0.01)	1.56 (r^2=0,71)	1.42 (r^2=0.72)

These results may suggest that the excess of SB, observed when the treatment was performed at physiological temperature, could derive from the first step of excision repair, whereas only DNA SB directly induced by MZ were detectable at the low temperature treatment. For 5 min, 0 °C treatment, the sensitivity was strongly increased when DNA is digested with ENDOIII with a clear dose-response relationship for both the pH levels. The data obtained after the short exposure are similar to those detected for long exposure. MZ can act with at least two timedependent mechanisms: an early oxidation of DNA bases and a later breaking of DNA strands irrespective of the treatment temperature. Oxidative stress seems to represent the main mechanism of DNA damage by MZ in human leukocytes.

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RALOXIFENE REDUCES INDUCIBLE NO SYNTHASE FORMATION AND INDUCES RAPID VASORELAXATION IN ISOLATED RAT AORTIC TISSUES

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The vascular wall is clearly one of the target organs of estrogens (E_2). Selective estrogenreceptor modulators (SERM) have the potential to retain most of the beneficial effects of estrogen while avoiding most of its adverse effects (1). Raloxifene is a SERM approved for the prevention and treatment of postmenopausal osteoporosis and has also been shown to have a favorable effect on inflammatory cardiovascular risk markers. A number of studies have shown that raloxifene acutely relaxes different arterial and venous vessel but the underlying mechanisms are unclear. We investigated the vascular effects of raloxifene using a dual approach. First, experiments were performed in isolated rat aortic smooth muscle cells (SMC) to test potential antiinflammatory effects following long-term treatment. Second, acute vasomotor effects were tested on aortic rings from estrogen-replaced ovariectomized (OVX) rats. The contribution of estrogen receptors (ER) to raloxifene action was assessed using the selective ERα antagonist MPP [1,3-bis (4-hydroxyphenyl)-4-methyl-5-(4-(2-piperidinylethoxy)phenol)-1H-pyrazole]. Inducible NO synthase (iNOS) and ERα proteins were detected by Western blotting in SMC stimulated with a cytokine mixture comprising IL-1β (10 ng/ml), TNF-α (25 ng/ml) and INF-γ (10 ng/ml) plus 1 μg/ml LPS for 24 h. Raloxifene was added at the same time as cytokines, while MPP was added 30 min before. The activity of iNOS was determined by the Griess' reaction in culture medium samples. Cumulative concentration-response curves for raloxifene and E_2 were obtained in aortic rings from estrogen-replaced OVX rats precontracted with noradrenaline (EC_{60}). After stimulation with cytokines for 24 h, iNOS synthesis became detectable in aortic SMC but was significantly reduced by incubation with raloxifene at concentrations greater than 0.1 μM. Similarly, raloxifene treatment significantly decreased cytokine-driven iNOS activity at the highest concentration tested of 1 μM ($p<0.05$). ERα protein expression was reduced by cytokines/LPS by more than 70% but was partially restored on treatment with increasing concentration of raloxifene. The negative regulation of iNOS by the drug was mediated by ERα as shown by MPP preatment. By contrast, preincubation of aortic rings with MPP had no effect on the rapid vascular relaxation evoked by raloxifene. For comparison, MPP reduced the relaxant responses to E_2, which was significantly stronger than that to raloxifene. This discrepancy was not related to variations in circulating E_2 levels, which were identical in all animals tested. In conclusion, raloxifene prevented cytokine-induced iNOS activation in isolated aortic SMC and relaxed aortic tissues of E_2-replaced OVX rats following acute administration with mechanisms different from E_2. These effects were raised at concentrations that may be relevant *in vivo* and involved at least in part ERα activation.

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CENTRAL AND PERIPHERAL ACTIVITY/EFFECTS OF UFP-112, A NOVEL POTENT AND SELECTIVE PEPTIDE AGONIST FOR NOCICEPTIN/ORPHANIN FQ RECEPTORS, ON *IN VIVO* GASTROINTESTINAL FUNCTIONS IN THE RAT

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UFP-112 ([(pF)$Phe^{4}Aib^{7}Arg^{14}Lys^{15}$]N/OFQ-$NH_2$), is a novel peptide ligand for the nociceptin/orphanin FQ (N/OFQ) peptide receptor (NOP). It has been generated by combining into the N/OFQ sequence four chemical modifications ([$Arg^{14}Lys^{15}$], [Aib^{7}], [(pF)Phe^{4}], and C terminal amidation) previously reported to increase N/OFQ potency. *In vitro* data obtained electrically stimulated isolated tissues demonstrated that UFP-112 behaved as a full agonist at NOP receptor approx. 100 fold more potent than N/OFQ. In the present study, we compared the effect of N/OFQ and its analogue UFP-112 on *in vivo* gastrointestinal functions (gastric emptying, colonic propulsion and gastric acid secretion), after central and peripheral administration in rats. When injected intracerebroventricularly, UFP-112 (10-250 pmol/rat) mimicked the effects of N/OFQ, inducing a dose related delay in the gastric emptying of a phenol red meal and a decrease of gastric acid secretion in water loaded rats after 90 min pylorus ligature; in both cases UFP-112 was approx. 100 fold more potent than N/OFQ, more effective and produced a longer lasting effect up to 120 min. UFP-112 (2.5-250 pmol/rat) also mimicked the effect of N/OFQ (0.01-10 nmol/rat) on colonic propulsion producing a dose response increase of the mean bead colonic expulsion time 10 fold higher than that induced by N/OFQ. Moreover the inhibitory effects of UFP112 on these gastrointestinal functions were not affected by naloxone and competitively blocked by the selective NOP antagonist, UFP-101. When injected intraperitoneally, UFP-112 (10-250 pmol/rat) produced a clear but not dose related stimulatory effect on gastric acid secretion and was 20-40 fold more potent than natural peptide, while showed no effect on gastric emptying. UFP-112 (2.5-100 pmol/rat) mimicked the action of N/OFQ (2.5-100 pmol/rat) on colonic propulsion producing an increase of the mean bead colonic expulsion time, with the same potency, but a longer lasting effect (more than 3 h). Collectively, these findings demonstrate that UFP-112 behaves in *in vivo* gastrointestinal functions as a highly potent ligand able to produce full and long lasting activation of NOP receptors.

MODULATION OF ENDOTHELIAL CELL FUNCTIONS FOLLOWING THE INTERACTION WITH BIOMATERIALS

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Vascularization, or angiogenesis, is an important process involved in development, growth and functioning of all tissues. Angiogenesis is a complex multistep event that includes migration, proliferation and differentiation of endothelial cells to form new sprouts from pre-existing vessels. A controlled angiogenesis process is fundamental for transplantation and reconstructive surgery with synthetic biomaterials, since vascular endothelial cell involvement occurs at every stage of biomaterial/tissue interaction. Thus it is important to study the biocompatibility of biomaterials for endothelial cells. In the last decade nanotechnologies have been under development as an innovative approach for the modification of biomaterials to be used in orthopaedic and cardiovascular surgery. The biocompatibility of nanostructured materials for vascular endothelium has been studied by using Coronary Venular Endothelial Cells (CVEC). The biomaterials have been characterized for the ability to mantain/improve endothelial proliferation (by MTT cell proliferation assay) and endothelial morphology (by optic microscopy, scanning electron microscopy (SEM) and immuno-histochemistry analysis) after different time of exposure to sterilized biomaterials. The involvement of biochemical pathways, endogenous factors and adhesion molecules important for the angiogenic process has been investigated by RT-PCR, Western blotting and immunofluorescence analysis (1). In particular the results obtained with nanocrystals of hydroxyapatite (HA) (commonly used as prosthetic material for bone replacement) documented their ability to preserve the angiogenic phenotype of capillary endothelium. Low HA concentrations (2-10 μg/ml) promoted cell survival and cell proliferation. Moreover, at these concentrations, HA contributed to retain endothelial morphology, which was confirmed by $\alpha_v\beta_3$-integrin expression and localization and by beta-actin organization. Investigation of key signalling pathways in angiogenesis (i.e. endothelial nitric oxide synthase (eNOS) and fibroblast growth factor-2 (FGF-2)) demonstrated that low concentration of HA increased their expression and activity, respectively. Furthermore, endothelial cells exposed to HA were more responsive to vascular endothelial growth factor (VEGF) in terms of Akt phosphorylation and NOS activity, and increased their ability to differentiate in to capillary-like structures when grown in 3D fibrin gel. In conclusion, synthetic preparation can be prepared integrating nanostructured HA in complex matrices to stimulate angiogenesis required for a finely controlled osteogenesis.

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A PROTEOMIC APPROACH TO IDENTIFY MOLECULAR MECHANISM REGULATING DENDRITIC SPINES MORPHOLOGY

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Synaptic activity regulates and promotes dendritic spines formation and maturation but the molecular mechanisms involved in these processes are still not well characterized. In this study we have developed a new proteomic approach, based on a conservative subcellular fractionation, to reveal changes in protein synthesis, degradation and post-translational modifications in hippocampal neuron cultures where a change in spines number and morphology has been induced by long-term stimulation/depression of synaptic activity. Experiments were performed on neurons fractionated by sequential exposition to different detergent-containing buffers after long-term treatment with Bicuculline or TTX. Four proteins fractions for each condition have been isolated and differentially analyzed by 2D-SDS-PAGE maps and MALDI-TOF. This analysis revealed how long-term stimulation/depression of synaptic activity regulates cell metabolic status, cytoskeletal dynamic, local protein synthesis, mitochondrial distribution, actin network organization and protein metabolism. Further studies were dedicated to dissect the contribute of translation machinery in neuron activity; a siRNA approach enlightened the key role of elongation factors in regulating spine shape and function. Interestingly we have also fond that phosphorylation of eEF2 by eEF2K/CamKIII can link glutamate receptors activation to an increase of local dendritic proteins synthesis required to induce spines maturation. .

VASCULAR ENDOTHELIUM AS A TARGET FOR ENDOGENOUS OUABAIN

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Treatment of malignant neoplasias is based on cytotoxic drugs which are not able to distinguish between the malignant cell and the normal one. In order to allow to treat exclusively the tumoral cells in a selective way, it's been studied hyaluronan acid (HA) as a vehicle: it's a biological compound of the extracellular matrix and it binds to CD44, a receptor overexpressed on tumoral cells. HA binds also to another receptor, RHAMM, which mediates cell motility. Several bioconjugates (BCs), characterized by an antineoplastic agent (FA) linked to HA and by different degrees of substitution, have been tested both in vitro an in vivo. The research has been based on HA molecules conjugated to an antitumoral drug which we'll call FA, because of a secrecy agreement we have with the pharmaceutical industry we're working with. First we tested CD44 expression and the compounds' cytotoxicity on several human cell lines. Later we checked bionconjugates' tolerability and efficacy on mice bearing TLX5 lymphoma, B16-F10 murine melanoma and on nude mice bearing human breast cancer MDA-MB-231. Finally, it was tested the effect of the treatment with HAs characterized by different molecular weight (HA150kDa and HA16kDa) and doses (from1 to 0,062mg/ml), on RHAMM expression on human multiple myeloma cell line RPMI8226. All the cell lines taken into consideration express CD44. The in vitro results show that the BCs don't increase the drug's cytotoxicity while in vivo results show that they are more toxic respect to FA alone. The treatment of mice bearing TXL5 lymphoma with HA-FA cause a statistically significant reduction of tumour growth and an increase of life-time expectancy. HA-FA is also effective on B16F10 melanoma where it causes the complete disappearance of liver metastases while on xenotransplant the treatment significantly reduces the primary tumour growth and it increases the life-time expectancy. These datas are statistically significant compared to the effect checked by the only FA. Finally, the treatment on RMPI8226 with HAs doesn't cause any variation on RHAMM expression and it doesn't cause apoptosis. HA-FA BCs present interesting antitumoral characteristics: in fact, even if they have a lower activity in vitro, in in vivo systems they show a therapeutic efficacy higher if compared to the antitumoral drug alone. On the other hand, the treatment with the only HA doesn't cause any variation on RHAMM expression and function.

