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INTERACTIONS OF NOVEL MDR REVERTING AGENTS WITH RAT SMALL INTESTINE MEMBRANE VESICLES ATPase ACTIVITY

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My doctoral work has dealt with the pharmacological characterization of novel compounds capable of reverting MDR in cancer cells. To investigate the interactions between drugs and MDR transporters, in search for novel effective MDR reverting agents, ATPase activity measurements on plasma membrane vesicles prepared from homogenates of rat small intestine mucosa (1) were performed with use of a spectrophotometric method based on continuous monitoring of ADP formation, regenerated in ATP by a coupled enzyme system (2). Western blot analysis indicated that MRP1, P-gp and MRP2 are expressed in these vesicles. MDR proteins exhibited both an ATPase activity correlated with drug transport and a basal ATPase activity in the absence of any drug. These enzyme activities were fully inhibited by μM concentrations of Na-orthovanadate, thus indicating that they are ascribable to P-gp and MRP1 (3). As reference substrates of these transporters both verapamil and epirubicin were used. Verapamil and epirubicin gave typical bell shaped concentration-activation curves with 30 μM and 1 μM maximum effective concentrations which stimulated basal ATPase activity by 55% and 250%, respectively. 3,5-Dibenzoyl-4-(3-phenoxy-phenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) has been shown to inhibit at sub μM concentrations P-gp mediated efflux of rhodamine 123 (R123) in LY5178 MDR1-transfected mouse lymphoma cells (4). DP7 inhibited concentration-dependently both basal and verapamil-stimulated ATPase activities, with IC_{50} values of about 1 μM. The inhibition mechanism of DP7 towards verapamil-stimulated enzyme was competitive in nature, with a K_i of 5.3 μM. Four novel isomeric N,N-dicyclohexane-4-ol-amine aryl esters, indirectly proven to be powerful inhibitors of P-gp in erythroleukemia K562 cells (5), were also tested for their effects on intestinal ATPase activity. The cis-cis isomer (MC185oxa) gave a typical bell shaped concentration-activation curve with 50 nM maximum effective concentration, thus behaving like an ATPase substrate. MC185oxa was tested also on 30 μM verapamil-stimulated ATPase activity showing a bimodal effect, at ≤25 nM stimulating while at higher concentrations inhibiting it. The trans-cis isomer (MC176oxa) inhibited in a typical U-shaped concentration-inhibition curve the basal ATPase activity, maximal inhibition (about 55%) being achieved at 5 nM. At higher concentrations the inhibition was reversed up to values comparable to those of control preparations. Moreover, at concentrations ≥5 nM, MC176oxa fully antagonised the portion of ATPase activity stimulated by either 1 μM epirubicin or 30 μM verapamil. The cis-trans and the trans-trans isomers did not exert significant effects. P-gp inhibiting properties of these compounds have been also evaluated by measuring the efflux of the P-gp specific, fluorescent substrate R123 in MDR1-gene transfected mouse T-limphoma L5178 cells in presence of different concentrations of the selected compounds. P-gp blocking activity was described by α_{max}, to express the efficacy and by IC_{50}, to measure the potency of the inhibitor. α_{max} varied between 0 (in the absence of the inhibitor) and 1 (when the amount of R123 found in L5178 MDR1 cells was equal to that determined in presence of 5 mM vanadate, which completely inhibited R123 efflux). In agreement with the results observed in K562 cells, cis-cis and trans-cis isomers were very potent and efficient inhibitors, with IC_{50}s in the low nanomolar range and α_{max} values very close to 1. In conclusion, trans-cis and cis-cis isomers exhibited both low nM potency and high efficacy in the tests used, thus appearing as novel inhibitors of MDR transporters and promising leads for the development of potent, efficient and safe MDR reverters.

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CHARACTERIZATION OF PRIMARY SMOOTH MUSCLE-LIKE CELLS FROM A *TSC2* RENAL ANGIOMYOLYPOMA. PROMOTER METHYLATION OF *TSC2* AS A NEW ONSET FOR THE PATHOGENESIS OF TUBEROUS SCLEROSIS LESIONS

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Tuberous sclerosis (TSC) is an autosomal-dominant disease characterized by hamartoma formation in various organs such as central nervous system, kidney and skin. The most common kidney lesion is the angiomyolypoma (AML). TSC is caused by mutations in two tumor suppressor genes *TSC1*, located on chromosome 9q34, and *TSC2*, located on chromosome 16p13, respectively encoding hamartin and tuberin. Tuberin and hamartin act together as a complex that inhibits phosphorylation of S6 kinase (S6K1) through mammalian target of rapamycin (mTOR). TSC is due to inactivating mutation in *TSC1* or *TSC2*, and follows the Knudson's two-hit tumor suppressor gene model. The first hit is a congenital lesion of one of the TSC genes, and the second hit is a defect occurring in the second allele causing loss of heterozygosity (LOH). In TSC, LOH frequency varied significantly among tumor types. In particular, LOH was found in 56% of renal TSC AML but only in 4% of TSC brain lesions (1). A number of different possibilities has been raised to explain, in many TSC tumors, the inability to identify a second, somatic event in *TSC1* or *TSC2*, for example the epigenetic silencing. However, no evidence of *TSC2* promoter methylation has been reported (2). We have isolated a homogenous cellular population from an AML of a male TSC patient. By immunofluorescence, the cells were characterized as smooth muscle-like cells (ASM cells) for the strong reactivity to α-actin antibody, a marker of smooth muscle cells, and the lack of labelling for vimentin, a marker of fibroblasts, and keratin 8-18, a marker of epithelial-like cells. The ASM cells were mostly positive for HMB45 and CD44v6, the markers of TSC and LAM cells (3,4). Genetic analysis by DNA sequencing showed a germline *TSC2* intron 8-exon 9 junction mutation. Although DNA analysis and PCR amplification, using a panel of microsatellite markers near the TSC *locus*, failed to demonstrate LOH, tuberin was undetectable by immunofluorescence and western blotting. These data lead us to assess whether the second hit could be an epigenetic lesion. Methylation of the *TSC2* promoter in ASM cells was found, and confirmed by transcriptional reactivation and tuberin expression following exposure to the histone deacetylase inhibitor, trichostatin A. Moreover, tuberin expression induced by trichostatin A incubation correlated with the insoppearance of HMB45 immunolabeling. These cells were, thus, named $TSC2^{-/meth}$ ASM cells. Their growth required epidermal growth factor (EGF), and insulin-like growth factor-I (IGF-I) did not replace it. $TSC2^{-/meth}$ ASM cells were very similar for their proliferative, morphological and biochemical characteristics to the smooth muscle cells with LOH that we previously isolated from an AML of a female *TSC2* patient ($TSC2^{-/-}$ ASM cells) (5). Similarly to $TSC2^{-/-}$ ASM cells, the blockade of EGF and IGF-I receptors with specific monoclonal antibodies, caused their death in 15 days. Rapamycin, an inhibitor of mTOR, and anti-EGFR antibody inhibited S6 and Erk phosphorylation in 24 h. Trichostatin A incubation for 72 h induced a reduction of S6 and Erk phosphorylation while, as expected, it was ineffective in $TSC2^{-/-}$ ASM cells. Our data show for the first time that a methylation in the *TSC2* promoter might cause LOH in TSC2 cells, and that the hamartoma pathogenesis also might be originated from epigenetic lesion in smooth muscle cells.

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LOW-DOSE NAPROXEN INTERFERES WITH THE ANTIPLATELET EFFECT OF LOW-DOSE ASPIRIN IN HEALTHY SUBJECTS

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Aspirin is the only cardioprotective nonsteroidal anti-inflammatory drug (NSAID) because it causes a complete and persistent suppression of cyclooxygenase (COX)-1-dependent thromboxane (TX)A_2 generation in platelets (1). However, naproxen, a balanced inhibitor of COX-1 and COX-2 with a long half-life (>12 h), can cause an aspirin effect on platelet COX-1, at least in some individuals, when it is administered at high-doses (500 mg BID) (2). However, a pharmacodynamic interaction between aspirin and high-dose naproxen has been detected and might translate into potential loss of aspirin cardioprotection (3). We performed a clinical study to investigate whether low-dose naproxen sodium (220 mg BID) administered 2 h before or after the administration of aspirin 100 mg daily interferes with aspirin's antiplatelet effect. Six healthy volunteers, received for 6 days 3 different treatments separated by 14 days of washout: i) naproxen 2 h before aspirin; ii) aspirin 2 h before naproxen; iii) aspirin alone. Blood samples were collected before and at 1, 2, 5, 12, 24, and 48 h after the first study drug on the 6_{th} day of different treatments to assess the inhibition of serum TXB_2, a capacity index of platelet COX-1 activity, and platelet aggregation induced by arachidonic acid (AA) (2 mM) and collagen (10 μg/mL) in plateletrich plasma (PRP). Urine samples were collected overnight (from 8 pm to 8 am) at baseline and on the 6_{th} day of the different treatments for 24 h for the assessment of the urinary excretion of 11-dehydro-TXB_2, a major enzymatic metabolite of TXB_2 that is an index of TXA_2 biosynthesis *in vivo*. The administration of low-dose aspirin for 6 days caused an almost complete inhibition of platelet COX-1 activity at 1 h after dosing which persisted up to 24 h after dosing (99.2+0.30% and 99.0+0.35%, respectively, mean+SEM, n=6). The profound and persistent suppression of platelet capacity to generate TXA_2 translated into a significant inhibition of platelet function induced by AA and collagen. The administration of naproxen 2 h before or after aspirin slightly mitigated the suppression of serum TXB_2 (95.8+2.9% and 98+0.43%, respectively) but it was associated with rapid recovery of collagen-induced platelet aggregation. However, in 1 subject out of 6, AA-induced platelet aggregation was completely recovered at 24 h after dosing with aspirin administered 2 h after naproxen. Moreover, we evidenced that higher degrees of suppression of platelet COX-1 activity are necessary to block collagen- than AA-induced platelet aggregation. In fact, a complete and homogeneous inhibition of platelet aggregation in PRP induced by AA occurred in the presence of >97% inhibition of platelet COX-1 activity *ex vivo*, while a more profound suppression of platelet COX-1 activity (>99.5%) was necessary to translate into a complete suppression of collagen-induced platelet aggregation. The reduction of urinary 11-dehydro-TXB_2 by aspirin was not affected by the coadministration of naproxen. These data suggest that the coadministration of naproxen with aspirin marginally mitigated the inhibition of COX-1 activity *ex vivo* caused by aspirin alone, at dosing interval. In conclusion, low-dose naproxen interfered with the irreversible inhibition of platelet function by aspirin. The consequences of this interaction at the clinical level remain to be tested in very large and well-controlled randomised clinical trials.

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TM4SF2: A GENE INVOLVED IN NON SYNDROMIC X-LINKED MENTAL RETARDATION AND ITS ROLE IN SYNAPSE DEVELOPMENT

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Non syndromic X-linked mental retardation (NS-XLMR) is a disorder characterized by significant limitations both in intellectual functioning and in adaptive behavior without any other distinctive clinical feature. A major challenge is to uncover the molecular causes of NS-XLMR and the underlying cellular mechanisms responsible for reduced cognitive function. Recently a novel form of NS-XLMR has been associated to mutations that alter TM4SF2, a member of the tetraspanins whose function in nervous system is still unknown. TM4SF2, when is overexpressed in COS-7 cells, localizes both in cytosol and plasma membrane. In the same cells TM4SF2 wild type, but not a mutated gene encoding a protein lacking the C-terminal tail, induces the formation of filopodia-like structures that are rich in actin. In hippocampal neurons, overexpressed TM4SF2 distributes in patches on dendrites as well as on axons; in particular the protein can be detected in dendritic spines of mature neurons and at the tip of axonal filopodia of young neurons. In young hippocampal neurons the overexpression of TM4SF2 causes an increase in axonal filopodia number compared to control neurons. By a two-hybrid screening of a human fetal brain cDNA library performed with the C-terminal intracellular domain of TM4SF2 as bait, we identified PICK-1 (protein interacting with C kinase I) as interactor of TM4SF2. In cotrasfected COS-7 cells TM4SF2, but not TM4SF2 lacking the C-terminal tail, partially colocalizes with PICK-1. *In vitro* the interaction has been validated by coimmunoprecipitation and GST pull down assay and involves the last four C-terminal amino acids of TM4SF2 and PICK-1 PDZ domain. The Cterminal tail of TM4SF2 is able to pull down PICK-1 from rat brain and GluR2/3, PICK-1 and β1-integrin from hippocampal neuron lysates. In hippocampal neurons TM4SF2 colocalizes with both PICK-1 and GluR2/3. These data suggest that TM4SF2 is implicated in the formation of the synaptic network during development.

TETRADECYLTHIOACETIC ACID INDUCES APOPTOSIS IN ANAPLASTIC THYROID CARCINOMA CELL LINE

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Anaplastic thyroid cancer (ATC) accounts for approximately 2-4% of thyroid carcinomas and is an extremely aggressive disease (1). Whereas 80-85% of thyroid carcinomas are well differentiated and most of them have a favourable prognosis, ATC is almost invariably fatal with a median survival ranging 4-7 months from diagnosis. Because of the relative rarity of the disease and the poor survival, the optimal therapeutic approach to ATC is uncertain. Some improvement has been reported in individual patients who have undergone multimodal therapy, including thyroidectomy, chemotherapy, and external radiation (1,2). However, the impact of chemotherapy on survival is poor and only very few patients survive longer than 5 years. Recently, ligand-activated transcription factors, peroxisomal proliferators-activated receptors (PPARs) that exist in three different isoforms α, β/δ, and γ, and belong to the nuclear hormone receptor superfamily, have emerged as regulators of cell proliferation, apoptosis and/or differentiation on several tumoral cell lines. Our study focused on tetradecylthioacetic acid (TTA), a modified saturated fatty acid analog pan activator of the PPARs that exhibits antiproliferative effects in several cancer cell lines such as native human acute myelogenous leukaemia blast cultures (3), rat glioma cells (4), and human breast cancer cells (5). Here we investigated the antiproliferative and proapoptotic effects of TTA on anaplastic thyroid carcinoma cell line (ARO) and PPARs involvement. The antiproliferative effects of TTA were evaluated by MTT assay and relevead a citotoxic activity in ARO cells with IC_{50} of 130.1 μM at 72 h. Apoptotic effect of TTA (30-100 μM; 24-48 h) in ARO cells, revealed by propidium iodide staining and flow cytometric analysis, was time and concentration related and accompanied by a significant ($P<0.05$) reduction of the antiapoptotic protein Bcl-2 expression and significant ($P<0.01$) activation of PARP-1, a DNA repair enzyme. Moreover, ARO cell treatment with 100 μM TTA induced both a time-dependent (6, 24, and 48 h) increase in the expression of PPARα and PPARγ reduction. Malonyldialdehyde level, an indicator of lipid peroxidation, was also significantly ($P<0.01$) increased by TTA treatment (100-200 μM, 48 h). Our data strongly supported an involvement of PPARs in the antiproliferative and pro-apoptotic effect of TTA.

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IMPACT OF CYP2C9 AND CYP2B6 ISOFORMS TOXICITY AND RESPONSE TO CHEMOTHERAPY IN BREAST CANCER PATIENTS

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The most commonly used drugs for the treatment of breast cancer include cyclophosphamide (CPA) as a single agent or in combination regimen with other drugs. Variability in response to treatment or in the development of toxicity could be explained by genetic polymorphisms (SNP) that alter the activity of enzymes such as cytochrome p450 (CYP). The hepatic bioactivation of CPA in 4-hydroxylation involves several isoforms of CYP, primarily CYP2B6 and CYP2C9. In this study, we have considered the SNPs of these isoforms that are the most frequent in Caucasian population. In particular, we have studied the variants: CYP2C9*2 (430C>T), CYP2C9*3 (1075A>C), CYP2B6*5(1459C>T) and CYP2B6*7 (516G>T, 785A>G and 1459C>T); all of these lead to a reduction of the enzyme activity. The aim of this study was to define the role of these polymorphisms in the development of severe toxicity (at the beginning and the end of treatment) and in survival in breast cancer patients after CPA treatment. Two hundred breast cancer patients were homogeneously treated with CMF (cyclophosphamide, methotrexate, and 5-fluorouracil) adjuvant regimen. Genomic DNA was extracted from peripheral blood mononuclear cells. The genotype analysis was performed using two PCR-based methods: RFLP (restriction fragment polymorphism) and pyrosequencing (mini-sequencing). Data from toxicity and survival was obtained from 185 patients. Development of hepatic toxicity was significantly correlated with genetic variants CYP2C9*2 and CYP2C9*3, both after the first cycle and at the end of chemotherapy. For CYP2C9*2 the association is confirmed when comparing patients carrying at least one 430T allele and grade 1-2-3 hepatic toxicity (p=0.0011, OR 3.55, CI 95% 1.676-7.527) with respect to the patients with 430CC genotype. A significant correlation was also found between CYP2C9*3 and hepatic toxicity (grade 0-3) at the end of chemotherapy (p=0.0298). Significant correlations with the overall survival were resulted coming out or CYP2C9*3 (p=0.0487; HR=1.5, 95% CI 0.89-5.65) and for CYP2B6*7 (p=0.0425; HR=6.21, 95% CI 0.83-46.9): for both these SNPs the presence of at least one variant allele is related to a reduced survival. This latest polymorphism was also significantly correlated with the development of hepatic toxicity at the end of therapy (p=0.0039). The presence of genetic variants in cytochrome P450 (CYP2C9*2, CYP2C9*3, and CYP2B6*7) could have an impact in the development of hepatic toxicity possibly due to a reduction in bioactivation of cyclophosphamide and leading to in accumulation of the parental drug. Moreover, two of these polymorphisms (CYP2C9*3 and CYP2B6*7) also show a significant impact in overall survival probably due to a reduction of cyclophosphamide action in patients with breast cancer treated with CMF chemotherapy.

EFFECTS OF THE PHYTOESTROGEN GENISTEIN IN AN EXPERIMENTAL MODEL OF POSTMENOPAUSAL METABOLIC SYNDROME

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Genistein, a soy derived phytoestrogen, has been demonstrated to be effective in reducing cardiovascular risk in postmenopausal women. We therefore investigated its effects in an experimental model of postmenopausal metabolic syndrome. Female ovariectomized spontaneously hypertensive obese rats (OVX-SHROB), a genetic model of syndrome X, and intact age-matched Wistar Kyoto (WKY) rats were used. Four weeks after surgery animals were randomized to receive either genistein (54 mg/human equivalent dose/day for 4 weeks), or vehicle. Body weight, food intake, systolic blood pressure (SBP), heart rate, plasma glucose, insulin resistance, total plasma cholesterol and triglycerides, and uterine weights were studied. Furthermore, we investigated acetylcholine (ACh)- and sodium nitroprusside (SN)-induced relaxation of aortic rings as well as NG-L-arginine (L-NMA: 10-100 μM) induced vasoconstriction in phenylephrine precontracted aortic segments. Liver expression of the peroxisome proliferator-activated receptor alpha (PPAR-α and gamma (PPAR-γ) was also assessed. OVX-SHROB animals had a slight increase in SBP, body weight, insulin resistance and plasma cholesterol. SHROB rats showed also impaired endothelial responses, blunted L-NMA induced contraction (L-NMA 100 μM: WKY=2.2±0.3 g/mg tissue; OVX -SHROB=1.1±0.4 g/mg tissue). Genistein treatment decreased SBP and plasma lipids, ameliorated endothelial dysfunction and insulin resistance, increased HDL-cholesterol and enhanced liver expression of PPAR-α and PPAR-γ. Our data strongly suggest that genistein is effective in improving cardiovascular profile in a experimental model of post-menopausal metabolic syndrome, likely stimulating the liver expression of PPAR-α and PPAR-γ receptors. These evidences would support the rationale for a clinical use of genistein in post-menopausal women affected by metabolic syndrome.

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INVOLVEMENT OF ANNEXIN A1 IN MOUSE MYOBLAST CELL DIFFERENTIATION

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Annexin A1 (ANXA1) is a calcium- and phospholipid-binding (1) protein involved in a broad range of cellular events including cell-cell communication, membrane aggregation and fusion, cell proliferation and differentiation (2-4). This study used molecular and microscopy approaches to explore the role of ANXA1 in C2C12 mouse myoblast cell differentiation. We found that the levels of ANXA1 expression increased during differentiation process and that the protein translocated from the cytoplasm to membrane surface. Moreover, electron microscopy experiments showed that the protein appeared more associated with membranes in differentiated than in non-differentiated cells. Our findings also show an attractive correlation between myogenic differentiation and ANXA1-S27-PO4 expression on membrane surface since highest levels of phosphorylated protein were detected at 3 days of differentiation. Immunofluorescence microscopy experiments confirmed this increase and showed small immunofluorescent patches on cell surface. To investigate the role of ANXA1 in regulating C2C12 differentiation, siRNAs direct against the protein were used. We found a partial, but significant, decrease of myogenic differentiation in C2C12 cells where ANXA1 was knocked down. The data reported here suggest for the first time a possible involvement of ANXA1 in C2C12 mouse myoblast cell line differentiation.

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HIGH MOBILITY GROUP BOX-1 EXPRESSION IN PATIENTS WITH CHEST TRAUMA: PRELIMINARY STUDY

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Chest trauma is frequently followed by pulmonary contusion and sepsis. High mobility group box-1 (HMGB-1) is a late mediator of severe sepsis that has been associated with mortality in experimental conditions. We studied the change in HMGB-1 mRNA expression in patients with chest trauma and its relationship with the severity of trauma and survival. A total of 24 consecutive patients with chest trauma, referring to the Intensive Care Unit of Messina University Hospital, were enrolled. Lung trauma was established on the basis of chest X-ray and computed tomography. Blood and broncho alveaolar lavage fluid (BALF) were withdrawn at admission and 24 h after the beginning of the standard therapeutic protocol. HMG-1 mRNA increases significantly in blood (r=0.84) and BALF (r=0.87) from patients with trauma and pulmonary contusion and is positively correlated with the severity of trauma and the final outcome. Our study suggests that HMGB-1 may be an early indicator of poor clinical outcome in patients with chest trauma.

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ENZYME-BASED ANTICANCER DIETARY MANIPULATIONS: FROM THE VARIED AND COLOURED "5 A DAY" TO THE SINGLE SERVING

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The reduced cancer risk and lack of toxicity associated with high intake of fruits and vegetables suggest that the antioxidant phytochemicals from these dietary sources be responsible for the positive outcome. This observation was initially translated in chemopreventive approaches focused on specific green variety consumption or even single nutrient supplementation. However, they not only failed to provide any health benefit but highlighted detrimental effects. Subsequently, public-health chemoprevention programmes in the USA and Europe were developed to increase whole vegetable consumption. Among these, one of the most famous was the NCI sponsored plan "5 to 9 a day for a better health". This campaign promoted wise food choice recommending the consumption of at least 5 to 9 servings a day of colourful fruits and vegetables. Aim of this study was to investigate the effects of the NCI suggested diet in the animal model in terms of metabolic profile modulation - focusing mainly on enzymes of both xenobiotic metabolism (XME) and of antioxidant defences - in comparison with the single colour mono-diet. The boost of "good" phase-II together with down-regulation of "bad" phase-I XMEs is, in fact, still considered one of the most evoked strategy of cancer control. Six male Sprague Dawley rats for each treatment group were used. According to the Italian Society of Nutrition, a serving of fruit, vegetables, and leafy greens corresponds to 150 g, 250 g, and 50 g, respectively, for a 70 kg man. Proportionally, rats received by oral gavage daily for 10 consecutive days one serving of lyophilized onion, tomato, peach, black grape or lettuce - for white, red, yellow, violet or green diet, respectively - or 5 servings of each green ("5 a day" diet). Liver subcellular fractions were tested for various cytochrome P450 (CYP) linked-monooxygenases, phase-II supported XMEs such as glutathione S-transferase (GST) and UDP-glucuronosyl transferase (UDPGT) as well as for some antioxidant enzymes. Routinary hematochemical parameters were also monitored. The treatments generated a complex pattern of CYP inactivation. In particular, the most pronounced modulations were exerted by the "5 a day" supplementation (up to 60% loss for CYP2E1-linked XME, $P<0.01$) accompanied by CYP content reduction (54%, $P<0.01$). The same hold true for lettuce diet with CYP content loss (43%, $P<0.01$). None or less modulation of phase-II supported XMEs were seen, with the exception of UDPGT for tomato diet (60% increase, $P<0.01$). Apart "5 a day" and lettuce which strongly induced DT-diaphorase (up to 141% and 171% increase, respectively, $P<0.01$), antioxidant enzymes were not significantly changed. Hematochemical parameters were mildly affected by such dietary manipulation. The results of this study show a general negative synergistic effect among thousands of different chemicals occurring in a varied diet (i.e. "5 a day" treatment) with respect to single colour/variety supplement. According to the classical chemopreventive theory, this could be of particular relevance: being the "5 a day" administration the most phase-I inactivating procedure, it would also be the most chemopreventive one. Without here entering in the long-term debate on the activating/detoxifying role of any XME, the simple consideration that such enzymes are involved in fundamental physiological processes, suggests a strong caution in the interpretation of these "apparently positive" results, admitting its reproduction in humans. Noteworthy, the initially promising inverse association between green intake and cancer risk has been less consistent or totally absent in the more recent literature. The latter rather suggests that, with respect to the vegan/vegetarian alimentary approach, only a diet rich in fruits and vegetables and poor in certain types of fat, along with moderate caloric intake, could be associated with reduced cancer risk.

MICROARRAY ANALYSIS OF THE CHRONIC ESCAPE DEFICIT MODEL OF DEPRESSION: EFFECTS OF ESCITALOPRAM TREATMENT IN HIPPOCAMPUS

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Currently, the biological bases of depression and the molecular mechanisms underlying antidepressant action are not completely understood. Behavioural models of depression and genome-wide gene expression analysis can be relevant to better understand the pathophysiology of this disease. Chronic escape deficit is a valid and useful model of depression and is based on the induction of an escape deficit after exposure of rats to unavoidable stress. This behavioural model allows to evaluate the capacity of a treatment to revert the escape deficit. The majority of antidepressant drugs need to be administered for at least 3-4 weeks in order to revert the escape deficit (1). In this study, we demonstrated that only one week of treatment with escitalopram, a widely used SSRI, is effective in the chronic escape deficit model of depression. Also, our study demonstrated that only 50% of the animals receiving escitalopram responded to the treatment. The mechanisms underlying the action of escitalopram are still poorly understood and the molecular targets and pathways involved remain to be identified. In order to identify the biological target involved in the response to escitalopram, we performed a microarray experiment using the chronic escape deficit model of depression after a 7 day treatment with escitalopram. Gene expression patterns in the rat hippocampus were analyzed using Affymetrix GeneChip Rat Exon 1.0 ST evaluating both gene-level and exon-level expression profiling on the whole genome. Total RNA extracted from hippocampus of each treated animal was utilized to chipping a single array using the Affymetrix protocols. Twenty single arrays were utilized for data analysis and divided into 5 replicates for each experimental group (naïve, stress, escitalopram responders and not responders). With 2 parallel analyses (gene level and exon level) of raw data files carried out in Expression Console software using iterPLIER algorithms, we identified various transcripts that were differentially regulated in each pairwise comparison. In order to identify biological processes and signalling networks regulated by escitalopram response, we performed a functional analysis using Ingenuity web tool. Functional annotation of selected genes reflected interesting different biological features between escitalopram responders and not responders. More specifically, the biological functions regard cellular growth and proliferation, gene expression, and signal transduction. We believe that this pharmacogenomic approach will be helpful to understand the molecular mechanisms involved in the pathogenesis of depression as well as in the response to antidepressant drugs.

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METABOLISM OF ADENINE NUCLEOTIDES AND NUCLEOSIDES *IN VITRO* BY RAT ILEUM

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Adenosine (Ado), together with its precursors and its degradation products, is well recognised as an important extracellular signalling molecule having a key role in the modulation of inflammatory responses. In this study we investigated the mechanisms responsible for the production and clearance of adenosine and adenine nucleotides by rat ileum *in vitro*. Longitudinally cut strips (3 cm) (with or without mucosal layer) from distal ileum of male Wistar rats were incubated in vials containing 2 ml of aerated (95% O_2, 5% CO_2) and warmed (36.5°C) Tyrode solution. Conditioned medium was obtained by removing the tissue after a 30 min incubation. ATP (50 µM), cAMP (50 µM), AMP (50 µM) or Ado (50 µM) were added 15 min after enzyme or transport inhibitors. An HPLC method (1) was used to quantify purine nucleotides and their metabolites in the samples. The reported data are means±SEM of the values obtained in 4 to 8 experiments. Statistical analysis of the data was performed using the unpaired Student's *t* test or one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. P values of <0.05 were considered statistically significant. Degradation of exogenously added Ado, AMP, and ATP and accumulation of their primary and secondary metabolites occurred following 60 min incubation with rat ileum as well as in conditioned medium. By contrast, cAMP concentration was reduced in the presence of ileum strips (25.29±1.52 µM) but not in conditioned medium. The adenosine deaminase (ADA) inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 1 µM) reduced Ado metabolism in 1 min of incubation by 55.5% and 72% in normal and conditioned medium, respectively. The ecto-5'-Nu inhibitor, alpha,beta-methylene-ADP (AOPCP; 200 µM) reduced AMP degradation in 5 min by 42% and 33% in normal and conditioned medium, respectively. Ileum strips were able to metabolize Ado and AMP also when deprived of the mucosal layer. At 2 min of incubation with 50 µM Ado its concentration decreased to 36.2±3.1 µM in the absence of mucosa as compared to 3.5±0.8 µM for intact ileum strips. After 3 min of incubation with 50 µM AMP in the two experimental models AMP concentration was 16.3±2.6 µM and 28.4±1.4 µM, respectively. Suramin (100 µM) did not affect ATP metabolism, indicating that enzymes responsible of ATP degradation in rat ileum are not those inhibited by suramin. S-(4-nitrobenzyl)-6-thioinosine (NBTI; 10 µM), an inhibitor of Ado equilibrative transporters (ENTs), did not affect the concentrations of Ado (50 µM) and its metabolites in samples incubated for 1 min or 60 min. These results indicate that in rat ileum Ado removal from the extracellular environment is primarily due to metabolic conversion by ecto- and exo-ADA. Ado concentration fell rapidly also in the absence of the mucosal layer, showing that ecto-/exo-ADA is abundant not only in the mucosa (2,3), but also in muscular cells. The nucleoside uptake through ENTs is irrelevant. The direct Ado precursor, AMP, is metabolized by ecto- and exo-5'-nucleotidase and the mucosal layer prevents enzymatic conversion of the nucleotide, possibly acting as a barrier to diffusion of the nucleotide into the deep tissue compartments or of the soluble enzyme into the medium. ATP and cAMP are metabolized to AMP and then adenosine by ecto-enzymes. We have demonstrated that exo-ATPases exist, that are insensitive to suramine, while no release of cAMP-PDEs occurs from the tissue. In the intestine, reparative effects of exogenous purine nucleotides and adenosine have been reported in different pathologies, such as diarrhea, enterocolitis and bowel resection (4). Agents that increase purine concentrations in the local environment such as hydrophilic, membrane impermeable inhibitors of purine metabolizing enzymes, might be useful for the treatment of inflammatory, autoimmune and ischemic bowel diseases.

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PERSISTENT DECREASE IN NEUROSTEROIDS LEVELS INDUCED BY NEONATAL ESTROGEN EXPOSURE IS ASSOCIATED TO CHANGES IN $GABA_A$ RECEPTOR SUBUNIT EXPRESSION AND SENSITIVITY TO ANXIOLYTIC DRUGS

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Neuroactive steroids exert a rapid, non genomic, effect on excitability of neurons through a direct modulation of the activity of membrane receptors. The progesterone reduced metabolites, allopregnanolone and THDOC, are potent positive modulators of $GABA_A$ receptor (1) and induce pharmacological effects similar to those induced by classical positive allosteric modulators like benzodiazepines. The gonadal steroids, in particular the estradiol, exert an important action during pre- and perinatal periods in the regulation of sexual dimorphism and neuronal plasticity, and in the growth and development of nervous system (2). It has been demonstrated that subcutaneous administration of estradiol during perinatal period, induces a marked neuroendocrine alteration that impairs gonadal activities and induces a significant reduction of the ovarian steroids concentrations, that persists in adult (3). On these evidences, the object of this study is to evaluate whether neonatal estradiol-benzoate administration in female rats, can affect brain neuroactive steroids concentrations, the expression of $GABA_A$ receptor subunits and the sensitivity to the compounds that act on receptor, like benzodiazepines and allopregnanolone. Female rats were injected with a single dose of estradiol-benzoate 4 h after birth. Steroids were measured by RIA. Proteins were analyzed by Western Blot. The elevated plus maze test was used as an animal model of anxiety. Data are given as means±SEM. Statistical significance of differences was assessed by ANOVA and Newman-Keuls test. Differences were considered significant at $P \leq 0.05$. Administration of estradiol-benzoate at birth results in a decrease of progesterone and allopregnanolone concentrations in cerebral cortex (-79% and -76%, respectively) and in hippocampus (-90% and -47%, respectively), measured 60 days after birth. The plasma concentrations of these steroids were also reduced although to a lesser extent. On the contrary, THDOC levels were not significantly modified in both brain areas. Treated females show an increase of the levels of α_1, α_2, and γ_2 $GABA_A$ receptor subunits in the cerebral cortex and α_4 level in hippocampus, revealing a regionspecific effect on $GABA_A$ receptor subunits expression. Moreover, in the elevated plusmaze test, diazepam (0.5, 1, or 2 mg/kg i.p.) induced a greater increase of the time spent in the open arms in neonatally estrogenized females respect to controls, suggesting an increased sensitivity to the anxiolytic action of this drug. Otherwise, allopregnanolone (5-8 mg/kg s.c.) induced a similar effect in both groups. Estradiol-benzoate neonatal administration induces a marked and persistent reduction of ovarian steroids levels. Allopreganolone reduction was associated to an increase of $GABA_A$ receptor α_1, α_2 and γ_2 subunits expression in the rat cerebral cortex and an increase of α_4 subunit expression in the hippocampus, suggesting different sensitivity of these areas to the effect of estradiolbenzoate. The different sensitivity to anxiolytic drugs could be also related to the different expression of $GABA_A$ receptor subunits. This treatment might thus represent a valid experimental model in which to further investigate the physiological role of these steroids in the modulation of GABAergic transmission.

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EFFECTS OF FENTANYL ON NOP AND MOP RECEPTOR GENE EXPRESSION IN SH-SY5Y CELLS

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A peptide termed nociceptin/orphanin FQ (N/OFQ) was identified as an endogenous agonist for the opioid receptor-like receptor, currently specified as NOP receptor. Despite many structural homologies with the opioid system, the NOP receptor shows low-affinity binding to selective opioid agonists or antagonists. At the same time, N/OFQ selectively activates the NOP receptor but not any opioid receptor subtype (1). This receptor/ligand system is widely expressed in the brain, the actions of N/OFQ resemble those elicited by opioid peptides and the pharmacological characterization of this neuronal system allowed to suggest that nociceptin acts as a functional antagonist towards the endogenous opioid system (2). N/OFQ elicits a unique range of responses, including a wide range of effects on pain processing such as hyperalgesia, analgesia, and allodynia, as well as anxiolytic actions, influences on learning and memory, and modulation of opioid-mediated processes. At this regard, previous studies reported the influence of acute and chronic morphine treatment upon proN/OFQ biosynthesis in the rat brain (3) and some report indicated that N/OFQ reversed systemic morphine antinociception in mice (4). Based upon these considerations, in order to better understand the neuronal pathways activated by chronic exposure to opiate ligands and with the aim to understand possible differences in the development of tolerance and dependence among different opiates, the present study investigated the effects of exposure to the opioid ligand fentanyl on MOP and NOP gene expression, in the human neuroblastoma SH-SY5Y cell line, costitutively expressing both receptors. The cells were exposed to 1 μM or 10 μM fentanyl for 24 h. The opioid receptor antagonist naloxone (500 μM) was added to the cell culture medium 10 min before to add fentanyl (10 μM). Fentanyl exposure resulted in a dose-dependent decrease of NOP mRNA levels (88.91±7.58, 69.56±7.43*, vs control 100±9.4, *P<0.05) and in a dose-dependent increase of MOP mRNA levels (101.30±12.98, 144.0±79.74*, vs control 100.0±2.44, *P<0.05). Preincubation of cells for 10 min with 500 μM naloxone blocked increase of MOP gene expression in cells exposed to 10 μM fentanyl for 24 h (87.30±2.90 vs control 100.0±2.44). These findings show that the μ opioid receptor agonist fentanyl is able to affect MOP and also NOP receptor gene expression in SHSY5Y cell line. Our data suggest the hypothesis that fentanyl can modulate nociception also through a mechanism of up-regulation of the MOP receptor and by inhibiting the expression of the NOP receptor that mediates functional antagonism toward classical opiate-induced analgesia.

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SYNERGISTIC ANTITUMOR AND ANTIANGIOGENIC ACTIVITY OF IRINOTECAN AND AXITINIB COMBINATION IN PANCREAS CANCER

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Pancreas adenocarcinoma is a leading cause of cancer death and represents a challenging chemotherapeutic problem even with the introduction of new chemotherapeutic drugs such as irinotecan (1), a topoisomerase I inhibitor. Axitinib (2), a novel and selective inhibitor of the VEGFR-2 tyrosine kinase, has been successfully used alone *in vivo* to inhibit tumour growth. Combination studies have not yet been performed to enhance the antiangiogenic/antitumor activity of these compounds in pancreas cancer. To provide a rationale for improving the therapeutic efficacy of irinotecan-based combination schedules in pancreas cancer, this study is designed to determine in various experimental settings the activity of axitinib alone and in combination with irinotecan on cancer and endothelial cell growth *in vitro* as well as the antitumor effects *in vivo*. *In vitro* microvascular endothelial (HMVEC-d) and pancreas cancer (MiaPaCa-2 and Capan-1) cell lines were treated with SN-38, the active metabolite of irinotecan, and axitinib alone or simultaneously combined for 72 h to evaluate the level of interaction (synergistic, additive or antagonistic) on the antiproliferative effects, the modulation of secreted pro-(VEGF) and anti-angiogenic (TSP-1) factors and the inhibition of ERK1/2 and Akt phosphorylation. Capan-1 human pancreas cancer xenografts in nude mice were treated with irinotecan (100 mg/kg weekly) and axitinib (50 mg/kg/day) alone or in simultaneous combination, and tumour volumes were measured. *In vitro* SN-38 and axitinib inhibited the proliferation of HMVEC-d (IC_{50}=13.32±4.71 nM and 30.47±12.90 nM, respectively; mean±SD). The IC_{50} of the pancreatic cancer cell line Mia Paca-2 and Capan-1 for SN-38 were 0.29±0.23 μM e 0.04±0.03 μM, respectively and for axitinib 0.80±0.35μM and 0.84±0.34 μM, respectively. A strong synergistic effect (combination index <1 and dose reduction index >1) on antiproliferative activity was found with the simultaneous combination of the two drugs on both endothelial and cancer cells. The secretion of TSP-1 was increased in endothelial and cancer cells treated at the IC_{50} of both drugs, whereas the expression of VEGF decreased. ERK1/2 and Akt phosphorylation were inhibited by axitinib in all the cell lines; in contrast, SN-38 failed to show the same profile. The *in vivo* therapeutic effect of axitinib regimen on tumour mass at day 36 of treatment was very effective (90.7 vs 1.597 mm_3 of control, P<0.05), and higher than CPT-11 alone (591.2 mm_3). Of note, the simultaneous combination of the two drugs obtained an almost complete regression of tumors (64.1 mm_3). *In vitro* and *in vivo* results show the highly synergistic properties of simultaneous combination of irinotecan and axitinib on pancreas cancer, suggesting a possible translation of this schedule into the clinics.

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EFFECT OF A CHRONIC TREATMENT WITH PENTOXIFYLLINE ON *IN VIVO* AND *EX VIVO* MARKERS OF DYSTROPHIC PROGRESSION IN MDX MOUSE MUSCLES

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Duchenne muscular dystrophy is a genetic muscle disease characterized by the absence of dystrophin. This condition leads to progressive muscle fiber death and impaired regeneration through complex and still unclear pathological mechanisms such as inflammation, fibrosis and altered calcium homeostasis. The phosphodiesterase inhibitor pentoxifylline has a wide anti-inflammatory, anti-ischemic and anti-fibrotic actions and we recently identified its ability to counteract the abnormal overactivity of voltage-independent calcium channels (1). In order to better evaluate pentoxifylline therapeutic potential on disease-sensitive parameters, a 4-8 weeks treatment (50 mg/kg/day i.p.) was performed in mdx mice, undergoing or not a chronic exercise on treadmill (1,2). *In vivo*, we evaluated the fore limb strength by mean of a grip strength meter. Other than the absolute maximal strength we evaluated, for each mouse, the normalized fore limb strength (strength/body weight) at the beginning (time 0) and at the end of 4 weeks of exercise (time 4). Pentoxifylline significantly counteracted the deleterious effect of exercise on mdx mouse force; in particular the increase in normalized strength was 1.10±0.12 in pentoxifyllinetreated exercised mdx mice vs 0.26±0.02 in untreated counterparts. Also the pentoxifylline enhanced resistance to treadmill running both in sedentary mdx mice (193.0±11.5 m; n=5 vs 116.3±13.2 m of untreated; n=5) and in exercised mice (114.58±15.11 m; n=6 vs 68.0±18.5 m of untreated; n=5). However the maximal distance run remained lower than that of wild-type. To evaluate the impact of pentoxifylline treatment on the function of dystrophic muscles, we measured *ex vivo*, by means of a functional test apparatus, the contractile properties of diaphragm, chosen because it is the most severely affected muscle in the mdx mouse (3,4). The main finding was that pentoxifylline enhanced isometric tetanic tension of diaphragm of exercised mdx mice with respect to the untreated counterparts (94.80±13.67 kN/m_2 vs 49.62±4.27 kN/ m_2 respectively). This parameter, although to a lesser extent, was also increased (by 22%) in treated sedentary mdx mice vs the untreated counterparts. The ability of pentoxifylline treatment to restrain the exerciseinduced muscle damage in mdx animals has been confirmed by the values of plasma creatine kinase (CK) and reactive oxygen species (ROS), two clear diagnostic indices for sarcolemma injury. The pentoxifylline treatment reduced the plasma levels of CK both in exercised mdx mice (3939.02±929.91 U/l vs 5690.69±1339.04 U/l of untreated) and in sedentary mdx mice (4680.58±1300.26 U/l vs 6812.77±1206.19 U/l of untreated). Also the ROS levels (measured in FORT unit: 1 FORT=7.6 mM H_2O_2) were lower both in exercised treated mdx mice (381.67±28.11 vs 518±11.82 of untreated counterparts; $P<0.005$) and in sedentary treated mdx mice (391.0±36.71 vs 460.50±45.22 of untreated counterparts). Thus pentoxifylline exerts ameliorative effects on muscle function of dystrophic mdx mouse, through its wide mechanism of action able to target multiple steps of the pathogenetic cascade.

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CARDIAC PROGENITOR CELLS THERAPY IN A MODEL OF DOXORUBICIN-INDUCED DILATED CARDIOMYOPATHY

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The objective of this study was to determine whether the progression of heart failure in an experimental model of doxorubicin-induced dilated cardiomyopathy (DCM) may be counteracted by the administration of cardiac progenitor cells (CPCs). CPCs are selfrenewing, clonogenic, and multipotent *in vitro* and are able to regenerate myocytes, endothelial cells, and smooth muscle cells *in vivo* (1). Anthracyclines represent the main class of anti-cancer drugs that are responsible for a high incidence of cardiovascular events which severely limit their anti-tumour efficacy. The cardiotoxic effects of anthracyclines, among which doxorubicin (DOXO) is the most powerful and widely used, are time- and dose-dependent and the most common cardiovascular complication is a development of chronic congestive heart failure (2). Fisher 344 female rats were injected i.p. over a period of two weeks with 6 equal doses of DOXO, each 2.5 mg/kg to reach a cumulative dose of 15 mg/kg; the control animals were injected with vehicle only (3). Within a week after last injection, rats exposed to DOXO had markedly reduced body weight in comparison with control animals and the mortality rate caused by DOXO was ~45% while the survival in the control group was 100%. Animals were lethargic, had scruffy and yellowish fur, red exudates around the eyes and enlarged abdomen indicating the presence of fluid in the peritoneal cavity. As expected at this time point, there was a significant impairment of left ventricular function in animals exposed to DOXO characterized by a decrease in ejection fraction and fractional shortening. Three weeks after first injection of DOXO, animals were divided into two groups: DOXO-CPCs group received intramyocardial injections of CPCs expressing enhanced green fluorescent protein (GFP) whereas the DOXO-saline group was injected with vehicle only. Three weeks after CPCs administration, cardiac function was evaluated again, and animals were euthanized and myocardial samples were analyzed by confocal microscopy. During the three weeks period after CPCs or saline injection, the mortality was significantly lower in DOXO-CPCs than in DOXO-saline group. All control animals were alive throughout the experiment. Additionally, three weeks after CPC therapy, there was an amelioration of the conditions of the animals; they were less lethargic, maintained body weight and had modest or none abdominal enlargement. The volume of peritoneal fluid in saline-injected animals was 6 times bigger than in DOXOCPCs group. In comparison with DOXO-saline rats, animals treated with CPCs had significantly higher ejection fraction and fractional shortening. Homing of injected CPCs to the myocardium was documented by the detection of clusters of GFP-positive cells in all CPCs-treated rats. The presence of GFP-positive cells was also documented by PCR that confirmed the immunohistochemical results. In addition, newly formed progeny of injected CPCs was marked with BrdU that was administered at the time of cell injection and continued thereafter. Three weeks after cell implantation, clusters of newly formed cardiomyocytes were detected throughout the wall of left ventricle. These cells were GFPlabeled and expressed the contractile protein α-sarcomeric actin. Majority of newly formed myocytes were labeled with BrdU, documenting active growth of the newly formed CPCsderived cells. Additionally, GFP-positive cells that expressed α-smooth muscle actin and von Willebrand factor were identified. Importantly, connexin 43 was detected between new myocytes demonstrating functional integration between newly formed cells. Thus, CPCs engraft differentiate and regenerate myocardium of animals suffering from DOXO treatment. In summary, our data demonstrated the treatment with CPCs positively interfered with clinically-relevant adverse effects of DOXO and increased survival of the animals exposed to DOXO. Thus CPCs differentiation can compensate, at least in part, the progressive cellular loss of the cardiomyopathic heart.

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INFLUENCE OF COCAINE ON A2A/D2 RECEPTORS AND LIPIDRAFT COMPOSITION IN CHO CELLS

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Growing evidence suggests that proteins can interact with each other, in view of their socalled *Lego property* giving rise to multimeric protein aggregates, i.e. Protein Mosaics (PMs) (1,2). In living organisms PMs respond to a general principle: they fulfil structural and/or functional tasks, hence during evolution topology (i.e. spatial arrangements) and modalities of monomer-monomer interactions have been optimised. The oligomers of G protein-coupled receptors (GPCRs) are examples of informational PMs [the so called: receptor mosaics (RMs)]. As a matter of fact, it is by now clear that receptor/receptor interactions (RRI) are one type of protein/protein interactions. RMs can operate as specialized input units in membrane associated molecular networks, the so-called horizontal molecular networks (HMNs), which are usually localized in specialized microdomains of the plasma membrane, e.g. lipid-rafts (LRs). In the last years it has been proven that formation of dimers is a general property of GPCRs leading to homomers, heterodimers and probably RMs. Our group has mainly been involved in the evaluation of the RRI between A2A receptors for adenosine and D2 receptors for dopamine. Among several evidences suggesting functional roles for these heteromers, recent results suggest that A2A/D2 receptor interactions may also be involved in cocaine addiction in a *in vivo* model of cocaine abuse. This prompted us to investigate the possible influence of cocaine on these receptor heteromers *in vitro*. Thus, effects of cocaine (150 nM, 150 μM, and 1.5 mM) on A2AR, D2R and LRs have been studied in Chinese Hamster Ovary (CHO) cells stably cotransfected with the two receptors. The results obtained in the present study show that low concentrations of cocaine (150 nM and 150 μM; exposition time 8 h) increase both D2R (percent of respective control Mean Gray Value (MGV), fiducial limits: 127.03-146.69, and 108.92-124.83, respectively) and A2AR densities (113.5-125.63 and 124.32-141.28, respectively) while at the highest concentration used (1.5 mM) it has no effect. As previously stated, RMs may be localized in specialized lipid micro-domains of the plasma membrane; therefore, we studied LRs in CHO cells exposed to the three different concentrations of cocaine for 8 h. Our data demonstrate that cocaine modifies the LR composition by dose-dependently increasing GM1 content and reducing, only at the highest concentration used (1.5 mM), CAV1 expression. LRs were also evaluated following exposition of cells to the three different concentrations of cocaine for 3 h. The presence of cocaine for a shorter period influences the LRs composition in an opposite fashion with respect to a longer exposition period (8 h). Therefore, it has been observed a reduction of GM1 content by the lowest concentration (150 nM) and an increase of CAV1 by the two highest concentrations (150 μM and 1.5 mM). The present data demonstrate for the first time that cocaine, at low concentrations and after an 8 h contact period, influences A2A/D2 heteromers *in vitro*. On the basis of our results, it may be hypothesized that this effect depends on cocaine action on LRs with a particular composition. Since it is known that A2AR/D2R internalisation is mediated by caveolin-1 (3), we might suggest that a high concentration of cocaine (1.5 mM) favours the formation of LRs enriched in GM1 reducing the invaginations enriched in caveolin-1. On the contrary, a low concentration of cocaine (150 μM) increases A2A/D2 heteromers by promoting planar LRs formation. At the lowest concentration (150 nM) only an increase of A2AR and D2R densities is observed without any effect on LRs. As previously stated, we observed opposite effects on LRs composition in CHO cells exposed to cocaine for 3 h, thus we are now performing experiments to evaluate the possible influence of cocaine on A2A/D2 receptor densities.

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IN VITRO EVALUATION OF THE BIOLOGICAL PROPERTIES OF THE ESSENTIAL OIL OBTAINED FROM *PISTACIA LENTISCUS*

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Pistacia shrubs of the *Anacardiaceae* family include various species among which there are *P. Vera*, edibile, *P. terebinthus* and *P. lentiscus*. Since ancient times, the phytocomplex from *Pistacia Lentiscus*, an essential oil extracted from leaves, fruits or trunk exudate, was used for treating several illness (i.e. gastralgia, diarrhoea, dyspepsia and peptic ulcer, even caused by *Helicobacter Pylori*) and for its antibacterical and antifungina activities. Recent data pointed out that the phytocomplex from *Pistacia Lentiscus* is also able to defend stomach from cancer. In this study the antitumor properties of the volatile oil from *Pistacia Lentiscus* twigs and leaves were investigated using human ovaric carcinoma cell lines (2008 and C13*) and human colon carcinoma cell line (LoVo) as *in vitro* models. Cell viability was determined using the MTT test. The results showed that after 3 h treatment, phytocomplex was able to inhibit the growth of each cell line at similar concentrations (about 150 µg\ml), while after 24 h of treatment the IC_{50} on 2008 and LoVo cells resulted about 3 folds higher. On C13* cells no appreciable differences were evidenced between 3 h and 24 h of treatment. Annexin V combined with propidium iodide (PI) was carried out to evaluate the apoptosis. Phytocomplex was able to induce late apoptosis and the percentage of apoptotic cells increased in a dose-dependent manner. Further molecular analysis showed, in every cell lines studied, a loss of mitochondrial membrane potential evaluated by rhodamine 123 staining. This confirmed the oil ability to stimulate apoptosis. The uptake of PI in treated but no permeated cells, investigated by microscopy, displayed a cellular membrane permeability alteration. Morphological changes induced by treatment were underlined using optical microscopy. The cell cycle distribution, analyzed by flow citometry, indicated that the phytocomplex induced G2M arrest in a dose-dependent manner.

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ROLE OF GSK-3β IN THE PROTECTIVE EFFECT OF INSULIN AGAINST CEREBRAL ISCHEMIA/REPERFUSION INJURY

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Diabetes is a leading risk factor for ischemic cerebrovascular diseases. Insulin administration may reduce brain damage evoked by ischemia/reperfusion (I/R) injury (1), but the molecular mechanisms underlying its effects are still poorly understood. The aim of my Phd project is to investigate the effects of insulin against cerebral I/R injury and the role of the glycogen synthase kinase (GSK)-3β pathway. I focused on GSK-3β being insulin a well-known inhibitor of this enzyme. GSK-3β is a serine/threonine kinase, originally identified for its key role in glucose metabolism (2). More recently, GSK-3β has been demonstrated to modulate several signal transduction pathways involved in neurodegeneration and inflammation (3). Unlike most kinases, GSK-3β is constitutively active and it is inhibited by phosphorylation at the residue of $Serine_9$ (Ser_9) (4). Binding of insulin to its receptor activates phosphatidylinositol 3-kinase leading to the subsequent activation of protein kinase B/Akt, and finally, the inhibition of GSK-3β. As the lack of insulin may drastically influence GSK-3β basal activity, I studied the effects of insulin administration against cerebral I/R injury in a rat model of insulinopenic diabetes. Streptozotocin-induced diabetic rats were subjected to 30 min occlusion of common carotid arteries followed by 1 h or 24 h reperfusion. Insulin was administered during reperfusion. In the first year, I focused on the identification of the pharmacological target. GSK-3β total expression and levels of Ser_9 phosphorylation were evaluated by RT-PCR and Western blot analysis in the rat hippocampus, being the brain area most sensitive to I/R injury. Total expression of GSK-3β was higher in the hippocampus of diabetic rats than that recorded in non-diabetic animals and not modified by I/R. Diabetic rats showed a stronger basal activation of the enzyme as the ratio of Ser_9 phosphorylated GSK-3β/total GSK-3β was lower in the diabetic group than in the control group. Levels of Ser_9 phosphorylation were not modified by I/R. Insulin administration (2-12 IU/kg) did not modify GSK-3β expression but it decreased its activity in a dose-dependent manner. During this second year, I demonstrated that GSK-3β inhibition by insulin was associated with protective effects. To weight the role of GSK-3β inhibition, a potent and selective inhibitor of GSK-3β, TDZD-8 (5), was used as a comparative pharmacological tool. TDZD-8 has been previously demonstrated in our laboratory to protect against cerebral I/R injury in nondiabetic rats (6). Mainly, after 24 h reperfusion, insulin or TDZD-8 similarly halved infarct volume which was larger in diabetic animal (34.9±4.8%; $P<0.05$) than in non-diabetic rats (23.4±3.9%; $P<0.05$). Insulin and TDZD-8 attenuated the I/R-induced rise in the levels of S100B, a calcium binding protein used as a marker of cerebral injury, without any significant difference between the two drug treatments. At 24 h reperfusion, both treatments significantly reduced (at the same level) plasma concentration of tumor-necrosis-factor-α, cyclooxygenase-2, and inducible-NO-synthase cerebral expression and neutrophil infiltration, measured as myeloperoxidase activity and intercellular-adhesion-molecule-1 expression. Overall, our data demonstrate that acute administration of insulin or TDZD-8 reduced cerebral I/R injury in diabetic rats. We propose that the inhibitory effect on the GSK-3β activity contributes to the protective effect. Further investigation is needed to better clarify the role of GSK-3β pathway in I/R injury.

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PROMOTING CONTINUING EDUCATION IN PHARMACOVIGILANCE: THE EXPERIENCE OF "PHARMASEARCH", A NETWORK OF GENERAL PRACTITIONERS REPORTING ADVERSE DRUG REACTIONS

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Underreporting of Adverse Drug Reactions (ADRs) is a major problem for all pharmacovigilance systems. Underreporting may be linked to lack of information and feedback provided to reporters. Given the positive results obtained in Italy and other countries, a network of Italian General Practitioners (GPs) was set up in collaboration with the Italian Society of General Practitioners (SIMG) under the coordination of the Institute of Pharmacology of Messina University (Coordinating Centre, CC) to improve ADRs spontaneous reporting among GPs and to provide them with continuing education on drug safety and rational use of medicines. The network of reporting GPs called "Pharmasearch" was set up in 2002 as collaboration between the CC and the SIMG. A group of GPs voluntarily forward to the CC a copy of each ADR reporting form filed in. The CC, after receiving the form, provides reporters with a personalised comment to the ADR reported, according to data retrieved from international literature. Moreover, GPs periodically receive reports about the activities of the network, including regular updates illustrating ADRs received, drugs implicated, etc. All cases of interest are studied in depth and spread to all participating GPs. Furthermore, a periodic bulletin (Inform@rete) is sent to participants, describing topics and news coming from international literature and regulatory agencies. This, in order to sensitise GPs about the importance of reporting ADRs and to keep them updated on the safety profiles of drugs and their correct use. To achieve this goal, a specific section of the website www.farmacovigilanza.org (SIMGxFarmacovigilanza) has also been created, containing case reports and periodical reports on network activity. Up to 30 May 2008, the number of reporting GPs was 261 (out of a total of 360 participants) and the total number of reports filed was 3094. Interestingly, reports for lansoprazole, enalapril and coamoxiclavulanate have risen and this increase coincides with patent expiration of their branded version. In order to promote GPs ADR reporting a drug safety project has been proposed (Progetto Under-reporting) that will involve 300 GPs already involved in SIMG networks (Pharmasearch and HealthSearch, a prescription database). In this project, every GP involved will have to actively monitor and report all ADRs one entire day per month for 6 consecutive months.

DIFFERENT CHANGES OF N/OFQ-NOP GENE EXPRESSION AFTER CHRONIC COCAINE IN THE RAT BRAIN

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Cocaine directly influences dopaminergic and serotonergic neurotransmission *via* inhibition of transmitter uptake and it also has indirect effects on opioid peptide systems. It has been shown that chronic administration of cocaine can increase μ-opioid receptor density in the nucleus accumbens (1) and can increase κ-opioid receptor density in caudate putamen and nucleus accumbens (2). Moreover continuous administration of cocaine elicited a significant decrease of prodynorphin mRNA levels in rat hypothalamus and increase in caudate-putamen (3). The neuropeptide nociceptin/orphanin FQ (N/OFQ) is the endogenous ligand for the opioid-like receptor NOP. This peptidergic system presents marked structural analogies with the three different opioid receptors MOP, KOP, DOP and their related peptides, nevertheless there is no cross-interaction between the neuropeptides and the receptors of the N/OFQ system and of the opioid system. Several studies have suggested that N/OFQ might regulate the behavioural effects of cocaine. The acute administration of N/OFQ attenuates cocaine-stimulated activity (4), intra-VTA injections of N/OFQ given daily in combination with cocaine diminish only on the first day the behavioural effects of cocaine (5). In contrast, if N/OFQ is given alone for 3 days, sensitization to a challenge dose of cocaine is evident 5-7 days later (5). A single injection of N/OFQ into the VTA or caudate putamen leads to an enhanced response to cocaine on locomotor activity 23 h later. However, if the rats receive a second intra-VTA injection of N/OFQ on day 2, prior to an injection of cocaine, the effects of cocaine are blocked (6). In addition, our recently studies have shown that N/OFQ levels are decreased in caudate putamen, nucleus accumbens, substantia nigra and VTA by treatment with cocaine (7). In order to clarify the complex interaction between cocaine and N/OFQ, the aim of the present study was to investigate if chronic cocaine produces alteration of N/OFQ-NOP gene expression in selective rat brain areas. Cocaine (50 mg/kg/die) or vehicle was infused continuously for 7 days *via* osmotic minipumps into male rats and the effects on mRNA levels were investigated using RT-PCR. We observed a significant decrease in N/OFQ gene expression in the substantia nigra, nucleus accumbens, and VTA (83.4±5.2%, 68.6±5.3% and 76.8±3.9% of controls, respectively) whereas there were no significant changes in the caudate putamen. About NOP receptor gene expression, another RT-PCR analysis showed a significant decrease in the medial caudate putamen and VTA (79.7±3.9% and 59.7±15.0% of controls, respectively), a significant increase in the lateral caudate putamen and nucleus accumbens (120.0±6.9% and 136.5±6.9% of controls, respectively) while there was no significant change in the substantia nigra. In conclusion, these studies show that N/OFQ levels are altered by treatment with cocaine. In particular, in the nucleus accumbens it is possible to hypothesize that the decrease of N/OFQ cause the up-regulation of NOP receptor, whereas in the VTA both N/OFQ and NOP gene expression significantly decrease. Furthermore, the changes in N/OFQ parallel those seen for κ-opioid receptors, suggesting that the interactions between cocaine and these systems might be similar. In summary, these data suggest that there is a complex interaction between cocaine and N/OFQ in that it appears that cocaine and N/OFQ coregulate each other effects.

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EFFECT OF AGING ON MEMORY, MAPK, AND GLIA ACTIVATION IN THE RAT

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Aging is accompanied by a decline in cognitive functions, along with a variety of neurobiological changes. The hippocampus, a structure that plays a critical role in memory formation is very sensitive to age-dependent cognitive impairments and is one of the structures more closely related to electrophysiological, structural, and morphological changes during aging. In the present study, we examined the effect of aging on memory encoding using a step-down inhibitory avoidance task, performing recall 1 h after acquisition in 3 months-old and 22 months-old Wistar rats. Latency times to step down the platform were measured, with a cut-off of 300 s. We also studied the effect of aging on ERK and p38MAPK pathways activation and on the presence of activated microglia and astrocytes in the hippocampus of adult and aged rats by immunohistochemistry on 40 µmthick slices. Quantization was performed counting blind immunoreactive cells. Aged rats did not acquire the step-down behaviour, as shown by the significantly shorter time (182 s) spent stepping down the platform in comparison to young rats (299 s; $P<0.05$) during recall of the task. Phospho-ERK positive neurons counts were similar in CA1 of young (70±14,n=9) and aged rats (61±9, n=4) in basal conditions, while 10 min after acquisition phospho-ERK positive neurons increased significantly in CA1 of young (+111% vs acquisition, n=11; $P<0.05$) but not of aged rats (-13%, n=4; n.s.). Phospho-p38 MAPK positive neurons were significantly more numerous in CA1 of aged (412±16, +292% vs young rats, n=16; $P<0.0001$) than young (105±25, n=6) rats. No differences were evoked by behaviour acquisition. The density of GFAP positive astrocytes was significantly lower in all hippocampal regions of aged rats, with a mean hippocampal density of 536±21 positive neurons/mm_2 in the young (n=7) and of 390±15 neurons/mm_2 in the aged rat (n=9; -27% vs young rats; $P<0.0001$). Confocal microscopy indicated that in the hippocampus of aged rats astrocytes were not only less numerous, but also smaller, with shorter branchings than in young rats. Only scarce activated microglial cells (OX-6 immunopositive) were present in the hippocampus of young rats (2.5±1.0, n=10). Substantial infiltration of activated microglial cells was visible in the CA3 and hylus of aged rats (91±11, n=14; $P<0.0001$ vs young rats), but not in CA1. The cells were hypertrophic, ranging from densely arborized cells to cells with a bushy appearance with swollen cell bodies and intensely stained short processes. These data show that senescence-induced dysregulation of ERK and p38MAPK activation as well as of glia in the hippocampus of aged rats underlie age-associated memory impairments.

ENDOTHELIAL CELLS PROMOTES MATURATION OF CORTICAL NEURONS IN CO-CULTURE MODEL

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The nervous and vascular systems share several anatomical and functional parallels, showing common signals to differentiate, grow, and specialize. Blood vessels are closely linked to neurons, so the cross-talk between neurons and vascular endothelial cells (ECs) is strictly controlled in the CNS. Vascular ECs form the blood brain barrier that protects the brain by rendering it impermeable to many biologically active macromolecules. Moreover, vascular ECs are not just inert tubes for delivery of blood, but they release trofic factors that can interact as a critical source of homeostatic support for neurons. The interactions between neurons and endothelial cells are relevant also for several diseases of CNS, including stroke, Alzheimer's disease, cerebral amyloid angiopathy, and CNS tumors. The aim of this study was to investigate the cross-talk between endothelial cells and neurons, and to assess the effect of ECs on cortical neurons (CNs) in a co-culture system. Neurons were isolated from the cortex of mice embryos (C57BL/6, E16). Neuron differentiation was assessed through immunofluorescence analysis of neuronal markers (β3-tubulin, synapsin, and NMDA-R). We also evaluated glia contamination on neuronal cultures through immunofluorescence analysis using GFAP antibody. Isolated CNs were analyzed at 3 days and 10 days of culture for markers. At 3 days CNs were sparse and did not express NMDAreceptors and synapsin, while CNs maturation was achieved. At 10 days of culture double staining GFAP for glia cells and β3-tubulin for neurons, indicated the absence of glia (<1%). When cortical neurons and ECs were co-cultured in a transwell for 2 days and immunofluorescence assay using β3-tubulin antibody evidenced the formation of neuron network. In the co-culture system, ECs were able to support and accelerate neuronal differentiation and maturation. In conclusion, methods for neuronal isolation and characterization were set up and validated. The cross-talk between neuronal and Ecs evidenced the capability of endothelium to contribute to neuronal differentiation.

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USING STREPTOLYSIN O PROTEIN FRAGMENTS AS POSSIBLE VACCINE CANDIDATES AGAINST *STREPTOCOCCUS* PYOGENES

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Streptolysin O (SLO) is one of the most important virulence factors of the human pathogen group A *Streptococcus* (GAS). This protein is a member of the super family of Gram positive toxins defined cholesterol-depending cytolysins (CDC), which bind cholesterol on the eukaryotic cell membranes and induce pore formation resulting in cell lysis. CDCs are all structurally correlated, being constituted by 4 functionally characterized domains, and their 3D structure has been well described (1). In addition to its cytolytic activity, SLO exerts its toxicity by promoting cytokine release by eukaryotic cells (2) and by activating the complement system (3). Following infection in humans, high anti-SLO antibody titers are measured, which are routinely used as a diagnostic marker of acute GAS infection. We have shown that SLO can induce a highly protective immune response in our mouse model of infection with GAS 3348 M1 strain, but the use of SLO as a potential vaccine candidate is hampered by its multiple toxic activities. By “surfome” analysis of different GAS strains, we identified 3 peptides that are localized either in N-terminal region or in the 2nd protein domain, two protein regions which are not supposed to be directly involved in SLO toxic effects. We therefore cloned and purified, as GST-fusion proteins, the first N-terminal 100 aminoacids (25_N-term), the whole 2nd domain (25_IId) and the fusion of these two protein regions (25_N-term.IId). We demonstrated that our peptides induce in mice antibodies that recognize SLO in a protein extract of GAS M1 3348 and that human sera of both pharyngitis patients and healthy adults, recognize the two domains in Western blot analysis. Moreover, mice immunized with the three SLO fragments are significantly protected against infection with a lethal dose of GAS M1 3348. Experiments are ongoing to test whether antibodies directed against one or more of the expressed SLO fragments are capable of inhibiting one of more of the toxin activities.

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ISOLATION AND PHENOTYPICAL CHARACTERIZATION OF HUMAN AMNIOTIC FLUID CELLS WITH POTENTIAL FOR THERAPY IN NEURODEGENERATIVE DISORDERS

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Embryonic, neural, and bone marrow stem cells have been successfully used for the treatment of spinal cord injury in animal models. However, in human diseases, the therapies involving embryonic and neural stem cells may present legal or ethical problems. Recent evidences have shown that amniotic fluid may be a novel source of stem cells for therapeutic transplantation (1). Amniotic fluid contains differentiated and undifferentiated cells arising from all three germ layers; indeed, previous studies showed that these cells display multilineage differentiation potential (for example adipocytes, osteocytes or neural cells) (2). The main goal of this study was to characterize cells isolated from human amniotic fluid as new source of therapeutic cells. In particular we are interested in using these cells in a mouse model of spinal cord injury. Samples of amniotic fluid were collected by a syringe during caesarean births (mean gestational age 40 weeks). Specimens were centrifuged, then the pellets were resuspended in Dulbecco's Modified Eagle medium (DMEM) and plated in culture dishes containing sterile slide cover slips. Cells were cultured in DMEM supplemented with 10% foetal bovine serum, 5 ng/ml fibroblast growth factor, glutamine and antibiotics, in a 5% CO_2 incubator with low O_2 tension (3). After 7 days, cover slips containing adherent cells were removed and placed in separate wells. When the cells reached confluence, they were treated with trypsin, collected and replated for subculture at a density of 10,000 cells/cm_2 in 6-well plates or 25-T culture flasks. Using this method, we isolated several different populations of adherent cells from three amniotic fluid samples. Each of these different cell cultures have been studied for *in vitro* proliferation potential and for expression profile by immunocytochemistry. We isolated only one population of polygonal cells from the first sample. The cells presented an exponential rate of growth, and the high proliferation capacity persists even after several passages *in vitro*. These cells resulted immunostained for nestin, βtubulin III and glial fibrillary acid protein (GFAP). Fewer cells also expressed cytokeratin 8-18. Cells derived from the second sample displayed a different morphology (large cells with irregular borders) and a lower proliferation rate than the first sample. These cells were strongly positive for nestin, βtubulin III and GFAP and sparsely positive for cytokeratin 8-18. The third sample showed the higher heterogeneity for sizes and morphologies of the cells. We obtained nine cell populations which consisted of three main cell types: polygonal, large irregular and fibroblast-like cells. Interestingly, two cultures (#5 and #6) presented fibroblast-like cells, a very high rate of growth, and high expression of nestin, βtubulin III and GFAP but no expression of cytokeratin 8-18. Another culture (#12) consisted in large irregular cells, sometimes multi-nucleated, presenting a low rate of growth that reached the plateau within three weeks. Besides the positive immunolabeling for nestin, βtubulin III and GFAP, these cells were also strongly positive for cytokeratin 8-18. To conclude, we were able to obtain different cell populations from amniotic fluid derived from caesarean births, which had different rates of growth and different morphologies but the same high expression of three neuronal markers nestin, βtubulin III and GFAP. This finding suggests that amniotic fluid may be a promising source of neural progenitor cells and led us to test the efficacy of amniotic fluid cells in an animal model of spinal cord injury. Up to now, ten mice subjected to a moderate contusion injury at T8 level have been transplanted *via* i.v. route with $1x10_6$ cells derived from the culture #6 from the third sample. Motor recovery of transplanted animals in comparison with a control group and the fate of grafted cells are under evaluation.

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TISSUE SELECTIVE ACTIVATING/BLOCKING ACTIONS OF 2H-1,4-BENZOXAZINE DERIVATIVES ON PANCREATIC AND SKELETAL MUSCLE K_{ATP} CHANNELS

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ATP-sensitive K_+ (K_{ATP}) channels couple cellular metabolism to electrical activity in skeletal and cardiac muscle, pancreatic β-cells and neurons. K_{ATP} channels are the targets for drugs of therapeutic interest such as sulphonylureas, that block the channel, and K_+ channel openers (KCO) such as diazoxide, which however lack of tissue selectivity. Previous studies demonstrated that synthetic 2H-1,4 benzoxazines (2H-Bz) are modulators of K_{ATP} channel activity. In skeletal muscle they show a dualistic action, inhibiting or opening the channel in the absence and presence of ATP, respectively (1). Substituents at position 2 of the Bz nucleus are pivotal in determining the potency of these compounds as agonist or antagonist in skeletal muscle (1). We have also demonstrated that the 2-propyl-Bz exerts an inhibitory action on β-cell K_{ATP} channels, while no pancreatic agonist has been found among these derivatives (2). In order to delineate the tissue selectivity for the activating/blocking actions of these compounds, here we investigated the effects of lengthening (2-hexyl-Bz) or branching (2-isopropyl-Bz) of the alkyl chain; we also examined the effects of the introduction of aliphatic or aromatic rings (2-methylciclohexyland 2-phenyl-Bz) on the activity of these molecules on pancreatic K_{ATP} channels. We performed *in vitro* patch clamp recordings on native pancreatic β-cells and HEK293 cells expressing Kir6.2ΔC36, that lacks the SUR subunit. In contrast to skeletal muscle (3), in whole cell perforated mode 2-hexyl-Bz did not augment K_{ATP} current (I_{KATP}) of native β-cells but it produced a dose-dependent inhibition of I_{KATP}; accordingly, in current clamp recordings, a concentration-dependent membrane depolarization was observed with this molecule. On the contrary, 2-isopropyl-Bz did not exert any effect on I_{KATP} in the same experimental conditions. The 2-hexyl-Bz, but not isopropyl-Bz, inhibited, in an ATPindependent manner, I_{KATP} from expressed Kir6.2ΔC36. The 2-phenyl-Bz had the same dualistic action on pancreatic K_{ATP} channels observed in skeletal muscle (3), that disappeared in patch clamp experiment performed on Kir6.2ΔC36, as this molecule showed an inhibitory effect. Finally, 2-methylciclohexyl-Bz caused activation and inhibition of I_{KATP} on native channels in perforated whole cell depending on the dose. These demonstrate that the substitution of the H at position 2 of the Bz nucleus with a linear aliphatic chain confers a K_{ATP} channel blocking activity in pancreas. The replacement of the linear with branched substitutes at position 2 totally abolishes the effect of this compound on pancreatic K_{ATP} channels; whereas in skeletal muscle it confers a KCO activity to the molecule both in presence and in absence of ATP (3). We demonstrated that the introduction of an aromatic ring and possibly of an aliphatic cycle determines the comparison of the dualistic effect on pancreatic K_{ATP} channel, recently demonstrated on muscular K_{ATP} channel (3). The inhibitory actions of these derivatives on pancreatic and skeletal muscle K_{ATP} channels reflects an interaction with an inhibitory site placed on Kir6.2 subunit. The agonist effect observed with the phenyl derivative on pancreatic K_{ATP} channels could depend on an interaction with a stimulatory site. 2-Hexyl-Bz could represent a potential anti-diabetic drug without peripheral side effects, as it blocks pancreatic K_{ATP} channel and fully activates the skeletal muscle subtype in the same range of concentrations. We also identify a muscular selective KCO (2-isopropyl-Bz), that could represent a lead compound to develop drugs for the treatment of some channelopathies, such as periodic paralysis. Selective pancreatic KCO, structurally different from diazoxide, could be effective in the hyperinsulinemia or to prevent the deterioration of β-cells mass in type 2 diabetic patients.