PURIFICATION OF L19-CYTOKINE FUSION PROTIEN, A POSSIBLE FUTURE RECOMBINANT MONOCLONAL ANTIBODY FOR CANCER TREATMENT

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Angiogenesis is a characteristic feature of many aggressive tumours and of other relevant disorders. Fibronectin (FN) is a large glycoprotein that is present in large amounts in the plasma and tissues. The Extra Domain B (ED-B) of fibronectin is a sequence of 91 aminoacids identical in mice, rats and humans, which can be either inserted or omitted in the FN molecule through a mechanism of alternative splicing. While ED-B is undetectable in healthy adult individuals, it accumulates around neovasculature structures and could represent a target for molecular intervention (1). The human recombinant antibody L19 single chain Fv displays a high binding affinity to ED-B (2) and it has been shown to selectively target neovasculature in animal models of angiogenesis-related diseases and in patients with cancer. When L19 is fused with cytokine having antitumour activity, such as IL2 or IL12 and TNF-α, a dramatic improvement of the therapeutic index is expected. In this work the overall L19-cytokine fusion protein manufacturing process is described, with particular attention at the level of the chromatographic purification steps of the fusion protein and contaminant removal. This protein is a recombinant fusion protein produced in a murine hybridoma cell line and cultured in biofermenters. The cell line used for the its expression is SP2/0-Ag14. The cell surnatant was collected and the fusion protein was isolated from the conditioned medium. To isolate the monoclonal antibody (mAb), three chromatographic purification steps are carried out: (a) affinity chromatography on a protein A resin packed in column for EBA technique (expanded bed absorption); (b) cation exchange chromatography is executed to separate different homoaggregates of L19-cytokine fusion protein; (c) preparative size exclusion chromatography able to change the buffer of cation exchange elution in the final formulation buffer. Chromatography experiments are also carried out to evaluate the DNA removal during the purification steps. Data obtained showed that the cells are able to produce a quite high protein concentration in conditioned medium when this protocol is followed. The chromatography step the results show a very appreciable process yield with affinity chromatography; a clear separation of homoaggregates during cation exchange chromatography; no loss of protein concentration in buffer exchange. Finally, it has been demonstrated that Dna residues are removed by the first two chromatography steps.

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ROLE OF REACTIVE OXYGEN SPECIES IN THE EFFECTS OF 7*β*-HYDROXYCHOLESTEROL AND 7-KETOCHOL ESTEROL ON HUMAN ENDOTHELIAL CELLS

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Oxidized low density lipoproteins (oxLDLs) are involved in the pathogenesis of atherosclerosis and cytotoxicity of oxLDLs has been linked to the formation of oxysterols (1,2) such as 7ketocholesterol (7-KC) and 7β-hydroxycholesterol (7β-OHC). A dual effect of oxLDLs on endothelial cells has been demonstrated: an antiapoptotic effect at lower concentrations (<40 µg/ml) and a proapoptotic effect at higher concentrations (>40 µg/ml). Both effects are mediated by different levels of reactive oxygen species (ROS) (3). In fact there is evidence about the role of ROS as signaling molecules regulating cell growth and migration in vascular cells (4). The main source of ROS is NADPH oxidase, a multicomponent membrane-bound oxidase regulated by several physiological and non-physiological stimuli (4). In endothelial cells NADPH oxidase is constantly activated at basal level, determining the intracellular physiological redox status (5). The purpose of this study was to investigate the role of ROS on the effects of 7-KC and 7β-OHC in human umbilical vein endothelial cells (HUVEC). HUVEC were isolated from human umbilical cord and used from passage two to six; cell viability was determined by the MTT reduction assay; apoptosis was determined by the cytofluorimetric analysis of the annexin V and propidium iodide binding; ROS production was determined in intact cells using the fluorescent cell-permeant probe CM-H_2DCFDA and the fluorescence was mesured with a multilabel plate reader at 10 min interval for 3 h; cell migration was evaluated with a modified Boyden chamber. An antiapoptotic effect on HUVEC was induced by lower concentrations (1-10 µg/ml) of 7-KC and 7β-OHC, while cell death was induced by higher concentration (20 µg/mL) of the two oxysterols. Both of them increased intracellular ROS production in a dose dependent manner (+50% and +100% at 20 µg/mL of 7-KC and 7β-OHC, respectively). Pretreatment of HUVEC with hydralazine (25 µM), a NADPH oxidase inhibitor (6), partially inhibited ROS production induced by only 7β-OHC. However hydralazine was not able to affect the antiapoptotic action of 7β-OHC, as demonstrated by the cytofluorimetric analysis of the annexin V and propidium iodide binding. HUVEC migration was inhibited by both oxysterols. The results show that the antiapoptotic effect of 7-KC and 7β-OHC is associated with an increase in intracellular ROS levels. NADPH oxidase is not involved in the antiapoptotic effect of the two oxysterols. Experiments are in progress to define the source of ROS and their role on the effect of both 7-KC and 7β-OHC on HUVEC viabilty and migration.

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MITHOCONDRIAL APOPTOSIS IN PERIPHERAL BLOOD LYMPHOCYTES AS PROGNOSTIC MARKER FOR SEVERE COPLICATIONS IN CIRRHOTIC PATIENTS WITH SEPSIS

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Infection and Sepsis are very frequent in cirrhotic patients and they are associated with a mortality spanning about 50%. Changes of apoptotic activity of leukocites take a part to immunodisfunction and immunosoppression in infection and sepsis. The Bcl 2 family members are major regulators of the mithocondrial apoptosis , they are known to be able to activating caspase-9.Many factors influence mithocondrial pathway of programmed cellular death, between these Heat shock protein 70 (Hsp70) has been shown to act as an inhibitor of apoptosis and p53, a transcription factor that regulates the expression of a wide variety of genes involved in cell cycle arrest and apoptosis in response to genotoxic or cellular stress. This work aims to examinate mithocondrial apoptosis in sepsis in cirrhotic patients in correlation to serum leptin levels. In this study were consecutively included 60 Cirrhotic Patients with ascites, with and without infection and 10 healthy donors. Lymphocytes were isolated from the blood by means of gradient. The proteins were extracted and the protein expression was estimated by means of Western Blotting. The Bcl 2 expression was correlated statistically with leptin levels. Leptin concentration was measured by a immunoenzimatic dosage (EIA). The level of caspase 9 protein was highly expressed as to healthy donors, with a slight decline of expression in septic patients as to cirrhotic patients without sepsis. The expression of the antiapoptotic protein Bax was increased in cirrhotic patients without infection as compared with healthy controls and it was significatly declined in septic patients compared with cirrhotic patients without sepsis($p=0,003$). Bcl 2 expression was reduced in cirrhotic patient as compared with healthy subjects and it was increased in cirrhotic patients with sepsis ($p = 0,04$). Analysis of the antiapoptotic protein Bcl XL show an increase in cirrhosis without infection as compared to controls and a decrease of expression in septic cirrhotics. Altrough this pattern seems in contrast with Bcl2 results. We showed that the electrophoresis profile of Bcl XL exibit a greater phosphorylation in cirrhotics without sepsis, which is known to alter the antiapoptotic function of this protein. ($p=0,009$). Levels of leptin measured by EIA test were increased with infection.

Levels of leptin were inversely proportional to Bax expression and directly correlated with antiapoptotic proteins. Levels of Hsp70 and p53 expression was increased in two groups of patients in comparison with healthy individuals, but did not differ significantly between them ($p = 0,008$, $p= 0,009$). This results show that meaningful alterations of expression and post traductional modifications are implicated in apoptotic regulation in infection of cirrhotic patients. It is possible to consider these indices as potential prognostic markers of infection in sepsis of cirrhotic patiens. It has been also discussed the usefulness of monitoring these activities as potential prognostic markers and the acquaintance of the molecular mechanism that govern the destiny of the leukocytes of the septic patients with cirrhosis and the individuation of a gene target could allow the development of new pharmacologic strategies to contrast the effects of the sepsis and apoptosis will catch up a better rationalization of the use of drugs.

BEHAVIORAL AND NEUROCHEMICAL MODIFICATIONS IN COCAINE AND MORPHINE SENSITIZED RATS 10-20 DAYS AND 6 MONTHS AFTER THE LAST DRUG ADMINISTRATION

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Behavioral sensitization to cocaine (CA) and morphine (MF) depends on a process of neuronal plasticity induced by their repeated administration. DARPP-32, a 32 kDa phosphoprotein enriched in medium spiny neurons in dorsal and ventral striatum, plays a critical role in dopamine (DA) signaling and DA signaling is crucial in mediating the effects of drugs of abuse. DARPP-32 is a major target protein for the cAMP-dependent protein kinase (PKA) and its function is determined by its phosphorylation state. Activation of PKA, increases phosphorylation at Thr34 DARPP-32 and decreases that at Thr75 DARPP-32. We have demonstrated that CA-sensitized rats show an increase in p-Thr75 DARPP-32 and Cdk5 levels in striatal areas that persists for at least 2 weeks after the last CA treatment. MF sensitized rats present intense stereotypies upon challenge with MF and are resistant to the consequences of unavoidable stress exposure; both these effects depend on DA D1 receptor activation. MF sensitized rats do not show modifications in DARPP-32 phosphorylation state. However, DARPP-32 phosphorylation pattern measured in MF-sensitized rats at different times after a MF challenge, was modified and we observed an increase in p-Thr75 DARPP-32 and a decrease in p-Thr34 DARPP-32 levels, 2 h after MF challenge. The aim of this study was to investigate whether the modifications in the neurochemical parameters observed in MF and CA sensitized rats were long-lasting and correlated to the behavioral expression of sensitization. Behavioral sensitization, DARPP-32 phosphorylation Cdk5 levels, PKA activity were studied in the NAc and CPu of CA or MF sensitized rats both 20 days and 6 months after the end of sensitization protocol. In these areas, we also studied the accumulation of the transcription factor ΔFosB. In order to evaluate the persistence of stress-resistance, MF-sensitized rats underwent a shock–escape test 20 days and 6 months after the protocol. CA–sensitized rats killed 20 days after the end of protocol confirmed the previously observed modifications in DARPP-32 phosphorylation state and Cdk5 level; moreover they showed a significant increase in ΔFosB expression. CA–sensitized rats killed 6 months after the end of protocol, did not show behavioral sensitization and no modifications in the neurochemical parameters were detectable compared to control rats. MF sensitized rats killed 20 days after the end of protocol showed increased ΔFosB expression and confirmed the absence of modifications in Cdk5 expression and DARPP-32 phosphorylation. The increase in p-Thr75 and decrease in p-Thr-34 DARPP-32 levels, observed 2 h after MF challenge were prevented by the acute administration of the D1R antagonist SCH23390 or the mGluR5 antagonist MPEP 5 min before or 1 h after MF challenge, respectively. These results suggest an involvement of DA D1 and mGluR5 transmission in the increased p-Thr75 expression induced by MF challenge. After 6 months rats still showed behavioral sensitization to MF and resistance to unavoidable stress exposure. However, these rats, did not show an increase in p-Thr75 expression 2 h after MF challenge or increase in ΔFosB expression, suggesting a lack of correlation between the behavioral component of sensitization and the modifications observed in DARPP-32 phosphorylation pattern and ΔFosB expression.

BACLOFEN AND D-CYCLOSERINE AS POTENTIAL TREATMENTS FOR AMPHETAMINE DEPENDENCE: LOCOMOTOR SENSITIZATION AND DRUG DISCRIMINATION STUDIES IN THE RAT

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Amphetamine (AMPH) abuse is now an epidemic of global proportions, second only to cannabis and more than cocaine and heroin combined. In spite of this, to date, no medications tested have shown efficacy to reduce AMPH use (1). The mesocorticolimbic dopaminergic pathway plays a fundamental role in the mechanisms underlying AMPH addiction and the neuroadaptive responses induced by chronic treatment may involve the modulatory role of GABA and glutamate (2). In order to clarify the role of gabaergic and glutamatergic systems in AMPH addiction, we tested the effects of the $GABA_B$ agonist baclofen (BCF) and the NMDA-glycine-site receptor agonist D-cycloserine (DCS) in two differents experimental models in rats: locomotor sensitization. As regards sensitization, we examined the effect of BCF coadministration on the acquisition and the expression of AMPH-induced locomotor sensitization; a further study was undertaken to test if chronic BCF treatment could prevent and/or reverse AMPH locomotor sensitization. Similar experiments were performed using DCS as a tool. Moreover we investigated the possible influence of BCF co-administration and BCF chronic treatment on the discriminative stimulus effects of AMPH. *Locomotor sensitization.* Male Sprague-Dawley rats were: I) chronically treated with BCF alone or in combination with AMPH and tested with AMPH; II) chronically treated with AMPH and tested with BCF plus AMPH; III) chronically treated with BCF before or after chronic AMPH and tested with AMPH. All the chronic treatments have been administered daily for 10 days. The same protocol was used to test the effects of DCS on the acquisition and expression of AMPHinduced locomotor sensitization and to test if a chronic DCS treatment could prevent and/or reverse it. *Drug discrimination.* Male Sprague-Dawley rats were trained to discriminate AMPH from saline under a two-lever fixed-ratio schedule of food reinforcement. In generalization studies BCF was co-administered with a full range of AMPH doses or chronically injected for 10 days before AMPH test sessions. as expected, the locomotor stimulant effect of AMPH underwent a strong sensitization in control animals. The effect was significantly reduced, both in the acquisition and in the expression phase, by a BCF dose that did not affect motor activity by itself. Chronic BCF treatment completely and permanently reversed AMPH sensitization, but did not prevent it. DCS decreased AMPH induced locomotor sensitization, both in the acquisition and in the expression phase. Moreover, chronic DCS treatment attenuated AMPH sensitization and, at least partly, prevented it. As regards drug discrimination, both BCF coadministration and BCF chronic treatment slightly, but significantly attenuated the cueing effects of AMPH. On the whole the data indicate that both $GABA_B$ and NMDA receptors are involved in the neuroadaptive responses induced by chronic AMPH treatment. BCF and DCS, which have a good safety profile in humans (3), may be useful to promote long term recovery in AMPH addicted subjects. Moreover, DCS might show efficacy to reduce AMPH relapses. However, further experiments are needed to confirm this suggestion.