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THERAPEUTIC PERSPECTIVES OF CANNABINOIDS IN NEUROPATHIC PAIN

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The pharmacological interest in cannabinoids, compounds derived from the *Cannabis sativa* plant, was revaluated thanks to the discovery of the endocannabinoid system (ECS), including the cannabinoid receptors CB1 and CB2, the endocannabinoids (EC) anandamide (AEA) and 2-arachidonylglycerol (2-AG), and their biosynthetic and metabolizing enzymes [the fatty amide acid hydrolase (FAAH) degrading AEA and the monoacylglycerol lipase (MAGL) the 2-AG]. Moreover, recent evidence demonstrated that AEA is also a ligand of the vanilloid receptor TRPV1, a non-selective cation channel expressed on primary afferent nociceptive neurons, so suggesting a cross talk between the EC and the endovanilloid system. The findings that the ECS has an important role in the modulation of pain provide a new therapeutical possibility in order to treat neuropathic pain, a pathological state with complex aetiologies and difficult to treat with common analgesics. Although CB1 agonists have long been considered as potential drugs, these agents produce substantial psychotropic effects that limit their clinical utility. On these bases, this study aimed to compare other different approaches to modulate the ECS in order to counteract the painful symptoms (particularly, thermal and mechanical hyperalgesia, assessed by plantar test and Randall-Selitto test, respectively) associated with a widely used animal model of neuropathic pain, the chronic constriction injury (CCI) of rat sciatic nerve. The ECS was modulated using: a) inhibitors of the EC metabolizing enzymes (URB597, a FAAH inhibitor, and URB602, a MAGL inhibitor) or the cellular transport (VDM11) that upregulate the EC tone only at the location where the EC synthesis has been stimulated, probably without causing significant side effects; b) an hybrid ligand compound (arvanil) with capability to block the EC transporter and to inhibit FAAH and that also possesses direct agonist activity at the CB1 and TRPV1 receptors; c) a controlled *Cannabis sativa* extract (eCBD) containing multiple cannabinoids (cannabidiol in large quantity, $\Delta_9$tetrahydrocannabinol, and other minor ones), and other non-cannabinoid fractions (terpens and flavonoids). All these drugs were administered for a week, starting from the 7$_{th}$ day following the surgical procedure. The repeated administration of URB597 (10 mg/kg i.p.) led to a partial antihyperalgesic effect, while the repeated administration of URB602 (10 mg/kg i.p.) resulted in a total relief of both thermal and mechanical hyperalgesia, so suggesting a major efficacy of MAGL inhibition over FAAH. VDM11 (10 mg/kg s.c.) completely inhibited pain already after 4 administrations, demonstrating that the EC transport inhibition is the most efficacious strategy. Arvanil (2 mg/kg i.p.) was able to counteract completely thermal hyperalgesia but only partially mechanical hyperalgesia, while the chronic treatment with eCBD (15 mg/kg p.o.) abolished both symptoms. Antagonism studies were then performed to better understand the mechanism of action of such drugs and to explain their different pharmacological effects. URB597, increasing AEA levels, acted *via* CB1 receptors, whose activation is, however, associated with the presence of psychoactive effects. In addition, since URB597 was not selective (different URB597 doses, able to evoke different antihyperalgesic effects, always produced the same inhibition of FAAH activity), its utility as analgesic is not promising. Also VDM11 acted through CB1 but, fortunately, it was devoid of side effects. The inhibition of the EC transporter increased both AEA and 2-AG, which, acting on upregulated CB1 during CCI conditions and strategically localized in key sites of pain pathway, give reason for VDM11 great efficacy. URB602 antinociceptive properties were mediated by both CB2 and PPARγ receptors (receptors involved in inflammation) for which 2-AG has affinity. On the other hand, both arvanil and eCBD activated only TRPV1 receptors. So, the involvement of other receptors, not only the cannabic ones, is essential in order to obtain a great pharmacological effect. In support of such hypothesis, we demonstrated that the expression of TRPV1 receptors was dramatically increased in the sciatic nerve of CCI rats, strongly supporting the TRPV1 as a new target in pain modulation. Also CB2 expression increased in CCI mice spinal cord, so contributing to URB602 antinociceptive efficacy.

CPVT-ASSOCIATED RYR2 MUTATION LEADS TO BASAL ECCOUPLING ALTERATIONS: INSIGHTS FROM A TRANSGENIC MOUSE MODEL

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Cathecolaminergic polymorphic ventricular tachycardia (CPVT) is a rare autosomic dominant inherited cardiac disease which leads to stress induced ventricular tachycardia and sudden death. It is associated with anomalous sarcoplasmic reticulum (SR) function and, up to date, mutations in CSQ2 (cardiac calsequestrin) and RYR2 (cardiac ryanodine receptor) genes have been found responsible of this disease in patients. One of the most common mutations, R4496C RYR2 mutation, has been intensively investigated in a transgenic knock-in mouse model. Previous data showed that RYR2 mutation leads to an arrhythmogenic substrate, both at cellular and tissue level. However, a detailed investigation of the consequence of SR dysfunction on excitation-contraction coupling has not been performed. Thus, we aimed to deeply characterize this model at a cellular level, trying to identify those subtle alterations which could help us to clarify the pathophysiology of CPVT and bring new insights on the SR properties in the heart. Wild Type (WT) C57 mice and C57 heterozygous mice carrying R4662C RYR2 mutation (RM mice) were used (n=4 for both). Ventricular cardiomyocytes were isolated by enzymatic dissociation and used for patch-clamp recordings using the perforated patch technique. Action potentials (APs) and calcium transients (CaT) were recorded simultaneously in cell pre-loaded with FLUO 3-AM. APs and CaT properties were recorded at different stimulation frequencies (0.5 to 4 Hz). Using 3 Hz as basal frequency, premature stimuli were given at different times and stimulation pauses of different lengths were imposed to evaluate post rest modifications of AP and transients. The same protocols were repeated after adding 100 nM isoproterenol (ISO), 50 μM tetracaine or ISO plus tetracaine. AP showed the expected adaptation to frequency both in WT and in RM mice, but average AP duration was increased in RM mice. CaT showed a slightly positive amplitude-frequency relationship in WT mice but a flat or negative relationship in RM mice. Recovery from premature stimuli was not different between the two groups. A clear post-rest potentiation of CaT was evident in WT mice (+110% on average after a 2 s pause), but hardly detectable in RM mice. ISO increased the amplitude of CaT in the WT by 40-60%, while it induced a small or no change in RM mice. Tetracaine slightly increased CaT amplitude under adrenergic stimulation in WT mice. Finally, ISO induced the appearance of arrhythmogenic mechanisms (spontaneous calcium releases and afterdepolarizations) in most of the cells from RM mice and only in a few WT cells. Changes in basal transient properties of RM cells are consistent with a “leaky SR” phenotype, already evident under basal conditions: a leaky SR cannot accumulate calcium during diastole and thus cannot respond properly to frequency changes and to stimulation pauses. ISO renders RYR channels even leakier: this explains the lack of transient amplitude increase in the mutated population. Likely, the mutated RYR generates many frequent small calcium releases under basal conditions, which are unable to generate DADs but alter the mechanical properties of myocytes; under adrenergic stimulation, bigger and longer spontaneous releases become possible, predisposing to arrhythmias.

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EFFECTS OF RACEMIC LUBELUZOLE AND PURE ENANTIOMERS ON VOLTAGE-GATED HUMAN SKELETAL MUSCLE Na^+ CHANNELS

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Lubeluzole is a chiral benzothiazole compound. It is a novel neuroprotective agent, currently in use in the management of acute ischemic stroke (1). The neuroprotective effects of lubeluzole have been attributed to inhibition of glutamate release, inhibition of nitric oxide (NO) synthesis and blockade of voltage-gated Na^+ and Ca^{2+} channels. In this study we tested the effects of racemic lubeluzole and pure R-(+) and S-(-) enantiomers on human voltage-gated Na^+ channel ($Na_v1.4$) expressed in a mammalian cell line, using patch-clamp technique. We evaluated the block of Na^+ channel by drug by measuring the reduction of Na^+ current (I_{Na}) elicited from the holding potential (hp) of -120 mV to -30 mV at 0.1 Hz and 10 Hz frequency stimulation. The concentration-response curves were fitted with a first-order binding function. The values of half-maximum inhibitory concentration (IC_{50}) at 0.1 Hz were 30.6±5.1 μM for the (R,S)-lubeluzole, 34.0±6.3 μM for the R-(+) enantiomer, and 33.0±14.4 μM for the S-(-) enantiomer. At 10 Hz, the IC_{50} values were 1.7±0.2 μM for racemic lubeluzole, 2.8±0.2 μM for the R-(+) enantiomer, and 1.3±0.1 μM for the S-(-) enantiomer. Thus, we observed no significant difference between R-(+) and S- (-) enantiomers and racemic compound, indicating that the action of lubeluzole on Na^+ channels is not stereoselective. Compared to known Na^+ channel blockers, such as mexiletine and flecainide (2), in the same experimental conditions, lubeluzole displays a 2.5-7 fold higher affinity at 0.1 Hz and a 18-fold greater affinity at 10 Hz, respectively, suggesting that Na^+ channel blockade may contribute significantly to the therapeutic effects of the drug. The block of I_{Na} by lubeluzole increased with the frequency of stimulation, indicating a use-dependent behaviour. According to the modulated receptor hypothesis, the use-dependence may arise from a higher binding affinity to open and/or inactivated channels with respect to closed channels (3). The use-dependence of lubeluzole may be critical for its therapeutic use.

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POSSIBLE INVOLVEMENT OF ANGIOTENSIN II IN MUSCULAR DYSTROPHY: MULTIDISCIPLINARY EVALUATION OF THE EFFECT OF A CHRONIC TREATMENT WITH ENALAPRIL IN DYSTROPHIC MDX MICE

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Duchenne muscular dystrophy (DMD) is the most common X-linked muscle disease characterized by progressive muscle weakness and degeneration. The mutation of the dystrophin gene leads to the absence of dystrophin in skeletal muscle fibers, a biochemical defect also observed in the mdx mouse, the murine phenotype of DMD. This leads to a complex and still not fully understood network of interconnected pathogenic events responsible for progressive muscle degeneration; these events involve the increased entrance of calcium, the activation of proteases, and the occurrence of a functional ischemic state. Cardiac involvement and respiratory failure are the most common causes of death in patients (1). Recent studies show that an early administration of perindopril, an inhibitor of angiotensin converting enzyme (ACEI), reduces mortality in children with DMD (2) and losartan, an AT_1 receptor antagonist, contrasts some of the pathological signs in mdx mice (3). Based on these observations, we have evaluated the potential therapeutic effect of enalapril (1 and 5 mg/kg/day i.p. for 4-8 weeks) in the exercised mdx mouse model, that shows a more severe progression of the pathology (4,5). A multidisciplinary approach, involving *in-vivo* evaluation of mouse strength, and *ex-vivo* electrophysiological, biochemical, histological, and immunohistochemical analyses, was used. After 4 weeks, the enalapril-treated mice were significantly stronger, in a dose-dependent manner, with respect to untreated ones. The value of the increment in normalized strength was 1.29±0.09, for 5 mg/kg (n=8) and 0.53±0.06 for 1 mg/kg treated mice (n=7) with respect to 0.035±0.003 of untreated counterparts (n=7). Two microelectrode current and voltage clamp recordings were used to measure *ex vivo* macroscopic chloride conductance (g_{Cl}), and mechanical threshold (MT), a calcium-sensitive parameter. We found that enalapril at 1 mg/kg and 5 mg/kg counteracted by 36% and 47%, respectively, the exercise-induced impairment of g_{Cl}, in extensor digitorum longus (EDL) muscle fibers. As opposite, the treatment was less effective on the spontaneously impaired g_{Cl} in diaphragm (DIA). As a result of the alteration of calcium homeostasis, the mdx EDL muscle fibers are characterized by about a 10 mV shift of the voltage threshold for MT toward more negative potentials. The treatment with enalapril at 5 mg/kg significantly shifted the strength-duration curve toward more positive potential (rheobase potential=-72.2±1.29 mV) with respect to that of untreated exercised EDL muscle fibers (-75.6±1.2mV). There was no significant decrease in plasma creatine kinase levels in enalapril-treated mdx mice compared to untreated animals. Enalapril at 5mg/kg ameliorated histological profile in gastrocnemius muscle (GC) and DIA. In fact, in GC there was a significant reduction of centronucleated fibers and significant decrease in degenerating area (1.2±0.2% vs 5.6±1.94%; $P<0.05$). Similarly a significant decrease in degenerating area was also observed in DIA of treated mice (1.80±0.71% vs 8.12±4.7%; $P<0.05$). Immunohistochemical determination of phospho-NFκ-B in GC muscles fibers showed a reduced number of NFκ-B labeled nuclei in enalapril-treated compared to untreated exercised mice (60.0±7.0% vs 28.0±13.5%). Thus, enalapril treatment produced, in a dose-dependent manner, a protective effect against dystrophic damage ameliorating both morphological and functional indices. Our results corroborate that ACEIs may have a direct beneficial effects on skeletal muscle other than on heart, supporting their interest for DMD therapy.

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NOVEL HYALURONIC ACID BIOCONJUGATES IN PHARMACOLOGICAL TREATMENT OF CANCER

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Targeting cytotoxic drugs selectively to tumoral cells is the goal of anticancer research. In fact, commonly used cytotoxic agents hit generally active-replicating cells, not discriminating between normal and tumour ones. In our laboratory we are focusing our attention on hyaluronic acid (HA) bioconjugates of different cytotoxic drugs. HA is, in fact, one promising molecule to be used as vehicle to hit selectively tumour cells. This polymer is constitutively expressed in the extracelllar matrix, and it can bind the CD44 receptor, a tumoral marker overexpressed in tumour cells. HA mediates through CD44 interaction many cellular activities such as adhesion, migration, invasive capacity and cellular survival. Moreover, it shows a promising activity as antiangiogenic compound, its potency and activity depending on its molecular weight, and increasing data suggest that HA is also capable to affect malignant cells ability to invade through tissues. Several HA bioconjugates containing different cytotoxics were tested both *in vitro* and *in vivo* in order to evaluate their efficacy against the growth of different cell lines (MIA PaCa2, NB4, MCF7, MDA MB231), compairing their effect to HA of the same molecular weight of the bioconjugates themselves. Also, the cytotoxic activity of HA of different mass weight was tested. Lines were initially screened for CD44 expression and for cytotoxic effect of bioconjugates, of drugs taken alone, and HA alone. Drug interaction with CD44 was verified using a CD44-blocking antibody. Invasive capacity of malignant lines was evaluated together with the inhibitory effect of bioconjugates with an invasion test that mimes extracellular matrix situation with transwell plates covered by matrigel. Bioconjugates tolerability and efficacy was checked *in vivo*: acute toxicity tests and survival curves were made using SCID xenograph models. All lines used did express CD44. The *in vitro* results showed different responses: we observed both an increase of *in vitro* cytotoxicity for some bioconjugates and a decrease in cytotoxicity for others, when compared to equal doses of the corresponding drug alone. The use of a CD44-blocking antibody confirmed a CD44 mediated uptake of all the bioconjugates. Bioconjugates were also effective in reducing invasive capacity of malignant cells and angiogenesis as well. Scarse or null activity both on invasion test and angiogenesis evaluation was observed for HA with molecular weight corresponding to that present in our bioconjugates. *In vivo* the use of bioconjugates resulted significantly more effective than the corresponding unbinded drugs in increasing life-time expectancy in xenograph SCID models. Thus, veicolation of cytotoxic through HA seems to be a winning strategy in the fight of tumours: decreases in *in vitro* activity may occur, but when bioconjugates were tested *in vivo*, they revealed to be less toxic and more effective than drugs taken alone. In our laboratory we are currently checking the involvement of CD44 activation through HA and bioconjugates binding in activation and regulation of Akt phosphorilation, focusing our attention on PI3K-PTEN signalling pathway. In fact, many researchers are now enlightening the connection between CD44 activation with the binding of HA and PI3K-Akt signalling system. It is well known that this system is heavily modified in malignant cells, and is involved both in invasivity and survival of tumoral cells.

ANTHOCYANIDINS MODULATE THE CYTOTOXICITY OF IRINOTECAN AND OXALIPLATIN IN HUMAN COLON ADENOCARCINOMA CELLS

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Colon cancer is the second leading cause of cancer death in most Western countries. Chemotherapy is used as a first-line treatment for metastatic colorectal cancer; the new agents incorporated into frontline therapies, irinotecan and oxaliplatin, have improved the prognosis, but still, drug resistance is the major cause of chemotherapy failure. Flavonoids, the most common and widely distributed group of polyphenols in plants, 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. The aim of this study was to evaluate the cytotoxicity of the anticancer agent irinotecan and oxaliplatin, alone or in combination with anthocyanidins, a class of flavonoids widespread in fruits and vegetables, and to study their apoptotic effect in LoVo, LoVo ADR, and CACO 2 human colon adenocarcinoma cells. Cytotoxicity assay (MTT test) was performed to determine IC_{50} of anticancer agents and anthocyanidins and to evaluate the effect of their combination in these cell lines. To determine apoptotic effect we analysed DNA integrity by agarose electrophoresis and changes in nuclear morphology using a fluorescence microscope. Anthocyanidins' antioxidant activity was assessed by measuring their ability to inhibit peroxyl radical-induced oxidation of DCFH to DCF. IC_{50} for the antitumor drugs in the LoVo cell line was 37.0±5.3 nM for camptothecin and 876.2±245.5 nM for oxaliplatin, respectively. IC_{50} values for anthocyanidins were: 37.6±3.3 μM (delphinidin), 46.9±1.8 μM (cyanidin), and 80.5±9.8 μM (malvidin). In the LoVo ADR cell line IC_{50} values were: 200±1.1 nM (camptothecin), 1.4±0.26 μM (oxaliplatin), 16.4±1.7 μM (delphinidin), and 26.2±6.2 μM (cyanidin). In the CACO 2 cell line only the antitumor drugs showed the cytotoxic effect - the IC_{50} value for camptothecin was 130.4±20.6 nM and for oxaliplatin 1.7±0.2 μM. The effect of the combination of anthocyanidins and antineoplastic drugs was also studied. In LoVo cells delphinidin (25 μM) increased significantly the cytotoxic effect of 0.01 μM camptothecin ($P<0.001$) and 0.1 μM oxaliplatin ($P<0.001$). The same concentration of cyanidin had similar effect in combination with camptothecin ($P<0.001$) but 50 μM cyanidin increased significantly cytotoxicity of both drugs ($P<0.001$). In LoVo ADR cells delphinidin (25 μM) increased significantly the cytotoxicity of 0.05 μM camptothecin ($P<0.001$) and 0.2 μM oxaliplatin ($P<0.01$) but cyanidin did not show any significant effect. The uptake study, performed with the aim to see if the possible mechanism of delfinidin action includes interaction with P-glycoprotein (P-gp; an ABC transporter), demonstrated that 50 μM delphinidin did not induce any significant effect on the concentration of 25 μM doxorubicin (a P-gp substrate) in LoVo and LoVo ADR cells, whereas 50 μM verapamil (a P-gp inhibitor) decreased significantly ($P<0.01$) doxorubicin extrusion from LoVo ADR cells and consequently increased its intracellular concentration. This suggests that delphinidin increases the cytotoxicity of the antineoplastic drugs through mechanisms that do not include the interaction with P-gp. When testing apoptotic potential we found that camptothecin (250 nM and 1 μM) induced nucleosomal DNA fragmentation typical of apoptosis in HL-60 cells (human promyelocytic leukemia cells) but not in LoVo or LoVo ADR cells. On the other hand, apoptotic cells with fragmented nuclei were observed in both HL-60 and LoVo cell lines 24 h after treatment with camptothecin (250 nM) and delphinidin (100 μM and 200 μM). When measuring antioxidant activity of anthocyanidins (1 h-incubation time) we found that cyanidin in LoVo and both cyanidin and delphinidin in CACO 2 cells acted as antioxidants whereas delphinidin in LoVo cells did not show any antioxidant effect. Our results show that: (i) anthocyanidins, in particular delphinidin and cyanidin, exert an important cytotoxic action in the LoVo and LoVo ADR colon cancer cell lines and increase the cytotoxicity of oxaliplatin and camptothecin; (ii) delphinidin increases the cytotoxicity of the antineoplastic drugs through mechanisms that do not include the interaction with P-gp; (iii) delphinidin and cyaniding show proapoptotic effect in LoVo cells; (iv) while cyanidin acts as an antioxidant in both less and more differentiated adenocarcinoma cells, delphinidin does it only in the latter.

EFFECTS OF THE PHYCOTOXIN YESSOTOXIN ON CULTURED RAT CARDIOMYOCYTES

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Yessotoxin (YTX) and its analogues are phycotoxins produced by the phytoplanktonic dinoflagellates *Protoceratium reticulatum*, *Lingulodinium polyedrum*, and *Gonyaulax spinifera*. When environmental conditions promote the growth of these species, yessotoxins accumulate in edible tissues of filter feeding shellfish exposed to these dinoflagellates and may possibly be ingested by humans through seafood consumption. Toxicological studies have revealed a high YTX toxicity after acute i.p. injection in mice, its LD_{50} values being lower than 1 mg/kg. Conversely, no lethality was found in mice after YTX acute or repeated oral administration. However, both oral and i.p. administration of YTX to mice caused tissue alterations at cardiac level, with swelling of muscle cells, separation of mitochondria, and cytoplasmic protrusions into the pericapillary space. Notwithstanding the cardiac tissue seems to represent the main toxin target, the effects on the functional cardiac properties remain unknown. Isolated cardiac cells are widely used to study the functional properties of the cardiac tissue at the single cell level. Hence, this study was performed on neonatal rat primary cultures of cardiomyocytes with the aim to investigate the effect of YTX on cardiac functionality. Patch-clamp recordings, Ca^{2+} imaging, and cAMP assays were performed to characterize YTX effects on the cardiomyocytes beating frequency. A time- and concentration-dependent reduction in the beating frequency was observed after 1, 5, and 24 h incubation with YTX (0.1-1 μM). In cells perfused for 1 h with a concentration of YTX that significantly reduced the beating frequency (1 μM), a decrease in the firing frequency (about 50%) was observed without any change in resting membrane potential or action potential amplitude (from 50.3±0.1 mV to 51.6±0.1 mV, n=30 events). Videoimaging measurements of the $[Ca^{2+}]_i$, performed at the same concentration and incubation time, showed no effect of YTX on both basal level and peak levels of $[Ca^{2+}]_i$ (from 1.9±0.1 to 1.8±0.2 mV, n=15 fields). Similar to control conditions, each action potential was always associated to a $[Ca^{2+}]_i$ transient in the presence of YTX. Moreover, 1 μM YTX did not modify basal cAMP levels in cardiomyocytes. These results indicate that the reduction in beating frequency is neither a consequence of the uncoupling between the membrane electrical activity and Ca^{2+} release from intracellular stores nor of the impairment of the mechanisms controlling Ca^{2+} and cAMP homeostasis. MTT and sulforhodamine B tests were carried out to determine YTX effect on cell viability, and revealed that incubation of cardiomyocytes with YTX (0.01-1 μM for 24, 48, and 72 h) caused a decrease in cell viability in a concentration- and time-dependent way. This effect was still evident in cardiomyocytes exposed to YTX for 1, 5, and 24 h and cultured up to 72 h in YTX-free medium. Cells pictures acquired by means of fluorescence microscopy, showed the presence of apoptotic nuclei in YTX-treated cells. In conclusion, the present study indicates that at nM concentrations, a short incubation with YTX causes an inhibition of beating activity and an irreversible reduction of cardiomyocytes viability *in vitro* and suggests that, although no human intoxication due to YTX contamination has been reported so far, the toxicological potential of this compound should be better investigated. Studying this toxin is limited by its non-commercial supply: YTX needed for these experiments was a kind gift of Professor T. Yasumoto (Tama Laboratory, Japan Food Research Laboratories, Tokyo).

ELECTROPHYSIOLOGICAL EVALUATION OF NOVEL BLOCKERS OF I_f CURRENT

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In the sino-atrial node (SAN), a major role in the initiation and autonomic control of rhythm generation is played by f-current (1), a mixed sodium-potassium inward current activated upon hyperpolarization and directly modulated by cyclic nucleotides. I_f is coded by HCN family genes and comprises four isoforms (HCN1-4) (2). In physiological condition, HCN expression is functionally relevant in the SAN region and other parts of the conduction system. I_f becomes upregulated in many cardiac disease at ventricular level (3). The overexpression of cardiac HCN channels may contribute to the increased propensity for cardiac arrhythmias. Thus, selective f-channel blockers have a potential for therapeutic use as bradycardic and antiarrhythmic agents. A few bradycardic agents such as zatebradine or ivabradine (4), have been developed but have side effects, especially on vision, likely related to the block of neuronal HCN isoforms. From this reason, the development of new compounds selective for the channel isoform typical of mammal sinus node cells, HCN45, is needed. Some zatebradine analogues, synthesized in collaboration with Prof. Romanelli of the Department of Pharmaceutical Chemistry, University of Florence, were tested on HEK293 cells, transfected with mHCN1, mHCN2 and hHCN4 cDNA and on cells from guinea-pig and rabbit SAN by patch-clamp technique. The compounds used for this study were tested for the first time on HEK293 cells expressing HCN1, HCN2, and HCN4 isoforms of f-channel and their activity was compared to that of ivabradine. All compounds (C1-C5) at the concentration of 10 μM, produced a reduction of maximal I_f amplitude, elicited by a step to -120 mV, although with different potency and selectivity as shown by their IC_{50}. C1 reduced I_f amplitude more in cells expressing HCN1 than in those transfected with HCN2 or HCN4. C2 displayed a major activity on HCN4 reducing I_f amplitude by 63%. For C3 current reduction was more marked on HCN1 and HCN4 than on HCN2- transfected cells. Compound C4 presented a greater activity on HCN1 isoform with respect to HCN2 and HCN4, reducing I_f by about 20%. Finally C5, the enantiomer of C4, had a low activity on all isoforms. The effect of all compounds, including ivabradine, was concentration-dependent and not reversed by washout. Moreover the decrease of the current amplitude was not associated with changes in the potential of half-maximal activation. The potency of the drugs was defined by IC_{50}, evaluated by fitting concentration-effect curves with the Hill equation. While the reference compound, ivabradine, did not show any isoform selectivity, C1 and C4 were more potent on HCN1 (2.31±0.40 μM and 0.60±0.07 μM, respectively) and C2 was more potent on HCN4 (9.74±7.70 μM). Finally, preliminary data obtained in SAN cells suggest that the effects on native I_f resemble those obtained on HCN4 isoform, in line with the hypothesis that HCN4 represent a major contributor in SAN cells. These findings indicate that the interaction with the different channel isoforms seems to have different structural requirements, which are presently under investigation, and, therefore, it should be possible to obtain a better selectivity of action than for zatebradine or ivabradine by suitably modification of the molecule. Work is underway to clarify the structural requirements necessary for the interaction with these molecular targets, and to better characterize the pharmacological profile of these substances.

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CORTICOTROPIN RELEASING HORMONE (CRH) INHIBITS CELL GROWTH OF HUMAN NEUROBLASTOMA AND MEDULLOBLASTOMA CELL LINES

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The hypotalamic peptide corticotropin releasing hormone (CRH) is the principal mediator of acute adaptive responses of the hypotalamo–pituitary-adrenal axis to stress. The effects of CRH on target tissues are mediated through the activation of two high affinity membrane receptors: CRH type 1 and type 2 (CRH-R1 and CRH-R2), which belong to the family of seven transmembrane domain receptors coupled to G proteins and activating adenilate cyclase. These receptors display different distribution within several tissues, reflecting distinct biological functions. CRH-R1 and CRH-R2 express different affinity for another family of mammalian CRH-related ligands: urocortin-I (UCN-I), urocortin-II (UCN-II) and urocortin III (UCN-III). The expression of both receptor subtypes has been reported in human cancers originating from tissues which may or may not express CRH receptors. Moreover, various human cancer cell lines derived from endometrial adenocarcinoma, breast cancer, neuroblastoma, small cell lung cancer, and melanoma also express CRH receptors. These findings suggest that CRH may play a role in the control of neoplastic cell growth. We have previously shown that CRH is able to inhibit the proliferation of a human endometrial adenocarcinoma cell line, Ishikawa (IK) cells, as well as of MCF-7 human breast cancer cells and IMR32 neuroblastoma cell line. Here we have investigated the possible antiproliferative effects of CRH on other cell lines derived from the central nervous system (CNS), namely human neuroblastoma SK-N-SH, and human medulloblastoma cell line DAOY. Cells were cultured in Eagles minimum essential medium with Earle's salts supplemented with 10% fetal bovine serum, 2 mM L-glutamine, Na^+ pyruvate, non essential aminoacid and 100 U/ml penicyllin and 100 µg/ml streptomycin. Cell lines were maintained at 37°C in a 5% CO_2 humidified atmosphere. For proliferation studies, cells were plated in 60 mm dishes at a density of $4x10_4$ cells/dish. The treatment of both SK-N-SH and DAOY with CRH induced a time- and concentrationdependent inhibition of cell growth with maximal effect after 8 days at the concentration of 100 nM (SK-N-SH 43.4±6.0% (SEM) inhibition, $P<0.001$ vs control; DAOY 33.7±9.4% inhibition, $P<0.05$ vs control). This effect was counteracted in a concentration-dependent manner by the CRH receptor antagonist astressin; the latter had no intrinsic effect on cell proliferation when given alone. The antiproliferative effect was not associated with induction of apoptosis, as shown by staining experiments with the fluorescent nuclear dye Hoechst 33258. The CRH related peptide urocortin was also able to significantly reduce cell proliferation of both cell lines. Having observed a functional, receptor-operated effect of CRH on cells proliferation we used the RT-PCR to demonstrate that these cells type express the CRH-R1α receptor subtype trascripts under basal conditions. These results provide further support to the hypothesis that CRH inhibits cell proliferation in human CNS tumour cell lines. Interestingly enough, preliminary results suggest that the effects of CRH on cell proliferation are mediated *via* the regulation of ciclin-dependent kinase activities, in particular P18; these findings are currently under thorough scrutiny for consistency and type(s) of cell line involved.

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PMA ALTERS PROSTACYCLIN/PGE_2 BALANCE THROUGH DOWNREGULATION OF PGI SYNTHASE IN HUMAN CORONARY ARTERY ENDOTHELIAL CELLS

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Prostacyclin and prostaglandin (PG) E_2 are generated in endothelial cells from arachidonic acid (AA) mainly through the expression of cyclooxygenase (COX)-2 (1). The product of COX-catalyzed metabolism of AA, PGH_2, is metabolized by specific synthases to generate prostacyclin and PGE_2, i.e. prostacyclin synthase, a member of the cytochrome P-450 (P450) superfamily CYP8A1 (2) and microsomal (m)-PGE synthase (PGES)-1, a member of the MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) superfamily (3), respectively. Prostacyclin plays an important role in protection of vascular function (4). In contrast, PGE_2 induces expression of matrix metalloproteinases (5) and may inhibit the production of macromolecules of the extracellular matrix, favouring plaque fragility (6). In addition, PGE_2 may promote angiogenesis (7) which plays a role in chronic inflammatory processes, such as atherosclerosis. In cells expressing both synthases, competition for PGH_2 could modify prostacyclin production under inflammatory conditions (8). The present work was conducted to study the expression of COX-2, COX-1, PGIS, mPGES-1, and 6-keto-$PGF_{1\alpha}$ (a non-enzymatic hydrolysis products of prostacyclin) and PGE_2 biosynthesis in human coronary artery endothelial cells (HCAEC) exposed to interleukin (IL)-1β (5 ng/ml), or phorbol 12-myristate 13-acetate (PMA) (10 nM), a trigger of endothelial dysfunction. HCAEC were cultured in EBM-2 supplemented with 5% FBS and vascular growth factor mixture. Expression of COX-1, COX-2, PGIS, and mPGES-1 were determined by Western blot analysis. Biosynthesis of prostanoids from endogenous substrate was analyzed on cellular media by specific radioimmunoassay techniques. IL-1β caused a comparable induction of both 6-keto-$PGF_{1\alpha}$ and PGE_2 (3300 and 2800 pg, respectively). In contrast, PMA induced higher levels of PGE_2 than 6-keto-$PGF_{1\alpha}$ (99000 and 3600 pg, respectively). IL-1β was associated with comparable levels of mPGES-1 and PGIS. In contrast, PMA was associated with a dramatic reduction of PGIS levels while mPGES-1 was not affected. In conclusion, our results show that in endothelial cells, down-regulation of PGIS expression in response to PMA (an activator of PKC) triggers a pro-inflammatory phenotype by shifting AA metabolism towards PGE_2 presumably by inducing a preferential coupling of COX-2 with mPGES-1. Interestingly, PKC pathway is activated by hyperglycemia and may participate in vascular disease in diabetes mellitus (9). Our results suggest that mPGES-1 may be a target for therapeutic intervention in this setting.

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INDUCTION OF PROSTACYCLIN BY STEADY LAMINAR SHEAR STRESS SUPPRESSES TUMOUR NECROSIS FACTOR-α BIOSYNTHESIS *VIA* HEME OXYGENASE-1 IN HUMAN ENDOTHELIAL CELLS

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Cyclooxygenase-2 (COX-2) is one of the vasoprotective genes up-regulated by steady laminar shear stress (LSS) in endothelial cells (1). However, they express constitutively also COX-1. Thus, the contribution of COX-isozymes to endothelial prostanoid generation, in physiologic conditions, is still controversial (2,3). The aims of this study were to explore in human umbilical vein endothelial cells exposed to steady LSS of 10 dynes/cm$_2$ for 6 h: 1) the contribution of COX-isozymes and down-stream specific synthases to the generation of different prostanoids; 2) the effects of the selective inhibitor of COX-2, NS-398 (1 μM) and the non-selective COX inhibitor aspirin (25 μM) on the biosynthesis of prostanoids and tumour necrosis factor-α (TNF-α), a pro-atherogenic cytokine, and on the induction of heme oxygenase-1 (HO-1), an antioxidant enzyme. LSS enhanced 6-keto-PGF$_{1α}$, PGE$_2$ and PGD$_2$ ($P<0.05$) vs static condition. LSS induced COX-2 (8-fold vs static condition, $P<0.05$) while COX-1 and the downstream synthases were not significantly modulated. LSS caused a significant reduction - vs static condition - of TNF-α released in the medium (3633±882 and 9100±2158 pg, respectively, $P<0.05$) or in cells lysates (1091±270 and 2208±300 pg, respectively, $P<0.05$). This was associated with HO-1 induction. NS-398 significantly ($P<0.05$) reduced 6-keto-PGF$_{1α}$ (by 30%) while did not affect the other prostanoids. Aspirin significantly ($P<0.05$) reduced 6-keto-PGF$_{1α}$, PGE$_2$, and PGD$_2$ levels by approximately 50%. In the presence of NS-398, aspirin or the prostacyclin receptor (IP) antagonist RO3244794 (4), the reduction of TNF-α by LSS was completely recovered and it was associated with down-regulation of HO-1 expression. In static conditions, the IP agonist carbacyclin induced HO-1. Finally, the HO-1 inhibitor QC15 (5) reversed TNF-α reduction by LSS. Our results enlighten the central role of LSS-dependent generation of prostacyclin to restrain inflammation in the endothelium through a mechanism that involves the action of HO-1. The finding of comparable pro-inflammatory effects by aspirin and NS-398 supports a dominant role of COX-2-dependent prostacyclin in vasoprotection induced by steady LSS in endothelial cells. These findings are provocative because propose that profound suppression of endothelial prostacyclin caused either by selective COX-2 inhibitors or traditional nonsteroidal antiinflammatory drugs may translate into acceleration of atherogenesis.

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EFFECT OF ANTIOXIDANT TREATMENT ON DISUSE-INDUCED SKELETAL MUSCLE ALTERATION IN THE HIND LIMBUNLOADED MOUSE MODEL

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We applied the model of hind limb unloading (HU) to male, 6-month-old C57BL mice to characterize the effects of disuse on muscle atrophy and sarcolemma resting conductance. After 14 days of HU, the *tibialis anterior* (TA), *extensor digitorum longus* (EDL), *soleus* (Sol) and *gastrocnemius* (Gas) muscles were removed from anesthetized mice and weighted. With respect to the control mice, no significant difference in muscle to body weight ratio was found for the EDL, whereas Sol, GAS, and TA showed a reduction of 22.5% (n=45 mice; P<0.0001), 11,8% (n=29; P<0.001), and 9.0% (n=26; P<0.025), respectively. Electrophysiological studies were made with the two endocellular microelectrodes technique to evaluate the resting sarcolemma chloride (g_{Cl}) and potassium (g_K) conductance in Sol, Gas, and EDL muscles, which are crucial for muscle excitability. No effect of HU was observed on the g_K in all the three muscles. In contrast, HU increased the g_{Cl} by 36.4±4.9% in Sol (n mice/n fibres=6/69; P<0.0001) and 11.9±2.7% in Gas (n/n=4/39; P<0.0025). In the EDL, the g_{Cl} increased not significantly by 9.4±4.4% (n/n=3/30; P>0.05) after HU. A number of evidences suggest that oxidative stress occurs during disuse (1). Thus we wondered whether there is a link between production of reactive oxygen species (ROS) and disused muscle atrophy. Consequently we measured oxidative stress markers, such as malondialdehyde (MDA) and reduced glutathione (GSH) levels in muscle tissue by absorbance and fluorescence assays. With respect to control mice, HU induced an increase of MDA levels and a decrease of GSH levels in Sol and Gas muscles homogenates, which confirms the presence of oxidative stress in disused muscles. We decided to start a treatment with Trolox, a water-soluble derivative of vitamin E that penetrates biomembranes and protects mammalian cells from oxidative damage. The mice received 45 mg/kg/day i.p. of trolox for one week in normal condition (free in the cage) and then for two weeks in HU condition. As expected, the MDA and GSH levels were restored toward control values, demonstrating the ability of trolox to prevent oxidative stress. However, no beneficial effect of trolox was found on muscle atrophy, since the muscle-tobody weight ratio were not different between HU mice and trolox-treated HU mice. In contrast, Trolox was able to fully prevent the changes in g_{Cl} associated with disuse in both Sol and Gas muscles. Furthermore *in vitro* application of 300 µM trolox showed no effect on g_{Cl} and g_K in Sol and Gas muscles, suggesting that trolox *in vivo* effects on the g_{Cl} is a long-term effect most probably related to oxidative stress prevention. All these findings suggest that oxidative stress is a consequence rather than a cause of atrophy in HU-induced muscle disuse.

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LIFESTYLES: STATE OF PSYCHO-PHYSICAL HEALTH OF WOMEN. EPIDEMIOLOGICAL RESEARCH AS A PROJECT OF FIELDWORK TRAINING FOR PHARMACISTS

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Life stressors contribute to the onset of depression in both men and women, but particular stressors affect the genders differently: women are also more frequently affected by physical and sexual abuse, which will significantly influence future episodes of depression (1). The most recent review of the literature suggests it is possible to reach an overall successful adherence improving strategy performed by pharmacists. More well-designed and well-conducted studies on the effectiveness of interventions by a community pharmacist to improve patient adherence need to be performed (2). The purpose of this study is to achieve several objectives, that can be summarize into two types: epidemiological objectives and educational objectives. Epidemiological objectives: 1) check which stressful events (on a list of stressful events extrapolated from the literature) are risk factors for the state of psycho-physical health of women treated or not treated with drugs for depression and anxiety; 2) check the level of satisfaction of therapy for depression and anxiety; 3) check the correlation between the use of other drugs, health products and the state of depression; 4) check how many women depressed and anxious recur to the psychologist/psychiatrist and/or the social assistant; 5) check the role of pharmacists in the management of anxiety and depression of women. Educational objectives: 1) introduce pharmacists to research; 2) educate and sensitize pharmacist on psycho-social problems associated with prescription of drug therapy of depression and anxiety disorders; 3) create a service point in pharmacies as a support and research on the "malaise" of women; 4) promote the growth of an image of pharmacy that is close to patients, in particular women, as a centre of health protection. Design: observational case-control study structered as a project of fieldwork training for pharmacists. Setting: two-hundred and sixty territorial pharmacists operating both in public and privat pharmacies in Veneto Region, Italy. Patients: on the basis of antidepressant and anxiolitic drug use in the Veneto Region, 12480 women will be interviewed (4160 cases and 8320 controls). Instruments: there are three types of questionnaire developed for this study: 1) a questionnaire (the same for cases and controls) that women fill out in pharmacy [this instrument is issued from the interview for Recent Life Events (IRLE) and the List of Threatening Experiences (LTE) (3,4) and it covers a comprehensive range of recent life events, their timing, and other important qualities]; 2) a questionnaire that pharmacist fill out about the pharmacotherapy of women selected as case and control; 3) a questionnaire filled out by pharmacist about his rule in the management of women who have mood disorders (2). Interventions: in the month of April, a preliminary educational meeting about the study was performed with pharmacists of the seven Provinces of Veneto Region. Two-hundred and sixty pharmacists who have joined the project will interview 24 women (8 cases and 16 controls) during the mount of May and 24 women (8 cases and 16 controls) during the mount of October. The cases consist of women who go to a pharmacy with a prescription for therapy of depression and anxiety disorders; controls, instead, consist of women who go to a pharmacy to buy any other product but not a drug for therapy of depression and anxiety disorders. During the month of June each pharmacist filled out his personal interview about his rule in the management of women who have mood disorders.

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ANTIMUTAGENICITY OF β-CARYOPHYLLENE IN AMES TEST AND MICRONUCLEUS ASSAY ON CULTURED HUMAN LYMPHOCYTES

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Chemoprevention by components of natural products has aroused lively interest in last years. Among natural substances, terpenoids seem to be potentially useful in prevention and treatment of several diseases, cancer included. β-Caryophyllene (β-CRY) is a sesquiterpene contained in several essential oils as those from *Eugenia caryophyllata*, *Salvia* spp., *Lavandula angustifolia* L., *Piper nigrum* L., and *copaiba* balsam (*Copaifera reticulata* Ducke); it is known to possess anti-inflammatory, anticarcinogenic and antioxidant properties (1,2). In this work, β-CRY has been evaluated for its potential antimutagenic activity against the genotoxicity induced by known mutagenic agents. The experiments were carried out by using two *in vitro* tests: the Ames test on *Salmonella typhimurium* TA 98 and TA 100 strains and on *Escherichia coli* WP2*uvr*A strain, with and without the extrinsic metabolic activation system (3); the micronucleus assay (MN) on cultured human lymphocytes (4). The antimutagenic activity of β-CRY has been studied against several known mutagens: 2-nitrofloruene (2NF), sodiumazide (SA), methylmethanesulfonate (MMS), and 2-aminoantracene (AA) for the Ames test (5); ethylmethanesulfonate (EMS) and colcemid (COL) for the MN assay. In the last test, β-CRY has been added to the lymphocyte cultures by three different treatment protocols, i.e. pre-, co- and post- treatment (6). Preliminary assays were performed to find the non-toxic concentration and to exclude a mutagenic effect of the test substance. In the Ames test, at non toxic concentrations (112.6-440.4 mM), β-CRY did not increase the number of revertant colonies, both with and without metabolic activation system (S9). The MN frequency in the human lymphocytes was not significantly increased by β-CRY in the range of the non-toxic concentration tested (0.0049-0.49 mM). β-CRY showed a strong antimutagenic activity against 2NF in TA 98; at the highest concentration tested (313.2 mM) the number of 2NF-induced revertant colonies was reduced by 83.9%. The sesquiterpene also induced a weak inhibition of the SA-mutagenicity in TA 100 (inhibition lower than 25%) and a moderate inhibion of the MMS- and 2AA-mutagenicity in WP2*uvr*A (up to 30.5%). In the MN assay, β-CRY (0.0049-0.49 mM) reduced significantly the frequency of MN induced by EMS in the preand co-treatment (up to 67% and 86% of reduction, respectively). No significant reduction of COL-induced MN frequency was found. In the range of concentrations tested, the nuclear division index (NDI) was not significantly reduced, thus no interference with the cellular proliferation was highlighted. The antimutagenic activity of β-CRY observed in both assay seems to be related to a desmutagenic mechanism as modification of the permeability of the bacterial membrane, or some extracellular physical, chemical or enzymatically catalysed interactions between the substance and the mutagen. No interactions between the substance and the components of an exogenous mammalian metabolic system can be supposed, since the effect was observed in absence of S9 mix. Moreover, the strong effect exerted in the MN assay, both in pre- and in co-treatment, suggests that β-CRY could act by chemical interaction with the substance in the medium of growth (co-treatment) or in the cytoplasm of the lymphocyte (pre-treatment), such as a desmutagen. Data obtained encourage further studies to investigate the potential role of this substance as chemopreventive agent.

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GLUCOCORTICOID-INDUCED LEUCINE ZIPPER (GILZ) TAKES PART TO MUSCLE DEVELOPMENT

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Myogenesis is a highly regulated multi-step model of terminal differentiation, in which myoblasts, undifferentiated mononucleated precursors, fuse into the multinucleated myotubes: this process is activated when cell critical density is reached and myoblasts exit from cell cycle. Myogenesis is driven by the muscle related factors (MRFs), which belong to the basic Helix-Loop-Helix superfamily (1); in parallel to their pro-myogenic action, MRFs induce the expression of a number of factors, such as TGF-β, which limit terminal differentiation in an autocrine manner, in order to preserve a population of undifferentiated precursors (2). Glucocorticoids (GCs) are important regulators of skeletal muscle metabolism. Among the genes that are positively modulated by GCs, GILZ is one of the most rapidly induced and mediates part of GC effects, such as NF-κB, AP-1, and Ras activity inhibition (3,4). We observed that GILZ is spontaneously expressed in murine skeletal muscle, as well as a newly identified isoform that we named L-GILZ, a 28 kDa protein encoded by a non-AUG starting transcript. In order to investigate the possible roles of GILZ and L-GILZ in myogenesis, we employed the murine myoblast cell line C2C12, which can reproduce differentiation process *in vitro*. We observed that GILZ and L-GILZ expression is developmentally regulated, with an induction during the terminal stages of differentiation. On the other hand, their over-expression resulted in myogenic inhibition, with counteraction of myogenin induction, a crucial step for cell-to-cell fusion. Since myogenin expression is driven by MyoD, the best described MRF, we focused our attention on potential interferences on its activity by GILZ and L-GILZ. We found that GILZ and LGILZ co-localized with MyoD during differentiation terminal stages: moreover, they interacted with MyoD and inhibited its transcriptional activity. Notably, abrogation of spontaneous GILZ and L-GILZ expression *via* gene silencing enhanced terminal differentiation with augmented myoblast recruitment into myotubes. Finally, we observed that both GILZ and L-GILZ are induced by GCs: GC treatment resulted in reduction of myotube formation and GILZ and L-GILZ knock-down *via* RNA interference dampened GC effects. Overall, our data indicate that GILZ and L-GILZ take part to the myogenic fine tuning *via* the direct inhibition of MyoD transcriptional activity and that they mediate antimyogenic effects of GCs.

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ROSIGLITAZONE INHIBITS THE INFLAMMATORY RESPONSE IN A MODEL OF VASCULAR INJURY IN RATS

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Rosiglitazone, a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, used clinically for its insulin-sensitizing activity, has been shown to exert antiatherogenic effects (1). Moreover, rosiglitazone regulates the transcription of numerous target genes that are involved in inflammation (2), thereby modulating the production of inflammatory mediators and inhibiting hyperplasia and restenosis after balloon-injury in rats. The aim of this study was to investigate the anti-inflammatory activity of rosiglitazone in our rat experimental model of carotid surgical injury (3), specifically examining the expression and the activity of some inflammatory markers such as nuclear factor-κB (NF-κB), cyclooxygenase 2 (COX-2) and phosphorylated p38 MAP kinase (pp38 MAPK). We also evaluated the effect of rosiglitazone on platelet aggregation, neointima formation and on the infiltration of the inflammatory cells. Rats were randomized in three groups: the rosiglitazone group (R) treated with rosiglitazone (10 mg/kg/day), the control group (C) treated with vehicle (0.9% w/v NaCl, pH 3) and the sham-operated group (SHAM). Drug or vehicle was administered by gavage for 7 days before carotid injury and up to 21 days after injury. Rats (5 for each time point) from C and R groups were killed at different times (0, 1, 2, 4, 48 h; 7, 14, 21 days) after surgical injury; uninjured and injured carotid arteries were removed and processed. Blood samples from all groups were drawn from the abdominal aorta and platelet aggregation was measured by aggregometer. The influx and behaviour of cells in response to injury by electron microscopy and immunohistochemistry were recorded. Vascular injury rapidly activated p38 MAPK (1 h); pp38 MAPK peaked at 48 h and slowly decreased thereafter. Rosiglitazone significantly inhibited p38 MAPK activation throughout the time-course of the experiment. Rosiglitazone also significantly reduced COX-2 expression 7 days after injury but not during the early inflammatory events following injury (4 h and 48 h) and it caused an early (2 h) and significant decrease of NF-κB/DNA binding activity in injured and uninjured carotids at all time points examined. Rosiglitazone treatment reduced significantly platelet aggregation at all time points as compared to the C group. In the C group, there was a well developed neointima in carotids 7 days after injury; the neointima became progressively thicker up to 21 days. Rosiglitazome treatment significantly reduced the thickness of neointima from 7 days after injury and until the end of experiment and the number of dendritic cells and the interactions of these cells with lymphocytes. Our results showed that a chronic treatment with rosiglitazone reduces significantly the inflammatory events induced by surgical injury of rat carotid by inhibiting activation and/or expression of the inflammatory markers p38 MAPK, NF-κB,and COX-2, platelet number and aggregation, and vascular remodelling (reduction of neointima formation, immune cell infiltration and recruitment and differentiation of repair cells).

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TRANSGENERATIONAL EFFECT OF SOCIAL ISOLATION ON NEUROSTEROIDS AND SENSITIVITY TO STRESS

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Social isolation of male rats both reduces the cerebrocortical and plasma concentrations of allopregnanolone and potentiates the effects of acute stress on the concentrations of this neuroactive steroid (1); furthermore, the steroidogenic effect of acute administration of ethanol was more pronounced in socially isolated than in group-housed rats (2). Moreover, isolated male rats showed an anxiety-like behaviour in the elevated plus-maze and Vogel conflict tests. At variance, female isolated rats challenged with acute foot shock stress or ethanol showed increases in brain and plasma levels of allopregnanolone similar to that induced in female group-housed animals. The effect of acute stress and ethanol on neuroactive steroid levels was evaluated in isolated and group-housed offspring; we also evaluated HPA axis function after dexamethasone challenge and the anxiety-like behaviour. For this purpose, isolated male and female were bred and male offspring were stabulated, at weaning, in groups of 8 rats/cage as group-housed offspring. Similarly to their parents, two month-old offspring of isolated rats showed a significant reduction in the brain and plasma concentration of allopregnanolone. On the contrary, the increase in brain and plasma concentration of allopregnanolone induced by foot shock stress or i.p. injection of ethanol was blunted in isolated rats offspring compared to the offspring of group-housed animals. In agreement, corticosterone levels in response to i.p. injection of dexamethasone were significantly lower in the offspring of isolated animals than in group-housed offspring. These results suggest a transmission across generation of changes in the neuroendocrine pattern caused by environmental chronic stress. Studies are in progress in order to establish whether effects observed in offspring are attributable to the inheritance by offspring of genomic information from parents or to alteration in the pattern of maternal care induced by social isolation.

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MACROMOLECULAR COMPLEXES IN ALZHEIMER DISEASE PATHOGENESIS

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Alzheimer's disease (AD) is a chronic neurodegenerative disease caused by a combination of events impairing normal neuronal function. Recently, it has been reported that synapseassociated protein-97 (SAP97), a protein involved in dynamic trafficking of proteins to the excitatory synapse, can drive ADAM10 (a disintegrin and metalloproteinase 10, the most accredited candidate for α-secretase) to the post synaptic membrane by direct interaction. This interaction can be disrupted by a cell-permeable peptide, Tat-Pro ADAM709-729, which mimics the proline-rich region of ADAM10, responsible for its association to SAP97. Here we administered this peptide *in vivo* in mice for 14 days, in order to avoid ADAM10 trafficking towards the membrane. The disruption of SAP97/ADAM10 interaction prevents ADAM10-mediated non-amiloidogenic cleavage of amyloid precursor protein (APP) and reduces the release of soluble APPα, one of the products of ADAM10 cleavage. Since AD is characterized by synaptic dysfunction, we investigated biochemically the effects of the reduction of ADAM10 activity for 14 days on the glutammatergic synapse's proteic composition. A specific decrease of NR2A subunit of NMDA receptor, but not of NR2B or GluR1 subunit of metabotropic glutammate receptor, can be showed after treatment. The autophosphorylation of Ca^{2+}-Calmodulin Kinase II, the most abundant kinase of the central nervous system, on the threonin residue in position 286 is also reduced by the treatment with Tat-Pro ADAM709-729. This decrease in APP physiological metabolism leads to an impairment of LTP induction. To strengthen these findings, we checked the reduction of the immunoprecipitation of SAP97 and ADAM10 *ex vivo* in hippocampus and frontal gyrus of AD patients and control subjects. The direct correspondence between the disruption of this complex in animals and humans highlights the importance of our results for a better understanding of AD pathogenesis.

ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE SIGNALLING PATHWAYS IN THE DEVELOPMENT OF SPINAL CORD INJURY

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Spinal cord injury (SCI) is a highly debilitating pathology. Although innovative medical care has improve patient outcome, advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration have been limited. The complex pathophysiology of SCI may explain the difficulty in finding a suitable therapy. Mitogenactivated protein kinase (MAPK) signalling pathways involve two closely related MAPKs, known as extracellular signal-regulated kinase 1 (ERK1) and ERK2. *In vivo* evidence of strategies directed to block the initiation of this cascade linking MAPK activation in posttraumatic pathophysiological process of SCI is not fully evaluated. The aim of the present study was to evaluate the contribution of MAPK3/MAPK1 in the secondary damage in experimental SCI in mice. To this purpose, we used 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), which is an inhibitor of MAPK3/MAPK1. Spinal cord trauma was induced by the application of vascular clips (force of 24 g) to the *dura via* a four-level T5-T8 laminectomy. SCI in mice resulted in severe trauma characterized by edema, neutrophil infiltration, and production of inflammatory mediators, tissue damage, and apoptosis. PD98059 treatment (10 mg/kg i.p.) at 1 h and 6 h after the SCI significantly reduced: 1) the degree of spinal cord inflammation and tissue injury (histological score); 2) neutrophil infiltration (myeloperoxidase activity): 3) nitrotyrosine formation: 4) proinflammatory cytokines expression: 5) nuclear factor-κB activation; 6) phospho-ERK1/2 expression; 6) apoptosis (terminal deoxynucleotidyl transferase dUTP nick-end labelling staining, Fas ligand, Bax, and Bcl-2 expression). Moreover, PD98059 significantly ameliorated the recovery of limb function (evaluated by motor recovery score) in a dosedependent manner. The results of the present study enhance our understanding of the role of MAPK3/MAPK1 pathway in the pathophysiology of spinal cord cell and tissue injury following trauma, implying that inhibitors of the activity of MAPK3/MAPK1 pathway may be useful in the therapy of spinal cord injury, trauma, and inflammation.

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EFFECT OF DE-ALCOHOLATED RED WINE IN ISOLATED RAT HEART: PROTECTIVE ACTION IN MYOCARDIAL ISCHEMIAREPERFUSION INJURY

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Epidemiological studies suggest that a moderate consumption of wine, particularly of red wine, reduces the incidence of developing coronary heart disease. This beneficial effect is mainly attributed to de-alcoholated extract, such as the occurrence of polyphenol compounds in red wine (1). Matrix metalloproteinases (MMPs) are a family of zincdependent endopeptidases that carry important roles in various pathophysiological processes. MMP-2 recently emerged as a key protein implicated in several cardiomyopathies, including myocardial infarction, heart failure and ischemia-reperfusion (I/R) injury (2). The aim of the present study was to investigate the effects of dealcoholated red wine (DRW) in isolated rat hearts, under control conditions as well as subjected to I/R injury. A possible interaction with MMP-2 activation has also been taken into account. The cardiac functions: left ventricular pressure (LVP), coronary perfusion pressure (CPP), coronary flow (CF), and surface electrocardiogram (ECG) of isolated rat hearts, perfused according to the Langendorff method, were monitored over 75 min of aerobic perfusion (control) or 25 min of aerobic perfusion followed by 20 min of global, no-flow ischemia and 30 min reperfusion (I/R). Lyophilized red wine, expressed as µg/ml of gallic acid equivalents, was resuspended in distilled water and infused during aerobic perfusion or for 10 min before and after ischemia. The level of MMP-2 in the coronary effluent and heart homogenates was determined by gelatin zymography and immunoblot analysis. DRW caused a concentration-dependend relaxation of coronary arteries (maximum decrease at 2.8 µg/ml DRW was 20% of control values; CPP=54.2±3.2 mmHg, control; 44.3±3.2 mmHg, 2.8 µg/ml RWPs, n=8) and elicited a negative inotropic activity. Both effects showed an “U” shape profile. N(ω)-nitro-L-arginine methyl ester (L-NAME 300 µM), a competitive inhibitor of endothelial NO synthase (eNOS), and wortmannin (100 nM), an irreversible inhibitor of phosphatidylinositol 3 (PI3)-kinase, prevented coronary dilation and the negative inotropic effect induced by DRW. None of the ECG parameters changed appreciably, when hearts were challenged with DRW up to the concentration of 28 µg/ml. On the contrary, second degree atrioventricular block occurred in the presence of 56 µg/ml DRW. Hearts subjected to I/R injury showed a significant impairment in the recovery of mechanical function that was partially reversed by 2.8 µg/ml DRW. In the coronary effluent, 10 µg/ml DRW counteracted the I/R-induced augmentation of 72 kDa MMP-2 activity released in the first min of reperfusion. Ten µg/ml of DRW also prevented the MMP-2 loss and troponin I degradation observed in homogenates of hearts subjected to I/R. In conclusion, components of DRW, at concentrations reached in human blood after intake of 100 ml of red wine (3), probably induce the redox-sensitive activation of the PI3-kinase/Akt pathway in coronary endothelial cells. This, in turn, causes phosphorylation of eNOS, resulting in NO formation and vasodilation. Furthermore DRW improve the recovery of the mechanical function and counteracts the activation and release of MMP-2 that occurs during myocardial I/R injury. The precise mechanism responsible for the interaction between DRW and MMP-2 however warrants further investigation. Finally, these results might explain, at least in part, the molecular mechanisms responsible for the prevention of cardiovascular diseases associated with a moderate consumption of red wine.

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INFLUENCE OF A CHRONIC TREATMENT WITH UFP-101, A POTENT NOP RECEPTOR ANTAGONIST ON BEHAVIOURAL AND CELLULAR EFFECTS AFTER EXPOSURE TO CHRONIC MILD STRESS IN THE RAT

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Nociceptin/orphanin (N/OFQ) and its receptor (NOP) constitute a peptide-receptor system that modulates several central and peripheral functions (1). The highly selective peptide NOP antagonist [Nphe$_1$, Arg$_{14}$, Lys$_{15}$]N/OFQ-NH2 (UFP-101) has proved to produce antidepressant-like effects in rodents that are prevented by the co-injection of N/OFQ (2). The chronic mild stress (CMS) paradigm appears suitable for the experimental investigation of depressive-like aspects, for predictive, face, and constructs validity (3). The aim of the present study was: 1) to confirm our previous results on the CMS impact regarding sucrose intake behavioural parameter in the rat; 2) to investigate the possible effects of a continuous treatment with UFP-101 on the CMS-induced anhedonia evaluating behavioural parameters [sucrose consumption, “despair” in forced swimming test (FST) and locomotion] and immunohystochemical parameters (cell proliferation by means of single series of 4 BrdU injections (50 mg/kg i.p.) 24 h before the rat’s sacrifice). Male Wistar rats were exposed to CMS for of at least 7 weeks to induce a condition of anhedonia (measured as reduced consumption of a sucrose solution). The UFP-101 effects were compared to those of fluoxetine (FL) used as a standard reference antidepressant drug. An additional group of rats was co-administered with N/OFQ for 10 days after UFP-101 treatment and submitted to the same tests and analyses. UFP-101 (10 nmol/rat, i.c.v. continuously infused by means of minipumps for 24 days) did not influence sucrose intake in non stressed animals, but reinstated the basal sucrose consumption in stressed animals (+40% vs non treated stressed rats), beginning from the second week of treatment as did FL (10 mg/kg, i.p.). Similarly, in the FST performed 2, 9, 16, and 23 days after treatment, UFP-101 reverted the effects of stress in a dose- and time-dependent manner, reducing the time of immobility of stressed rats to that of non stressed controls (-78% vs non treated stressed rats); FL produced the same effect from day 9 onwards. Repeated coadministration of N/OFQ (5 nmol/rat i.c.v., from day 15 to 24) completely prevented the behavioural effects of UFP-101. On day 24 rats were sacrificed, and brains and blood were collected for biochemical evaluations. The present results confirmed our previous data showing that UFP-101 reverses also the biochemical effects of CMS, such as increase of serum corticosterone levels, 5-HIAA/5-HT ratio alteration in cerebral cortex and pons. Moreover, we have previously observed that the CMS procedure did not reduce brainderived neurotrophic factor (BDNF) mRNA or protein in hippocampus (through RNAse protection assay) in comparison to non-stressed animals. Experiments are now in progress to determine the effects of the CMS model and of the UFP-101 treatment on hippocampal proliferation, on neurogenesis, and on the signaling of BDNF and FGF-2 in brain by means of histochemical techniques. Preliminary results indicate that CMS reduced proliferation of cells in the adult rat hippocampus. The UFP-101 treatment did not produce any primary effect on cell proliferation and did not affect the reduced proliferation observed in stressed animals. The mentioned findings support the view that blockade of the NOP receptor signalling in the brain produces antidepressant-like effects in the CMS test, and indicate that the effect of the NOP receptor antagonist UFP-101 is as rapid as that of classical antidepressants. This effect does not appear to be mediated by increased hippocampal cell proliferation. Finally, the N/OFQ reversion of all effects induced by UFP-101 suggests a mechanism that acts at the receptor level. These findings provide further support to the hypothesis that NOP receptor may represent a candidate target for innovative antidepressant drugs.

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METRONOMIC IRINOTECAN IN COLORECTAL CANCER

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The aim of the present study was to rationally develop CPT-11 metronomic chemotherapy (1) regimens in pre-clinical and clinical settings of colon cancer treatments, investigating the related pharmacokinetic and pharmacodynamic parameters. *In vitro* cell proliferation, apoptosis and thrombospondin-1/vascular endothelial growth factor (TSP-1/VEGF) expression analyses were performed on both endothelial and colorectal cancer cells exposed to low concentrations (metronomic) of SN-38, the active metabolite, for 144 h (2). The HT-29 human colorectal cancer xenograft model was used and tumour growth, microvessel density, VEGF, and TSP-1 quantification was performed in tumours of mice treated with various metronomic schedules. Based on the preclinical data, 20 consecutive patients with a diagnosis of irinotecan-resistant metastatic colorectal carcinoma were treated with continuous infusion of CPT-11 at three dose levels (1.4, 2.8, and 4.2 mg/m_2/day). A pharmacokinetic analysis of CPT-11 and its metabolites was performed; whereas ELISAs and real time RT-PCR were used for plasma VEGF/TSP-1 and gene expression in peripheral blood mononuclear cells. SN-38 preferentially inhibited endothelial cell proliferation and induced apoptosis, increasing the expression and secretion of TSP-1. Metronomic CPT-11 significantly inhibits HT-29 tumour growth in the absence of toxicity, which was accompanied by decreases in the microvessel density and increases in TSP-1 gene expression in HT-29 tumour tissues. Four patients (20%) obtained a stabilisation of disease that lasted a median period of 3.9 months; toxicities >grade 1 were not observed. Pharmacokinetic analysis demonstrated that the Css of SN-38 were 1.00±0.52, 2.29±0.87, and 3.33±0.96 ng/ml, respectively for the three doses and statistically different ($P<0.05$). Higher TSP-1 plasma levels were found during the metronomic CPT-11 infusion when compared to the baseline values as well as to the increased gene expression in the PBMC compartment, especially at the lower CPT-11 doses. Metronomic irinotecan, at the doses used, is a non-toxic and clinically feasible therapy with potential, antiangiogenic and antitumor activity in colorectal cancer.

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LITHIUM CLORIDE REDUCES AMYLOID BETA PEPTIDE (Aβ) LOAD AND AMELIORATES LEARNING AND MEMORY IN TGCRND8 MICE

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Glycogen syntase kinase-3β (GSK-3β) has been postulated to mediate Alzheimer's disease (AD) tau hyperphosphorilation and amyloid β peptide (Aβ)-induced neurotoxicity, so a drug able to reduce its activity might have great therapeutic relevance. LiCl is a widely used drug for bipolar disorder and, among its various functions, captured our interest for its characteristic to inhibit GSK-3β. Here, we investigated the effects of chronic LiCl administration in TgCRND8 mice that express the human APP695, harbouring the Swedish (KM670/671NL) and the Indiana (V717F) familial AD mutations, and exhibit amyloid deposition and cognitive deficits since 3 months of age. LiCl was administered at two groups of mice of two different ages. The first group is constituted by TgCRND8 (tg; n=16) and wild type (wt; n=16) mice of 2 months of age and represents the early/pre-symptomatic stage of AD. The second group instead represents the late/symptomatic stage of AD and was constituted by 6-month-old tg (n=16) mice and age-matched control (n=16) mice. All the animals were daily i.p. injected with 0.6 M LiCl (10 μl/g of b.w.) or sterile NaCl (10 μl/g of b.w.) for a period of 5 weeks. No evidence of adverse effects and changes in b.w. was noticed in mice during the treatment. At first, investigation on lithium actions was conducted on the pre-symptomatic age group of mice. In this group no difference was found between steady-state levels of total GSK-3β in treated and untreated mice. Conversely in lithium treated tg mice inactive phospho-GSK-3β (Ser_9) levels was found significantly increased both in the cortex and hippocampus and the active phospho-GSK-3α (Tyr_{279}) and phospho-GSK-3β(Tyr_{216}) immunoreactivity was found decreased compared to vehicle treated mice, confirming inhibition of GSK-3 activity by lithium. In addiction, a significant increase of phospho-Akt (Ser_{473}) levels was found in the brain of LiCl treated tg mice compared to saline treated tg mice, demonstrating that GSK inhibition by lithium is linked to Akt activation. To evaluate whether this treatment have effects on Aβ load, immunohistochemical analysis was performed with an antibody against Aβ(1-42) and quantification of Aβ deposits revealed that LiCl treatment significantly reduced Aβ plaque burden both in the motor cortex (-33.3% for plaque number, $P<0.05$, and -48% for maximum plaque area, $P<0.02$; unpaired Student's *t*-test) and in the hippocampus (-56% for maximum plaque area, $P<0.001$; unpaired Student's *t*-test) of TgCRND8 mice, compared to vehicle-treated tg mice. Moreover, LiCl treatment significantly improved working memory performances in the "step down" inhibitory avoidance test ($P<0.001$, two-way-ANOVA+Bonferroni's post-test) and ameliorated visual and spatial learning abilities in the Morris water maze task ($P<0.01$, one-way-ANOVA+Bonferroni's post-test). Effects of lithium treatment on the group of elderly TgCRND8 mice are still under investigation.

Cl-IB-MECA ENHANCES TRAIL-INDUCED APOPTOSIS IN ANAPLASTIC CANCER CELLS

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TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) is a member of the tumour necrosis factor family of cytokines that can trigger apoptosis by its cell-membrane death receptors TRAIL-R1 (DR4) and TRAIL-R2 (DR5) (1) in most cancer cells. The thyroid cancer cell lines are differently sensitive to TRAIL-induced apoptosis (1,2). Particularly, follicular thyroid carcinoma cells and medullary thyroid carcinoma cells are resistant to TRAIL-induced apoptosis. Recent reports have demonstrated that resistance among some thyroid cancer cell lines can be reversed by a protein synthesis inhibitor, cycloheximide (1), suggesting a role for (a) short-lived apoptosis inhibitor(s). Several studies showed that Cl-IB-MECA and other analogues, known as potent and selective agonists of adenosine receptor A_3 subtype (A_3AR), induce an inhibitory effect on tumour cell growth. The effects of Cl-IB-MECA in cancer cells are concentration- and cell type-dependent. In our previous report, we have identified the A_3AR expression in thyroid carcinomas and demonstrated that Cl-IB-MECA inhibits thyroid papillary carcinoma cells (NPA) (3). In this study we propose to evaluate thyroid anaplastic carcinoma susceptibility to TRAIL/Apo2L in presence of Cl-IB-MECA and the possible mechanism affecting the downstream signalling of apoptosis induced by TRAIL/Apo2L. The aim of this study was to evaluate whether Cl-IB-MECA is able to modulate the apoptotic activity of TRAIL/Apo2L in anaplastic thyroid cancer cell lines which are differently sensitive to TRAIL/Apo2L, and thus to investigate the possible molecular mechanism underlying the effects of Cl-IB-MECA in combination with TRAIL/Apo2L. FRO and ARO cells were co-treated with both reagents for 24 h. Flow cytometry analysis showed that Cl-IB-MECA enhanced TRAIL-induced cell death in FRO cells, whereas in ARO cells it was not able to enhance apoptosis. To confirm these data, caspase-3 and caspase-9 activation was assessed. As expected, cleaved forms of caspase-3 (p21 and p17) and caspase-9 were increased in FRO cells after Cl-IB-MECA and TRAIL stimulation. Combined treatment with Cl-IB-MECA and TRAIL induced also proteolytic cleavage of caspase substrate PARP. To determine whether the TRAIL-sensitizing effects of Cl-IB-MECA were mediated by A_3AR activation, MRS1191 (0.5 μM) or FA385 (1 μM) were used as A_3AR antagonists and flow cytometry analysis was performed. The latter did not have any effects on the synergism of Cl-IB-MECA in TRAIL-induced apoptosis in FRO cells. These finding show that the TRAIL-sensitizing actions of Cl-IB-MECA are A_3AR -independent. Cl-IB-MECA in combination with TRAIL (0.1 ng/ml) reduced significantly Bcl-2, Bcl-XL, XIAP, and cFLIP proteins expression, suggesting that the increased apoptosis rate in FRO cells was partially mediated by reduction of these antiapoptotic proteins. We have also investigated DR5 protein expression in both cell lines after Cl-IB-MECA and/or TRAIL treatment. The latter, in our experimental conditions, increased only in FRO cells. These data suggest that pharmacological modulation of apoptosis induced by TRAIL/Apo2L may offer a new therapeutic strategy in human malignancies.

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RAT INTESTINAL PRECISION-CUT SLICES AS TOOL TO STUDY XENOBIOTIC INTERACTIONS WITH TRANSPORT PROTEINS

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Intestinal metabolism (phase I and/or phase II) and drug transporters (phase III) have been recognized as major physiological mechanisms that protect organisms from toxic compounds. Several phase III proteins commonly known as ATP-binding cassette (ABC) play key roles in tissue maintenance by transporting metabolic waste and toxic chemicals out of cells (1). To study xenobiotic interactions with ABC proteins like multidrugresistance (MDR) protein and multidrug-resistance associated protein (MRP) transporters, intact cell systems are required. The aim of the present study was to set up an intestinal precision-cut slice technique to study the interaction of ATP-dependent transporters with xenobiotics. Slices were prepared as described by De Kanter et al. (2). Slices were individually incubated in RPMI 1640 under 95% O_2 5% CO_2 atmosphere at 37°C in 12 well plates in presence of 0.5 μM calceinAM and various concentrations of the well known MDR or MRP inhibitors verapamil, indomethacin, and glibenclamide. The intracellular deesterification product of calceinAM, calcein, was measured spectrofluorimetrically. The presence of transport inhibitors increased the intracellular concentration of calcein in a time-dependent fashion and the optimum time of incubation was at 30 min. Furthermore verapamil, indomethacin, and glibenclamide promoted a concentration-dependent accumulation of calcein (EC_{50} 3.28 μM, 14.5 mM, and 190 μM, respectively). This data suggest that the precision-cut intestinal slices are a reliable, simple, and fast system for evaluating xenobiotic interactions with ABC transporters. Together with data already appeared in the literature (3), these results indicate that the model is also suitable for studying phase I and phase II drug metabolism. We suggest that precision-cut slices may be used to check phase I, II, and III reactions, all involved in intestinal detoxifying mechanism.

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POSSIBLE INVOLVEMENT OF α-SYNUCLEIN IN THE PATHOGENESIS OF PARKINSON'S DISEASE

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Parkinson's disease (PD) is a progressive and complex neurodegenerative disorder with a prevalence of 30-90/100,000 in North America and Europe. It is characterized by severe motor symptoms such as tremor, bradykinesia, and muscular rigidity in addition to various signs of cognitive impairment. The etiopathogenesis causes of PD have not been defined; it may be caused by environmental factor, genetic factor (15-20% of PD patients) or combination of both. The presence of cytoplasmatic filamentous inclusions, known as Lewys bodies (LBs), is a common feature at the cellular level. Recent studies have shown that the main molecular component of LBs is a small (19 KDa, 140 amino acids) and abundant synaptic protein named α-synuclein (α-syn), which interacts with a variety of proteins, including those involved in regulating the vesicular release of dopamine. Two missense mutations (A30P and A53T) in the gene for α-syn, located on chromosome 4, cause familiar PD. Furthermore increasing evidences suggest that abnormal metabolism of α-syn in dopaminergic neurons could play a role in the pathogenesis of familiar as well as sporadic PD. However it is not clear whether neurodegeneration in PD, as well as in other neurodegenerative syndromes characterized by LBs-like formations, is mediated by toxic effects of α-syn aggregates or is the consequence of the free native α-syn decrease and its related, but still unknown, physiological functions. The complexity of the mechanisms underlying α-syn-induced neurotoxicity has made difficult the development of animal models that faithfully reproduce human PD pathology. To elucidate the possible role of α-syn in neurodegenerative processes, in this study, we used a 6-hydroxydopamine- (6-OHDA)-induced rodent model of PD and an antisense oligonucleotide approach. In particular the α-syn levels in *substantia nigra* (SN) and *striatum* of rats were evaluated with unilateral lesion either in SN or *striatum* or nigrostriatal bundles through Western blot analysis. Behavioural studies to evaluate the effects either of antisense oligonucleotide for α-syn or 6-OHDA lesion were performed. Our data show an important alteration of α-syn levels in nigrostriatal pathway, suggesting a possible function of this protein in the pathogenesis of PD.