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SYNERGISTIC CYTOTOXICITY, INHIBITION OF AKT AND C-KIT PHOSPHORYLATION AND MODULATION OF GENE EXPRESSION BY SORAFENIB AND GEMCITABINE IN HUMAN PANCREATIC CANCER CELLS

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Pancreatic cancer is one of the most lethal human tumours and, although the first-line agent gemcitabine produces a clinical response, there has been little improvement in prognosis in the last 20 years. Therefore, research effort has focused on target-specific agents, such as sorafenib, which blocks both the RAF/MEK/ERK signaling pathway and receptors involved in neovascularization and tumour progression, including VEGFR-2 and c-Kit. The present study investigates whether sorafenib would be synergistic with gemcitabine against pancreatic cancer cell lines. Cells were treated with sorafenib and gemcitabine, alone or in combination and the cytotoxicity was assessed with direct cell count. Pharmacologic interaction was studied using the combination index (CI) method, while cell cycle was investigated with flow cytometry. Moreover, the effects of drugs on Akt and c-Kit phosphorylation, and on apoptosis induction were studied with ELISA and fluorescence microscopy, respectively. Finally, quantitative PCR analysis was performed to assess whether sorafenib, at IC_{50} concentration levels, modulated the expression of the gemcitabine activating enzyme deoxycytidine kinase (dCK) and the drug target ribonucleotide reductase (RR). Sorafenib was cytotoxic against MIA PaCa-2, Capan-1, PANC-1 cells with IC_{50}s of 3.48±0.27, 0.61±0.16, and 4.56±1.32 µM, respectively. A dose dependent inhibition of cell growth was also observed after gemcitabine treatment with IC_{50}s of 0.08±0.01 (MIA PaCa-2), 0.10±0.02 (Capan-1), and 0.178±0.039 (PANC-1) µM. The CI analysis showed synergism for both sequences. Flow cytometry demonstrated that gemcitabine enhanced cellular population in the S phase. Cell exposure to gemcitabine resulted in a significant Akt phosphorylation inhibition, whereas sorafenib exposure reduced c-Kit phosphorylation. Fluorescence microscopy demonstrated that cells treated with drugs and their combinations presented typical apoptotic morphology; in particular, drug combinations significantly increased apoptotic index with respect to single agents in Capan-1 and MIA PaCa-2 cells. PCR showed that sorafenib reduced the expression of RRM1 and RRM2 in MIA PaCa-2 and Capan-1 cells, enhancing the dCK/(RRM1xRRM2) ratio ($p<0.05$). These data demonstrate that sorafenib and gemcitabine synergistically interact against pancreatic tumour cells, through suppression of Akt and c-Kit phosphorylation, induction of apoptosis and reduction of RRM1 and RRM2 gene expression, thus providing the experimental basis for developing this combination for the treatment of pancreas cancer.

REGULATION OF NEURONAL NACHRS IN NEUROBLASTOMA CELLS AFTER LONG-TERM EXPOSURE TO THE NEW NICOTINIC ANTAGONIST 1,2-CYTISINYLETHANE

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Nicotinic drug treatments can affect nAChR expression both *in vitro* and *in vivo* through molecular mechanisms not fully understood. Post-mortem brain tissues from human smokers or animals chronically exposed to nicotine contain more density of nicotine binding site; this increase varies among different brain regions and involves several nAChR subtypes. In the present study, we studied the effect of the novel cholinergic drug 1,2-bisN-cytisinylethane (CC4) on the neuronal nAChR expression in the neuroblastoma cell line SH-SY5Y. Binding assays and pharmacological experiments on SH-SY5Y were performed on control or CC4 treated cell membranes by incubation with ^{3}H-Epibatidine or ^{125}I-αbungarotoxin. Binding to whole cells was performed on 12-well plates as described in . Binding to 2% Triton X-100 extracts, immunoprecipitation of ^{3}H-Epibatidine-labelled receptors by subunit-specific antibodies and Western blotting were performed as described in. Electrophysiological experiments were performed as described in. Treatment for 48h of SH-SY5Y with 1mM CC4 up-regulates 3H-Epi receptors and to less extent 125I-αBgtx receptors; this up-regulation occurs both on the receptors present on cell surface and in intracellular pools and is not due to transcriptional events nor to stabilisation of rate turnover. The up-regulated receptors have a different subunit composition with a large increase in receptors containing the β2 subunit (α3β2β4 or/and α3β2). The CC4 up-regulated receptors are functional and their pharmacological profile is slightly different from that of receptors present in control cells.The present study reports the long-term action of the novel nicotinic drug 1,2-bisN-cytisinylethane (CC4) on native nAChR of SH-SY5Y cells and demonstrates that, unlike standard nAChR blockers, high concentration of CC4 could differentially upregulate functional nAChRs.

THE EXPRESSION OF HEART-RELATED GENES IS ALTERED IN RAT HEARTS PERFUSED WITH HIGH LEVELS OF GLUCOSE

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Diabetes mellitus is characterized by an increased risk of death after cardiovascular diseases. This complication is related to hyperglycemia, insulin resistance and hypertension (1). In order to investigate the pathogenetic mechanisms underlying cardiovascular complications related to high glucose concentration we studied, by microarray technique, gene expression modifications in isolated diabetic and non diabetic rat hearts perfused with high glucose solution. Diabetic and non diabetic rat hearts were cannulated *via* the aorta and perfused under constant flow for 2 h with control Krebs solution (glucose 11.1 mmol/l) and high glucose Krebs solution (glucose 33.3 mmol/l). Total RNA was extracted from total hearts using the RNeasy kit (Qiagen, Hilden, Germany). Analysis of gene expression has been carried out by cRNA Microarray technique with Applied Biosystems 1700 Chemiluminescent analyzer and TaqMan gene expression assays (Applied Biosystems, Foster City, CA). Statistical analysis in microarray experiments has been performed on the complete series of experiments using Spotfire software. Biological processes, molecular functions and pathways have been studied with PANTHER classification system (http://www.pantherdb.org).In our microarray studies we identified a series of genes significantly up and down regulated ($p<0.05$). In particular, we observed a change of expression of 167 genes in non diabetic rat hearts (control vs. high glucose concentration), a change of expression of 368 genes in diabetic rat hearts (control vs. high glucose concentration) and a change of expression of 2,742 genes in diabetic rat hearts vs. non diabetic rat hearts both exposed to high glucose concentration. Among them we selected only those genes with a fold change > 2 and studied their biological processes and molecular functions by Panther classification system. In particular, in our experimental model we observed a significative change of expression of genes involved in: ion trasport, mRNA transcription signal transduction, cytokine and chemokine mediated signaling pathway, protein, fatty acid and steroid metabolism and modification, apoptosis and immunity defense. Data by Real Time PCR (in non diabetic rat hearts) confirmed gene expression changes of two genes involved in ion transport, three genes involved in cytokine and chemokine mediated signaling pathway, and two genes involved in carbohydrate metabolism. We observed a significative upregulation of: Ucp1 (uncoupling protein 1) (microarray change: +5.6; real-time PCR change: +261.4); Il6 (interleukin 6) (microarray change: +4.05; real-time PCR change: +3.81); Ccl7 (chemokine (C-C motif) ligand 7) (microarray change: +3.5; real-time PCR change: +2.51); Ccl2 (chemokine (C-C motif) ligand 2) (microarray change: +3.6; real-time PCR change: +5.49); Has1 (hyaluronan synthase1) (microarray change: +2.1; real-time PCR change: +1.94); Has2 (hyaluronan synthase2) (microarray change: +3.5; real-time PCR change: +2.38) and down-regulation of TRPC3 (transient receptor potential cation channel member 3) (microarray change: -0.33; real-time PCR change: -0.60). Others real-time PCR experiments are in course to validate microarrays results.Our results demonstrate that both in diabetic and non diabetic rat hearts, high glucose modifies the expression of some genes involved in the regulation of important cell functions. These genes could shed light on new therapeutic targets for cardiovascular desease induced by high glucose levels or by diabetes.

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THE ROLE OF GKAP IN MAINTAINING THE POSTSYNAPTIC DENSITY STRUCTURE AND DENDRITIC SPINES NUMBER

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The postsynaptic density (PSD) consists of a network of interacting proteins that anchors and links glutamate receptors and other postsynaptic membrane proteins to cytoplasmic, cytoskeletal elements and signaling pathways. PSD95, GKAP and Shank families of scaffold proteins are core components of the PSD that interact directly. We previously demonstrated that PSD-95 and GKAP interaction is fundamental for the correct folding and synaptic localization of Shank (1), a scaffold protein involved in dendritic spine's maturation (2) and that the formation of a post-synaptic complex constituted by PSD95, GKAP, Shank and NLG1 is able to recruit a pre-synaptic terminal leading the maturation of an excitatory synapse (3). Here we further investigate the role of GKAP using an RNA interference approach in primary hippocampal neuronal cultures. The specificity of several siRNA was first tested in heterologous transfected cells. We then proceed transfecting the specific siRNA in developing and mature hippocampal neurons. When the GKAP expression is inhibited in mature neurons we observed a decrease in both post-synaptic scaffold proteins clusters and dendritic spines numbers and a morphological changes of the spines heads. Our results suggest a role for GKAP in maintaining PSD structure and dendritic spines number in mature neurons.

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HYPERPHOSPHORYLATION OF TAU PROTEIN IN THE BRAIN OF A HUMAN APP TRANSGENIC MOUSE MODEL

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Hyperphosphorylation and accumulation of tau in neurons is one of the main pathological hallmarks involved in the dynamic of neurodegeneration in Alzheimer's disease(AD). The AD brain is marked by the buildup of two aberrant protein aggregates: senile plaques, comprising the fibrillary β-amyloid peptide (Aβ), and intraneuronal neurofibrillary tangles (NFTs), consisting of hyperphosphorylated microtubule associated protein tau, accumulating as aggregated paired helical filaments. Although the disease pathophysiology is still a matter of debate, the prevailing hypothesis, attemping to elucidate it, has been that β-amyloid deposition occurs first, being the "iniziator" of AD pathogenesis; while cell death and tau pathology are considered to be downstream events. Hyperphosphorylation of tau is regulated by DKK-1 and by several kinases that phosphorylate tau on specific sites. In the present study, the expression of DKK-1 and of the kinases GSK-3β and phospho-SAPK/JNK have been investigated in selected brain regions of TgCRND8 mice, by means of single and double labelling immunohistochemistry, histochemistry and western blotting analysis. TgCRND8 and non-Tg littermates control mice of 7 and 12 months of age were used. Hyperphosphorylated tau protein was revealed by means of AT-8, AT-100, PHF-1 and CP-13 antibodies. In the cortex and hippocampus of TgCRND8 mouse brain, in the surrounding of amyloid plaques, numerous PHF-1-immunopositive cells were present. PHF-1 immunoreactivity was localized in the cytoplasm and processes of neurons and in numerous degenerating neuritis. Western blotting analysis revealed that PHF-1 immunoreactivity was clearly increased in the cortex and hippocampus of TgCRND8 mouse brain with respect to wt controls. In the II and III layer of secondary motor cortex and in the dentate gyrus of the hippocampus of TgCRND8 mouse brain, numerous AT-100 immunopositive neurons were detected and some neurons, randomly located, were AT-8 positive. The immunohistochemical data were confirmed by Western blotting analysis. In the cortex, hippocampus and thalamus of TgCRND8 mouse brain an increased phospho-SAPK/JNK immunoreactivity respect to controls was found and SAPK/JNK staining showed a granular morphology within PHF-1-positive neurons. Western blotting revealed that phospho-SAPK/JNK bands in the cortex, hippocampus and thalamus of the transgenic mouse brain were more intense than in the wt mouse brain. Western blotting with GSK-3β antibody revealed the presence of thicker immunopositive bands in the cortex and hippocampus of the transgenic mouse brain than in control mouse brain. In the II, III, V and VI layer of secondary motor cortex, in the piriform cortex and in the CA1 area of the hippocampus of TgCRND8 mouse brain, numerous DKK-1 immunopositive neurons were detected. DKK-1 immunoreactivity was localized in the cytoplasm and processes of neurons. The immunohistochemical data were confirmed by western blotting analysis.