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

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Irritable bowel syndrome (IBS) affects approximately 15-20% of general population, causes abdominal pain, discomfort, altered bowel habits, and it is more common in women. Serotonin (5-HT) is a key mediator of intestinal peristalsis and visceral sensation. After its release, it is effectively removed from the biophase by 5-HT transporter (SERT). Gene expression of SERT is modulated by a promoter polymorphism (44-base pair insertion/deletion; 5HTTLPR), which gives rise to long (L) and short (S) alleles. The S allele is associated with a decreased SERT expression, leading to reduced efficiency of cellular 5-HT reuptake, a condition which seems to affect the response of psychiatric disorders to drugs acting as SERT blockers. Since SERT inhibitors have been proposed for treatment of IBS, this study has been designed to evaluate possible associations of 5HTTLPR polymorphism with different clinical forms and symptom severity in IBS. IBS patients were selected according to Rome II criteria, and grouped into diarrhoea predominant (D-IBS), constipation predominant (C-IBS), and alternating IBS (A-IBS). Symptom severity was estimated by Francis-Whorwell score (1). Healthy volunteers were also enrolled. Genomic DNA was extracted from whole blood or saliva, and the SERT gene promoter region containing 5HTTLPR polymorphism was amplified by polymerase chain reaction. One hundred seventy nine IBS patients (41 males, 138 females; mean age 38.9 years; age range 18-75 years) and 177 healthy volunteers (47 males, 130 females; mean age 41.3 years; age range 19-84 years) were genotyped. All subjects were Italians of Caucasian origin. Frequencies of 5HTTLPR genotypes in IBS patients (L/L 31.8%, L/S 53.6%, S/S 14.5%) did not differ significantly from healthy volunteers (L/L 29.9%, L/S 49.7%, S/S 20.3%; Chi-square test: P=0.351). When stratifying patients by clinical variants, the genotype distribution was: D-IBS (n=64), L/L 32.8%, L/S 50.0%, S/S 17.2%; C-IBS (n=64), L/L 39.1%, L/S 50.0%, S/S 10.9%; A-IBS (n=51), L/L 21.6%, L/S 62.7%, S/S 15.7%. Comparison of genotype frequencies in bowel habit subgroups vs healthy volunteers did not indicate a significant difference in D-IBS, C-IBS or A-IBS patients. However, there was a trend towards a lower prevalence of S/S genotype in C-IBS patients (10.9% vs 20.3%). Mean symptom severity score values in IBS patients with L/L (282.7±76.8), L/S (290.3±77.6) and S/S (303.6±92.5) genotypes did not differ significantly (ANOVA: F=0.620, P=0.539). Mean symptom severity score values in patients with D-IBS (271.9±84.9), C-IBS (312.1±73.1), and A-IBS (284.4±74.9) differed significantly (ANOVA: F=4.405, P=0.014). Current evidence about a possible involvement of 5HTTLPR polymorphism in the pathophysiology and/or clinical presentation of IBS is conflicting. The results obtained in our Italian cohort of IBS patients do not suggest an association between 5HTTLPR polymorphism and IBS or its clinical variants. In addition, no relationship appears to exist between 5HTTLPR genotypes and symptom severity in IBS patients.

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DNA DAMAGE IN UVB-IRRADIATED HUMAN KERATINOCYTES IS REDUCED BY THE MAIN CONSTITUENTS OF LIQUORICE ROOT

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UV radiation, in particular its UVB component, is an important environmental factor in the pathogenesis of skin aging and cancer. The harmful effects of UV radiation are always associated with generation of reactive oxygen species (ROS). Many natural compounds prevent the occurrence and reduce the severity of UV-induced photoaging and diseases of the skin (1). In the last years there are increasingly reports in the international literature of naturally occurring triterpene saponins being used successfully in *in vitro* tests or as antiproliferative or anti-tumour agents. In particular, many studies show that the main constituents of liquorice root act both as antimutagens and as inhibitors of the onset and progression of some murine tumours (2,3). The aim of our study was to investigate the potency of the main constituents of liquorice root, glycyrrhizin (GL) 18β-glycyrrhetinic acid (18β-GA) and glabridin, on UVB irradiated human keratinocytes. In particular we examined in cultured epidermal cells the effects of UVB doses at 50 mJ/cm$_2$ and 75 mJ/cm$_2$. MTT test and [$_3$H]-thymidine incorporation were performed as a test of the proliferation rate, whereas 2'7'-dichlorodihydrofluorescein diacetate (DCF) assay was employed to determine formation of intracellular ROS. Keratinocyte apoptosis plays a critical role in regulating epidermal development and restraining carcinogenesis. Keratinocyte apoptosis may be triggered by UVB (4). Here, we show that pre-treatment of human keratinocytes with 18β-GA and glabridin inhibited UVB mediated apoptosis through the activation of p53, down-regulation of bcl-2 and inhibition of PARP cleavage. On the contrary, GL is not involved in UVB apoptotic inhibition. Further studies must be performed to understand the mechanism of the protective effect.

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MENOPAUSAL TRANSITION: A POSSIBLE RISK FACTOR FOR NEURODEGENERATIVE EVENTS

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Incidence and prevalence of Alzheimer's disease (AD) is higher in postmenopausal women than in aged matched men. Since at menopause the endocrine system and other biological paradigms undergo substantial changes, we have evaluated whether (and how) the balance between some biological parameters allegedly neuroprotective and others pro-neurotoxic varies during lifespan in either normalcy or neurodegenerative disorders. We evaluated the leukocyte expression of estrogen receptors (ERs), glucocorticoid receptors (hGRs), interleukin-6 (IL-6) and CD36 (a class B scavenger receptor, playing a key role in the proinflammatory events associated with AD), and the plasma levels of estrogens, cortisol and dehydroepiandrosterone sulfate (DHEA-S), in a wide population of healthy subjects and AD patients of either sex. Studies were performed in peripheral leukocytes, since these cells: 1) are easily obtainable by a simple blood sampling; 2) express many molecules and multiple receptors which are under the same regulatory mechanisms as those operative in the brain; 3) some of them, e.g. monocytes, share many functions with microglial cells. Moreover, we analyzed the cerebral expression of CD36 in triple transgenic mice, a valid model of AD. In healthy men all the study parameters were quite stable during lifespan. In women, instead, at menopausal transition, some changes that may predispose to neurodegeneration occurred. In particular, there was: 1) an up-regulation of ERs and a concomitant increase of IL-6 gene expression, events likely due to the loss of the inhibitory control exerted by estradiol [E(2)]; 2) an increase of hGR-α/hGR-β ratio, indicative of an augmented cortisol activity on hGRα not sufficiently counteracted by the inhibitory hGRβ function; 3) a reduced CD36 expression, directly related to the increased cortisol activity; 4) an augmented plasma cortisol/DHEA-S ratio, widely recognized as an unfavourable prognostic index for the risk of neurodegeneration. In AD patients of both sexes, the expression of the study parameters was similar to that found in sex- and age-matched healthy subjects, thus indicating their unrelatedness to the disease, and rather a better correlation with biological events. According to our results, menopausal transition is a critical phase of women's life where the occurrence of an unfavourable biological milieu would predispose to an increased risk of neurodegeneration. Collectively, the higher prevalence of AD in the female population would depend, at least in part, on the presence of biological risk factors whose contribution to the development of the disease occurs only in the presence of possible age-dependent triggers.

TOXICITY ASSESSMENT OF *FUSARIUM* TOXINS IN "PRECISION CUT SLICES" OF RAT LIVER AND INTESTINE

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Fusarium species infestations of cereal crops occur worldwide. *Fusarium* toxins such as deoxynivalenol (DON), zearalenone (ZEN), and fumonisin B1 (FB1) have been shown to cause diverse toxic effects in animals and are also suspected of disease causation in humans. *Fusarium* toxins also have different effects on cells: protein synthesis inhibition, lipid peroxidation induction, and sphingolipid metabolism inhibition. The aim of the present study was to assess the toxic effect of ZEN on liver and small intestine precisioncut slices (1). Precision-cut rat liver slices of 250 µm of thickness were prepared from fresh tissue core (8 mm) using a Krumdieck tissue slicer filled with oxygenated, ice-cold Krebs-Henseleit buffer (2). To prepare agarose-filled slices of 400 µm of thickness, the ileum was cut into 10 cm parts subsequently legated on one side, filled with 3% (w/v) low melting agarose solution in 0.9% (w/v) NaCl at 37°C, and were allowed to gel in 4°C ice-cold Krebs-Henseleit buffer. The cylinders obtained were used to prepare precision-cut slices using a Krumdieck tissue slicer filled with oxygenated, ice-cold Krebs-Henseleit buffer (3). After a pre-incubation period of 30 min, slices were incubated in RPMI1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 wellplates with continuous gentle shaking. Liver slices viability was checked measuring LDH release. MTT test was used to assess small intestine slices viability based on the capacity of viable cells to metabolise a tetrazolium colourless salt to blue formazan in mitochondria (1). Finally we performed experiments incubating liver and small intestine slices with RPMI1640, red wine (1:10) and *Fusarium* toxins (ZEN 40, 120, and 240 µM) to investigate the possible protective effects by red wine flavonoids. In liver slices incubated with 240 µM ZEN we observed an increase in lipid peroxidation, statistically significant after 24 h of incubation. MTT assay results showed that red wine protected against 240 µM ZENinduced damage in small intestine slices with a statistically significant difference after 2, 4, and 24 h of incubation.

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IDENTIFICATION OF AMINOACIDS INVOLVED IN ACTIVATION OF KIDNEY CLC-Ka CHLORIDE CHANNEL

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CLC-K proteins, classified as CLC-Ka and CLC-Kb in humans, and CLC-K1 and CLC-K2 in rodents, are Cl^- channels essential in the mechanism of water diuresis and NaCl reabsorption in the kidney and also for the hearing process. They are implicated in several human genetic diseases, as type III and type IV Bartter syndrome. For their correct expression and function, CLC-K channels require the presence of the barttin β subunit. Pharmacological tools able to modulate the activity of CLC-K channels are interesting in the treatment of several pathological conditions. Specific modulators of CLC-Ka can regulate water diuresis without affecting osmotic diuresis while CLC-Ka openers might be useful to treat patients with Bartter syndrome type III or type IV. In a previous study (1), we identified the binding site for the CLC-Ka blockers, such as 3-phenyl-CPP and flufenamic acid. It was close to N68 and according to the structure of the bacterial homologue CLC-ec1, it lies in the channel pore (2). Now, we focus our attention on the characterization of the activating binding site. In fact, recently, we demonstrated that niflumic acid (NFA) was able to increase CLC-Ka sustained current, suggesting the presence of a hypothetical activating binding site. Particularly, NFA increases CLC-Ka currents at concentrations up to ~1 mM whereas at higher concentrations it has an inhibitory effect suggesting the presence of two distinct binding sites for NFA with different affinity. The occupation of the high affinity site would produce the potentiation of the current whereas binding to both sites would induce block (3). In order to identify the NFA binding site, we performed extensive site-directed mutagenesis on CLC-Ka. Mutations were introduced by recombinant PCR. All constructs were co-expressed with the barttin. RNA was prepared and injected into *Xenopus* oocytes (4). All electrophysiological data were acquired using voltage-clamp and patch-clamp techniques. Among tested mutants, only seven mutations significantly reduced the effect of NFA. Three of these (G167A, F213A, and F426A) drastically altered general gating properties but they did not affect NFA binding. The other four mutations (L151A, L155A, G345S, and A349E) greatly reduced the effect of NFA. Based on the crystal structure, L151 and L155 are located on the extracellular side of helix E, whereas G345 and A349 are positioned in the loop connecting helices K and L. These two regions are expected to be exposed to the extracellular side of the channel, relatively close to each other; for this reason they are good candidates for being part of the potentiating NFA binding site. Together with the drug modelling techniques, the identification of the amino acids involved in the NFA binding site can significantly contribute to the development of therapeutic drugs useful for Bartter syndrome.

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RECOMBINANT SONIC HEDGEHOG PRODUCED IN *E. COLI* WITH MODIFICATIONS THAT RESTRICT ITS BIOLOGICAL ACTIVITY

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Proteins of the Hedgehog (Hh's) family are important signalling molecules during embryonic development of animals. Members of this family have been identified in both vertebrates and invertebrates, and three Hh's, referred to as Sonic, Indian, and Desert, with different tissue distributions, have been identified in rodents and humans. Sonic Hedgehog (SHH), one of three Hh's, is the most intensively studied genes in the development biology, where SHH functions as a morphogen: it acts at a distance from source of signal to establish cell identities in the ventral spinal cord in a concentration-dependent manner. This protein plays a central role in the development of the nervous and skeletal system, where patterning the brain, the spinal cord, craniofacial elements, the axial skeleton, limbs, and digits. SHH, expressed in the embryonic notochord, induces floor plate formation at the ventral midline of the neural tube and promotes the subsequent differentiation of ventral neurons in a region-specific manner, e.g. dopaminergic neurons in the midbrain and motor neurons in the spinal cord (1). SHH signalling pathway is also involved in regulating granule cell precursor (GCP) proliferation in the cerebellum by Purkinje cell, where SHH is produced (2). Natural SHH is synthesized as a long inactive 45 kDa precursor protein and converts itself into an active molecule, through autocatalytic internal cleavage, to yield a 20 kDa N-terminal fragment (SHH-N) that is responsible for all its biological activity. SHH-N remains membrane associated through the addition of two lipids, a palmitic acid at its N terminus and cholesterol at its C terminus. The palmitate addition is catalyzed by putative acyltransferase and the cholesterol moiety of SHH-N is acquired as the SHH precursor undergoes intramolecular cleavage reaction. The palmitoylation of SHH appears to be important to augment SHH activity, while the cholesterol moiety of SHH-N is, probably, required for SHH to move away trough cell membrane, for example to move away from cells close to the zone of polarizing activity (ZPA) for long-distance signalling. Many studies also reveal an essential role for cholesterol moiety in restricting rather than promoting the SHH activity gradient during limb development (3). In order to evaluate the activity of this protein, here we have cloned rat SHH by RT-PCR using rat mRNA as template in a procariotic expression vector under the control of the T7 promoter. The mature domain of rat SHH (23-197) has been mutagenized at its N terminus by adding two additional amino acid Ile Ile in the place of Cys_{23} in order to mimic the presence of palmitic acid, while six His residues and a TAT traslocation sequence was added at C terminus. Histidine residues permits an easy purification of protein using an Imac Columm, while the TAT translocation domain composed by 8 basic amino acids residues, (YGRKKRRQRRR), permits an efficients translocation of the protein inside the cells. In this way we would evaluate our recombinant protein Ile Ile (24-197) SHH His-TAT *in vitro* and *in vivo* studies also compared to recombinant protein, Ile Ile (24-197) SHH His(6), without these modifications. Preliminary data shows that our recombinant protein Ile Ile (24-197) SHH His(6) is biologically active *in vitro* on neural progenitor cells isolated from postnatal cerebellum. Now we started to examined the possible role played by our protein Ile Ile (24-197) SHH His-TAT *in vitro* on muscle satellite cells and *in vivo*, in the ZPA, during limb development, where the spatial restriction of inductive signal plays a crucial role in patterning of vertebrate tissue.

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CLIOQUINOL TREATMENT IMPROVES COGNITIVE DEFICITS AND REDUCES AMYLOID BETA PEPTIDE (Aβ) BURDEN AND NEUROINFLAMMATION IN TGCRND8 MICE

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Several studies indicate the amyloid beta peptide (Aβ) as an important causative agent in Alzheimer's disease (AD), although the common mechanistic links between Aβ and other critical elements of AD are still poorly understood. *In vitro* studies show that Cu_{2+} and Zn_{2+} play an important role in the Aβ aggregation and neurotoxicity. Development of therapeutic agents designed to modulate metal bioavailability, as clioquinol (CQ), has provided promising results in the treatment of AD. In this study, TgCRND8 mice expressing human APP695, harbouring the Swedish (KM670/671NL) and Indiana (V717F) familial AD mutations, were used. This mouse model exhibits deposition of Aβ and robust cognitive deficits by the age of 3 months. Twelve TgCRND8 (tg) and 12 wild type (wt) mice, at 4 months of age, were dosed orally once/day for 5 weeks with CQ (30 mg/kg) or vehicle (CMC 0.05%). No differences in general health and body weight parameters were observed between CQ- and CMC-treated tg and wt animals. MALDI-TOF-TOF analysis of brain coronal sections showed that CQ, 20 min after administration, was mainly distributed within the cortex and the hippocampus. CQ-treated tg showed a statistical significant improvement of learning capabilities in the “step down” inhibitory paradigm (one-way-ANOVA+Bonferroni post test: $P<0.001$) and in the Morris water maze task ($P<0.01$). The disease modifying effect of CQ was associated with a statistically significant reduction in Aβ plaque burden (number, size, and total area) in the frontal (-24%, -38%, and -33%, respectively) and the parietal cortex (-36%, -47%, and -52%, respectively) and in the hippocampus (-43%, -33%, and -56%, respectively), in tg mice as compared to CMCtreated tg mice. Moreover, thioflavine-S staining revealed substantial differences in plaques morphology between CMC- and CQ-treated tg mice in the cortex and in the hippocampus. CQ chronic treatment was also associated with a modest but statistically significant increase in Zn_{2+} and $Fe_{2+/3+}$ levels in the parietal cortex and in Cu_{2+} levels in the hippocampus of tg as compared to CMC-treated wt mice.

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MODULATION OF CANNABINOID SYSTEM IN COGNITIVE DEFICIT AND PSYCHOTIC-LIKE SYMPTOMS INDUCED BY PHENCYCLIDINE

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Recent advances in the neurobiology of cannabinoids have renewed interest in the association between cannabis and schizophrenia. We recently demonstrated that chronic phencyclidine (PCP) treatment, an experimental protocol that reproduces the so called “hypofrontality” observed in patients and induces cognitive deficits (1), altered cannabinoid system in term of CB1 receptor functionality and endocannabinoid levels mainly in the prefrontal cortex, and that chronic co-treatment with THC worsened this picture. Thus, in the present study, we tested the effect of CB1 receptor antagonist on cognitive impairment induced by repeated injections of PCP. Chronic co-treatment with PCP+AM251 significantly improved recognition memory and CB1 receptor efficiency indicating that the CB1 receptor antagonist could be relevant to ameliorate the cognitive aspect of schizophrenia. In parallel, we used an acute PCP injection (3.5 mg/kg) in order to reproduce positive schizophrenic-like symptoms (hyperlocomotion, ataxia, and stereotyped behaviour) and tested the hypothesis that cannabinoid compounds would modulate these symptoms. Rats received an acute administration of the CB1 receptor agonist Δ_9-THC (0.5 mg/kg) and the anandamide uptake inhibitor AM404 (3 mg/kg), respectively, 30 and 60 min before PCP. Both these compounds were able to counteract the PCP-induced psychotic-like symptoms in activity cage, inhibiting the increase of the locomotor activity and ataxia, and reducing the time spent in stereotyped behaviours observed for 40 min. The relative involvement of cannabinoid or vanilloid receptors in the protective effects of AM404 was assessed through the pre-treatment with the CB1 antagonist AM251 (0.5 mg/kg) or TRPV1 antagonist capsazepine (10 mg/kg) 30 min before PCP. AM251 reversed the protective effect of AM404 only on locomotion whereas capsazepine reversed AM404 protective effects on stereotypes and ataxia. This study demonstrated a dual role of CB1 and TRPV1 receptors in modulating positive effect of cannabinoids. Taken together these findings put forward that pharmacological modulation of the cannabinoid system could differently affect positive and cognitive symptoms present in schizophrenia.

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PREGNANCY IMPROVES VASCULAR AND INFLAMMATORY PARAMETERS IN SPONTANEOUSLY HYPERTENSIVE RATS

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During pregnancy physiological adaptation in the vascular and hormonal setting is necessary for maternal well-being and foetal growth to avoid maternal complications or foetal programming. The spontaneously hypertensive rat (SHR) represents a genetic animal model of hypertension widely used in medical research because shares features with idiopathic hypertension in humans (1). Hypertension is associated with an increased risk for tissue injury that may be mediated by endothelium dysfunction, the ongoing of inflammatory process, with overproduction of O_2^- and other reactive oxygen species in cardiovascular system and kidney (2,3). In fact, previous studies have demonstrated that treatment with either antioxidant or immunosuppressive/anti-inflammatory agents improve hypertensive state in SHR (4). Here, the adaptative mechanisms underling the improvement of hypertensive status observed during pregnancy in SHR were investigated. We hypothesized that at the end of pregnancy (20_{th} day) the modification of renal reninangiotensin system plays a pivotal role in this protective effect associated to a reduction in inflammatory parameters and oxidative damage. Non pregnant (-NP) or pregnant (-P) SHR and Wistar Kyoto normotensive rats (WKY) were used. Arterial blood pressure and heart rate were measured in conscious rats. In kidney AT1, AT2, NF-κB, and IκB-α protein expression were determined. Angiotensin II (Ang II) response in mesenteric bed of these animals was also evaluated. Malondialdehyde (MDA) levels in kidney and heart were determined as an indicator of lipid peroxidation. For the first time we determined, evaluating Ang II receptors in the kidney of pregnant SHR, a decrease of AT1 and an increase of AT2 expression. In SHR-P mesenteric vascular response to Ang II became similar to that of WKY. In SHR pregnancy is associated to a marked decrease of p65 NF-κB and a significant increase in IκB-α and to an improvement of oxidative stress tissue damage, reducing MDA content. The pathophysiological alterations and the increased proinflammatory parameters, evidenced in SHR, are strongly ameliorated by pregnancy. Our results indicate that the improvements induced by this status could be related to the modification of AT1 and AT2 expression, the reduction of inflammatory status due to the downregulation of NF-κB, as well as to the reduction of oxidative stress.

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METABOLIC PRECURSORS FOR THE BIOSYNTHESIS OF SURFACTANT DISATURATED-PHOSPHATIDYLCOLINE IN PRETERM INFANT WITH RESPIRATORY DISTRESS SYNDROME

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Pulmonary surfactant is a tensioactive agent synthesized by the lung type II cells. It reduces the alveolar superficial tension providing an adequate lungs expansion and gas exchange. Surfactant is a complex mixture of phospholipids and proteins, respectively 90% and 10%. Among the lipid fraction the most abundant molecule is disaturated-phospatidylcoline (DSPC). Pulmonary surfactant deficiency is the hallmark of respiratory distress syndrome (RDS), the most common respiratory disease of preterm infants. The lung function is impaired and the surfactant quantity and composition, included DSPC, is reduced. Exogenous surfactant was introduced as treatment for RDS in the early 1990s and it has greatly reduced morbidity and mortality in preterm infants with RDS (1). The synthesis and metabolism of the surfactant phospholipids have been widely studied in animal models with radioactive tracers. However, this approach is not acceptable in humans for ethical reasons. Recently stable isotopes have been used to investigate surfactant synthesis and turnover *in vivo* in preterm infant with lung injury (2-6). In these studies molecules labelled with stable isotope were used as metabolic precursors of DSPC to measure the incorporation rate of the tracer palmitic acid (PA) into surfactant DSPC (4-6). They show that plasma free fatty acids (FFA), plasma glucose, and body water are all suitable metabolic precursors for DSPC synthesis. Our objective was to investigate preferential metabolic substrate for surfactant DSPC synthesis in preterm infant with RDS. During the last three years our research group collected 46 DSPC studies in 23 preterm infants who required exogenous surfactant and prolonged mechanical ventilation for RDS. Eight infants received a simultaneous infusion of U_{13} C-Glucose (GLUC) and (16-16-16)-$_3H_2$-Palmitate (PA), 8 infants U_{13} C-GLUC and deuterated water (D_2O), and seven infants U_{13} C-PA and D_2O for 24 h. Samples of the first two groups have been already analyzed by other members from the research group. I performed the analysis of DSPC derived from seven infants who received U_{13} C-PA and D_2O. DSPC extracted from sequential tracheal aspirates was isolated by thin layer chromatography and its isotopic enrichment was measured by massspectrometry. Plasma FFA-PA DSPC secretion time (ST), fractional synthesis rate (FSR), peak time (PT) and half-life (HL) will be calculated for each metabolic precursor. Seven infants who received PA and D_2O simultaneously showed a mean DSPC FSR of 22.3±14.8% per day from PA. My results will be compared with DSPC FSRs from plasma glucose, from D_2O and $_3H_2$PA in order to find a correlation among the three different metabolic precursors kinetics. Further studies are also in progress for the application of the mass isotopomer distribution analysis (MIDA) approach. The basic principle is that the pattern of excess isotopomer frequencies reveals the enrichment of the precursor subunits that entered the product through probability analysis based on the binomial distribution (7). I will analyze the same 46 studies already examined to measure the isotopomer frequencies of GLUC, PA, D_2O to deeply investigate the role of the three different isotopic administered precursors.

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THE ROLE OF CHOLESTEROL IN SYNAPSE STABILITY AND ACTIVITY

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Previous studies using rat brain synaptosomes, demonstrated that SNARE proteins and P/Q type calcium channels ($Cav_{2.1}$) colocalize in membrane subdomains enriched in cholesterol. This localization seems to be important for the physical and functional coupling of the secretory machinery. We have recently investigated in cultured neurons whether cholesterol-enriched microdomains may play a role in presynapse formation and activity. Firstly, we demonstrated the distribution of SNARE proteins and $Cav_{2.1}$ channels in detergent resistant membranes isolated from cultured neurons. Secondly, we showed that $Cav_{2.1}$ and SNAP-25 patches detected by immunocytochemistry at presynaptic buttons have properties of cholesterol-enriched domains being insoluble to Triton-extraction but completely soluble after saponin pre-treatment. To analyze the role of these microdomains in the stability of presynapses, we treated neurons at 14 DIV with fumonisin B1 (an inhibitor of sphingolipid synthesis) and mevastatin or squalestatin S1 (two drugs know to affect the synthesis of cholesterol by inhibiting HMG-CoA reductase or squalene synthase). In fumonisin, mevastatin or sqalestatin treated cultures, the density of puncta immunolabeled for presynaptic proteins was reduced compared to controls and the size of the remaining puncta appears increased (~1.5 fold to control). To analyze whether the morphological modifications observed with the drugs were associated with alteration of neurotransmitter release, the exo-endocytic recycling of synaptic vesicles was monitored by two way: i) the selective uptake in synaptic vesicles of the fluorescent styryl dye FM1-43, and ii) the internalization of an antibody direct against the luminal epitope of the synaptic vesicle protein synaptotagmin. The results demonstrated that squalestatin, but not fumonisin and mevastatin, induces a reduction of the uptake of both FM1-43 and synaptotagmin antibodies. These data suggest that cholesterol play an important role in maintaining the morphology and stability of presynapses and that its depletion may impair synaptic vesicle exo-endocytosis.

A POSSIBLE INVOLVEMENT OF CORTICAL GLUTAMATERGIC NEUROTRANSMISSION IN WORKING MEMORY PROCESSES

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The present study was performed to validate a spatial working memory task using pharmacological manipulations. The water escape T-maze, which combines the advantages of the Morris water maze and the T-maze while minimizing their disadvantages, was used. Scopolamine, a drug that affects cognitive function in spatial working memory tasks, significantly decreased the rat performance in the present delayed alternation task. Since glutamate neurotransmission plays an important role in the maintaining of working memory, we evaluated the effect of ionotropic and metabotropic glutamatergic receptors antagonists, administered alone or in combination, on rat behaviour. As the acquisition and performance of memory tasks has been linked to the expression of the immediately early gene cFos, a marker of neuronal activation, we also investigated the neurochemical correlates of the water escape T-maze after pharmacological treatment with glutamatergic antagonists, in various brain areas. Moreover, we focused our attention on the involvement of perirhinal cortex glutamatergic neurotransmission in the acquisition and/or consolidation of this particular task. The perirhinal cortex has strong and reciprocal connections with both specific cortical sensory areas and some memory-related structures, including the hippocampal formation and amygdala. For its peculiar position, perirhinal cortex has been recently regarded as a key region in working memory processes, in particular in providing temporary maintenance of information. The effect of perirhinal cortex lesions with ibotenic acid on the acquisition and consolidation of the water escape T-maze task was evaluated. In conclusion, our data suggest that the water escape T-maze could be considered a valid, simple, and quite fast method to assess spatial working memory, sensible to pharmacological manipulations. Following execution of the task, we observed cFos expression in several brain regions. Furthermore, in accordance to literature, our results suggest that glutamatergic neurotransmission plays an important role in the acquisition and consolidation of working memory processes.

ROLE OF CANNABINOID SYSTEM IN THE MODULATION OF MICROGLIAL PLASTICITY IN NEUROPATHIC PAIN

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Neuropatic pain is a debilitating disease which involves different neurotransmitters and several cytotipes of the CNS. However, the mechanisms underlying neuropathic pain are still poorly understood. Recent studies highlighted the role of microglia in the pathophysiological mechanisms of neuropatic pain (1). In physiological conditions microglial cells are in a resting state, also called ramified microglia. After peripheral injury microglia switches toward the active state by changing morphology and releasing proinflammatory cytokines such as IL-1β. Moreover, recent data suggest that activated microglia over-express the cannabinoid receptor CB2 (2). In this study we have evaluated the analgesic properties related to a new synthetic CB2 selective agonist (NESS400) by using behavioural analysis. Moreover we investigated the role of microglia in both spinal and in supra spinal levels with an immunohistochemical approach in a mouse model of neuropathic pain, in naïve and neuropathic mice. Mononeuropathy was induced by spared nerve injury (SNI). Sensory changes were evaluated by testing the mouse paw withdrawal thresholds (PWTs) to mechanical stimulation using a Dynamic Plantar Aesthesiometer (Ugo Basile, Italy). Chronic treatment with NESS400 (4 mg/kg) prevented mechanical allodynia and thermal hyperalgesia 3, 7, and 14 days post-SNI. This effect was reverted by both the CB1 selective antagonist AM251 (1 mg/kg) and the CB2 selective antagonist AM630 (1 mg/kg). The development of SNI-induced allodynia was associated with an increase of microglia cells number at spinal level (iba-1 labelling, mean±S.E., 12.479±0.187 vs 4.861±0.816 cells/104 μm_2; $P<0.001$ compared to naïve mice, n=3 mice/group). Moreover, the injury led to a significant increase of the pro-inflammatory cytokine IL-1β immunostaining (5.111±0.444 vs 2.164±0.436 cells/104 μm_2; $P<0.001$ compared to naïve mice, n=3 mice/group). Surprisingly, NESS400 treatment increased the microglial cells number compared to vehicle treated neuropathic animals (16.396±0.230 vs 12.479±0.187 cells/104 μm_2; $P<0.001$). On the other hand, NESS400 treatment determined a reduction of the IL-1β immunostaining compared to the only vehicle treated SNI mice (4.119±0.131 vs 5.111±0.444 cells/104 μm_2; $P<0.001$). These changes were not revealed at the supra spinal level in any of the animal group, suggesting that microglia activation does not occur at brain levels. Intriguingly, NESS400 treatment also in this case determined an increased number of microglia cells. These data are consistent with a role of cannabinoid system in the modulation of microglial plasticity in neuropathic pain conditions.

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POSSIBLE INVOLVEMENT OF apoE4 GENOTYPE IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by loss of memory, alteration of mental functioning (thinking and speaking), confusion, changes of mood, time and space disorientation. The etiopathogenesis is still unknown and there are no diagnostic markers or effective therapies available. Extracellular deposits of amyloid-β peptide (senile plaques) and intracellular deposits of abnormally hyperphosphorilated Tau protein (neurophibrillary tangles) are the cerebral pathological signs of AD. Moreover, loss of cholinergic neurons and synapses is widespread. Whilst some cases have a genetic component (familiar AD), the majority of cases (sporadic AD, 85-90%) is probably caused by several both genetic and environmental risk factors. The major genetic risk factor for AD and for cognitive deficits associated with aging is an allelic variant of apolipoprotein E (apoE). In order to explore the influence of genotype apoE4 on cognitive functions, apoE4 Target Replacement model and apoE wild-type mice were submitted to different behavioural tests (Morris Water Maze, object recognition, and passive avoidance) predictive of cognitive deficits in hippocampus, an area mostly affected in AD. Possible gender differences were assayed using both male and female. The elevated plus maze was used in order to control the anxiety-like behaviour of the two experimental groups as anxiety is a common symptom in AD.

ADAM10 REGULATES DENDRITIC SPINE MORPHOLOGY AND GLUTAMATE RECEPTOR COMPOSITION THROUGH NCADHERIN CLEAVAGE

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Cell-cell adhesion molecule N-cadherin is involved in many important physiological events such as synapse formation during development and activity-dependent spine remodelling; N-cadherin is also essential for the correct functioning of excitatory synapse, i.e. induction of long-term potentiation and long-term depression. More recently, it has been shown that α-secretase ADAM10 is capable for N-cadherin cleavage occurring at the membrane level. Here we demonstrate that synapse-associated protein-97 (SAP97) mediated trafficking of ADAM10 is essential in modulating N-cadherin metabolism in the postsynaptic compartment both in primary hippocampal neurons and *in vivo* in mice. Inhibition of ADAM10 trafficking/localization at synaptic sites and, consequently, its α-secretase activity, using a cell-permeable peptide able to disrupt its interaction with SAP97 leads to a decreased ADAM10 mediated N-cadherin metabolism. This event is paralleled by a significant modification of spine morphology and molecular composition of AMPA receptors. Thus, our data show that ADAM10 plays an important role for spine morphogenesis and can influence glutamate receptor composition of post synaptic density, suggesting an implication in functional (plasticity) events of the glutamatergic synapse.

DIABETES AFFECTS ADENOSINE METABOLISM BY RAT VASCULAR SMOOTH MUSCLE CELLS

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In the cardiovascular system extracellular ATP, ADP, AMP, and adenosine are signaling molecules, involved in several physiological processes, such as cell proliferation, platelet aggregation, vasodilatation, and inflammation (2). Vascular inflammation is a fundamental feature of diabetes complications, including retinopathy, neuropathy, and foot ulceration (1). Considering the vasoprotective effect of adenosine, we investigated the pathways that are responsible for its extracellular degradation by vascular smooth muscle cells (VSMCs) from diabetic in comparison with normoglycaemic rats. Diabetes was induced in Sprague-Dawley rats by i.v. injection of streptozotocin 4 weeks before sacrifice. VSMCs obtained from normal and diabetic rat aortas were incubated for 24 h in the presence of LPS (1μg/ml) combined with IL-1β (10 ng/ml), TNF-α (25 ng/ml), and INF-γ (10 ng/ml) to mimic the *in vivo* environment of some vascular inflammatory events in vascular inflammation. An HPLC method (3) was used to quantify adenosine and its metabolites in the culture medium of VSMCs. When VSMCs from normoglycaemic or diabetic rats were incubated under basal conditions or in the presence of LPS plus cytokines, adenosine and inosine were detectable in trace amounts only in some of the samples and the concentration of hypoxanthine (11.1±2.8 μM in control, and 9.1±2.1 μM in diabetes) was not significantly affected by the inflammatory stimuli. At the end of the 24 h incubation with adenosine (0.01-1 mM), only sub μM concentrations of this nucleoside were still present in the culture medium of VSMCs obtained from control rats. Hypoxanthine accumulation fully accounted for the loss of 0.01 and 0.1 mM exogenous adenosine. At 1 mM adenosine, also inosine concentration increased (224±51 μM) above control level (1.4±0.9 μM). Using diabetic VSMCs again the recovery of adenosine was very low (1 μM at 1 mM exogenous adenosine) and hypoxanthine accumulation was directly related to the concentration of added adenosine. The increase of inosine level was already evident at 0.1 mM adenosine and at 1 mM of the nucleoside it even exceeded that of hypoxanthine (714±114 μM and 435±86 μM, respectively). When cells were incubated with exogenous inosine (1 mM), the amount of this nucleoside remaining in the medium after 24 h as well as the accumulation of hypoxanthine were quantitatively comparable to the ones measured after cell exposure to an equimolar concentration of adenosine. By contrast, for diabetic cells the proportion of inosine still present in the medium was half than that measured after exposure to 1 mM adenosine, even though hypoxantine accumulation was not statistically different. Moreover, the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA; 1 μM) allowed a higher recovery of exogenously added adenosine (1 mM) in diabetic (358±30 μM) as compared to control cells (195±34 μM). These results show that, in an *in vitro* model of vascular inflammation, diabetes alters the activity of enzymes involved in adenosine clearance by VSMCs and the consequent formation of its degradation products, most importantly hypoxanthine, a very well known source of superoxide radicals. The higher recovery of exogenous adenosine elicited by EHNA in diabetic cells is in keeping with the previously reported increase in adenosine deaminase activity associated with diabetes (4,5). Moreover, the lower inosine to hypoxanthine ratio found in VSMCs isolated from diabetic rats, possibly reflecting a reduced purine nucleoside phosphorylase activity, may represent a preventive mechanism against oxidative injury.