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GENERATION OF A DROSOPHILA MODEL FOR JUVENILE MYOCLONIC EPILEPSY (JME) BY GENETIC MANIPULATION OF TWO FLY GENES HOMOLOGOUS TO MYOCLONIN, A NEWLY IDENTIFIED JME GENE

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In 2004, EFHC1 was identified as a novel gene causing Juvenile Myoclonic Epilepsy (JME). This gene encodes myoclonin/EFHC1, a novel non-ion channel protein whose function is currently unknown. The protein contains 3 DM10 domains, a motif with unknown function and an EF-hand, a Ca2+ binding motif. Human myoclonin was shown to be ubiquitously expressed and in patch-clamp analysis in HEK cells EFHC1 specifically increased R-type Ca^{2+} currents. Despite this information, the normal role of myoclonin and how its mutation leads to disease are still unclear. We have identified two Drosophila homologs (CG8959 and CG11048) of myoclonin/EFHC1 and we are now using Drosophila as a model to study the functions of this gene in normal development and in pathology to define the mechanisms whereby mutation of myoclonin causes human disease. We extracted total mRNA from adult fly heads and performed RT PCR to clone the CG11048 cDNA, a construct containing the cDNA was used to create transgenic flies. A number of genetic techniques were used to modulate the expression of the CG11048 encoded protein. We performed immunoistochemistry on Drosophila third instar larva preparations to determine the endogenous expression pattern of CG11048 and to analyse potential morphological phenotypes of the neuromuscular junction caused by ectopic expression of CG11048. To address the function of myoclonin/EFHC1 proteins we searched for Drosophila homologs and identified two proteins, CG8959 and CG11048, that share extensive similarity with their human counterpart. We have cloned the CG11048 cDNA, and generated transgenic flies for its. I exploited the GAL4/UAS expression system to promote expression of CG11048 both globally and in a tissue specific fashion. Preliminary data show that high ubiquitous levels of CG11048 are lethal. Similarly, flies strongly expressing CG11048 specifically in neurons do not survive. However, transgenic lines exhibiting lower levels of expression allow viability and the observation of adult phenotypes. Surviving flies overexpressing CG11048 ubiquitously and neuronally display similar behavioural and motor phenotypes: inability to fly, severely uncoordinated and impaired walking and reduced viability. In addition, overexpression of CG11048 produces significant morphological alterations of synaptic terminals at the larva neuromuscular junctions. I have also raised an antibody against the CG11048 protein and demonstrated that CG11048 is expressed in all neuromuscular junction terminals of third instar Drosophila larvae. To understand the function of CG11048 is crucial to determine the consequence of the absence of this protein. Since there are no available mutants we used homologous recombination to generate knock-out mutants of CG11048. Absence of the protein allows viability, and a more detailed phenotypic analysis is currently under way. EFHC1 is a protein of unknown function. Our preliminary data from Drosophila support a role for CG11048 in synaptic structure or function or both. This information is extremely important because it could suggest a mechanism for the pathogenesis of this form of Juvenile Myoclonic Epilepsy. It is easy to imagine that mutation of EFHC1, a synaptic protein, may lead to synaptic dysfunction which in turn would provide a mechanistic explanation for epilepsy in humans.

POSSIBLE ROLE FOR SEROTONIN IN THE NOCICEPTIN/ORPHANIN FQ INHIBITION OF PARACETAMOL-INDUCED ANTINOCICEPTION IN THE RAT

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Nociceptin/orphanin FQ (N/OFQ) is an endogenous peptide involved in nociceptive sensitivity at supraspinal levels, exerting an antagonistic activity toward opioid-induced analgesia. We observed that paracetamol exerts an antinociceptive effect in the hot-plate test that is accompanied by a decrease in the serotonin levels in some brain areas. On this basis, the purpose of this study was to find out whether N/OFQ is also able to counteract paracetamolinduced antinociception and whether this possible antagonistic effect could be mediated by the serotonergic system in the rat brain. The brain areas investigated were the frontal cortex, the temporal-parietal cortex and the pons. Moreover, we studied the effect of UFP-101 (an antagonist of NOP receptors) on N/OFQ and PARA interaction. The antinociceptive effect of PARA was evaluated by means of the hot-plate test and the serotonin levels were measured by reverse-phase high-performance liquid chromatography (HPLC). The rats were randomly divided in groups of eight animal each. The antinociceptive effect of PARA (400 mg/kg i.p.) was completely abolished by nociceptin/orphanin FQ (N/OFQ, 10 nmol, i.c.v.). N/OFQ alone did not modify the basal levels of serotonin, but significantly reverted the increase evoked by PARA in the frontal cortex, temporal-parietal cortex and pons. UFP-101 (20 nmol i.c.v.) significantly prevented both the behavioral and the biochemical actions of N/OFQ on PARA. The data from the hot-plate and serotonin concentration experiments were expressed as means±S.E.M. A three-way ANOVA, using the program package SPSS 11.0 (SPSS Chicago, IL, USA) was performed to examine the effect of paracetamol, nociceptin/orphanin FQ or UFP-101 treatment and their interaction in the hot-plate test and in serotonin level determinations. The Bonferroni *post-hoc* test was used following three-way ANOVA when the effects of nociceptin/orphanin FQ, UFP-101 and paracetamol had been separately evaluated. The level of significance was set at 0.05. The results obtained in this study suggest that serotonin is involved, at least in part, in the mechanism underlying the N/OFQ antagonism of PARA-induced antinociception. The UFP-101 blockade of N/OFQ antagonism of PARA induced behavioral and biochemical effects indicates the involvement of the NOP receptor in this neuropeptide action.

BROAD-SPECTRUM ANALGESIA THROUGH ACTIVATION OF THE NUCLEAR RECEPTOR PPAR-A

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Peroxisome proliferator-activated receptor-α (PPAR-α) is a nuclear receptor that serves important functions in lipid nutrient utilization (1) and inflammation (2, 3). Like other members of the nuclear receptor superfamily, PPAR-α is activated through ligand binding, which causes changes in receptor conformation, heterodimer formation with the 9-cis-retinoic acid receptor (RXR), recruitment of coactivators into a multiprotein complex, and regulated transcription of responsive genes (1). Here we report that agonists of the nuclear receptor PPAR-α suppress pain behaviours induced in rodents by nerve injury, inflammation or chemical tissue damage. Male Swiss mice were obtained from Charles River, male wild-type C57BL6 and PPAR-α $^{-/-}$(B6.129S4-SvJae-Ppara^{tm1Gonz}) mice, previously backcrossed to C57BL6 mice for 12 generations, were from Taconic (Germantown, New York). *Sciatic nerve ligation* was performed in mice following an adaptation of the method of Bennett and Xie (4). *Adjuvant-induced arthritis:* 0.1 ml of CFA (Mycobacterium tuberculosis, Sigma) was intradermally injected into the base of the tail of male Swiss mice. Withdrawal thresholds were measured 30 min after i.p. drug administration; mechanical hyperalgesia was determined by measuring paw withdrawal thresholds to a constant pressure (15g), thermal hyperalgesia was assessed as reported by Hargreaves (5). *Formalin test* (6): formalin (10 µl, 5%) was injected into the plantar surface of mice using a 27-gauge needle fitted to a microsyringe.) . Drugs and formalin were dissolved in the vehicle (0.9% sterile saline/ 5% PEG-400/ 5% Tween-80).Following injection, animals were immediately transferred to a transparent observation chamber where pain behavior (time spent licking and biting the injected paw) was continuously monitored for 45 min (phase-I: 0-15 min; phase-II: 15-45 min). On days 7 and 14 after surgery, administration of PEA (30 mg-kg^{-1}, i.p) or GW7647(30 mg-kg^{-1}, i.p), caused a rapid reversal of both mechanical and thermal hypersensitivity, whereas injections of vehicle had no such effect. Similar results have been observed also in CFA inflammatory model. In PPAR-α -/- null mice PEA or GW7647 were ineffective in reducing thermal and mechanical hyperalgesia. On formalin test, when cooadministered with formalin, the PPAR-α agonists GW7647 (50 µg -10µl^{-1}) or PEA (50 µg -10µl^{-1}) reduced both early and late phase of irritant-induced nociception. In conclusion we have shown that PPAR-α agonists exert rapid and profound analgesic effects in animal models of acute, persistent inflammatory and neuropathic pain, which are contingent on PPAR-α expression. Thus, our results reveal an unexpected role for PPAR-α in pain regulation and suggest that agonists of this nuclear receptor may represent a novel class of broad-spectrum analgesics.

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HIPK2 RESTRAINS CYTOSOLIC-PHOSPHOLIPASE-A$_2$-DEPENDENT PROSTA-GLANDIN-E$_2$ GENERATION: A NOVEL MECHANISM TO CURB TUMOUR CELL GROWTH

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Homeodomain-interacting protein kinase-2 (HIPK2), a co-repressor for homeodomain transcription factors, is a multifunctional kinase whose role in tumor cell survival is not completely clarified. We addressed whether HIPK2 restrains colon tumorigenesis by turning off cytosolic-phospholipase A$_2$ (cPLA$_2$)-dependent prostaglandin E$_2$ (PGE$_2$) generation in the light of overwhelming evidence suggesting the contribution of this prostanoid in a variety of cancers. In human colorectal cancer cell line RKO, we studied the effect of RNA-interference for HIPK2 (HIPK2i) on prostanoid biosynthesis both in the absence and in the presence of the cPLA$_2$ inhibitor arachidonyl trifluoromethyl ketone. We evaluated the role of HIPK2 in the *cPLA$_2$* gene regulation by reverse transcriptase-PCR (RT-PCR), transcriptional activity, and chromatin-immunoprecipitation analyses. The involvement of HIPK2 in tumorigenicity *in vivo* was studied by tumor growth of HIPK2-interfered cells in nude mice. We compared the gene expression of HIPK2 and cPLA$_2$ in human colorectal cancer specimens by RT-PCR. HIPK2 silencing was associated with rousing PGE$_2$ biosynthesis that was profoundly suppressed by the cPLA$_2$ inhibitor. HIPK2 overexpression along with histone deacetylase 1 (HDAC1) inhibited cPLA$_2$-luc promoter that resulted strongly acetylated in HIPK2i cells. The tumours derived from HIPK2i cells injected in nude mice showed noticeably increased growth compared to parental cells. HIPK2 mRNA levels were significantly higher in colorectal cancers of familial adenomatous polyposis patients which showed undetectable *cPLA$_2$* levels, compared to sporadic colorectal cancer expressing *cPLA$_2$*. Our findings enlighten a novel mechanism of HIPK2 to restrain progression of human colon tumorigenesis, at least in part, by turning off cPLA$_2$-dependent PGE$_2$ generation.

TIMING OF ESTROGEN REPLACEMENT INITIATION AFFECTS *EX VIVO* VASCULAR REACTIVITY IN THE AORTA OF OVARIECTOMIZED RATS

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17 β-Estradiol (E_2) exerts beneficial effects on the cardiovascular system and is known to cause vasodilation through both genomic and nongenomic activation of endothelial nitric oxide synthase (eNOS) (1). *In vitro* experiments showed that ERα mediates the acute aortic relaxation induced by E_2 and that circulating estrogen levels influence the extent of this response (2). Experimental evidence and observational studies also suggest that postmenopausal hormone therapy protects against cardiovascular disease, whereas randomized clinical trials are not consistent with these findings. It has been hypothesized that the timing since estrogen treatment initiation is crucial for cardiovascular protection (1). The aim of this work was: i) to investigate the effect of acute treatment with the selective ERα agonist PPT and E_2 on aortic preparations from long-term ovariectomized (OVX) rats; ii) to evaluate eNOS protein in aortic endothelium from long-term OVX rats by Western blot and immunohistochemistry (IHC). At 1, 4 or 8 months since surgery, OVX rats received subcutaneous implant of sylastic capsules containing vehicle or E_2 for 5 days to yield normal proestrus E_2 levels. Cumulative concentration-response curves to E_2 and PPT ($1x10^{-13}$-10^{-7} M) were obtained in tissues precontracted with noradrenaline (EC_{60}). Two aortic rings from 3 to 5 rats were used for each set of experiments. Acetylcholine (ACh)-induced response was also tested ($1x10^{-9}$-$1x10^{-5}$ M). The ERα agonist PPT relaxed by about 23% aortic rings from 1month OVX rats after estrogen replacement (pD_2=10.2±0.3), as did E_2, whereas neither PPT nor E_2 induced vasodilation in tissues from untreated OVX rats. Similar responses to PPT and E_2 were obtained in vessels from estrogen-replaced rats after 4 months since ovariectomy (pD_2=10.2±0.1 for PPT). Conversely, neither PPT nor E_2 affected relaxation in aortic rings from 8-months OVX rats even after estrogen treatment ($p<0.05$ vs. 1-month OVX+E_2). In vessels from 1- and 4-months OVX rats, Ach induced similar vasorelaxation (about 100%) in both estrogen-replaced and vehicle-treated group. By contrast, response to ACh was significantly impaired in aortas from 8-months OVX rats (E_{max}=73.4±0.1) and *in vivo* estrogen treatment only partially restored aortic relaxation (E_{max}=81.4±0.1). eNOS protein was detected by IHC in aortic rings from all experimental groups. However, eNOS expression as measured by Western blot was higher in aortic endothelial lysates from estrogen-replaced compared with vehicle-treated rats at all time points. In conclusion, both PPT and E_2 induced acute aortic relaxation in vessels from estrogen-treated 1- and 4-months OVX rats but this effect was abrogated in aortas from 8-months OVX rats even after estrogen replacement. These results indicate that the timing of estrogen replacement initiation influenced the vascular reactivity in aortas from OVX rats, suggesting that early estrogen treatment could preserve endothelial function.

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PROGNOSTIC FACTORS FOR THE SUCCESS OF PHARMACOTHERAPY WITH ANTIDEPRESSANTS IN ONCOLOGY AND IN PSYCHIATRY: ADAPTATION TO STRESSFUL LIFE EXPERIENCES AND POLYMORPHISM OF SEROTONIN TRANSPORTER AND OF MONOAMINE OXIDASES

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Extensive studies have investigated the association of psychiatric disorders and mental suffering with biochemical mechanisms related to mental functions. Considerable attention has been recently given to the gene encoding for the proteins implicated in the regulation of monoaminergic neurotransmission, and for their polymorphisms, particularly the serotonin transporter (SERT) and monoaminoxidase-A (MAO-A). The genetic polymorphism 5HTTLPR (gene liked polymorphic region) has been identified for SERT, consisting in the insertion/deletion of 44 bp in the promoter region producing the long or the short alleles. This polymorphism has been found to moderate the influence of stressful life events on the development of depression. Individuals with one or two copies of the short allele exhibited more severe depressive symptoms, and a higher rate of suicides after exposure to stressful life events, in comparison with individuals homozygous for the long allele. Similarly, the functional VNTR (variable number tandem repeat) polymorphism in the promoter region of the MAO-A gene was found to moderate the effect of maltreatment in children on subsequent antisocial behaviour; subjects with a genotype conferring high levels of MAO-A expression (alleles with › 3 copies of the 30-bp repeat sequence) were less likely to develop antisocial problems later in life. The aim of the research has been therefore to examine the role of these polymorphisms in a population of subjects which unsuccessfully committed a variable number of suicide attempts and which were exposed to a different number of stressful life events. Moreover, the role of these polymorphisms in relation to development of depression and to mental adjustment to cancer has been examined in a cohort of women with newly diagnosed breast cancer, and in a cohort of terminal patients with various advanced cancers; in these patients, the response to the treatment with SSRI antidepressant drugs was also evaluated. The severity of suicidal behaviour significantly depends on the number of life events experienced in the 6 months before each suicide attempt. The analysis of the genotype indicates that the differences in functional activity of SERT caused by 5-HTLPR polymorphism play a significant role, alone or in combination with the gender of the patients. Contingency tables show that female patients with 5-HTTLPR polymorphism of SERT conferring low functional activity, have an increased number of unsuccessful suicidal attempts when exposed to stressful life events. The analysis of the genotype of women with early breast cancer indicates that subjects with the VNTR polymorphism conferring high MAO activity have significantly higher scores for HADS (Hospital Anxiety and Depression Scale) anxiety. In neoplastic terminal patients the analysis of the genotype reveal that the antidepressant effects of Citalopram and Sertraline, as evidenced using HADS, depends significantly on the polymorphism of SERT; the effects of Sertraline are significant also on anxious preoccupation, hopelessness-helplessness and fighting spirit as determined using mini-MAC (Mental Adjustment to Cancer). These findings appear to encourage the further study of the genetic polymorphisms of monoaminergic neurotransmission in relation to the drug treatment effectiveness in neuropsychiatry.

SULFORAPHANE CAN RESTORE SENSITIVITY TO DOXORUBICIN IN MOUSE FIBROBLASTS CHARACTERIZED BY P53^{SER220} MUTATION-RELATED CHEMORESISTANCE

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p53The major factor that limits the effectiveness of chemotherapy in patients with advanced cancer is the acquisition of chemoresistance. The biochemical and molecular mechanisms explaining this resistance are not completely know (1). Most probably this resistance is a multifactorial phenomena including alteration in drug accumulation, increased activity of glutathione Stransferases, binding to protein or non protein thiols, increased removal of lethal adducts by activated DNA repair enzymes, and loss of p53 function (2-4). One promising strategy for reversing drug resistance in cancer is the concomitant use of chemopreventive agents and standard chemoterapeutic agents. In some combinations of two agents with different presumed mechanisms of activity, synergistic or additive effects may be seen and this should allow the reduction of the doses and of the toxicity associated with the treatment. In this context, sulforaphane (SUL) has been largely studied. This molecule is abundant in many cruciferous vegetables such as broccoli, cabbage, cauliflower and others. In the last years many studies have shown that SUL possesses different interesting properties such as induction of phase II drug metabolizing enzymes (5), inhibitions of phase I drug metabolizing enzymes, induction of apoptosis and modulation of the cell cycle (6-7). Mice fibroblasts characterized by a different p53 status (p53 wild type, p53 knock-out, and $^{Ser\ 220}$ mutation) were treated with doxorubicin (DOX) and with the association of DOX and SUL and analyzed for cell viability, DOX accumulation, and apoptosis induction. Cell viability was analyzed by the trypan blue dye esclusion test. Apoptosis and DOX accumulation were detected by flow cytometry. Very high concentrations of DOX were necessary to decrease the viability of p53$^{Ser\ 220}$ and p53 knock-out (but not wild type) cells. Treatment of p53$^{Ser\ 220}$ and p53 knock-out cells with DOX did not induce apoptosis, also at very high concentrations (10 μM) thereby demostrating a direct role of p53$^{Ser\ 220}$ in DOX chemoresistance. SUL restored chemo-sensitivity in DOXresistant and p53$^{Ser\ 220}$ and p53 knock-out cells, irrespective of p53 status. The SUL-mediated reversal of DOX resistance was not related to an increased intracellular accumulation of DOX. Taken together, our data could prompt innovative clinical studies designed to investigate whether the coadministration of SUL can enance the efficacy of DOX-based regimens.

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AN APPROACH TO EVALUATE APPROPRIATENESS IN THE ANTIBACTERIAL THERAPY IN ITALIAN HOSPITALS

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Inappropriate use of antimicrobial agents in hospitals has resulted in the emergency of resistant microorganisms, increased costs and unnecessary exposure of patients to drugs. Up to date only very limited data on the consumption of antibacterials in hospitals are available. The aim of this study was to investigate the pattern and trends of antibiotic consumption over the period 2002 to 2004 in Italian hospitals and to evaluate the variability between different hospitals and clinical specialities inside them also with the development of indicators of appropriateness. The study was carried out in five hospitals of Emilia Romagna region and data on the use of antimicrobial drugs and on the bed days in the period 2002 to 2004 were obtained from the pharmacy services. Antibiotic usage was analysed by clinical areas (surgery, medicine, intensive care unit and paediatrics) and single specialities. The consumption of antibiotics was analysed by ATC classification and daily defined doses for 100 bed days. The antibiotic consumption increased in the three age period (18%), the total amount ranging between 64.9 to 76.8 DDD/100 bed days. The intensive care unit showed the highest consumption of antibacterials, while pediatrics presented the lowest ones in the three years. Combinations of penicillins and β-lactamase inhibitors were the most used drugs, followed by fluoroquinolones and third generation cephalosporins. Co-penicillins were also the antibacterials with the highest increase in all the clinical areas between 2002-2004 (more than 40%). Fluoroquinolone utilisation sharply increased over the years in medicine area (+28%), particularly in oncology and nephrology specialisations (more than 50%). The use of third generation cephalosporins remained fairly stable during the study period in paediatrics and surgery, while increased in medicine (+15%). First generation cephalosporins consumption was high in surgery (+13%). In all the five hospitals in 2004, glycopeptide use was higher in intensive care unit (range, 12.1-27,9) and in surgery (range, 1.8-8.9).The ratio of injectable vs.. oral antibiotics ranged between 1.07-1.47 in 2002, decreasing in 2003 (0.90-1.31) and in 2004 (0.87-1.23). The number of different antibacterials used in 2002 ranged between 45 for Ferrara and 66 for Bologna, while in 2004 was 42 and 62 respectively. In all the three years the general medicine and surgery had the highest value of number of different antibacterials while hematology had the lowest ones. We found a great variability between both areas and specialities of different hospitals and this could be an indicator of poor appropriateness of drug use. On the other hand, we identified two reliable and suitable indicators for evaluating antibiotic use and they could be useful for an antibiotic management programme aiming to provide a high standard of care for patients.

IS ETHANOL A PRO-DRUG? *IN VIVO* MICRODIALYSIS STUDY IN THE RAT

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Ethanol (EtOH) is a well-known psychoactive compound and is considered responsible for the euphoriant and addictive properties of alcoholic drinks. Relevant evidence demonstrates that acute ethanol administration increases the electrophysiological activity of dopamine(DA) neurons in the ventral tegmental area (VTA) and elevates extracellular DA levels in the nucleus accumbens (NAcb) (1). This increment in DAergic transmission is thought to play an important role in mediating the rewarding effects of several drugs of abuse. Alcohol metabolism involves several enzymes including alcohol dehydrogenase (ADH) and cytocrome P450 in peripheral, and H_2O_2-catalase in the central nervous system. Acetaldehyde (ACD), the first metabolite of EtOH is an active compound possibly implicated in many of the behavioural, toxic, and psycostimolant effects of ethanol (2). Indeed, recent evidence support the hypothesis that ACD may mediate some of the reinforcing effects of EtOH (3), suggesting that EtOH should be considered as a pro-drug whose psychoactive properties are mediated by it first metabolite (4). In the present study we used *in vivo* microdialysis to measure extracellular DA levels in the NAcb shell. Male albino Wistar (body weight 280-300 g), were implanted with a I-shape dialysis probe aimed at the NAcb shell. DA extracellular levels where monitored in freely moving animals after administration by gavage of ACD (20-40 mg/kg) and EtOH (1-2 g/kg). To investigate the role of ACD produced by ethanol metabolism, 4-methylpyrazole (4-MP) was administered 24 h before ethanol (1-2 g/kg). During the experiments, the probe was perfused with a Ringer's solution; dialysates where collected every 15 min and analysed for DA using HPLC with electrochemical detection. Both etoh and ACD when administered by gavage were able to increase DA extracellular concentrations in the shell of the NAcb, while no effects was observed after ethanol administration in rats pre-treated with 4-MP. Remarkably, ACD seemed more effective than ethanol in stimulating DA release in the NAcb shell; in fact administration of ACD 20 mg/kg resulted in an increase in DA levels by 35-40%, a value comparable with the one obtained with the administration of EtOH 1 g/kg (25-30%). Data are expressed as mean ± S.E.M. percent of change from baseline (n = 4 in each group). *$P<0.05$ for comparison with baseline values (ANOVA, followed by Dunnett's test). Our results definitely support the hypothesis that ACD may mediates some of the reinforcing effects of EtOH. Furthermore, the lack of ethanol effects in animals pretreated with 4-MP, suggests that ACD derived from EtOH metabolism, produces DA release in the nucleus accumbens shell. Therefore, in this circumstance etoh function as a pro-drug being able to stimulate the activity of the mesolimbic D system, *via* its main metabolite ACD.

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GRAM POSITIVE BACTERIA *VERSUS* GRAM NEGATIVE BACTERIA EFFECT IN THE HUMAN AIRWAY EPITHELIAL CELLS.