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NO-DONORS, SULFHYDRYL (SH), AND DISULFIDE (SS) REAGENTS INFLUENCE ON PLATELET FUNCTIONALITY

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Blood platelets are central to haemostasis and platelet aggregation is a direct index of platelet function. Nitric oxide (NO)-donors, sulfhydryl- and disulfide-acting reagents may interfere with platelet activation, but the existence of their functional relationship has been scarcely investigated. Inhibition of platelet activation is an important function of endothelium-derived NO and is supported by NO synthesized in the platelets themselves. NO-donors are drugs that releasing NO act through guanylate cyclase activation (cGMPdependent pathway) and/or other complex mechanisms (cGMP-independent pathway). There are conflicting data in the literature regarding the mechanism of action of the various donors (1,2). In the case of cGMP-independent mechanisms, NO seems to be involved in different pathways, one of which is the inhibition of cell-surface proteins involved in platelet activation by nitrosation or nitration of cysteine or tyrosine residues. Although we traditionally think of cysteine residues and disulfide bonds as structural components in proteins, current evidence also points to the possibility of thiol-disulfide rearrangement as a dynamic process in stimulus-response coupling. Sulfhydryl groups on the platelet surface are necessary for platelet aggregation (3) and a variety of proteins containing sulfhydryl groups (PSH) and protein disulfides (PSSP) are present in the platelet surface. In particular, disruption of specific PSSP in platelet's integrin αIIbβ3 causes its activation (4). In turn, activation of αIIbβ3 by agonists like ADP or collagen facilitates the binding of soluble fibrinogen, leading to the formation of a platelet aggregate. In this study, in *in-vitro* tests of human platelets aggregation, we compared the anti-aggregating effect of three NO-donors [sodium nitroprusside (SNP), S-nitrosoglutathione (GSNO), and 3-morpholinosydnonimine (SIN-1)]. To clarify their mechanism of action (cGMP-dependent and/or cGMP-independent), we also evaluated the anti-aggregating effect of these NO-donors using platelets pre-incubated with 1H-[1,2,4]oxadiazolo[4,3a]quinoxalin-1-one (ODQ), a selective inhibitor of soluble guanylate cyclase (sGC). SNP, GSNO, and SIN-1 inhibited platelet aggregation with different power, GSNO having the most powerful effect. In the presence of ODQ, GSNO exhibited a cGMP-independent mechanism of action, whereas SNP and SIN-1 inhibited platelet aggregation by cGMP-dependent and independent mechanisms. In the second part of this study, in order to evaluate the contribution that PSH and PSSP have in platelet function, we tested the effect of two reagents, dithiolthreitol (DTT) and N-ethylmaleimide (NEM), on platelet aggregation and PSH levels on the surface of human platelets. NEM is a sulfhydryl reagent used to block PSH reactivity, thus inhibiting the formation of cystine linking in proteins, whereas DTT is a strong reducing agent frequently used to reduce PSSP. DTT induced *per se* platelet aggregation and increased PSH labelling in the platelet surface. In contrast, NEM inhibited platelet aggregation induced by a dose of platelet agonist which by itself caused irreversible aggregation. Furthermore, NEM decreased sulfhydryl labelling in the platelet surface. Since GSNO platelet inhibition may be due to PSH blockage, and a similar action is supported by NEM, a potent thiol blocker, we suggest that critical PSH blockage regulate platelet inhibition. On the other hand, since DTT, a potent reducing agent that cleaves PSSP, is *per se* an aggregating agent, we suggest that PSSP reduction, deliberating critical PSH, may activate platelets.

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CENTRAL ROLE OF THE CELL-ECM INTERACTIONS IN THE Ru(II)$Cl_2(\eta_6$-$C_7H_8)$(PTA) (RAPTA-T) MECHANISM OF ACTION

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Distant metastases of solid tumours still remain a hurdle in cancer treatment and selective anti-metastatic agents are needed to face up with this aspect of cancer disease. Among metal compounds, ruthenium derivatives are emerging as promising anti-tumour and antimetastatic agents. A series of representative compounds was evaluated for their interference with some steps of the metastatic progression by appropriate *in vitro* tests (cell detachment, migration, invasion, adhesion to a new growth substrate) and in an experimental murine tumour model. From these studies, the selective behaviour of the organometallic compound Ru(II)$Cl_2(\eta_6$-$C_7H_8)$(PTA) (RAPTA-T) against highly invasive cells emerged *in vitro* and *in vivo*. In particular, RAPTA-T showed the greatest activity in tests such as migration and cell detachment, two phenomena requiring the interaction of tumour cells with ECM components and the reorganization of the actin cytoskeleton. The aim of this study was to understand the mechanism of action of RAPTA-T. For this purpose, RAPTA-T was studied on the highly invasive breast cancer cell line MDA-MB-231, evaluating how 1 μM, 10 μM, and 100 μM RAPTA-T can influence the activation of the β1 integrin (with immunocytochemistry techniques) when cells are grown on ECM components, such as fibronectin and collagen IV, or during the adhesion process to the same substrates. In parallel, the effect of the same doses of RAPTA-T were studied on the actin cytoskeleton (labelled with phalloidin) after 15 min or 1 h of treatment and 4, 16, and 20 h of cell recovery in complete medium. Given that Rho GTPases are involved in actin cytoskeleton reorganization it was also analysed whether 100 μM RAPTA-T could influence the activation of RhoA (pull down assay, SDS PAGE, and Western blot analysis). The analysis of the effect of RAPTA-T on β1 integrin showed the increase of its active form only when cells were grown on fibronectin independently whether they were in the step of adhesion or already attached to the substrate, whereas there were no changes on the other substrates considered. Moreover RAPTA-T caused morphology changes consisting of extended lamellipodia and filopodia leading cells to acquire a spread phenotype in a dose- and timedependent manner. On cells seeded on collagen IV, RAPTA-T treatment lead to a transitory reorganization of the actin cytoskeleton in stress-fibers: this phenomenon was transient, i.e. it was evident after 1 h treatment and it disappeared 4 h later. On the contrary, RAPTA-T treatment of the cells seeded on fibronectin caused the concentration of the actin close to the cell membrane, with the formation of an actin cortical ring, already after the 1-h treatment and 1 μM RAPTA-T; this modification persisted up to 16 h after the end of treatment. The analysis of the activation of RhoA showed that RAPTA-T was unable to induce variations in the active form of this GTPase in cells grown on plastics. However, since previous experiments highlighted the central role of the ECM substrates and of their interaction with the cells in the activity of this ruthenium compound, it will be interesting to evaluate the effects of RAPTA-T treatment on RhoA activation in cells seeded on fibronectin and collagen IV. These data put new light in the potential action of the class of ruthenium complexes represented by RAPTA-T for the treatment of metastatic tumours.

PRECISION-CUT LIVER SLICES TO STUDY DRUG METABOLISM: CYTOCHROME P450-DEPENDENT METABOLISM OF *l*-DEPRENYL

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Several *in vitro* systems are available for studying the metabolism and toxicity of novel compounds in the liver. Although subcellular fractions, such as microsomes, and isolated hepatocytes, have been most extensively used, precision-cut liver slices could be an alternative and useful tool as it presents some typical advantages: no proteolytic enzymes are necessary for preparation, and cell heterogeneity, cell-cell interactions, and multienzymes system are maintained (1). The aim of the present study was to set up the preparation of rat liver slices in order to improve the tools for studying xenobiotic metabolism. Precision-cut rat 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 RPMI 1640 complete medium under carbogen atmosphere and incubations were carried out at 37°C in 12 well plates with continuous gentle shaking (2). The slices retained good cell-viability for 48 h of incubation (as measured by glutathione content, MTT test, and LDH leakage). These slices possessed relatively stable metabolic functions, measured by the time-dependent metabolism of 7-ethoxycoumarin, CYP dependent enzyme analysis, and Western blot. In a second part of the studies, the kinetic analysis of the well-known drug *l*-deprenyl was performed both in the precision-cut liver slices model and in microsomes. The formation of *l*-deprenyl metabolites was determined by GLC (3) in the incubation medium. A preliminary assay of *l*-deprenyl N-dealkylation by rat liver microsomes and rat precisioncut liver slices gave rise to the primary metabolites *l*-methamphetamine and *l*-nordeprenyl, the amount of products formed increased linearly with time up to 45 min and the initial rates were proportional to the amount of microsomal protein added. The formation of low amounts of the secondary metabolite *l*-amphetamine was also evident. In the precision-cut slices, the kinetic study of *l*-nordeprenyl and *l*-methamphetamine formation showed atypical Michaelis-Menten kinetics. The same analysis performed in microsomal fractions of rat liver revealed that both metabolites possess a biphasic Michaelis-Menten kinetics. These data, together with the different V_{max} values obtained, could be the result of the complex multi-enzymatic system represented in the slices that may interact with the *l*deprenyl, modifying its concentration in the enzymatic site. In conclusion, the precision-cut liver slices represent an alternative *in vitro* model to microsomes for studying drug metabolism. Further studies are necessary to clarify the differences between the two systems.

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ENDOTHELIAL DYSFUNCTION IN MICE WITH STREPTOZOTOCIN-INDUCED TYPE I DIABETES IS OPPOSED BY COMPENSATORY OVEREXPRESSION OF COX-2 IN THE VASCULATURE

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Cardiovascular complications are the leading causes of increased morbidity and mortality in diabetic patients (1). Under healthy conditions, cardiovascular homeostasis is maintained by several integrated pathways regulating endothelial synthesis and release of vasoconstrictors such as endothelin-1 (ET-1) and vasodilators including nitric oxide (NO) and cyclo-oxygenase (COX)-dependent prostacyclin (PGI_2). Endothelial dysfunction is characterized by reduced bioavailability of NO secondary to increased oxidative stress or elevated expression of vasoconstrictor and pro-thrombotic factors that lead to abnormal vasoreactivity. In diabetes, persistent fasting and post-prandial hyperglycaemia induces a chronic pro-inflammatory state that may directly contribute to endothelial dysfunction by altering expression of genes important for vascular homeostasis (2). Since in many disorders of cardiovascular and metabolic homeostasis compensatory responses are often present to maintain or restore physiological function (3,4), we investigated whether increased expression of COX-2 in the vasculature of a mouse model of type 1 diabetes may mediate a compensatory response that opposes the endothelial dysfunction induced by persistent hyperglycemia. Balb/c mice were treated with vehicle (control, citrate buffer) or streptozotocin (STZ, 240 mg/kg, i.p.) to induce type 1 diabetes (T1D). In mesenteric vascular beds (MVB) isolated *ex vivo* from mice 1 week after STZ treatment, dosedependent vasorelaxation in response to either acetylcholine (ACh; 0.01-100 μM/30 s) or sodium nitroprusside (SNP; 0.1-10 μM/30 s) was comparable to that in MVB from agematched control mice injected with vehicle alone (control). By contrast, MVB from mice 8 weeks after STZ treatment had severely impaired vasodilator responses to ACh ($P<0.005$ vs control), but not to SNP, consistent with endothelial dysfunction. Pre-treatment of MVB from control mice with the NO synthase inhibitor L-NAME (100 μM, 30 min) nearly abolished the vasodilator action of ACh ($P<0.001$ vs respective basal). Conversely, in MVB from mice 1 week after STZ treatment, vasodilator action of ACh was only partially impaired by L-NAME pre-treatment. This suggests that vasculature of mice with T1D may have compensatory NO-independent mechanism to augment vasodilator actions of Ach and oppose endothelial dysfunction. Indeed, pre-treatment of MVB isolated from mice 1 week after STZ treatment with the selective COX-2 inhibitor NS-398 (10 μM, 30 min) unmasked endothelial dysfunction not evident in MVB from control mice pre-treated without or with NS-398. Expression of COX-2 in MVB, aortic endothelial cells, and aortic vascular smooth muscle cells isolated from STZ-treated mice was significantly increased when compared with samples from control mice ($P<0.05$). Moreover, concentrations of the COX-2 dependent vasodilator 6-keto PGF-1α was substantially elevated in conditioned media from aorta isolated from STZ-treated mice ($P<0.01$ vs respective basal). Endothelial dysfunction in a mouse model of T1D may be opposed by compensatory up-regulation of COX-2 expression and activity in the vasculature. Our findings may have important implications regarding the safe use of selective COX-2 inhibitors in diabetes and may contribute to developing novel therapeutic strategies for diabetes and its cardiovascular complications.

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NEUROTENSIN SYSTEM MODULATION OF GLUTAMATERGIC SIGNALLING: RELEVANCE FOR THE TREATMENT OF NEURODEGENERATIVE DISEASES

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Neurotensin (NT) acts in the mammalian brain as a primary neurotransmitter or modulator of classical neurotransmitters. Several *in vitro* and *in viv*o studies have demonstrated the existence of close interactions between NT, dopamine, and glutamate both in limbic and striatal brain regions. The extracellular accumulation of glutamate and the excessive activation of glutamate NMDA receptors have been postulated to contribute to the neuronal cell death associated with several chronic neurodegenerative disorders. NT significantly enhances glutamatergic signalling in the basal ganglia were there is probably an antagonistic NTS1/D2 interaction that can lead to an enhancement of glutamate signalling contributing to the neurodegeneration of nigro-striatal dopaminergic neurons found in Parkinson's disease. In the cerebral cortex (1-4), the same amplification of NMDA-receptor signalling probably occours *via* NTS1 activation/interaction (5). These findings suggest a reinforcing action of NT on several functions exerted by glutamate on CNS, in particular on the glutamate-mediate excitotoxicity in several brain areas (5-7). Primary cultures of rat cortical neurons have been prepared from 1 day old SD rats. For oxygen-glucose deprivation (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, LDH levels, mitochondrial dehydrogenase activity (MTT), determination of endogenous extracellular glutamate levels, nuclear staining with Hoechst 33258, caspase-3 activity, annexin V staining, and MAP-2 immunoreactivity 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. Significant alterations of both biochemical and morphological parameters evaluated were observed: increase of LDH efflux, impairment of oxidative ability of mitochondria (MTT levels), increase of caspase-3 activity, increase in the number of the apoptotic nuclei, AN(+)/PI(-) immunoreactive cells, and in the number of MAP-2 aggregations in dendrites, as measured 24 h later the ischemic insult, with respect to sister cell cultures not exposed to OGD. The addition of 100 nM NT to the cultures was associated with a significative enhancement of all the OGD-dependent alterations, whereas pre-exposure of the cells to the NTS1 antagonist SR48692 (100 nM) prevented both the effect of the neuropeptide and OGD, alone or in combination, with the sole exception of caspase-3 activity and annexin V staining that were only partially counteracted by SR48692. The results obtained with this *in vitro* model of cerebral ischemia point out the involvement of NT in the pathological events induced by OGD thus suggesting a potential role for the NTS1 antagonist, SR48692, against this acute neurodegenerative process (8).

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NEUROPROTECTIVE EFFECTS OF GUANOSINE AGAINST AMYLOID-β PEPTIDE-INDUCED TOXICITY IN NEURONAL AND GLIAL CELLS

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The amyloid-β (Aβ) peptide is the major component of senile plaques that are one of the hallmarks of Alzheimer's disease (AD). It is well recognized that Aβ exists in multiple assembly states, such as soluble oligomers or insoluble fibrils, which affect neuronal viability and may contribute to disease progression (1). In particular, common Aβ-neurotoxic mechanisms are Ca^{2+} dyshomeostasis, reactive oxygen species (ROS) formation, altered signalling, mitochondrial dysfunction, and neuronal death such as necrosis and apoptosis (2). A recent study shows that the ubiquitin-proteasome pathway plays a crucial role in the degradation of short-lived and regulatory proteins that are important in a variety of basic and pathological cellular processes including apoptosis (3). Guanosine (GUO) is a purine nucleoside present extracellularly in brain that shows a spectrum of biological activities, both under physiological and pathological conditions. Recently, it has become recognized that both neurons and glia also release guanine-based purines (4). However, the role of GUO in AD is still not well established. In this study, we investigated the mechanism basis of neuroprotective effects of GUO against Aβ peptide-induced toxicity in neuronal (SH-SY5Y) and glial (C6) cells, in terms of mitochondrial dysfunction and translocation of phosphatidylserine (PS), a marker of apoptosis, using MTT and annexin-V assay, respectively. In particular, treatment of SH-SY5Ycells with GUO (12.5-75 μM) in presence of monomeric $A\beta_{25-35}$ (neurotoxic core of Aβ), oligomeric, and fibrillar $A\beta_{1-42}$ peptides, showed a strong dose-dependent inhibitory effects on Aβ-induced toxic events. The maximum inhibition of mitochondrial function loss and PS translocation was observed with 75 μM of GUO. Subsequently, to investigate whether neuroprotection of GUO can be ascribed to its ability to modulate proteasome activity levels, we used lactacystin, a specific inhibitor of proteasome. We found that the antiapoptotic effects of GUO were completely abolished by lactacystin. To rule out the possibility that these effects resulted from an increase in proteasome activity by GUO, the chymotrypsin-like activity was assessed employing the fluorogenic substrate Z-LLL-AMC. The treatment of SH-SY5Y with GUO (75 μM for 0-3 h) induced a strong increase, in a time-dependent manner, of proteasome activity. In parallel, no increase of ubiquitinated protein levels was observed at similar experimental conditions adopted. We then evaluated an involvement of anti and proapoptotic proteins such as Bcl-2, Bad, and Bax by Western blot analysis. Interestingly, both Bad and Bax levels decreased after 2 h treatment of SH-SY5Y with GUO. By contrast, treatment with GUO did not modify the Bcl-2 levels. Similar results have been obtained with the treatment of C6. Taken together, these results demonstrate that GUO neuroprotective effects against Aβ-induced apoptosis are mediated, at least partly, *via* proteasome activation. In particular, these findings suggest a novel neuroprotective pathway mediated by GUO, which involves a rapid degradation of pro-apoptotic proteins by the proteasome. In conclusion, the present data raise the possibility that GUO could be used as an agent for the treatment of AD.

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EFFECT OF SHORT- AND LONG-TERM TREATMENT WITH RISPERIDONE AND OLANZAPINE ON PROLACTIN LEVELS IN CHILDREN AND ADOLESCENTS WITH PSYCHIATRIC DISORDERS

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Hyperprolactinemia is a well-known effect of the currently marketed antipsychotics, due to dopamine D2 receptor antagonism on the pituitary gland. It occurs frequently with conventional antipsychotics and some atypical antipsychotics, such as risperidone and amisulpride, but is rare with other newer agents including olanzapine. Few studies have evaluated changes in prolactin levels in children and adolescents during treatment to risperidone vs olanzapine. The aim of the present study was to report on serum prolactin levels in children and adolescents with psychiatric disorders treated with risperidone or olanzapine. Thirty-seven patients (26 males and 11 females; age 7 to 17 years), with the diagnosis of autism, schizophrenia or mental retardation with behavioural disorders participated to the study. Twenty-six patients (20 males and 6 females) started a treatment with risperidone (dose range 0.5-4 mg/day) and 11 (6 males and 5 females) received olanzapine (dose range 2.5-20 mg/day) and were monitored for 12 months. Plasma levels of prolactin were determinated at baseline and at months 1, 3, 6, and 12. Risperidone and olanzapine concentrations were measured by HPLC. In children and adolescents treated with risperidone, prolactin levels were 147±58 mU/l at baseline, 425±198 mU/l ($P<0.01$) after 1 month, 576±345 mU/l ($P<0.01$) after 3 months, 481±287 mU/l ($P<0.01$) after 6 months, and 350±180 mU/l ($P<0.01$) after 12 months. In the group treated with olanzapine, prolactin levels were 170±71 mU/l at baseline, 274±95 mU/l ($P<0,05$) after 1 month, 399±160 mU/l ($P<0,05$) after 3 months, 413±202 mU/l after 6 months ($P<0,05$), and 294±116 mU/l ($P<0,05$) at month 12. The elevation in prolactin levels was more evident among risperidone than olanzapine recipients, both during acute treatment and during maintenance. Female patients showed greater prolactin elevations than males regardless of drug or treatment duration. There was no correlation between plasma concentrations of risperidone or olanzapine and percent increase in prolactin levels. Only an adolescent female, treated with risperidone, showed galactorrhea, a symptom related to hyperprolactinemia. In children and adolescents, risperidone treatment induced hyperprolactinemia which tended to diminish with time. In few cases elevated prolactin levels were related with side effects. Olanzapine treatment was associated with a mild and transient elevation of prolactin concentrations. The differential effect of risperidone and olanzapine on prolactin levels is presumably due to a different receptor affinity for D2 receptors.

THE THIOREDOXIN INHIBITOR PMX290 SENSITIZES HYPOXIC COLON CANCER CELLS TO 5-FLUOROURACIL: ROLE OF HIF-1 MODULATION

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Solid tumours invariably develop hypoxic areas as actively proliferating cells rapidly outgrow oxygen and nutrient supply provided by existing vessels. Tumour hypoxia is a powerful driving force for malignant progression and an adverse prognostic factor in cancer patients (1). Clinical and preclinical studies have firmly established that hypoxia is associated with impaired response to both radiotherapy and chemotherapy. This latter effect is due in part to poor perfusion and restricted drug access to hypoxic areas (2); however, a major role is played by activation of a family of hypoxia-inducible transcription factors (HIFs), orchestrating a coordinated adaptive response (3). HIFs act as heterodimers, consisting of an oxygen-dependent α and a constitutively expressed β subunit and binding hypoxia response elements (HREs) throughout the genome (3). HIF-1 regulates the expression of more than 100 genes encoding key factors in cell proliferation and survival, glucose metabolism, invasion and angiogenesis (4). HIF-1 upregulation has been shown to induce expression of drug efflux transporters, to alter the activity of DNA repair mechanisms, and to shift the balance between pro- and anti-apoptotic factors towards cell survival, thereby decreasing the effectiveness of a number of currently used anticancer agents (5). Based on these observations, a number of small molecule and nucleotide-based agents inhibiting HIF-1, mostly by specifically targeting HIF-1α synthesis and/or degradation, have been identified and developed (4). Among these, thioredoxin-1 inhibitors, such as the quinol compounds PMX290 and PMX464, have been shown to decrease HIF-1α levels and/or HIF-1 activity in a number of different cancer cell lines (6). In the present study we investigated the role of HIF-1 in the response of HCT116 human colon adenocarcinoma cells to 5-fluorouracil (5FU), a cornerstone in the polychemotherapeutic management of this tumour type, and we assessed the ability of the fluorinated, indole-substituted quinol compound PMX290 to increase the cytotoxic activity of 5FU in this cell line following HIF-1 upregulation. Our results indicate that when HIF-1 activity is increased, either by exposing cells to hypoxia, or by forced expression of a degradation-resistant form of HIF-1α, cell response to 5FU is significantly impaired; conversely, knockdown of HIF-1α by RNA interference partially prevents hypoxia-induced resistance to 5FU. PMX290 significantly inhibits HIF-1 activity and concomitantly increases 5FU cytotoxicity in hypoxic cells. These observations suggest that quinol compounds, by targeting cells that present increased HIF-1 activity, might help to improve the success rate of 5FU-containing regimens for the management of colon cancer.

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SPECIFIC PKCδ INHIBITOR PROTECTS VASCULAR ENDOTHELIAL CELLS FROM OXIDATIVE ASSOCIATED INJURY

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Oxidative stress plays a central role in endothelial dysfunction and significantly contributes to cardiovascular diseases. Among the multiple molecular events, increased levels of reactive oxygen species (ROS) production, released during ischemia/reperfusion injury, are responsible for the protein kinase C delta (PKCδ) activation in endothelial cells. PKCδ is implicated in mediating oxidative stress, apoptosis, and inflammation, hallmarks of reperfusion injury. Recent studies demonstrate that the inhibition of PKCδ translocation in cardiomyocytes through a new selective peptide inhibitor, δV1-1, reduces the damaged area of the heart after ischemia/reperfusion. We set up an *in vitro* model to study δV1-1 effect on vascular endothelial cell. Cells maintained in a stressed condition, characterized by medium containing low serum concentration (0.1% BCS), were rescued by δV1-1 which reduced ROS production and promoted cell survival. In this project we investigate the molecular mechanism involved in δV1-1 protective effect on coronary endothelial cells. We focused our attention on enzymes involved in maintaining endothelial cell functions such as eNOS, or implicated in cell survival as the protein kinase PI3K/Akt or MAPK. Treatment with δV1-1 modified eNOS site specific phosphorylation, in particular it induced phosphorylation in Thr_{495} and Ser_{114} and de-phosphorylation in Ser_{1177} which were then involved in PI3K/Akt or MAPK activity. Moreover, inhibition of PKCδ translocation promoted a rearrangement of the localization/activity of the above enzymes which were responsible for endothelial cell survival. In conclusion, the δV1-1 pro-survival effect resulted in a mechanism characterized by a new correlation among PKCδ/eNOS/Akt signalling pathway.

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STUDY ON THE POTENTIAL ANTIDEPRESSANT ACTIVITY OF ZIPRASIDONE

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The antipsycotic ziprasidone besides being an antagonist of dopamine D_2 and serotonin 5-HT_2 receptors also has an antagonistic effect at norepinephrine and serotonin transporters and intrinsic activity at serotonin 1_A receptors. This pharmacological profile could characterize this drug as an antipsychotic also effective on symptoms of mood disorder. In order to study the possible antidepressant activity of ziprasidone, I utilized two experimental protocols that model in rats some symptoms of depression. The 2 symptoms reproduced in rats are: a) hyporeactivity to aversive stimuli (escape deficit, ED) and b) anhedonia, characterized by a reduced motivation to operate in order to earn a reward. Both behaviours are induced by exposure of the animals to single or repeated sessions of an unavoidable stressor. Rats exposed to a noxious, avoidable stimulus, rapidly learn how to avoid it, and a "naïve" rat easily escapes 25-28 out of 30 consecutive electric tail-shocks. This avoidance competence can be disrupted by previous exposure to an unavoidable stressor; in this condition, when tested for escape 24 h later, the animal avoids an average of 3-5 out of 30 tail-shocks (ED). In order to maintain the ED condition for long periods of time, rats showing a clear ED are subjected to the chronic stress procedure consisting in brief shock or restraint exposures on alternate days for 3 weeks. The anhedonia model exploits the fact that exposure to repeated, unavoidable stress prevents the acquisition of an appetitive behaviour aimed at consuming a palatable food (vanilla sugar, VAB) in satiated rats. I first studied the possible preventive effect of ED development by a 2-week treatment with different doses of ziprasidone (2.5, 5, and 10 mg/kg). In a second experiment, I evaluated whether repeated ziprasidone treatment (5 mg/kg) was able to revert the hyporeactivity induced by chronic exposure to unavoidable stress (fluoxetine treatment was used as positive control of antidepressant activity). Ziprasidone treatment at the 3 doses used was able to prevent the development of ED induced by unavoidable stress exposure. ANOVA analysis indicates a difference between groups ($F_{7.48}$=5.75; P<0.001). Post-hoc Dunnett's test showed that the number of escapes in the naïve and ziprasidone treated groups was significantly higher compared to the stress group (P<0.01). The results show that the repeated treatment with ziprasidone, differently from fluoxetine treatment, failed to reverse the escape deficit in chronically stressed rats. In another series of experiment, control animals and chronically stressed rats with and without a concomitant ziprasidone treatment underwent the 3 week protocol of VAB acquisition. The results obtained indicated that rats exposed to the chronic stress protocol failed to acquire the appetitive behaviour while the group of stressed rats treated with ziprasidone acquired the behaviour and their performance reached the same levels of control rats treated with saline or ziprasidone. Ziprasidone does not satisfy all the criteria to be considered a real antidepressant in experimental models of depression, however its protective activity on ED and anhedonia development that shares with classical antidepressants suggest that this drug could be very useful in psychoses associated with mood disorders and psychotic depression.

INHIBITION BY NOVEL N,N-DICYCLOEXANE-4-OLAMINE ARYL ESTERS OF PGP-MEDIATED RHODAMINE 123 EFFLUX IN L5178Y MDR-1 TRANSFECTED MOUSE LYMPHOMA CELLS

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Many tumour cells become resistant to commonly used cytotoxic drugs due to the overexpression of ATP-binding cassette (ABC) transporters. Two proteins, Pgp (MDR-1, ABCB1) and MRP-1 (ABCC1), have been demonstrated to pump a wide selection of the most commonly used cancer drugs and their overexpression correlates broadly with negative treatment response characteristics of many different forms of cancer (1). Several generations of pharmaceutical inhibitors of Pgp have been examined in preclinical and clinical studies. The main problems associated with the development of these drugs is due to their poor specificity, low potency, and interference with physiological functions (2). A new series of Pgp-dependent MDR inhibitors having a N,N-bis(cyclohexanol)amine scaffold has been designed, on the basis of the frozen analogous approach (3). The use of this scaffold results in four geometrical isomers when the N,N-bis(cyclohexanol)amine moiety is esterified with two different aryl acids. The Pgp inhibiting properties of compounds **a**, **b**, **c**, **d** (Figure 1) have been evaluated by measuring the efflux of Pgp specific fluorescent substrate rhodamine 123 (R123) in MDR1-gene transfected mouse Tlimphoma L5178 cells in presence of different concentrations of the selected compounds and quantified as fluorescence increase by flow cytometry (4). Their effects were compared to those of verapamil, the well known calcium channel blocker able to inhibit Pgp function. Pgp blocking activity was described by α_{max}, which expresses the efficacy, and by IC_{50}, which measures the potency of the inhibitor. α_{max} varied between 0 (in the absence of the inhibitor) and 1 (when the amount of R123 found in L5178 MDR1 cells was equal to that determined in presence of 5 mM vanadate that completely inhibited R123 efflux). Results demonstrated that all the aforementioned compounds inhibited Pgp-mediated R123 efflux in a concentration-dependent manner although with different potency. Isomers **c** and **d** were very potent and efficient inhibitors, with the IC_{50} in the low nanomolar range (IC_{50}=1.9 nM and 0.72 nM, respectively) and an α_{max} value very close to 1. Isomers **a** and **b** showed IC_{50} values two orders of magnitude lower than that of the former compounds, i.e 130 nM and 230 nM, respectively. On the contrary, verapamil was a very week inhibitor of Pgpmediated R123 efflux since its maximum effect (α_{max}=0.22) was observed at 100 μM concentration. Finally, the reversibility of the inhibition of Pgp pump by the tested compounds was assessed by determining the reduction of fluorescence upon dilution of the cells/inhibitor mixture. Results emphasized a partially reversible inhibition of Pgp by all of these four isomers. In conclusion, compounds **c** and **d** appear to be useful pharmacological tools for studying Pgp and sister proteins and very promising lead compounds for the development of safe and efficient MDR reverters.

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ROLE OF SPHINGOSINE-1-PHOSPHATE PATHWAY IN AIRWAY INFLAMMATION AND HYPER-REACTIVITY

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Sphingosine-1-phosphate (S1P) is a pluripotent lysophospholipid signalling molecule that has been implicated in regulation of several cellular processes. Biological effects of S1P are mediated by binding and signalling through a family of 5 differentially expressed Gprotein-coupled receptors located in the plasma membrane, namely S1P1-5 (1). The function of S1P has been extensively investigated and S1P has been shown to be involved in many biological functions including cell growth, survival, differentiation and motility, and calcium homeostasis (2). A pivotal role of S1P in human asthma has been suggested on the basis that S1P levels are elevated in the airways of asthmatic individuals after segmental allergen challenge. It has recently shown that S1P is involved in mast cell chemotaxis and in the eosinophil recruitment *in vivo* further suggesting its importance in airway hyperreactivity and smooth muscle contraction. Here we assessed the role of S1P/sphingosine kinase (SPK) pathway in regulation of bronchial tone. Our objective was to determine, using an integrated pharmacologic and molecular approach: 1) the role of S1P as endogenous modulator of the bronchial tone; 2) the linkage between S1P pathway and bronchial hyper-responsiveness. We evaluated S1P effects on isolated bronchi and whole lungs, harvested from Balb/c mice sensitized to ovalbumin (OVA) vs vehicle-treated mice, by measuring bronchial reactivity and lung resistance. We found that S1P administration on nonsensitized mouse bronchi does not cause any direct effect on bronchial tone, while a significant increase in ACh-induced contraction occurs after S1P challenge. Conversely, in OVA-sensitized mice S1P/SPK pathway triggers airway hyper-responsiveness. Indeed, S1P causes a dose-dependent contraction of isolated bronchi. Similarly, in the whole lung system, S1P increased airway resistance only in OVA-sensitized mice. The action on bronchi of S1P is coupled to an enhanced expression of SPK1 and SPK2 as well as of S1P2 and S1P3 receptors. In these experiments the key role for S1P/SPK in hyper-reactivity has been confirmed by pharmacologic modulation of SPKs. S1P/SPK pathway does not seem to play a major role in physiologic conditions, while it may become critical in pathologic conditions (3). These results open new windows for therapeutic strategies in diseases like asthma.

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USE OF ANTIEPILEPTIC DRUGS IN ELDERLY IN THE YEARS 2004-2007: A POPULATION BASED STUDY IN SOUTHERN ITALY

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An increasingly use of antiepileptic drugs (AEDs) was observed in the last years, particularly among elderly people. On this basis, the aim of this study was to assess the trend of use of older and newer AEDs in elderly people in a general practice setting of southern Italy. Data were extracted from the Arianna database in the years 2004-2007. This database collects information about a population of almost 150,000 individuals living in Caserta and registered in the lists of 88 general practitioners (GPs). Patients aged 65 or more, who received at least one AED prescription, were identified. Utilization of newer and older AEDs was calculated as one-year prevalence and incidence of use. Stratification by gender, age, and indication of use were performed. Prevalence of older AEDs use slightly increased during the observational period (from 14.4/1,000 in 2004 to 19.8/1,000 in 2007). Conversely, a strong increase of newer AEDs use was observed until 2006, followed by marked reduction in 2007 (from 22.5/1,000 in 2004 to 41.0/1,000 in 2006, and to 25.5/1,000 in 2007). Concerning the incidence of use of both older and newer AEDs, a similar trend was observed. Most of the users of older AEDs were treated because of epilepsy (57.8%), while users of newer AEDs were mainly treated because of pain (79.5%). Gabapentin and pregabalin were the drugs mostly used as a new AED treatment. However, the incidence of gabapentin use decreased from 12.5/1,000 in 2004 to 2.6/1,000 in 2007, while pregabalin, marketed in July 2004, rose from 5.5/1,000 to 18.1/1,000 in 2006, decreasing to 6.7/1,000 in the following year. An increasing use of AEDs in elderly has been observed in the last four years, mostly due to the prescription of newer compounds in indications other than epilepsy. In January 2007, the Italian Drug Agency revised reimbursement criteria of newer AEDs drugs, as reported in “Nota 4”, according to updated scientific evidence. This could explain the reduction of prevalence and incidence of use of newer AEDs drugs during 2007. A different prescribing pattern of AEDs in elderly patients was shown in a general practice of southern Italy. Indeed, older and newer AEDs are mainly used respectively in the treatment of epileptic disorders and neuropathic pain. The increased use of pregabalin starting from 2005 confirms the trend of new marketed drugs to be widely prescribed in general practice after their introduction in drug market, while the reduction of newer AEDs use reflects the introduction of new health policy intervention.

DIFFERENTIAL EFFECT OF THE QUASI-IRREVERSIBLE CYP3A4 INHIBITOR ERYTHROMYCIN ON THE PHARMACOKINETIC PARAMETERS OF TOTAL AND UNBOUND QUININE IN HEALTHY SUBJECTS AND PATIENTS WITH CIRRHOSIS

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In vivo inhibition of cytochrome P450 (CYP) 1A2 by the reversible inhibitor fluvoxamine causes a reduction in the clearance of CYP1A2 substrates, the magnitude of which decreases in proportion to the degree of liver dysfunction, regardless of the clearance characteristics (flow-dependent or capacity-limited) of the drug involved (1,2). A main question remains to be addressed in order to assess whether this is a general phenomenon, i.e. whether the magnitude of the inhibitory effect is dependent on liver functional status, irrespective of the mechanism (reversible or irreversible) of CYP inhibition. In order to resolve this question, we evaluated the effect of liver cirrhosis on the inhibition of the metabolic disposition of quinine, a probe of CYP3A4 (3), by the mechanism-based, quasiirreversible inhibitor erythromycin. The study was carried out in 10 healthy volunteers and 20 cirrhotic patients, 10 with mild (Child grade A) and 10 with severe (Child grade C) liver dysfunction, according to a randomized, double-blind, 2-phase, crossover design. In one phase all participants received placebo for 5 days; in the other phase they received three 600-mg doses of erythromycin ethylsuccinate, 8 h apart, for 5 days. On day 2 of both phases, quinine sulphate (500 mg) was administered orally 1 h after the morning erythromycin dose. Concentrations of quinine and its metabolite 3-OH-quinine were measured by HPLC (3) in plasma and urine up to 96 h. Free quinine concentration was determined in all plasma samples by ultrafiltration (4). Erythromycin co-administration significantly reduced quinine clearance in healthy subjects and in patients with mild liver dysfunction (by 33% and 30%, respectively), whereas it had virtually no effect on quinine clearance in patients with severe liver function impairment. Erythromycin also caused a marked increase in free quinine fraction, particularly in Child class C cirrhotics, in which unbound fraction was almost doubled. At variance with total quinine clearance, unbound clearance was significantly reduced (by 35%) also in patients with severe cirrhosis. Total and unbound formation clearances of 3-OH-quinine were reduced to similar extents (about 60% and 75%, respectively) in the three study groups. The effect of erythromycin on total quinine clearance is the result of two opposing actions: inhibition of the intrinsic metabolic activity of the liver and increase in free quinine concentration which, in Child C cirrhotics, is such as to completely mask the inhibition of intrinsic clearance. The observations that unbound quinine clearance and 3-OH-quinine formation clearance are inhibited to a very similar extent in controls and cirrhotic patients indicated that, unlike reversible inhibitors, the effect of irreversible inhibitors does not depend on liver functional status.