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The airway epithelium, in addition to its barrier function, plays a prominent and active role for the immune response in the lung. Many bacterial species such as *E. coli*, *C. pneumoniae*, *S. aureus* are capable of causing severe inflammation in lung diseases like pneumonia and cystic fibrosis. Airway epithelial cells express TLRs and their activation has been shown to induce the production of several cytokines, chemokines and antimicrobial peptides. Although there are numerous reports of various bacterial products capable of inducing CXCL-8 secretion, it is unclear if the activation of more than one TLR can inhibit or increase chemokine production. The purpose of this study was to investigate the effect of Gram positive and negative bacteria on human lung epithelial cells. A549, human lung adenocarcinoma cells, were cultured in DMEM and plated in 96-well plates and treated with different pathogen associated molecular patterns (PAMPs). After 24 hours, the supernatant was recovered for the CXCL-8 ELISA assay. Then, cells were treated and collected (1x106 cells/well) for real time PCR for measuring CXCL-8 mRNA. *S.aureus*, Gram positive bacteria, did not induce a markedly elevated levels of the CXCL-8, however, *E.coli*, Gram negative bacteria, did increase the chemokine production from A549 in a concentration-dependent manner at 24 hours. Interestingly, the co-administration of *E.coli* (10^8 CFU/ml) and S. aureus (10^7-10^8 CFU/ml) induced a significant increase of CXCL-8 compared to *S.aureus* alone ($P<0.05$, $P<0.0001$ respectively). This synergistic effect was confirmed by the RT-PCR for CXCL-8 mRNA ($P<0.0005$). The next step was to understand the involvement and the interaction of TLRs and Nucleotide-oligomerization binding proteins 1 and 2, intracellular proteins, implicated in bacteria inflammatory response, by using specific ligands. To evaluate and mimic Gram positive bacteria we used Pam3CSK4 (0.1µg/ml) and/or FSL-1 (0.1µg/ml), TLR2/1 and TLR2/6 ligands, respectively, co-administered with *E.coli*. FSL-1 had an additive effect on CXCL-8 release whilst Pam3CSK4 had an inhibitive effect ($P<0.05$) versus *E.coli* (10^8 CFU/ml) alone. The same results were further confirmed by the coadministration of LPS (1µg/ml), TLR4 ligand, and FSL-1 or Pam3CSK4 alone either at the protein release or at the mRNA level. The addition of MDP Lys 18 (1µM), Nod2 ligand, to FSL-1 and Pam3CSK4 increased in a very significant ($P<0.0001$) way the chemokine release after *E.coli* costimulation. In contrast, the co-administration of FK565 (1µM), Nod1 ligand, and LPS to *S.aurues* (10^8 CFU/ml) revealed an inhibitory effect. Furthermore, we co-administered LPS and FK565 and MDP Lys 18 in order to mimic the effect of *E.coli*, able to activate either TLR4 or the intracellular Nods proteins. The activation of TLR4 by LPS synergizes with S.aureus ($P<0.01$) but the addition of MDP Lys 18 reduced the chemokine production either if administered alone or if they were added to the *S.aureus* curve. In contrast, FK 565 induced an additive effect on CXCL-8 release after the co-administration with LPS *plus* MDP Lys18 on *S.aureus* curve. In conclusion, the synergistic effect of *S.aurues* with *E.coli* on CXCL-8 release could be explained through the co-activation of TLR2 heterodimers, TLR4 and Nod2 protein. This study could further elucidate the importance of TLRs and Nods in epithelial cells and their importance as future and innovative pharmacological targets for the clearance of bacteria after lung infections.

ROLE OF NO ON THE MITOGENIC EFFECT OF IL-1β IN GLIOMA CELLS

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Inflammatory factors such as chemokines and cytokines play a critical role in the host defense against disease and injury. Glial cells of the central nervous system (CNS) produce chemokines and cytokines when activated and may be the primary source of CNS disease and injury (1, 2). Chronic expression of chemokines and cytokines has been reported to occur in the CNS in a number of neurological disorders including multiple sclerosis/experimental autoimmune encephalomyelitis and Alzheimer's disease (3). To assess a potential role for cytokines in the CNS dysfunction associated with these conditions, we have investigated the effect of IL-1β on the regulation of cell growth. Earlier observations in our laboratory establishing a role for nitric oxide (NO)/Ca^{2+} signalling in IL-1β induced pyrogenic effect (4), prompted us to study this signalling in the IL-1β regulation of cell proliferation. Pretreatment of C6 glioma cells with different doses of IL-1β, resulted in a dose-dependent increase in cell growth. Data showed that both unspecific [N-ω-nitro-l-arginine methyl ester (L-NAME)] and selective {N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W)} iNOS inhibitor, significantly reversed the proliferative response induced by IL-1β. On the contrary, the guanyl ciclase inhibitor, 1H(1,2,4) oxadiazole (4,3-a) quinoxalin 1-one (ODQ), failed to reverse this effect. Either preventing release of Ca^{2+} from endoplasmic reticulum with ryanodine plus 2-Aminoethoxydiphenylborane (2APB) or inhibiting calmodulin activity with N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7), antagonised the mitogenic effect of IL-1β. Regulation of ERK (extracellular signal-regulated protein kinase) activity by IL-1β was also investigated. Data showed that IL-1β induced ERK phosphorilation in a dose dependent manner and this effect was antagonized by L-NAME, 1400W, ryanodine plus 2APB and W7. All together these data indicated that IL-1β upregulated cell proliferation by a mechanism which was cGMP-independent and occurred via the NO/Ca^{2+}/calmodulin/ERK signalling pathway.

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ENZYME-BASED ANTICANCER DIETARY MANIPULATIONS: "ORGANIC" vs. "INTEGRATED" FRUIT

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Epidemiological and animal studies, linking high and varied fruit and vegetable intake to lower cancer risk, suggested the theoretical possibility that regular, long-term mass administration of isolated, naturally occurring, dietary constituents, as well as the exclusive consumption of single greens, can provide a way of controlling cancer incidence. Although no exact mechanism of molecular chemoprotection is known, it is believed that the up-regulation of phase-II and the down-regulation of phase-I drug metabolizing enzymes by phytoalexins could protect from mutagens and neoplasia. Particular attention has been devoted to the intake of apples, as proposed by the "prophet" Edgar Cayce. Swiss Albino CD1 male mice were daily injected by gavage with either "organic" or "integrated" lyophilized apples in repeated (7 or 14 consecutive days) fashion and subcellular preparations from different organs (liver, kidney, lung) were tested for various monooxygenases, using either single substrates as probes of different CYPs or the regio and stereo-selective hydroxyilation of testosterone. Data were processed using the Wilcoxon's rank method. Both "organic" and "integrated" apples showed a complex pattern of induction (up to ~ 700% for the deethylation of ethoxyresorufin in kidney, 7 days treatment) and suppression (up to ~ 85% loss, same probe, lung, 14 days treatment) ($p<0.01$) of various CYP-linked microsomal monooxygenases. While no substantial differences between "organic" and "integrated" apples were seen, a manipulation of murine microsomal metabolism is actually present. This study suggests that caution should be exercised in using apple-base diet and, more in general, the exclusive extensive consumption of a single type of plant. It is not without means that the American National Cancer Institute has adopted the slogan "5 a day for better health", recently updated to "5 to 9 a day for better health", meaning to educate the population to a very varied diet, rich in fruits and vegetables.

ADHERENCE TO CHRONIC CARDIOVASCULAR THERAPIES: PERSISTENCE OVER THE YEARS AND DOSE COVERAGE

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To evaluate adherence to chronic cardiovascular drug treatments, in terms of long-term persistence and dose coverage. We collected general practice prescription data of antihypertensives, lipid lowering agents, oral hypoglycaemic agents and nitrates over a five-year period (1998-2002) in a Northern Italian district (Ravenna, 350,000 inhabitants). We selected subjects (> 40 years) receiving at least one prescription of the above drugs in December 1999. For each patient, we documented the regimen at the time of selection and evaluated adherence to treatment during the following 3 years in terms of persistence (at least one prescription per year) and daily coverage (recipients of an amount of medication consistent with daily treatment). Less than 10% of the 32,068 selected subjects were naïve to treatment. Antihypertensives were the most represented therapeutic category. Among patients already on treatment in December 1999, persistence was virtually complete, whereas more than 40% of naïve patients withdrew within one year, except for nitrates. The rates of coverage were always much lower than the corresponding values of persistence. Coverage resulted significantly higher in older patients (chi-square for trend 69.41; $p<0.001$), males (OR 1.30; 95% CI 1.25-1.36) and users receiving more than one therapeutic category. Lack of adherence to chronic cardiovascular treatments represents an important matter of concern: although most people continued treatment over the years, less than 50% received an amount of drugs consistent with daily treatment, thus jeopardising the proved beneficial effects of available medications.

EFFECTS OF RANOLAZINE ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF RAT HYPERTROPHIED VENTRICULAR CARDIOMYOCYTES

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Ranolazine is an active piperazine derivative that has been approved as a novel anti-anginal drug by the US Food and Drug Administration for the treatment of patients with chronic angina. Early studies in the 90's suggested that ranolazine exert its therapeutic action by inhibiting free fatty acid oxidation and by increasing oxidation of glucose and lactate. However, results of recent studies show that ranolazine reduces calcium overload in the ischemic myocyte by the inhibition of the late sodium current (INa). Therefore, we aimed to investigate the effect of ranolazine on ventricular cardiomyocytes isolated from the hypertrophic heart of aged spontaneously hypertensive rats (SHR). In this model, alterations in cellular electrophysiological properties have been extensively documented. **Material and** We used SHR as a genetic model for chronic hypertension and Wistar Kyoto normotensive rats (WKY) as a control; SHR progressively develop myocardial hypertrophy that increases with aging and finally evolves toward heart failure. SHR and WKY rats aged 12 -15 months were used. Isolation of left ventricular cardiomyocytes (VCMs) was obtained by enzymatic digestion on a Langendorff apparatus. The isolated cells were re-suspended in Tyrode's solution containing 0.5 mM calcium. The electrophysiological effect of 3 and 10 µM ranolazine (RAN) was assessed by patch-clamp recording of action potential (AP) in perforated configuration. APs were elicited at 0.2, 0.5 and 1 Hz in the current-clamp mode and AP duration (APD) measured at –50 mV (APD-50) and 90% of repolarization (APD90). Because RAN is reported to block late sodium current, we tested whether 1µM tetrodotoxin (TTX), a selective blocker of sodium channels, could mimic the effect of RAN on APD. APD90 in SHR cells was significantly increased with respect to WKY cells (220.9±21.4 ms vs 157.9±19.7 ms; $p<0.05$ n=9). The effect of RAN on AP consisted in a clear-cut dose-dependent decrease of APD in SHR cells. In fact, AP elicited at 0.2 Hz showed a significantly APD reduction after exposure to 3 and 10 µM RAN. APD-50 was 170.3±21.0 ms in control and 132.7±17.4 ms and 106.1±18.3 ms with 3 µM and 10 µM RAN, respectively ($p<0.05$, n=13); APD90 was 211.3±21.9 ms in control vs 182.1±17.1 ms and 156.9±17.7 ms in 3µM RAN and 10 µM RAN, respectively ($p<0.005$, n=13). The effect was similar in myocytes stimulated at higher driving rates (0.5 or 1 Hz). In WKY cells, only 10 µM RAN decreased APD at 0.5 and 1 Hz . At 0.2 Hz the effect of 10 µM RAN was higher in SHR cells with respect to WKY cells, APD90 being reduced to 74% and 80%, respectively. The effect of 1µM TTX at 0.2 Hz was similar to that of RAN in SHR cells: APD50 was 201.4± 24.1ms vs 139.6±11.5 ms and APD90 was 232.7±20.8 ms vs 180.4± 9.3 ms in control condition and after 1µM TTX exposure, respectively ($p<0.05$, n=7). In WKY cells APD50 was 186.6±11.2 ms vs 126.1±17.1 ms and APD90 was 223.8±10.3 ms vs 174.4±16.4 ms in control condition and after 1µM TTX exposure, respectively ($p<0.005$ n=4). Ranolazine reduces AP duration in SHR ventricular cells, to a greater extent than in WKY cells. Thus, increased Na entry through non-inactivating Na channels may contribute to delay in repolarization of AP in hypertrophied myocytes, an alteration which is specifically counteracted by exposure to ranolazine.

PROTEIN-THIOL SUBSTITUTION OR PROTEIN DETHIOLATION BY SH/SS EXCHANGE REACTIONS: THE ALBUMIN MODEL

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Protein *S*-thiolation occurs in all biological environments by two mechanisms that implicate thiol /disulfide (SH/SS)[§] exchange reactions: XSSX+PSH$\rightleftharpoons$XSH+XSSP (1) and/or sulfenic acid (PSOH) formation, e.g., PSOH+XSH$\rightarrow$XSSP+H_2O (2). It protects the SH enzyme from ROS inactivation by irreversible oxidation in sulfinic or sulfonic acids, activates cell signalling, buffers disulfide excesses and regenerates NPSH (namely GSH) by thiol/disulfide (SH/SS) exchange reactions (1-6) Protein dethiolation, the reverse of reaction, (1), is in plasma different than in cellular environment because not supported by enzymes (glutaredoxin) and by the GSH regenerating system (hexose monophosphate shunt) (1-3). In addition, the plasma protein thiolation/dethiolation process is almost exclusively sustained by albumin. Albumin is good protein model for studying the mechanisms of protein dethiolation and redox form distribution of plasma thiols. Moreover, these studies could contribute to solving the putative toxicity of Hcy redox forms, as this thiol is present, at about 80% of the total, in plasma as a mixed disulfide with albumin. We used biochemical approach to understand the kind of SH/SS exchange (substitution and dethiolation) of thiolated albumin with TNB and molecular simulation of differently thiolated albumin and computational chemistry to better interpret our biochemical data to understand which factors influence the plasma thiol redox form distribution by SH/SS exchange reactions Thionitrobenzoic acid was subjected to rapid substitution after thiol addition with the formation of the corresponding XSSP (peaks at 0.25-1 min) that in turn was dethiolated by the excess of remaining thiols, up to reaching an equilibrium at 35 min. Dethiolation of XSSP was accompanied by XSSX and PSH formation. In these experiments the reaction sequence related to the pKa difference of reactants underscores the importance of pKa to determine substitution or dethiolation. In addition, structures by molecular simulation of NPSHs thiolated albumin, carried out for understanding the importance of sulfur exposure of mixed disulfides, confirmed the pKa difference as an important factor to determine the leaving groups in SH/SS exchange reactions. At the contrary the exposure resulted to be the most relevant factor for the reacrion rate.

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GENE SILENCING OF COX-1, eNOS AND FGF-2 IN ENDOTHELIAL CELLS DURING ANGIOGENESIS

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Angiogenesis is a physio-patological process characterized by the formation of a new vascular network. Microvascular endothelial cells are the key players of angiogenesis process. This process is crucial for embryonic development, wound repair, inflammation and tumor growth. A number of growth factors and cytokines control this process. Cyclooxygenases (COXs) are key enzymes for inflammatory diseases and are known to modulate angiogenesis by releasing prostanoids. COX-1, constitutively expressed in endothelial cells, is responsible for vascular tone control and function through the production cytoprotective prostaglandins. Fibroblast growth factor 2 (FGF-2) plays an important role in vascular responses by increasing proliferation, and migration of endothelial cells *in vitro* and promoting angiogenesis *in vivo*. FGF-2 acts on endothelium in an autocrine/paracrine way inducing endothelial cell survival. Endothelial nitric oxide synthase (eNOS) is a crucial gene inducing angiogenesis either at tumoral or and physiological level. It has been demonstrated that nitric oxide is upstream of COX-1 and FGF-2 expression in vascular endothelial. In order to investigate the relative importance of FGF-2, COX-1 and eNOS in the control of endothelial functions required during angiogenesis, we studied the experimental conditions to silence each gene by RNA interference (RNAi). RNAi is a sequence-specific, post-transcriptional gene silencing mechanism initiated in animals and plants by the introduction of double stranded RNA (dsRNA) homologus in sequence to the silenced gene. The mediators of messenger RNA degradation are nucleotide called siRNA. Cell lines: Bovine, coronary postcapillary venular endothelial cells. Assays: Western Blotting; siRNA for COX-1, eNOS and FGF-2; proliferation and immunofluorescence. Our results show that all the mRNA under investigation can be silenced; COX-1 silencing resulted toxic for endothelial cells; silencing of eNOS and FGF-2 allowed a modulation of the functional responses; treatment of the cells with siRNA for eNOS blocked both trancription and protein synthesis of FGF-2, thus reinforcing the central autocrine axis NOS/FGF-2 in angiogenesis.

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PURINERGIC RECEPTOR ANTAGONIST MODULATES HYPERALGESIA AND ALLODYNIA IN A MOUSE MODEL OF NEUROPATHY: INVOLVEMENT OF NO SYSTEM AT CENTRAL AND PERIPHERAL NERVOUS SYSTEM LEVEL

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Neuropathic pain, consequent to peripheral injury, has been associated with upregulation of NOS expression and subsequent NO overproduction at level of both injured sciatic nerve and spinal cord. Animal models of nerve injury provide a reliable tool for investigating the mechanisms underlying neuropathic pain. First aim of this work was to study NO and its associated biosynthetic enzymes, at different pain transmission anatomic steps, in mononeuropathy induced by chronic constriction injury of C57BL/65 mice sciatic nerve. The paw withdrawal latency and the sensitivity to tactile stimuli were evaluated at 3, 7 and 14 days after injury by the plantar test and the dynamic plantar aesthesiometer, respectively. At the same times, thalamus, L4-L6 dorsal spinal cord, corresponding ipsilateral dorsal root ganglia (DRGs) and ipsilateral sciatic nerve, proximal to injury, were removed using a dissecting microscope. Neuronal and inducible nitric oxide synthase (i and nNOS) content was measured by western immunoblotting. Nitric oxide production was assessed on the basis of nitrite/nitrate, which are the oxidation end products of nitric oxide, using a fluorimetric procedure. Hyperalgesia and allodynia were already maximal at 3 days and they remained elevated up to 14 days. On 3rd day, the expression of iNOS was unmodified both locally and centrally and only at subsequent evaluation times increased in central and peripheral nervous system. In contrast, nNOS was precociously elevated in contralateral thalamus and only later it was upregulated in the peripheral nervous tissues. Nitric oxide content increased in all tissues, except for contralateral thalamus, at 7 and 14th day. These results support the hypothesis that NO-NOS system is involved in neuropathic pain, particularly nNOS seems to have a role on its development and maintenance; instead iNOS in later phases. So it appears to be important marker of painful neuropathy both in the central and peripheral nervous system. ATP activates cation ion channels, P2X receptors, and G-protein-coupled P2Y receptors and it is now recognised as an endogenous mediator of pain. The second objective was to evaluate the possible role of purinergic signalling in hyperalgesic and allodynic responses. Therefore we determined the effects of a non-selective P2 purinergic receptor-antagonist, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), on pain behaviour and increase in NONOS system. The administration in mice of PPADS (6.25, 12.5 and 25 mg/kg, i.p.), once a day for eleven days, from day 3 after the nerve injury, dose and time dependently attenuated both mechanical allodynia and hyperalgesia. The complete antiallodynic and antihyperalgesic efficacy of PPADS 25 mg/kg was associated with 1) reduction in nNOS content of contralateral thalamus, DRGs and sciatic nerve, 2) decrease in overexpressed iNOS in all tissues and, consequently, 3) inhibition of NO overproduction. Finally, we can hypothesize that PPADS is therapeutically effective, because it binds the different subtypes of purinoceptors (P2X and P2Y) involved in neuropathic pain.

GENDER-RELATED DIFFERENCES IN GUINEA-PIG URINARY BLADDER DAMAGE CAUSED BY ANOXIA-GLUCOPENIA/REPERFUSION CONDITIONS

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The aim of the present study was to investigate the effects of anoxia and glucopenia on both male and female guinea-pig urinary bladder. In whole bladders placed in an organ bath containing oxygenated Krebs' solution, intrinsic nerves undergone selective electrical field stimulation (EFS) and smooth muscle was stimulated with carbachol, ATP and high potassium. The effect of 1, 2 or 3 h anoxia-glucopenia (A-G) on the contractile response and the ensuing recovery in normal Krebs' solution, was monitored. In another series of experiments the glycogen content in whole male or female urinary bladder was measured. Results: under different stimuli, male urinary bladder proved to contract more efficiently than female organ. After 1 h of A-G the EFS response of the male urinary bladder was virtually abolished but had returned to 60% of the control response in the recovery phase; in female urinary bladder the EFS responses, markedly diminished during the A-G, fully recovered during the reperfusion phase. Full recovery of the response to carbachol, ATP and high potassium stimulations was observed in both male and female urinary bladder. A-G had to be extended to 2 h to cause smooth muscle impairment, which was more evident in male as compared to female urinary bladder. Under these conditions, a higher neuronal impairment was also observed in male than in female urinary bladders. When A-G was extended to 3 h, the intrinsic nerves response in the reperfusion phase was very poor. When 2-DG, an inhibitor of anaerobic glycolysis, was added during 1 h A-G phase, both neuronal and smooth muscle damage were significantly enhanced in male urinary bladder, as well as, though to a lesser extent, in female urinary bladder. A significantly higher, basal glycogen content was observed in female as compared to male bladders, and in both genders it was inversely related with the duration of exposure to A-G. In conclusion, the higher resistance of female urinary bladder to ischaemic-like conditions, can be partly ascribed to the higher glycogen content.

HEPATIC ADVERSE DRUG REACTIONS: A CASE/NON-CASE STUDY IN ITALY

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Adverse drug reactions ADRs can involve all tissues and organs, and liver injuries are considered among the most serious and are a cause of concern among physicians and patients. The aim of this study, was to assess the extent of drug-induced liver injuries in Italy by comparing the number of cases of hepatic ADRs with the reports of all the other drug-related reactions present in same database. The spontaneous reports collected from January 1990 to May 2005 were analysed. The adverse reactions were classified according to the WHO Adverse Reaction Terminology for causality assessment and only those with "certain, "probable" or "possible" causality assessment were included. Probable association between drugs and hepatic ADRs was assessed using a case/non case method. On May 2005, the database contained 35,767 reports of ADR, of which 11,829 were excluded because unclassifiable or unlikely in terms of causality assessment. Therefore, the analysis was carried out on 23,938 reports, of which 1,069 concerning hepatic ADRs (cases) and 22,869 non-cases. The percentage of the serious ADRs was about 40% in the overall database, and about 74% among the cases. Statins, antiplatelet agents, non-steroidal anti-inflammatory drug and macrolides were the drug classes with the highest number of cases. Hepatic adverse drug reactions remain an important concern for several widely used drugs in clinical practice. Monitoring hepatic enzymes on a monthly basis for the first six months of treatment has been suggested for patients taking medications that are known as hepatotoxic. A better knowledge of epidemiology and mechanisms of hepatic ADRs may contribute to minimize their occurrence.

NEUROPATHIC PAIN AND THE ENDOCANNABINOID SYSTEM IN THE DORSAL RAPHE: PHARMACOLOGICAL TREATMENT AND INTERACTIONS WITH THE SEROTONERGIC SYSTEM

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Cannabinoids have been proven effective for the treatment of neuropathic pain. Here we have used a model of neuropathic pain, consisting of rats with chronic constriction injury (CCI) of the sciatic nerve, to investigate whether endocannabinoid levels are altered in the dorsal raphe (DR) and to assess the effect of repeated treatment with WIN 55,212-2, a synthetic cannabinoid agonist, or AM404, an inhibitor of endocannabinoid reuptake, on DR serotonergic neuronal activity changes and on behavioural hyperalgesia. CCI resulted in significantly elevated anandamide, but not 2-arachidonoylglycerol, levels in the DR. Furthermore, along with thermal and mechanical hyperalgesia, CCI caused serotonergic hyperactivity (as shown by the increase of basal activity of serotonergic neurons, extracellular serotonin level, and expression of 5-HT_{1A} receptor gene). Repeated treatment with either WIN 55,212-2 or AM404 throughout the development of neuropathic pain reverted the hyperalgesia and enhanced serotonergic activity induced by CCI in a way attenuated by daily co-administration with SR141716A, a selective cannabinoid subtype 1 (CB_1) receptor antagonist. Despite the elevated levels of anandamide following CCI, SR141716A did not produce hyperalgesia nor any effect on serotonergic neuronal activity when administered alone. Furthermore, the effects of AM404 were not accompanied by elevation of endocannabinoid levels in the DR. In conclusion, following CCI of the sciatic nerve, the endocannabinoid and serotonergic systems are activated in the DR, where repeated stimulation of cannabinoid CB_1 receptors with exogenous compounds restores DR serotonergic activity, as well as thermal and mechanical nociceptive thresholds, to pre-surgery levels. However, elevated endogenous anandamide in the DR does not necessarily participate in the CB_1-mediated tonic control of analgesia and serotonergic neuronal activity.