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IN VIVO ANALYSIS OF MITOFUSIN FUNCTION

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Charcot-Marie-Tooth disease (CMT) comprises a frequently occurring, genetically heterogeneous group of peripheral neuropathies. CMT falls into two main forms: the demyelinating CMT type 1 with decreased nerve conduction velocities and the axonal form, CMT type 2. In contrast to the well-known molecular genetic defects causing the CMT1 phenotype, the genes associated with CMT2 have only recently been identified (1). Mutations in the mitochondrial protein mitofusin 2 (Mfn2) are the most commonly identified cause of CMT2. Mfn2 is ubiquitously expressed and it is localized to the outer mitochondrial membrane. Homozygous Mfn2 knockout mice die in midgestation owing to placental defects. Although heterozygotes were reported to have a normal phenotype, mouse embryonic fibroblast cultures from Mfn2-deficient mice had markedly lower mitochondrial mobility and displayed fragmented mitochondria, due to a severe reduction in mitochondrial fusion (2). Mobility and transport of mitochondria are key elements to the functional health of the extended neuronal axons, particularly in peripheral nerves. This could be a clue to a possible mechanism of action in CMT2 (3). To better understand the function of mitofusin as well as the mechanism responsible for the disease, we are conducting a study of the *Drosophila* homolog of mitofusin, named Marf. A blast search of *Drosophila* databases, using the human protein sequence, has identified a fly gene product that reveals an extensive homology with the human protein. We are using *Drosophila* as a model to conduct a detailed analysis of the loss and gain of function phenotypes, aimed at defining a functional role of this protein. Knock-down of Marf in the nervous system induces muscular plaque defects at the third instar larva stage, with lack of mitochondria in the distal axon. The overexpression of Mfn2 in fruitflies is larval lethal, causing clusterization of mitochondria in the perinuclear regions of neuronal cell bodies. We will discuss the role of Marf in the control of mitochondrial dynamics, in order to understand its involvement in the function of the larval nervous system. These studies should help to advance our knowledge of the molecular mechanisms responsible for CMT that are presently not understood.

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USE OF DISEASE-MODIFYING DRUGS FOR MULTIPLE SCLEROSIS IN NEUROLOGICAL CENTRES OF EMILIA ROMAGNA

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Current treatment of multiple sclerosis (MS) with disease-modifying drugs (DMD) includes interferon-β (IFN-β), glatiramer acetate (GA), and immunosuppressive drugs [e.g.mitoxantrone (MIT), azathioprine (AZA), cyclophosphamide (CYC)]. Knowledge on effectiveness of each DMD in clinical practice and optimal pattern of their use (especially, when to start drug therapy) are still lacking. Databasing may help to optimise patient care by providing valuable long-term information in treated patients and by overcoming limitations of RCTs (1). A monitoring project of DMD use in MS has been set up in collaboration with 19 Neurological Centres in Emilia Romagna. The project aimed to identify criteria for drug choice in MS and to fill the incomplete knowledge about optimal pattern of use in clinical practice. For each patient under DMD therapy, information on subject history, disease details [MS course, disability degree in terms of Expanded Disability Status Scale (EDSS), relapses, Magnetic Resonance Imaging (MRI) results] and drug therapies (drugs, doses, switches, side effects) were recorded in a regional database. Data collected from May 2006 to January 2008 were analysed. For each diagnostic parameter a cut-off value was set in order to stratify the patients according to the severity of MS. Afterwards, the relationship between prescribed drug and diagnostic parameters was analysed by non-adjusted odds ratio (OR, 95% CI), with IFN-β1a 30 mg per week (the most frequently used regimen) set as reference. Moreover, the drug choice was analysed according to the strength of disease progression by the MS Severity Score (MSSS; 2), a combined parameter of EDSS disability and disease duration. Data of 934 patients (71% females) were collected. MS was diagnosed by the most recent McDonald's Criteria in 43% of cases. The relapsing remitting course was the most frequently observed (64%) and the majority of patients (51%) had a quite low degree of disability (EDSS ≤2.5). IFN-β was prescribed in 73% of cases, followed by GA (12%), AZA (8%), and MIT (6%). IFN-β1a represented the most frequent drug choice (67%) in relapsing remitting patients, whereas in progressive forms IFN-β1b (27%), MIT (25%), and AZA (20%) were significantly preferred to IFN-β1a once a week. In patients with high disability (EDSS ≥3), the drugs with higher frequency of administration were significantly preferred to IFN-β1a once a week. No significant relationship between MRI results and drug choice was found. The MSSS analysis showed a relationship between drug choice and disease progression: immunomodulatory drugs (IFN e GA) were preferred in slow progression (MSSS ≤5.0), whereas immunosuppressive drugs (MIT, AZA, and CYC) were preferred in fast progression (MSSS >5.5). IFN-β1a and GA were preferred in patients with stable and moderate MS, whereas IFN-β1b and immunosuppressive agents were especially used in those experiencing worsening of the disease. The database is an efficacious and helpful tool to monitor and to manage DMD in MS: it provided information about DMD efficacy, safety and use in clinical practice. In addiction, periodical meetings of the Emilia Romagna SM Group allow a critical comparison of prescription habits among the participant clinicians. In the future, with a larger dataset, this tool will provide more solid information about the role of each DMD in MS and therefore, evidence based recommendation could be defined in order to guide prescribers.

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DRUG-INDUCED *TORSADES DE POINTES*: DATA MINING OF THE FDA ADVERSE EVENT REPORTING SYSTEM (AERS)

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Regulatory concern on drug-induced *torsades de pointes* (TdP) recently culminated in drug withdrawal from the market (e.g. astemizole, grepafloxacin, and thioridazine) or restriction of use (e.g. terfenadine and haloperidol). Given the rarity of an adverse drug reaction (ADR) such as TdP (usually less than 1 in 100,000) (1), post-marketing monitoring and especially the spontaneous reporting system are valuable tools to detect signals which may deserve further investigation. The aim of the present study was: (a) to identify cases of TdP associated with non-antiarrhythmic drugs submitted to the FDA Adverse Event Reporting System (AERS); (b) to perform qualitative and quantitative analysis in order to detect a signal that may require further investigation. AERS is a computerized information database designed to support the FDA's post-marketing safety surveillance program for all approved drug and therapeutic biologic products. Health care professionals, manufacturers, and consumers send reports voluntarily through the MedWatch program. Reports from January 2004 through December 2007 were retrieved from the AERS database. For each quarter data files, tables including drug/biologic information for as many medications as were reported for the event - DRUG file - and adverse events coded by "Medical Dictionary for Regulatory Activities" (MedDRA) terms - REACTION file - were considered. A unique number identifying an AERS report allowed linking all information from the different tables. All reports of TdP were selected from REACTION files and the relevant "primary suspect drug" (PS) was identified from DRUG files. To each trade name of drugs, the generic name and the ATC code were assigned, in order to group all cases of TdP for each drug. The qualitative analysis was performed by the case/non-case method (2). Cases were represented by TdP reports without the co-administration of antiarrhythmic agents (i.e. amiodarone, dofetilide, flecainide, ibutilide, mexiletine, propafenone, propranolol, quinidine, and sotalol) whereas non-cases were all reports of ADRs other than TdP. For each drug retrieved as PS, all trade names were identified and used to collect all non-cases. Proportional reporting ratio (PRR), a measure of disproportionality, with 95% confidence interval (95% CI) and chi-square with Yale correction were performed for each drug as a quantitative method to detect a signal. (3,4). According to current opinion, concomitant presence of PRR>2, chi-square>4 and case count>2 was used to assess disproportionality (5). PRRs and 95% CIs were calculated using the statistical package Epi Info, version 3.3.3-2005. Validation of the method was performed using sotalol as positive control. Among 1,301,839 reports of ADR, 1,436 cases of TdP were selected. Of these, 380 reports were excluded because of the co-administration of antiarrhythmic drugs. Thus, the remaining cases of TdP were 1,056 with 199 active substances identified as PS. Twenty-six drugs with at least 10 reports were detected. Among these, antibacterials (8 molecules) and antipsychotics (4 drugs) were the most represented therapeutic classes. The highest number of TdP reports (85) involved methadone. Significant PRR was found for almost all drugs although remarkable differences should be outlined between agents within each therapeutic class. Relevant and unexpected disproportionality signal was observed for cetirizine, cilostazol, donepezil, and famotidine. An extremely high disproportionality was also observed for cisapride (PRR 114.6, 95% CI 91.1-144.1) and methadone (PRR 94.3, 95% CI 76.1-116.8). Sotalol generated the expected signal (PRR 77.5, 95% CI 56.4-106.4) and turned out to be an appropriate positive control. The higher than expected signal generated by cetirizine, cilostazol, donepezil, and famotidine deserves further investigation. The strong signal associated with methadone prompts careful monitoring of methadone use.

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PIOGLITAZONE PROTECTS NEURONAL CELLS AGAINST GLUCOSE DEPRIVATION BY STIMULATING MITOCHONDRIAL BIOGENESIS

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Peroxisome proliferator-activated receptors (PPAR)γ is a ligand-activated transcription factor and two PPARγ agonists, rosiglitazone and pioglitazone, are currently in clinical use for the treatment of type II diabetes (1). A growing body of experimental and clinical data indicates that PPARγ might be a promising therapeutic target in neurological diseases. In fact, PPARγ agonists exert protective effects in animal models of stroke (2), Parkinson's disease (PD) (3), Alzheimer's disease (AD) (4), amyotrophic lateral sclerosis (ALS) (5), and two clinical trials have shown that rosiglitazone reduces the cognitive impairment in a subset of patients (those lacking ApoE ε4 allele) with mild-to-moderate AD (6,7). However, the mechanisms underling these effects remain to be elucidated. Previous findings have demonstrated that PPARγ agonists stimulate mitochondrial biogenesis in several peripheral tissues (8). Similar effects have recently been shown in the brain of mice and in neuronal-like cells treated with long-term rosiglitazone and pioglitazone, respectively (9,10). While mitochondrial biogenesis has been proposed to contribute to the effects of PPARγ agonists in diabetic patients by ameliorating cellular glucose utilisation (11), to date there is no evidence that it can contribute to the protective effects of these drugs. Based on these data, the aim of this study was to evaluate, *in vitro*, whether pioglitazone reduces neuronal cell death induced by glucose deprivation and how mitochondrial biogenesis contributes to this effect. SH-SY5Y human neuroblastoma, differentiated into neuronal-like cells, were pretreated with increasing pioglitazone concentrations (10 pM-1 μM; 5 days) and, after 24-h washout, they were glucose deprived in the absence or presence of malonate, a mitochondrial complex II inhibitor. Cell death was measured both by MTT assay and trypan blue exclusion test. Mitochondrial biogenesis was studied by measuring the expression of peroxysome proliferator-activated receptor γ coactivator (PGC)-1α, master regulator of mitochondrial biogenesis, and the level of three mitochondrial markers: mitochondrial DNA (mtDNA), cytochrome c oxidase subunit I (COX I) and subunit IV (COX IV). Cell pretreatment with pioglitazone prevented glucose deprivation-induced neuronal cell death in a concentration- and time-dependent manner. During the pretreatment period, pioglitazone increased the expression of PGC)-1α, COX I, COX IV, and the mtDNA amount, thus indicating that this drug stimulates mitochondrial biogenesis. Finally, pioglitazone was unable to protect cells against malonate-induced cell death, therefore suggesting that the protective effects of this drug require functional mitochondria. In conclusion, our data indicate that a long-term exposure to low pioglitazone concentrations stimulates mitochondrial biogenesis thus contributing to the increased cell resistance against metabolic stress.

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SYNERGISTIC CYTOTOXICITY, INHIBITION OF SIGNAL TRASDUCTION PATHWAYS, 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 tumours and, although gemcitabine produces a clinical meaningful response, there has been little improvement in prognosis. Therefore, research effort has focused on target-specific agents, such as sorafenib, which blocks both the RAF/MEK/ERK signalling pathways and receptors involved in neovascularization and tumour progression, including VEGFR-2 and c-Kit. We investigated whether sorafenib would be synergistic with gemcitabine against pancreatic cancer cell lines. Cells were treated with sorafenib and gemcitabine, alone or in combination and pharmacologic interaction was studied using the combination index (CI) method. Cell cycle and apoptosis were investigated with flow cytometry and fluorescence microscopy, respectively. Moreover, the effects of drugs on Akt (S473), c-Kit (Y823), ERK (pTpY185/187, and VEGFR2 (pY1059) phosphorylation was studied with specific ELISA. Finally, quantitative PCR analysis was performed to assess whether sorafenib and gemcitabine modulated the expression of targets related to drug activity. Sorafenib was cytotoxic against MIA PaCa-2, Capan-1, PANC-1, and BxPc3 cells with IC_{50}s of 3.48, 0.61, 4.56, and 1.33 μM, respectively. A dose dependent inhibition of cell growth was observed after gemcitabine and sorafenib treatment; the CI analysis showed that both schedules of two drugs exhibited synergism in all cell lines. Flow cytrometric studies demonstrated that gemcitabine enhanced cellular population in the S phase, whereas sorafenib was not able to significantly modulate cell cycle distribution. Cell exposure to gemcitabine resulted in a significant Akt phosphorylation inhibition, whereas sorafenib exposure reduced c-Kit, ERK, and VEGFR2 phosphorylation in all cell lines. Fluorescence microscopy demonstrated that cells treated with drugs and their combinations presented typical apoptotic morphology; in particular, drug combinations significantly increased ($P<0.05$) apoptotic index with respect to single agents. PCR showed that sorafenib enhanced the dCK/(RRM1xRRM2) ratio in MIA PaCa-2, Capan-1, and PANC-1 cells and that gemcitabine induced RKIP gene expression in all cell lines ($P<0.05$). These data demonstrate that sorafenib and gemcitabine synergistically interact against pancreatic tumour cells, through suppression of Akt, c-Kit, ERK, and VEGFR2 phosphorylation, induction of apoptosis and modulation of dCK, RRM1, RRM2, and RKIP gene expression, thus providing the experimental basis for developing this combination for the treatment of pancreas cancer.

INVESTIGATING INFLAMMATORY RESPONSE IN CARDIOVASCULAR PATHOLOGY

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Cardiovascular diseases (CVD) are the main cause of death in western societies and they could become the main cause of death globally in the next 15 years. Inflammation plays a key role in CVD and much of the current research is focused on understanding what drives this inflammation and how it is regulated. Our goal was to study pathogenetic mechanisms underlying inflammatory response in CVD in several pathological scenarios, applying high sophisticated techniques in molecular biology and imaging such as Microarray (MC) (Applied Biosystems 1700 Chemiluminescent analyzer) and Multiphoton Laser Scanning Microscopy (MPLSM) (Radiance 2000MP, Bio-Rad Laboratories). In particular we used MC technique to identify gene expression profile: 1) in a model of high glucose concentration stress on the heart, using rat isolated hearts perfused for 2 h with control Krebs solution (11.1 mM glucose) and high glucose Krebs solution (33.3 mM glucose) (1,2); 2) on human IL-17-producing CD4+ T isolated using CD4 isolation kit II (Miltenyi Biotec, Bergisch Gladbach) from patients affected by pathologies as atherosclerosis. Moreover we are focusing on multiphoton microscopy potentialities in cardiovascular medicine, with particular emphasis on tracking individual cell lineages allowing for the first time using real time imaging of vascular inflammation. MC data were obtained in the laboratories of Excellence Centre for Cardiovascular Diseases, Second University of Naples, while MPLSM images were produced in the Centre for Biophotonics, University of Strathclyde in Glasgow. MC data were validated using Real Time PCR TaqMan assays (Applied Biosystems, Foster City, CA). Statistical analysis in MC experiments has been performed using Spotfire software. We studied biological processes, molecular functions, and pathways with PANTHER classification system. Proteic expression was analyzed by Western blot. Gene expression profile studies were valued across three replicate experiments and we selected only known genes with fold change more than 1.7, $P<0.05$ and a biological function linked to our goal. In the high glucose concentration experiments we observed a significant upregulation of several genes involved in immunity defense as interleukin 1b (IL1b), interleukin 6 (IL6), chemokine (C-C motif) ligand 7 (Ccl7), chemokine (C-C motif) ligand 2 (Ccl2), chemokine (C-C motif) ligand 3 (Ccl3), chemokine (C-C motif) ligand 12 (Ccl12), chemokine (C-X-C motif) ligand 2 (Cxcl2), and an increasing phosphorilation, at different time of perfusion, of important transcriptor factors involved in the expression of them, as p38, NF-κB and Akt. These results showed that high glucose concentration is able per se to activate pro-inflammatory gene expression changes in perfused hearts by important mechanism as the activation of transcription factors. MC results performed on human CD4+ T-cell IL-17 producing cells clones showed a significant up regulation of CD161 gene in comparison with either TH-1 or TH-2 clones. A series of important experiments performed in the University of Florence on the basis of MC results, have identified CD161 as the most important surface marker of human IL-17-producing cells (3). These important results shed light on new targets for the study of immune response in many pathologies such as CVD disease. Finally, by MPLSM we were able to obtain the 3D imaging of an atherosclerotic plaque in an isolated ApoE$^{-/-}$ mouse aortic arch, and identification of fluorescent adoptively transferred leukocytes in an atherosclerotic prone carotid artery. These data show homing of lymphocytes to atherosclerosis-prone arterial sites using real-time imaging of intact arteries and for the first time 3D reconstruction of the *en face* atherosclerotic plaque at a cellular level. At the least these results give us the possibility to propose MPLSM as a powerful tool for imaging immune cells in CVD.

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POSSIBLE ROLE OF NOCICEPTIN/ORPHANIN FQ-NOP RECEPTOR SYSTEM IN THE MODULATION OF NOCICEPTION AND MOOD ALTERATIONS

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Nociceptin/orphanin FQ (N/OFQ) and its receptor (NOP) are widely distributed in the central nervous system (CNS), where they modulate several functions such as pain, anxiety, stress, learning, memory, food intake, and drug addiction. On this basis, the purpose of the present research was to investigate, in the rat, the role of NOP ligands in: a) modulation on nociception in paracetamol-induced analgesia; b) anxiety-related behaviours after development of tolerance to hypolocomotor effects; c) exposure to chronic stressful conditions which cause depression. As regards point a), we have demonstrated that both the antinociceptive effect of paracetamol (400 mg/kg, i.p.), evaluated by means of the hot-plate test (1) and the changes in central serotonin content were completely abolished by administration of N/OFQ (10 nmol/rat, i.c.v.) and restored by a pre-treatment with the NOP antagonist UFP-101 (20 nmol/rat, i.c.v.). In anxiety experiment (point b), a double i.c.v. injection on N/OFQ dose-dependently decreased the expression of anxiety-related behaviour in both the elevated plus maze and the conditioned defensive burying tests without affecting locomotor activity. UFP-101 significantly reduced the effects of N/OFQ to control values in either test (2). In the stress paradigm (point c), rats were exposed to chronic mild stress (CMS; 3) for a period of at least 6 weeks to induce a condition of anhedonia, measured as reduction of 1% sucrose solution intake. The stressed groups were treated, once a day, with UFP-101 (5, 10, and 20 nmol/rat, i.c.v.), imipramine (IMI, 15 mg/kg, i.p.), or saline for 21 days. UFP-101 reinstated sucrose solution intake within the first week of treatment following the highest dose; at 10 and 5 nmol/rat it abolished the reduction in sucrose intake from the second and third week of treatment, respectively. The restoration of sucrose consumption, once induced, remained stable up to the end of the experiment for all treatments. Rats were submitted to the forced swimming test (FST) on day 22, 24 h after the last treatment. On day 23 rats were decapitated, their blood collected to measure corticosterone (CORT) content and brains removed for 5-HIAA/5-HT analysis. In the FST, 24 h after the last administration, all UFP-101 treatments decreased the time of immobility to that of non stressed controls. IMI produced similar effects on sucrose intake and on the FST. Pre-treatment with either UFP-101 at the higher doses or with IMI completely abolished the increase in CORT induced by CMS. 5-HT turnover was increased by CMS in the frontal cortex and decreased in the pons; UFP-101, as well as IMI, was able to revert these changes to values comparable to those of non stressed controls. Repeated coadministration of N/OFQ (5 nmol/rat, from day 12 to day 21) completely prevented the behavioural and biochemical effects of UFP-101 (10 nmol/rat). Our results showed that UFP-101 reversed the CMS-induced changes in behaviour, HPA axis control, and central 5-HT turnover. On the whole, the present findings support the view that the N/OFQ-NOP system represents an important candidate target for the development of innovative therapeutics for several neurological conditions, including nociception, and psychiatric diseases, chiefly involving anxiety and depression.

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NOCICEPTIN/ORPHANIN FQ-NOP RECEPTOR SYSTEM IN SENSITIZED MOUSE LUNG BRONCHOCONSTRICTION

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Nociceptin/Orphanin FQ (N/OFQ) is a heptadecapeptide derived from a larger precursor protein prepro-N/OFQ (1). This peptide is the endogenous ligand of an opioid like Gprotein coupled receptor recently named the N/OFQ peptide receptor (NOP) (2). This receptor has an overall 60% homology with the classical MOP, DOP, and KOP (μ, δ, κ) opioid receptors (3). Recent studies indicate that N/OFQ has a broad spectrum of physiological functions and pharmacological effects in both the central and peripheral nervous systems as well as in some non-neuronal tissues (2). In the central nervous system, NOP receptor activation by N/OFQ inhibits the release of several neurotrasmitters including noradrenaline and glutamate (4). In the periphery N/OFQ is able to inhibit excitatory non-adrenergic non cholinergic responses in isolated bronchi *via* an inhibition of tachykinin release from non myelinated C-fibres of afferent sensory terminal nerves that innervate all compartments of pulmonary wall from trachea to bronchiole. The aim of the current study was twofold: first, to evaluate the role of the N/OFQ-NOP receptor system in bronchoconstriction induced by sensory nerve activation using the novel and selective NOP receptor agonist/antagonist UFP-112/UFP-101 and knock out mice for the NOP receptor ($NOP^{-/-}$); second, using actively sensitized and ovalbumin (OVA) challenged mice, to investigate the role of the endogenous N/OFQ in allergen sensitization mechanisms. We used 9-12 week old C57BL/6J ($NOP^{+/+}$ and $NOP^{-/-}$) and Balb/C mice sensitized or not to OVA. By using an isolated perfused mouse lung model, bronchopulmonary function in presence or in absence of NOP selective agonists/antagonists coupled with measurements of endogenous N/OFQ levels before and after capsaicin induced bronchoconstriction were evaluated. N/OFQ significantly inhibited capsaicin induced bronchoconstriction in both naïve and sensitized mice, these latter animals displaying airway hyper responsiveness to capsaicin. The inhibitory effect of N/OFQ were mimicked by the selective NOP agonist UFP-112 and abolished in $NOP^{-/-}$ and by the selective NOP receptor antagonist UFP-101. UFP-101, when used alone, induced airway hyper responsiveness to capsaicin in naïve but not in sensitized mice. Endogenous N/OFQ levels significantly decreased in sensitized with respect to naïve mice. Confirming the role of the N/OFQ-NOP receptor system in the inhibition of capsaicin induced bronchoconstriction, for the first time we have documented different airway responsiveness to capsaicin between naïve and sensitized mice due, at least in part, to decreased endogenous N/OFQ levels in the sensitized mice. Because airway hyper responsiveness is a crucial pathological process in asthma, this study could add the N/OFQ-NOP system to the list of possible targets in the therapy of asthma.

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ANTIHYPERTENSIVE DRUGS USE IN ITALIAN GENERAL PRACTICE

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The aim of the present study was to evaluate the use of antihypertensives in general practice. Data source was Arianna database "Caserta-1" Local Health Service. Among 127,389 individuals >15 years, registered in 93 general practitioners' lists during 2003-2005, patients affected by hypertension receiving at least one prescription of antihypertensives (ACE-inhibitors, α-blockers, β-blockers, calcium channel blockers, diuretics, sartans) were selected and analysed. Prevalence and incidence of use/100 inhabitants and defined daily doses (DDD) for 1,000/inhabitants were calculated. One-year prevalence of use (approximately 20%) and one-year incidence of use of antihypertensives (3.3%) were close all through the periods considered. Nonetheless, DDD/1,000 inhabitants increased from 226.5 in 2004 to 258.7 in 2005. ACE-inhibitors were the most prescribed drugs, in more than 50% of treated patients (20% associated with hydrochlorotiazide); no variation between 2003 and 2005. Conversely, sartans prevalence increased from 23.6% in 2003 to 26.8% in 2005, due to the improved prescription of diuretic-fixed association (10.8% in 2003; 14.8% in 2005). For the most part new users started with ACE-inhibitors both in 2004 and 2005. Nevertheless, a slight decline was observed in 2005 (42.1% vs 40.7%). On the contrary, sartans new users increased in 2005 (15.1% vs 16.7%) due to diuretic-fixed association. Although prevalence and incidence of antihypertensives use was unmodified from 2003 to 2005, DDD/1,000 inhabitants increased. ACE-inhibitors prescriptions and users were reletively stable. On the contrary, sartans use and, in particular, fixed association, increased also as starting treatment, even though the high cost and lack long-term effectiveness. This data could be considered in developing strategies aimed to increase the appropriateness of hypertension treatment.

SET UP OF A CO-CULTURE MODEL AS A TOOL FOR THE STUDY OF NEUROVASCULAR DISEASES

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Interactions between endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) play an important role in the maintenance of normal vascular structure, function and homeostasis (1). It has been reported that ECs modulate VSMCs proliferation and migration and consequently vessel diameter and thickness (2). Furthermore cell-cell interaction of ECs and VSMCs affects the expression of regulatory components such as fibrinolytic, coagulation, and angiogenic factors (1). In several pathologies the interaction between ECs and VSMCs plays a role and contribute to the onset and development of disease. Among all the pathologies in which the vascular compartment is involved my interest is focused on cerebral amyloid angiopathy (CAA). It refers to pathological changes occurring in cerebral blood vessels caused by deposition of amyloid protein (3). CAA is commonly associated with normal ageing and Alzheimer's disease and it is also the principal feature of hereditary haemorrhage with amyloidosis (4). My project is the development of an ECs/VSMCs co-culture model reproducing *in vitro* the vessel wall of the brain. The cell lines used along all the experiments are of human origin; both ECs and VSMCs are from brain microvessels and umbilical cord vessels. In order to evaluate the reciprocal cell response and direct interaction, ECs and VSMCs were plated on the surfaces of the same co-culture insert, named Transwell, one opposite to the other. Alternatively ECs were plated on the well bottom of a multiwell plate, while VSMCs on the inner surface of the insert, thus allowing an indirect interaction due to the mediators released by ECs. Preliminary data, in the matter of CAA, suggest that ECs, upon administration of amyloid protein both at different concentrations and at different times, exert a toxic effect on VSMCs impairing their proliferation and viability.

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SOCIAL ISOLATION ALTERS NOX EXPRESSION IN RAT CENTRAL NERVOUS SYSTEM

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Schizophrenia is a multidimensional mental disorder, involving emotional and cognitive dysfunctions (1). Recent evidence suggests that loss of fast-spiking interneurons might be an important biological feature of the disease. There is also increasing evidence that reactive oxygen species (ROS) play an important role in the development of schizophrenia (2). Progress in the understanding between oxidative stress and psychotic disease requires animal models. In a mouse model of ketamine-induced psychosis, a role for NOX-derived superoxide production in the loss of phenotype of fast-spiking interneurons has been shown. Another interesting animal model of schizophrenia is social isolation rearing, which also leads to the loss of fast spiking interneurons (3). In this study we have investigated the expression of NOX genes in the central nervous system of normal rats and rats after social isolation rearing. Virtually no NOX2 expression was found in the central nervous system of normal rats, while NOX1 was found in nucleus accumbens, prefrontal cortex and striatum, but not in amygdala and hippocampus. Social isolation rearing dramatically changed the expression patterns of NOX enzymes. Generally speaking NOX2 expression increased, while NOX1 expression decreased. More specifically, social isolation induced moderate expression of NOX2 in amygdala and hippocampus, and a strong expression of NOX2 in the nucleus accumbens. NOX1 gene expression was strongly decreased in the prefrontal cortex of isolated rats. Our results demonstrate that social isolation rearing has a striking effect on the expression of NOX enzymes in the central nervous system. The localization of the most striking changes is of major interest with respect to psychotic disease. Indeed, in schizophrenia patients and models, the nucleus accumbens is the most affected area of the brain in terms of neuronal signalling, while in the prefrontal cortex there is a decreased synaptic plasticity. Further research will be necessary to establish whether there is a causal link between the altered expression of NOX enzymes and the pathological changes in these brain areas. While the role of NOX1 and NOX2 gene is still not well understood, these preliminary data open new perspectives about the involvement of oxidative stress in schizophrenia.

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AGONISTS OF THE CANNABINOID RECEPTOR TYPE 1 (CB1) INDUCE A PROLIFERATIVE RESPONSE IN CEREBELLAR NEURAL PROGENITOR CELLS

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Cannabinoids and their endogenous counterparts, endocannabinoids, have been shown to control proliferation and differentiation of neural stem/progenitor cells in the hippocampal subgranular zone and in the subventricular zone of the embryonic and adult mammalian brain (1). To elucidate the cellular and molecular mechanisms underlying cannabinoid neurogenic action, we used neural progenitor cells isolated from primary cultures of rat post-natal cerebellum as an *in vitro* model of neural cell proliferation. Phenotypical and genotypical characterization of these cells by immunocytochemistry and RT-PCR, respectively, has shown that they share some of the properties of stem cells (2). The functional presence of the two cannabinoid receptors CB1 and CB2 in cerebellar neural progenitor cells at 10 days *in vitro* (DIV) was assessed with immunocytochemistry and Western blot analysis. To evaluate the proliferative effect by [$_3$H]-thymidine incorporation assay, cerebellar neural progenitor cells at 10 DIV were incubated for 24 h with increasing concentrations (1-1000 nM) of the non-selective synthetic cannabinoid agonists WIN-55,212-2 or CP-55,940 in the absence or in the presence of the selective antagonists at CB1 and CB2 receptor, AM 251 (10-1000 nM) or AM 630 (10-1000 nM), respectively. WIN-55,212-2 (100 nM) and CP-55,940 (1 μM) significantly increased [$_3$H]-thymidine incorporation by 35.3±9.5% and 20.2±7.9%, respectively; both proliferative responses were completely abolished by AM 251 treatment but not by AM 630. The direct involvement of CB1 receptor in cannabinoid induced cerebellar neural progenitor cell proliferation was confirmed using ACEA, a potent CB1 selective agonist. ACEA (1 nM and 10 nM) significantly increased [$_3$H]-thymidine incorporation (by 37.9±19.2% and 37.8±13.6%, respectively) and this effect was completely reverted by 10 nM AM 251. Western blot analysis showed that the incubation of cerebellar neural progenitor cells for 5-60 min with ACEA (1 nM and 10 nM) produced an ERK activation suggesting the involvement of the MAPK/ERK cascade in CB1-induced proliferation. Research are in progress to define a possible cross talk between PI3K/Akt/GSK-3 and MEK 1,2/ERK signalling pathways in mediating the cannabinoid proliferative effect.

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AN *IN VITRO* STUDY ON THE EXPRESSION AND ROLE OF UROTENSIN-II AND ITS RECEPTOR IN HUMAN VASCULAR ENDOTHELIAL CELLS

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Urotensin-II (U-II) is a cyclic peptide, originally isolated from the urophysis of the goby *Gillichthys mirabilis*. The human form of U-II (hU-II) is a cyclic undecapeptide. Mature hU-II is synthesized from a large precursor molecule, the prepro-U-II, whose mRNA has been found in many tissues. U-II has been identified as the endogenous ligand of a specific high-affinity receptor, recently identified as the orphan receptor GPR14 (1) which has been renamed urotensin receptor (UT-R). The human UT-R isoform belongs to the class A superfamily of G-protein-coupled receptors. The principal physiological role of U-II in mammals is in the cardiovascular system, where it exerts a potent systemic vasoconstrictor and hypertensive effect and several lines of evidence suggested that U-II might be involved in the pathophysiology of the cardiovascular system (2). On endothelial cells (ECs) of animal origin, U-II has also been shown to exert a clear-cut pro-angiogenic effect (3), but few experimental data are presently available clarifying the effect of U-II in human endothelium. Thus, in the present study, *in vitro* models based on human vascular ECs of both venous and arterial origin have been used to further evaluate the angiogenic properties of U-II. Immunocytochemical analysis showed that human ECs expressed VEGF and VEGF receptors. ECs of venous origin also expressed UT-R, but not the U-II peptide. An opposite pattern of expression was observed in ECs from artery, in which U-II resulted expressed while UT-R was undetectable. The presence (or absence) of these markers was always confirmed by RT-PCR. From a functional point of view, some heterogeneity was observed among the human vascular ECs analysed. In the Matrigel assay, ECs from different vascular beds exhibited at baseline different properties to self-organize, the arterial ones generating a pattern of capillary-like structures of lower extension and complexity as compared to the pattern generated by ECs of venous origin. The angiogenic assay, however, provided unequivocal evidence that, on the ECs from human veins, U-II exerted a potent angiogenic action *in vitro*, comparable to that of FGF-2. Moreover, the activity of UII resulted specifically triggered by the binding of U-II to its receptor, being suppressed by the specific antagonist palosuran. Consistently with the above mentioned pattern of UT-R expression, U-II was unable to induce any pro-angiogenic effect on arterial ECs. Remarkably, the pro-angiogenic action of U-II was not associated with a proliferogenic effect on human ECs, in contrast with that of FGF-2. Altogether, the results of the present study suggest that U-II, in addition to regulating cardiovascular function, also exerts a direct action on the development and remodelling of the venous vascular network.

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ENDOCANNABINOIDS MODULATE CXCR4-EVOKED TNFα RELEASE AND GLUTAMATE EXOCYTOSIS FROM ASTROCYTES

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During the past few years, a number of papers have suggested that chemokines have physiological functions in addition to their roles in neuroinflammatory diseases. The best evidence concerns the CXC-chemokine stromal cell-derived factor (SDF-1α or CXC12) and its receptor CXCR4, whose signalling cascade is also implicated in the Ca^{2+}-dependent process leading to glutamate exocytosis from astrocytes (1). The glutamate release process activated by both the endogenous ligand SDF-1α and the HIV-1 glycoprotein gp120, was described as a complex signalling pathway involving the extracellular release of glutamate, TNF-α and prostaglandins (2) that, in the presence of activated microglial cells was dramatically amplified leading to neurotoxicity (3). Interestingly, recent studies have suggested the involvement of the endocannabinoids system in the neuronal apoptosis and neuroinflammatory pathways caused by gp120 (4) and in the neuron-astrocyte fast communication systems in the brain (5). In particular it has been shown that hippocampal astrocytes express functional CB1 receptors, which upon activation lead to Ca^{2+} mobilization and glutamate release (5). Here we investigated the role of endocannabinoid system (ES) in the CXCR4-mediated TNF-α and glutamate release from astrocytes. In the first part of our work we exposed human astrocytoma cells (U373MG) to gp120 (200 pM) for 1 h and measured the activity of enzymes of the ES, the endogenous levels of endocannabinoids, together with the levels of TNF-α in the extracellular medium. We found that exposure of cells to gp120: a) increased the activity of the specific enzyme involved in the degradation of anandamide (AEA), i.e. fatty acid amide hydrolase (FAAH; about +80%, $P<0.05$), with a parallel reduction of ~40% ($P<0.05$) of the endogenous levels of AEA; b) increased the extracellular levels of TNF-α (about +85%, $P<0.05$). Interestingly, when cells have been pre-incubated with a FAAH inhibitor (URB509, 1 μM, 15 min), the release of TNF-α was abolished (about -85%, $P<0.05$), consistent with the involvement of endogenous AEA in the mechanism that controls the release from astrocytes of TNF-α and, possibly, of glutamate. In the second part of our work we wanted, therefore, to elucidate whether the Ca^{2+}-dependent glutamate exocytosis from astrocytes represents a target of (endo)cannabinoids. To investigate whether primary cultures of cortical astrocytes express functional CB1/CB2 receptors (CBRs) we performed: a) double immunolabelling experiments with antibodies against CBRs together with GFAP; b) singlecell Ca^{2+} imaging experiments by preincubating cells with the Fluo4-AM calcium indicator and by studying the intracellular Ca^{2+} increase upon the activation of CXCR4 (SDF-1α, 3 nM) and of CBRs (with the agonists methanandamide and ACEA, and the antagonist AM251). We then studied CXCR4-mediated exocytosis in astrocytes by taking advantage of a state-of-the-art imaging technology recently developed to investigate synaptic vesicles turnover in the nerve terminals (6). We: a) devised a strategy to specifically load vesicles expressing vesicular glutamate transporters 2 (VGLUT2) with markers of fusion events such as FM 4-64; b) combined epifluorescence and total internal reflection fluorescence (TIRF) imaging (1) to study the CXCR4-mediated glutamate exocytosis in the absence and in the presence of CBRs agonist and antagonists.