DOXORUBICIN CARDIOTOXICITY: EXPOSURE TIMING AND APOPTOSIS

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Experimental data support the presence of cardiomyocyte apoptosis (CA) in doxorubicin (DOX) induced cardiomyopathy; in particular, a significant induction of CA has been demonstrated shortly after DOX administration with a peak 24-48 h after the first injection (1). It is known that DOX-cardiotoxicity is more severe when the same cumulative dose is given for short period by administration of high doses than for a long period low doses (2). Aim of this study was to assess if variations of exposure times to the same DOX cumulative dose influence cardiac apoptosis in rats. Wistar rats were randomly assigned to either controls (CTL) (n=10) or to each DOX-treated (TRT) groups (n=20). DOX was administered intraperitoneally four times per week at the following doses: a) 5 mg/kg/day over a period of one week; b) 2.5 mg/kg/day over two weeks; c) 1.25 mg/kg/day over four weeks. Control animals received injections of comparable volumes of saline. DOX cumulative dose administered to all groups (20 mg/kg) was estimated as being sufficient to induce progressive cardiomyopathy (3). 48 h after first DOX administration and at the end of treatments, rats were sacrificed, hearts were excised and homogenated to be used for western blotting analysis of protein expression. All values were expressed as the mean ± SE and evaluated by paired Student's *t* test. A *P* value of <0.05 was considered significant. Forty-eight h after first DOX administration, western blot analysis showed that:

- 5 mg/kg/day DOX significantly ($p<0.05$) increased expression levels of proapoptotic and antiapoptotic proteins: a) procaspase 3 (TRT 1.81±0.1 vs. CTL 1.30 ± 0.08) and its cleaved form (TRT 7.41 ± 1.33 vs. CTL 0.6 ± 0.2); b) procaspase 9 (TRT 0.94 ± 0.12 vs. CTL 0.18 ± 0.06) and its cleaved form (TRT 0.61 ± 0.09 vs. CTL 0.10 ± 0.02); c) Bax/Bcl_2 *ratio* (TRT 0.46 ± 0.03 vs. CTL 0.22 ± 0.02).
- 2.5 mg/kg/day DOX and 1.25 mg/kg/day DOX did not affect expression levels of pro- and antiapoptotic proteins.

Moreover, at the end of treatments:

- 5 mg/kg/day DOX significantly ($p<0.05$) increased expression levels of: a) procaspase 3 (TRT 3.9 ± 0.2 vs. CTL 2.8 ± 0.13) and its cleaved form (TRT 2.5 ± 0.1 vs. CTL 1.6 ± 0.07); b) procaspase 12 (TRT 6.83 ± 0.3 vs. CTL 4.31 ± 0.4) and its cleaved form (TRT 8.82 ± 1.15 vs. CTL 0.23 ± 0.11); c) procaspase 8 (TRT 3.3 ± 0.1 *vs.* CTL 2.6 ± 0.2); d) Bax/Bcl_2 *ratio* (TRT 0.2 ± 0.01 vs. CTL 0.08 ± 0.02).
- 2.5 mg/kg/day DOX significantly ($p<0.05$) increased expression levels of: a) procaspase 12 (TRT 7.95 ± 0.1 vs. CTL 4.83 ± 0.53).
- 1.25 mg/kg/day DOX did not affect expression levels of pro- and antiapoptotic proteins.

It is concluded that 20 mg/kg DOX administered over a period of four weeks induced minor apoptotic effects respect to the administration of the same cumulative dose in shorter period. This could be useful to establish a treatment scheme with best efficacy and less toxicity. Further studies are in progress to elucidate these mechanisms.

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DEVELOPMENT OF NEW CELL CULTURE SYSTEMS: THE BIOREACTORS

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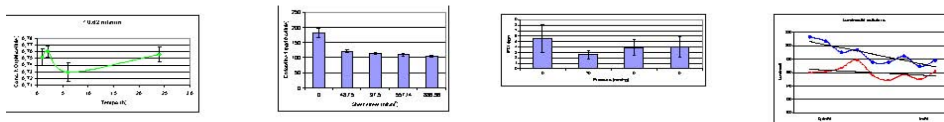
Static cell cultures have provided a great deal of information about cell biochemistry but show some limits, particularly for the simulation of a physiological environment. The aim of this work is to exploit new research instruments developed at the Faculty of Engineering of University of Pisa for evaluating the possible use in pharmacological testing. Our bioreactors, flexible in the use and customizable to work demand, are composed of two parts: one is the control system performed by a pc and the second part consisting of the cell culture chamber. We have developed four different type of cell culture chambers useful to understand different types of chemical and physical stimuli on various cell lines. The four bioreactors are: *Laminar Flow Bioreactor*: specially designed for fluid dynamic studies to obtain controlled laminar flow conditions was used test endothelial cells under different shear stress; this force produces, during experiments, a particular trend in Nitric Oxide (NO, Fig. 1a) and Endothelin1 (ET-1, Fig 1b) production.



Hydrostatic Pressure Bioreactor: absence of flow and possibility to generate desired pressure impulses let us to analyze only effect of pressure Figure 2 on cells for example to simulate the effects of hypertension on the endothelium; variation in ET-1 (Fig. 2) production let us to suppose a role for this endogenic molecule in regulation of vascular tone. *Concentration Gradient Bioreactor*: this type of chamber geometry creates a gradient of concentration of xenobiotics and was tested on endothelial cells using H2O2 with two different concentrations and analising, by imaging techniques, its apoptotic effect (Fig. 3). Figure *Multi-Compartmental Bioreactor (MCB)*: developed on the basis of allometric laws, with this bioreactor we can realise connected cultures; in this system different type of cells are not physically in contact but there is media culture that flows, "connects" and let a cross-talk by biomolecules produced. Connected cultures of HepG2 or murine hepatocytes and HUVECs were tested. Samples collected during experiments was tested for different substrates like albumin (Fig. 4a, b), urea (Fig. 5a,b), NO, glucose. Results show a non-physical interaction between these two cell types.

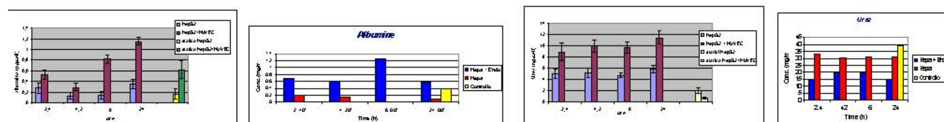


Figure 4a Figure Figure Figure These cell culture systems have demonstrated the feasibility of using bioreactors in pharmacology and physiology studies and their possible use for support in testing of chemical substances to understand better their possible effects on human body.

H. PYLORI CLARYTHROMICIN RESISTANCE: A MAJOR DETERMINANT OF ERADICATION THERAPY FAILURE

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The eradication of *Helicobacter pylori* is an important prevention tool and an efficacious treatment for severe upper gastrointestinal disorders associated with the bacterial infection. Current treatment strategies utilizing a proton pump inhibitor (PPI) and two antibacterial agents (triple therapies), yield an eradication rate of around 80-90%. Both host and bacterial genetic determinants affect the outcome of the treatment. In particular, genetically determined differences in the activity of the cytochrome P450 (CYP)2C19 isoenzyme, influencing the pharmacokinetics of many PPIs, have been demonstrated to affect the *H. pylori* eradication rate. The aim of the present study was to verify, among *H. pylori* infected subjects, any correlation between the eradication outcome and:1. the host polymorphisms of *CYP2C19*, *IL1B* and *IL-1RN* genes; 2. *H. pylori* clarithromycin resistance (Cla^R) and virulence gene *cagA*. Patients infected by *H. pylori* (culture and/or histology positive) underwent a 7 days triple therapy (omeprazole 20 mg b.d., amoxycillin 1g b.d. and clarithromycin 500mg b.d.). Eradication success was assessed by ^{13}C Urea Breath Test two months after therapy. DNA was extracted from gastric biopsies and bacterial isolates. Host *CYP2C19* -806 C>T and +681G>A, *IL-1 β* -31 T>C and *IL-1RN* intron 2 VNTR polymorphisms were studied by means of PCR and TaqMan chemistry, RFLP analysis and agarose gel electrophoresis respectively. *H. pylori cagA* gene presence was assessed by PCR and agarose gel electrophoresis while all five 23S rDNA mutations known to cause Cla^R were assayed by a PCR and reverse hybridization approach. Forty-seven eradicated and fifty-five not eradicated patients were enrolled in the study. The overall genotype frequencies were: C/C 51.5%, C/T 42.6% and T/T 5.9% for *CYP2C19* -806; G/G 74.5%, A/G 23.5% and A/A 2.0% for *CYP2C19* +681; T/T 39.2%, C/T 54.9% and C/C 5.9% for *IL-1β* and 1/1 49.0%, 1/2 33.3%, 2/2 6.9%, 1/3 3.9%, 1/4 1.0%, 1/5 1.0% and 2/3 4.9% for *IL-1RN*. *H. pylori* eradication success was significantly associated with Cla^R (χ^2=10.826, p<0.01), being 23S rDNA mutations detected in 0/18 and 18/53 eradicated and not eradicated cases respectively. *CagA* positive and negative strains were eradicated at similar rates (χ^2=0.174, p:ns). The *CYP2C19* -806 T allele (associated with an increase CYP2C19 trancriptional activity) was slightly more frequent among not eradicated than in eradicated subjects (32.3% vs 23.4% respectively). However, treatment outcome was not significantly associated with the genotype or allelic frequencies of any polymorphism studied both considering patients overall (χ^2=2.402, p:ns and χ^2=1.297, p:ns for *CYP2C19* 806; χ^2=0.013, p:ns and χ^2=0.001, p:ns for *CYP2C19* +681; χ^2=1.721, p:ns and χ^2=1.193, p:ns for *IL-1B*; χ^2=3.797, p:ns and χ^2=2.690, p:ns for *IL-1RN* respectively) or only those infected by clarithromycin sensitive *H. pylori* strains (χ^2=1.909, p:ns and χ^2=1.488, p:ns for *CYP2C19* 806; χ^2=0.093, p:ns and χ^2=0.014, p:ns for *CYP2C19* +681; χ^2=2.952, p:ns and χ^2=1.120, p:ns for *IL-1 β*; χ^2=6.819, p:ns and χ^2=4.521, p:ns for *IL-1RN* respectively). In conclusion, clarithromycin resistance of the *H. pylori* infecting strain appears to be a major determinant of *H. pylori* eradication failure following triple therapy. Host genetic polymorphisms seem to play only a minor role.

CHARACTERIZATION OF NEUROPROTECTIVE MECHANISMS OF GUANOSINE AND SIGMA RECEPTOR LIGANDS

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Systems that are involved in achieving neuroprotection comprehend the adenosine receptor family and the those able to reduce oxidative stress, but the quest for new neuroprotective pathways is still open. Recent findings indicate that guanosine, another purine derivative, can exert trophic effect in glial cells, whereas the neuroprotective effects and the purported mechanisms of this molecule on neurones are largely unexplored. So, we are investigating the putative role of guanosine in cell proliferation and differentiation, and the signal transduction pathways involved using the human-derived neuroblastoma SH-SY5Y cell line. The proliferative effect of guanosine was initially investigated using the MTT test. In these experiments, guanosine significantly and concentration-dependently rescued cells exposed to starvation for 24 (+23% for 75 µM guanosine), 48 or 72 h. Both the MAPK and the PI3-K pathways seem to be involved in these effects: a 30-min pre-treatment with selective inhibitors (10 µM PD-098,059 and 30 µM Ly-294,002, respectively) significantly antagonized 100 µM guanosine-induced cell proliferation and, in parallel, guanosine-induced phosphorilation of ERK1/2 and AKT as determined with the ELISA CASE™ Kit. The involvement of PKA and PKC will be determined measuring the ability of guanosine to induce changes in cAMP and IP_3 levels with binding protein assays based on the displacement of [^{3}H]cAMP and [^{3}H] IP_3 by cell samples. The effects of guanosine on cell cycle will be confirmed using the flow citofluorimetry technique. Preliminary experiments indicate that guanosine can also affect cell differentiation. Finally, radioligand binding saturation experiments with [^{3}H]guanosine will be performed on both intact cells or cell-derived membranes in order to establish the presence of specific guanosine recognition sites on cells and to proceed to its pharmacological characterization. Another interesting target for neuroprotection are the sigma receptors. These receptors have no homology with other receptor systems, have no known natural ligands, but appear to play a critical role in a large diversity of cell functions. σ_1 receptor subtypes are involved in cell survival, in the modulation of neurotransmitter release and neural firing and behaviourally in learning and memory processes. Utilizing radioligand binding assays we are thus screening different series of newly synthesized compounds in order to identify potent and selective ligands. With the same technique the modulatory activity of phenytoin on agonist binding will be used to discriminate between σ_1 receptor agonists and antagonists. Finally, the most promising σ_1-agonists will be tested as putative neuroprotective agents using the SHSY5Y cell line, that express both sigma receptors.