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ROLE OF NITRIC OXIDE/Ca^{2+}/ERK1/2 MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY IN INFLAMMATION-INDUCED ASTROCYTE ACTIVATION

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In a wide range of neuroinflammatory conditions accompanying brain injury, hypoxia/ischemia or neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and multiple sclerosis, astrocytes undergo a remarkable transition from quiet nursing cells to a state, referred to as "reactive" (1,2), characterized by cell hypertrophy with upregulation of glial fibrillary acidic protein (GFAP) (3) and hyperplasia (astrogliosis) with predominant increase in astrocytic proliferation and formation of astroglial scar, a process which is considered the major impediment to axonal regeneration after brain damage (4). Despite reactive astrocytes represent a consistent feature in all acute and chronic brain inflammation, a clear characterization of the astrocytic hypertrophic vs hyperplastic response is still lacking. To achieve this, in the present work we used human astrocytoma U-373 MG cells stimulated with IL-1β, a major neuroinflammatory cytokine (5,6) as an *in vitro* inflammation model to study the time-course profile of GFAP expression vs proliferation and the signalling pathway underlying these effects. Serum starved cells were treated with IL-1β for 1 h, washed and then reincubated for different periods in the absence of IL-1β. Thereafter, cells were fixed and stained with DAPI for cell counting. In another series of experiments, p42/44 extracellular signal-regulated kinases (ERK) and GFAP activities were assessed: cells were lysed, protein separated by 10% SDS-PAGE, and Western-blotted with anti-phospho-p42/44, or fixed in 4% paraformaldehyde and incubated with anti-GFAP primary antibody, followed by immunofluorescent secondary antibody. GFAP positive cells were determined by imaging and expressed as fluorescence intensity per unit area. Apoptosis was determined in 4% paraformaldehyde fixed cells after treatment with active anti caspase-3, followed by immunofluorescent secondary antibody. The percentages of caspase-3 positive cells were expressed relative to DAPI positive cells. Data showed that 24 h after treatment, low IL-1β concentrations induced a dose-dependent upregulation of cell proliferation (124.6±7.4% and 221.8±5.9 above control, for 0.1 and 1 ng/ml, respectively) which paralleled ERK activation (43±12% and 155±8.9 above control, for 0.1 and 1 ng/ml, respectively) whereas high IL-1β levels reversed both these effects by promoting apoptosis. Pre-treatment with the unspecific or the selective iNOS inhibitor, N-ω-nitro-l-arginine methyl ester (L-NAME) and N-[[3-(aminomethyl)phenyl]methyl]-ethanimidamide dihydrochloride (1400W), respectively, antagonised cell proliferation and ERK activation induced by IL-1β. Functionally blocking Ca^{2+} release from endoplasmic reticulum with ryanodine and 2-aminoethoxydiphenylborane (2APB), or inhibiting ERK activity with 1,4- diamino-2,3-dicyano-1,4-bis[2-aminophenylthiol]butadiene (U0126) downregulated IL-1β-induced cell proliferation as well as ERK activation. IL-1β induced an increase in GFAP expression which reached the peak value (70.0±13.1% above control) after 12 h, at which time the proliferative response was absent. Pretreatment with U0126 antagonized the increase in GFAP expression induced by IL-1β. All together these data show that the hypertrophic and hyperplastic responses represent two temporally separated processes of the astrocyte activation, and identified the NO/Ca^{2+}/ERK signalling pathway as a novel mechanism possibly mediating both these effects. Since abnormal astrocyte activation serves detrimental effects in brain repair after damage, our data raise the possibility that modulating or partly blocking this signalling pathway could turn out to be valuable as a neuroinflammatory disease therapy.

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NOVEL PHARMACOLOGICAL APPROACHES TO THE TREATMENT OF ISCHEMIC CARDIOMYOPATHY: FOCUS ON IVABRADINE

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Despite substantial advances in medical therapy, heart failure (HF) still remains a leading cause of global morbidity and mortality in the world. Epidemiological studies indicate that mortality rate due to heart failure increases according to severity of prognosis. Of note, approximately 50% of these deaths are sudden and unexpected, and presumably the consequence of lethal arrhythmias. During my PhD course I have focused my studies to investigate new therapeutic drugs and target for the treatment of HF. In the first year, I focused my attention on late sodium current and its inhibitor (ranolazine) as a potential target/drug for treatment of ischemic cardiomyopathy. In the second year, I studied 3-iodothyronamine, an endogenous derivative of thyroid hormone that through a new signalling pathway is involved in heart failure. In this last year, I studied the effect of ivabradine (IVA), a selective bradycardic agent approved for therapeutic use, on chronic treatment of post-myocardial infarction (MI) rats. The results of this study are reported thereafter. Seven days after coronary legation, MI rats were treated with 10 mg/kg/day IVA (MI+IVA) or vehicle (MI) (drinking water) for 90 days. SHAM rats were used as controls. Heart rate (HR) was measured by echocardiography at 0, 1, and 3 months. Rats were sacrificed at 3 months and isolated cells from left atria (LA), left ventricle (LV), and right ventricle (RV) used for functional and molecular investigation. The f-current (I_f), transientoutward (I_{to}) current, and action potential (AP) were recorded by patch-clamp. Quantitative RT-PCR was used to investigated mRNA level of HCN2, HCN4 (encoding for f-channel), $Kv_{4.2}$ (encoding for transient-outward channel) isoforms, and ANP. HR was significantly reduced (-12%) in MI+IVA vs MI or SHAM at 1 and 3 months. mRNA level of ANP, an index of fetal gene re-expression during LV remodelling, which was below detection limit in SHAM, was markedly increased in MI (13 fold vs SHAM). This up-regulation was attenuated by 50% in MI+IVA group (6.5 fold vs SHAM). Electrophysiological data show that in LV cells, action potential duration (APD) measured as percentage (30, 50, 70, and 90%) of repolarisation, was homogeneously and significantly prolonged in MI (APD_{30}: 45±8 ms vs 23±5 ms; APD_{50}: 72±13 ms vs 32±6 ms; APD_{70}: 108±18 ms vs 51±7 ms; APD_{90}: 177±34 ms vs 88±12 ms, $P<0.05$, MI vs sham); this effect was attenuated by -20% in MI+IVA at 30% and 50% of repolarisation and by -13% both at 70% and 90% of repolarisation. In LA and RV cells, no significant differences were observed with IVA among all groups. Down regulation of I_{to} current plays a pivotal role in APD prolongation in HF. In LV cells, peak I_{to} (measured at +50 mV) was significantly reduced in MI to 53% of SHAM. This effect was partially but significantly reverted (76% of sham) in MI+IVA. According to AP measurements, I_{to} density was not significantly different in LA and RV cells vs SHAM or MI treated or not-treated with IVA. Consistently with electrophysiological measurements, quantitative expression of $Kv_{4.2}$ was significantly decreased in LV samples by 43% ($P<0.05$) vs SHAM and partially recovered to 80% ($P=0.057$) vs SHAM in MI+IVA rats, but in LA and RV myocytes, like I_{to} density, $Kv_{4.2}$ expression was similar in MI+IVA and MI rats. I_f density in LV and LA cells was higher in MI with respect to SHAM but not in MI+IVA (in pS/pF, LV: 67±12, n=21, vs 27±6, n=12; LA: 18±6, n=9, vs 53±14, n=13, $P<0.05$). Activation curve of I_f was similar in all groups. mRNA HCN2 and HCN4 increased in MI and returned toward control values in MI+IVA (HCN2: 0.9±0.1, n=6, 1.9±0.2*, n=5, 1.3±0.2 n=5; HCN4: 1.0±0.1, n=6, 5.9±1.2*, n=5, 2.0±0.5, n=5,*$P<0.05$; SHAM, MI and MI+IVA, respectively). Functional expression of fcurrent was rarely detected in SHAM RV myocytes. In a well-established rat model of heart failure, a 3-month treatment with IVA partially counterbalances structural and electrophysiological remodelling in LV, LA, and RV myocytes. These results support a beneficial effect of HR lowering thus suggesting a rationale for IVA use in the treatment of HF.

HYDROXYTYROSOL: A NEW NATURAL ANTIANGIOGENIC COMPOUND?

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Prostaglandin E_2 (PGE_2), the major product of cyclooxygenasese-2 (COX-2), is an important promoter of tumour growth and is also a potent angiogenic factor. Recent studies on human colon cancers have demonstrated that expression of COX-2 and the increased production of PGE_2 are correlated with vascular endothelial growth factor (VEGF) expression and angiogenesis, which is transcriptionally mediated by hypoxia inducible factor-1α (HIF-1α). Differences in lifestyle among populations over the world may play a significant role in the risk of developing a colon cancer. Epidemiological studies support the beneficial effects of the Mediterranean diet in human health, particularly in the prevention of cardiovascular disease and cancer. In particular, the beneficial effects of virgin olive oil could be linked to both its monounsaturated fatty acid and its antioxidant content. Among the antioxidants in olive oil, hydroxytyrosol or 2-(3,4-dihydroxyphenil) ethanol (DPE) has protective effect against oxidative stress-related damage. Many *in vivo* studies show the biological, for example, anti-inflammatory and anti-thrombotic, activities of DPE. In this study, we investigated the effect of DPE on PGE_2 induced HIF-1α expression and angiogenesis in human colon adenocarcinoma cells. Our results show that DPE inhibits PGE_2-induced HIF-1α and angiogenesis. Pre-treatment of cells with DPE significantly reduces PGE_2-induced production and VEGF expression at protein level and the PGE_2-induced pseudocapillary like structures in an *in vitro* model of co-colture of endothelial and colon cancer cells. These data suggest the potential antiangiogenic role of a Mediterranean diet component, hydroxytyrosol, specifically targeting HIF-1α.

MOLECULAR AND FUNCTIONAL ANALYSIS OF *DROSOPHILA* ATLASTIN GENE

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The hereditary spastic paraplegias (HSP) encompass a diverse spectrum of neurodegenerative disorders in which the predominant feature is the progressive spasticity associated with mild weakness of the lower limbs. The SPG3A gene, encoding atlastin-1 protein, has been identified as the locus responsible for a form of HSP characterized by the earliest onset. Atlastin-1 protein sequence shows homology to large GTPases of the dynamin superfamily: it contains a large GTPase domain and two transmembrane domains targeting the protein to either Golgi (1) or endoplasmic reticulum (ER) (2,3) membranes, and is capable of oligomerization (1). Atlastin-1 has been supposed to be implicated in ERGolgi vesicle trafficking (2) and in Golgi and ER morphogenesis (4), but its precise function remains unexplained and consequently the pathological mechanism underlying disease remains unknown. The *Drosophila* genome contains a much conserved atlastin-1 ortholog (D-atlastin) making the fly a valuable system to study its fuction. *In vivo* analysis in *Drosophila* reveals that at the subcellular level D-atlastin is highly enriched in and colocalizes with ER markers. To clarify D-atlastin biological role, transgenic flies for its overexpression and knockdown were generated: loss of D-atlastin function causes fragmentation of the ER network, while the overexpression modifies tubular ER profiles into expanded cisternae. Because D-atlastin is capable of homo-oligomerization, which can stimulate the GTPase activity of dynamine superfamily members (5), our hypothesis is that D-atlastin is involved in the process of homotypic fusion of ER membranes. To better understand D-atlastin role at the ER membranes, we are looking for interacting proteins. Forced overexpression of D-atlastin in the developing eye of *Drosophila* results in a rough eye phenotype. We are screening a large collection of deficiencies for dominant suppressor and enhancers of the D-atlastin induced rough eye phenotype. In addition, we are isolating proteins that bind to D-atlastin using the co-immunoprecipitation technique.

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ANTAGONISM OF ADENOSINE A_{2A} RECEPTORS DURING *IN VITRO* ISCHEMIA DELAYS ANOXIC DEPOLARISATION AND AMELIORATES NEURONAL SURVIVAL IN THE RAT CA1 HIPPOCAMPUS

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Anoxic depolarization (AD) is an event observed during cerebral ischemia both *in vivo* and *in vitro* and it has been strictly correlated with the extent of brain damage in this pathological condition (1). Ischemia is accompanied by an increase in adenosine outflow throughout the brain (2). In the hippocampus, adenosine acts by stimulation of the specific receptors A_1, A_{2A}, A_{2B}, and A_3 (3). Whereas the neuroprotective role of adenosine A_1 receptors is well defined, the function of the other receptors subtypes is still controversial. The aim of this study was to asses the involvement of adenosine A_{2A} receptors on the electrophysiological changes induced by oxygen and glucose deprivation (OGD) and on cell vitality and astrocytic response in the CA1 region of rat hippocampal slices. Extracellular field excitatory post-synaptic potentials (fepsps) and the negative voltage shift, as signature of AD, were recorded from the CA1 dendritic layer. *In vitro* OGD was induced by switching to an artificial cerebrospinal fluid solution without glucose in which oxygen was replaced by nitrogen. Each slice was subsequently stained with histochemical or immunohistochemical methods and visualized with a confocal laser-scanning microscope. In the absence of any pharmacological treatment, 7-min OGD always elicited an irreversible loss of neurotransmission and were invariably followed by the appearance of AD, with a mean latency of 6.6±0.1 min and a mean peak amplitude of 7.0±0.5 mV (n=24). The application of the selective adenosine A_{2A} receptor antagonist, 4-2-[7-amino-2-(2-furyl) [1,2,4]triazolo-[2,3-a][1,3,5]triazin-5-yl-amino]ethylphenol (ZM 241385, 100 nM, n=22) before, during, and after 7-min OGD, prevented AD appearance and elicited a consistent fepsp recovery of 94.4±5.0% (n=22, $P<0.001$), in comparison to that found in untreated OGD slices (5.4±2.8%, n=24). Moreover, fepsp recovery was maintained for several h after the end of OGD (n=11). When tested on OGD episodes of longer duration (30 min), ZM 241385 (100 nM, n=11) delayed significantly the latency of AD to 9.0+0.4 min in comparison to 7.2+0.2 min found in the absence of the antagonist. Conversely, the selective A_{2A} agonist 2-[4-(2-*p*-carboxy-ethyl)phenylamino]-5'-N-ethylcarboxamidoadenosine (CGS 21680, 50 nM, n=9) did not modify AD latency. In order to assess the cell damage caused by ischemia, 3 h after OGD, hippocampal slices were stained with propidium iodide (PI, 5 µg/ml), a fluorescent compound that enters cells with disrupted plasma membrane. A substantial CA1 pyramidal neuronal damage after OGD was detected (n=4), an effect significantly reduced by 100 nM ZM 241385 ($P<0.05$, n=4). Moreover, glial fibrillary acidic protein (GFAP) immunostaining showed, 3 h after OGD, an evident astrogliosis that was not substantially modified by ZM 241385 (100 nM) application. Our results indicate that in the CA1 region of rat hippocampal slices, the selective blockade of adenosine A_{2A} receptors exerts a protective effect during ischemia by delaying AD appearance and increasing neuronal survival, therefore reducing damage propagation from the ischemic core to the salvageable surrounding area.

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GENETIC POLYMOPRPHISMS IN IMATINIB TRANSPORTERS AS DETERMINANTS OF THE PHARMACOLOGICAL RESPONSE

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Chronic myeloid leukaemia (CML) is genetically characterized by chromosome Philadelphia (Ph), the result of the reciprocal translocation between chromosome 9 and 22. Ph produces an aberrant protein (Bcr-Abl) with constitutive tyrosine kinase (TK) activity. The knowledge of the unique genetic lesion responsible for the pathogenesis of CML as paved the way for the development of targeted therapies. TK Inhibitors (TKI) blocking the deregulated Bcr-Abl gene product is the first successful example of targeted therapy. The first TKI entering the clinical practice - imatinib mesylate (IM) - is now the first-choice treatment of CML. Though the enormous success of CML therapy with IM, refractoriness and resistance have been observed in a proportion of patients. Some cases of resistance are due to over-expression of Bcr-Abl, which is surmounted increasing IM dosage; other to mutation in Bcr-Abl, abrogating IM binding site, for which TKIr of second generation (i.e. nilotinb, sunitinib, etc.) are under study. However, for a large fraction of cases, the origin of resistance is still unknown. It is now clear that blood and tissue concentration of most drugs are influenced by interindividual variation in genes encoding drug metabolising enzymes and transporters. Differential expression of influx (hOCT1) and efflux (MDR1) transporters may be a critical determinant of intracellular drug levels and, hence, resistance to IM. Purpose of this study was to investigate the role of genetic variants (SNPs) in genes encoding transporters of IM, as candidate of IM responsiveness. A population of 184 patients, 121 responder and 63 non responder, was included in the study. Genomic DNA was extracted using DNA-Blood mini Kit (QIAGEN); genes variants were assessed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and real-time-PCR (Applied Bio-System 7300). Deviation from Hardy-Weinberg equilibrium was checked by χ_2 test. Differences in genotypes distribution between responders and non responders was estimated using logistic regression analysis. Polymorphisms in the MDR1 gene, *8 and *6, were under represented in non responder as compared to responders, however the differences are not statistically significant. Analysis of MDR1 haplotypes revealed that responder had a marginally significant lower frequency of wild-type MDR1 *6 and *8 compared to non responder (24.8% vs 33.3%; P=0.049). We did not find any differences in genotype frequencies between responder and non responder for the other SNPs studied. In conclusion SNPs in MDR1 may support the identification of individuals at risk of IM resistance. Currently other SNPs in MDR1 and hOCT1 polymorphisms are under study in order to identify a panel of SNPs with predictive value allowing optimising the therapeutic use of IM in CML patients.

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MICROARRAY ANALYSIS ON THE HIPPOCAMPUS OF A RAT MODEL OF DEPRESSION

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Although the neurobiological basis of depression has not been fully elucidated, accumulating evidence suggests that neuroplasticity and stress are key factors of the pathophisiology of depression. The coordinated behavioural, endocrine, and autonomic responses to a stressor are adaptive and integral to survival. The neuroplasticity is the brain's ability to adapt and change over time and the regulation of gene expression represents a major component in long term plastic changes through which the nervous system physiologically respond to stimuli such as stressors. However, chronic or repeated engagement of these adaptive responses incurs an allostatic load that is expressed as an increased risk for pathogenesis of stress-related disorders including major depression. The hippocampus is a limbic structure that has received increasing attention in depressive disorder. The hippocampus is responsive to stress stimuli because it expresses high levels of glucocorticoid receptors and plays a significant role in negative feedback regulation of the HPA axis, a principal system in the response to stress. Numerous evidences demonstrate that neuronal atrophy and loss of plasticity occurs in hippocampus in response to stress and depression. Therefore the highly plastic, stress-sensitive hippocampal region may play a central role in depressive illness. In order to better understand the genes and the mechanisms underlying the effects of stress on the neural plasticity of the hippocampus, we used a behavioural paradigm of depression, the chronic escape deficit model (1), which is based on the modified reactivity of rats to external stimuli induced by exposure to unavoidable stress. The model of chronic escape deficit begins as an acute escape deficit which can be indefinitely sustained by repeated administration of mild stressors. This has proved to be a valid and useful model of depression and considers depressive symptoms like behavioural despair. In order to investigate the changes in gene expression profile associated with stressful condition we compared stressed to naïve animals, in which typical escape deficit behaviour was not induced, and we performed a gene expression profiling on hippocampal RNA using GeneChip Rat Exon Array (Affymetrix). Briefly, the analysis of raw data, normalized using the iterPLIER method, was performed by Expression Console software (Affymetrix). The Welch *t*-test was used to assess statistical significance of differential expression between the stressed group and the naïve group. The results showed that 148 transcripts were differently expressed. The microarray results were validated by relative quantitative real time PCR. Ingenuity Pathway Analysis was employed to analyze the functions and roles of these genes in biological processes. These functional analyses indicate that multiple pathways are involved in the underlying mechanisms of a stress condition associated with escape deficit. Among the cellular functions and biological processes, the most relevant pathways affected in the chronic escape deficit model were apoptosis, gene expression regulation, cell cycle control, cellular growth and proliferation, and signal transduction pathways. Our results suggest that stressors modify the neuroplastic ability of the hippocampus. Among the differently expressed genes, we selected a list of transcripts which were analyzed at the exon level in order to evaluate the occurrence of alternative splicing events. It is clear that new advances in our understanding of disease will come not from the study of single genes but from the analysis of whole transcriptomes where genes act in concert. We believe that these novel findings will be of general biological significance, will allow a comprehensive understanding mechanism of stress action and will provide unexpected biological insights into depression.

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OLIGOPHRENIN-1 REGULATES SYNAPTIC ACTIVITY DEPENDENT SHUTTLING BETWEEN SYNAPSES AND NUCLEUS OF Rev-erbAα

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Oligophrenin-1 (OPHN1) is a synaptic RhoGTPase-activating protein involved in X-linked mental retardation that regulates dendritic spines shape and outgrowth of axons in brain (1). By using the C-terminal fragment of oligophrenin 1 as bait in a two-hybrid screening of a human fetal brain cDNA library we identified as an interactor, NR1D1 (Rev-erbAα). ReverbAα is an orphan nuclear receptor that constitutively suppresses gene transcription and regulates the circadian clock in the CNS (2). We confirmed the interaction *in vitro* and *in vivo* by co-immunoprecipitation and GST-pull down. In COS-7 cells we observed that overexpressed oligophrenin-1 is able to recruit Rev-erbAα (normally localized in the nucleus) to the cytoplasm. Furthermore, overexpression of oligophrenin-1 induces an accumulation of endogenous Rev-erbAα in dendritic spines in hippocampal neurons. The oligophrenin-1δC (deleted of C-terminus), a mutant that mimics the mutation present in the XLMR patients, was not able to induce this effect in COS-7 and in hippocampal neurons. In HEK cells we observed that oligophrenin-1 protects Rev-erbAα from degradation by GSK3β. We also have found that RNAi knockdown of oligophrenin-1 inhibited the translocation of Rev-erbAα into the dendritic spines. More remarkable, synaptic activity mediated by AMPA receptors induces translocation of Rev-erbAα to the dendritic spines. RNAi experiments showed that oligophrenin-1 is required for the translocation of ReverbAα in spines induced by synaptic activity. Moreover we observed an alteration of ReverbAα expression in oligophrenin-1 knock out mouse, i.e. there is a reduction of Rev-erbAα in hippocampus. Our results demonstrate for the first time the interaction between an orphan receptor (Rev-erbAα) and a synaptic protein (oligophrenin-1). This interaction regulates the localization of Rev-erbAα between synapse and nucleus induced by synaptic activation.

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ANTIINFLAMMATORY, ANTIOXIDANT, AND ESTROGEN RECEPTOR β-MEDIATED MECHANISMS IN PHYTOESTROGEN GENISTEIN-INDUCED PAIN RELIEF IN A MOUSE NEUROPATHY MODEL

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In a previous study we demonstrated that the phytoestrogen genistein (4',5,7-trihydroxyisoflavone), a nutraceutical present in soybean, reduced time- and dosedependently nociceptive hypersensitivity in a mouse model of mononeuropathy, induced by sciatic nerve chronic constriction injury (CCI). Simultaneously, the phytoestrogen dose able to reverse neuropathic pain (3 mg/kg), following 11 s.c. administrations, reduced the neuropathic inflammatory and oxidative stress state: it reversed the iNOS and nNOS overproduction in peripheral and central nervous system steps involved in pain development and transmission, the peripheral NF-κB over-activation, and the increased ROS and malondialdehyde (MDA) content in injured paw tissues. The first aim of the present work was to investigate the estrogen receptor (ER) involvement in the genistein-induced pain relief. So, we examined the ability of the ERβ selective antagonist 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo [1,5-a]pyrimidin-3-yl]phenol (PHTPP), and the ERα selective antagonist methyl-piperidino-pyrazole (MPP), to reverse the phytoestrogen antihyperalgesic and antiallodynic ability. PHTPP (4.7 mg/kg, s.c.) or MPP (2 mg/kg, i.p.) was co-administrated with genistein (3 mg/kg) once a day, for 3 or 2 days, respectively, from the 11_{th} or 12_{th} day after nerve injury. We suggested the involvement of ERβ in genistein effect, since the ERβ-selective antagonist reversed antihyperalgesic and antiallodynic activity, whereas the specific ERα antagonist was ineffective. The second objective was to analyse more deeply the involvement and the receptor mediation of antioxidant effects in phytoestrogen antinociceptive efficacy. We showed that PHTPP did not reverse the genistein ability to decrease the paw tissue oxidative stress (ROS and MDA) induced by CCI: so, the antioxidant effect seems not to be ERβ-mediated. We also dosed in paw tissues the GSH content and the activity of some antioxidant enzymes, such as glutathione-related enzymes and catalase, through fluorimetric and spectrophotometric procedures. To compensate the tissue oxidative damage, the antioxidant system was activated in the neuropathic mice paw tissues and the genistein repeated treatment further increased all these enzymatic activities. Neuropathy is also associated with neuroinflammation and particularly with the proinflammatory cytokines overexpression at the peripheral and central nervous system pain steps. Genistein blunted the overexpression (RT-PCR) of both proinflammatory cytokines, IL-1β and IL-6, demonstrating the genistein neuroimmunomodulatory action involvement in its antiallodynic and antihyperalgesic efficacy. These results suggest altogether that soy phytoestrogen ameliorates the CCIinduced nociceptive hypersensitivity by ERβ-mediated antiinflammatory and antioxidant mechanisms.

DISCOVERING THE ROLE OF NEW GLUCOCORTICOIDINDUCED LEUCINE ZIPPER (GILZ) ISOFORM (L-GILZ) ON CELL DEATH AND PROLIFERATION

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Glucocorticoids (GC) are of extraordinary therapeutic value in a wide range of autoimmune/inflammatory diseases and are able to modulate cell death and proliferation. These effects are consequent to genomic and non-genomic signals activated by GC interaction with GC receptor (GR). Moreover, mRNA and/or protein synthesis inhibition counters GCs-induced apoptosis as well anti-proliferative activity thus indicating that gene transcription is required. In the past few years, with the aim to deeply analyze the molecular mechanisms of GC action, we have identified a number of GC-induced genes including glucocorticoid-induced leucine zipper (GILZ), a protein rapidly induced by GC treatment. GILZ induction represents one of the mechanisms contributing to GC-induced cell growth inhibition. Here we describe the cloning of a new GILZ transcript variant, long-GILZ (LGILZ), that uses a different first exon, as compared to GILZ, for the initiation of gene transcription. Interestingly, translation initiation site starts from a non-canonical non-AUG (CUG) codon. This new transcript gives an open reading frame (ORF) of 705 bp and encode for a protein of 28 kDa molecular weight containing 234 amino acids. Moreover, LGILZ is expressed mainly in testis, but also in spleen, thymus, and skeletal muscle. The two transcripts, GILZ and L-GILZ, differ only in the N-terminal part and share an identical leucine zipper domain and C-terminal part, containing a proline and glutamic acid rich (PER) region. All together, analysis of GILZ genomic locus shows that three isoforms could be codificated: GILZ, L-GILZ, and short-GILZ (S-GILZ), a 80 amino acid long protein. Considering that GILZ and L-GILZ share functional domains (leucine zipper, PER domain) important for interaction with Ras and NF-κB, we investigated the role of GILZ and of the L-GILZ new isoform on cell death and proliferation. Results showed that GILZ over-expression is able to induce apoptosis in different cell types, including T lymphocytes. Moreover, GILZ also inhibits cell proliferation consequent to T cell activation, *via* inhibition of Ras signaling cascade and NF-κB transcription activity. L-GILZ expression, as GILZ, is induced by GC treatment and is capable to interact with and inhibit Ras and NF-κB. Consequently, L-GILZ induces apoptosis and inhibits cell proliferation. Overall, our data demonstrate that GILZ and L-GILZ are crucial mediators of GC-induced effects on cell death and proliferation.

MOLECULAR AND FUNCTIONAL ANALYSIS OF *DROSOPHILA* EFHC1 GENE

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Juvenile myoclonic epilepsy (JME), the most common cause of grand mal seizures, accounts for 3-12% of all epilepsies. This pathology is a collection of seizure patterns that are clinically distinct from those in other forms of idiopathic generalized epilepsies (IGEs), with the major characteristic being adolescence-onset myoclonic seizures. In 2004, Suzuki and collaborators (1) found 6 missense mutations in the EJM1A gene in chromosome 6p12 segregating in 25 epilepsy affected members of six unrelated families. This gene encodes for a protein called EFHC1. EFHC1, or myoclonin 1, is a protein of 640 amino acids containing three DM10 domains, whose function is unknown, and an EF-hand motif, a typical domain of calcium modulators (2). We have identified two *Drosophila* homologues (CG8959 and CG11048) of myoclonin/EFHC1.We are now using *Drosophila* as a model to study the functions of CG8959 gene in normal development and in pathology to define the mechanisms whereby mutation of myoclonin causes human disease. We have generated transgenic flies for overexpression and knock out of CG8959 gene. We are conducting a detailed analysis of the loss and gain of function phenotypes at the neuromuscular junction level aimed to neuromuscular junction defining the functional role of this protein. The *Drosophila* neuromuscular junction is a valuable model system to dissect the mechanisms underlying neurological disorders.

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NEUROPROTECTIVE AND DIFFERENTIATING EFFECT OF GUANOSINE IN HUMAN NEUROBLASTOMA SH-SY5Y CELLS

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The role of a guanine-based purinergic system in the mammalian central nervous system has gained a wide acceptance in the recent years, based on the increasing number of studies demonstrating several effects of guanine-based purines in the central nervous system, in particular their implication in neuroprotection. Recent findings indicate that guanosine can exert trophic effect on glial cells, whereas the neuroprotective effects and the purported mechanisms of this molecule on neurones are largely unexplored. Therefore, we have investigated 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. Using the MTT test, we have demonstrated that guanosine significantly and concentration-dependently rescued cells exposed to serum deprivation (0% FBS) for 24, 48, and 72 h. These data have been confirmed by cell flow cytometry. Indeed, guanosine decreased the percentage of serum-deprived cells on G_0 phase whereas increased the percentage on S phase. One mechanism possibly involved in the guanosine-induced neuroprotective effect is the activation of MAPK cascade, since guanosine caused a timeand concentration-dependent phosphorylation of ERK1/2, measured by CASE™ Kit (Cellular Activation of Signaling ELISA). Accordingly, the selective ERK1/2 inhibitor PD 098,059 (10 μM) significantly counteracted 100 μM guanosine-induced cell proliferation. Once established the neuroprotective effect of guanosine in serum-deprived neurons, we focussed our attention on the morphological changes induced by guanosine in cells cultured in 10% FBS. Under these conditions, guanosine was able to induce differentiation in SHSY5Y cells. First, neuritogenesis was assessed by a visual method based on the evaluation of neurite extension: cells were considered differentiated if they had at least one process longer than the cell body. Retinoic acid (RA) was used as positive control. The ability of guanosine to enhance neurite extension was found to be time- and concentration-dependent increasing the percentage of differentiated cells, with respect to controls, by 64% and 107% at 10 μM and 150 μM, respectively, after 72 h exposure to the purine. Concomitantly, neurites length, measured by the computerized program Scion Image, was increased by 51% and 90% by 10 μM and 150 μM guanosine, respectively. We have also quantified guanosine differentiating effect by marking cells with phalloidin, a fluorescent molecule which binds to actin filaments. To better characterize these morphological changes we have identified specific markers involved in differentiation by immunocytochemistry. In particular, we have studied changes of expression and organization of microtubular proteins β-tubulin and MAP2 and we have confirmed the expression of a specific neuronal nuclear protein as NeuN at different length of treatment. Since differentiation and cell growth are mutually exclusive phenomena, we analysed cell proliferation by colouring a cell membrane protein with a specific dye (sulforhodamine B assay). Our results confirmed that there is a time- and concentration-dependent decrease in cell proliferation after guanosine treatment, as well as after exposure to RA. In the attempt to identify the mechanisms that underlie guanosine-induced differentiation, we started to investigate different signal transduction pathways. First, we demonstrated that guanosine (30-300 μM) concentrationand time-dependently activates ERK1/2 MAP kinases with a peak at 2.5 min (100 μM). Then we demonstrated that guanosine slightly stimulate intracellular cAMP production and excluded calcium involvement in guanosine-induced differentiation by analyzing $[Ca^{2+}]_i$ movement in neuroblastoma cells loaded with FURA-2. In conclusion, these results demonstrate that guanosine plays an important role in the neuronal homeostasis, acting as a neuroprotective and differentiating endogenous controller. Alike RA, guanosine seems to act through well established intracellular second messenger pathways, such as ERK1/2 MAPK cascade. However, the starting point of its mechanism of action is still unknown and requires further investigation efforts.

INVOLVEMENT OF κ-OPIOID AND ENDOCANNABINOID SYSTEM ON SALVINORIN A-INDUCED REWARD

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The recreational drug, salvinorin A, derived from the plant of *Salvia Divinorum*, is a potent and selective-opioid receptor agonist. The abuse of selective κ-agonists is a novel phenomenon, the mechanism of which is not fully understood. We investigated salvinorin A given s.c. on the conditioned place preference (CPP) (0.05-160 μg/kg), in a twocompartment chamber, and i.c.v. self-administration (0.01-1 μg/infusion) paradigms, in Wistar rats. Furthermore, with the *in vivo* microdialysis technique in freely moving rats, the effect of salvinorin A on dopamine (DA) levels in the shell portion of *nucleus accumbens* was studied. In addition, the effects of salvinorin A on the zebrafish (Danio rerio) model were investigated through its swimming behaviour and CPP task. Swimming activity was determined in a squared observational chamber after an i.m. treatment of salvinorin A (0.1-10 μg/kg). For the CPP test, zebrafish were given salvinorin A (0.2 and 1 μg/kg) or vehicle and evaluated in a two-compartment chamber. Since an interaction between κ-opioid and cannabinoid system has been recently reported (1), we also investigated the role of cannabinoid and opioid system on salvinorin A effects. The present results demonstrate the rewarding effects of salvinorin A in a range of doses between 0.1 and 40 μg/kg s.c. in the CPP and 0.1-0.5 μg/infusion in i.c.v. self-administration tests. The highest dose (160 μg/kg employed in CPP test, and 1 μg/infusion in the i.c.v. self-administration) was aversive. Moreover, in the shell of *nucleus accumbens*, DA extracellular levels were increased after salvinorin A (40 μg/kg s.c.), reaching a maximum value of about 150%. In the zebrafish, salvinorin A (0.1 and 0.2 μg/kg) induced accelerated swimming behaviour in comparison with vehicle, whereas a "trance-like" effect, at doses between 5 and 10 μg/kg, was observed. The rewarding effect was antagonized by i.p. pretreatment with the cannabinoid CB1 receptor antagonist rimonabant (1 mg/kg), or the κ-opiate receptor antagonist nor-binaltorphimine (nor-BNI) (10 mg/kg), either in rats or in zebrafish. Taken together, these results indicate that salvinorin A, as is sometimes reported in humans, exhibits rewarding effects, which appear mediated by activation of both κ-opioid and cannabinoid CB1 receptor. The employment of the zebrafish addiction model, as previously reported for cocaine (2) and amphetamine (3), appears a promising tool to uncover the potential abuse of new drugs.

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HERPES SIMPLEX VIRUS-1 INFECTION ALTERS RECEPTORDEPENDENT AND INDEPENDENT CONTRACTIONS IN RAT SMALL INTESTINE

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Neurotropic viruses are implicated in digestive neuropathies but the mechanisms through which such viruses affect the enteric nervous system (ENS) are unknown. *Herpes simplex* virus-1 (HSV-1), orally inoculated to laboratory animals, targets neurons in the ENS (1). This study aims to assess the effects of HSV-1 infection on the mechanical responses of the small intestine to Ca^{2+} and to purinergic receptor stimulation. Rats were inoculated intranasally (103 pfu) and after 4 weeks (W) again i.g. (108 pfu) with HSV-1. Infected or mock infected rats were sacrificed 1 and 6 W after the i.g. inoculation. The presence of HSV-1 infection was determined by PCR amplification of HSV-1-tk gene, RT-PCR for HSV-1 latency associated transcripts (LATs), and early gene ICP-4. In isolated ileum segments, mounted vertically in organ baths, changes in muscle tension were recorded using isometric transducers. Contractions were evoked by $CaCl_2$ 5 mM in ileal preparations maintained in depolarizing Ca^{2+}-free Tyrode solution (in mM: NaCl 60, KCl 60, $MgCl_2 \cdot 6\ H_2O$ 0.49, NaH_2PO_4 0.32, $NaHCO_3$ 12, glucose 5) in the presence or absence of verapamil (0.1 μM). Concentration-response curves to adenosine (ADO; 0.1-1.25 mM), ATP (0.1-1.25 mM) and R(-)-N^6-(2-phenylisopropyl)adenosine (R-PIA; 0.1-15 μM) were obtained cumulatively. HSV-1 infected rats did not show any clinical and histological abnormalities in the ileum. In the brain and ENS, HSV-1 established a latent infection demonstrated by the presence of viral tk-DNA and LAT mRNA. In ileum segments maintained in a depolarizing salt solution, $CaCl_2$ caused a sustained contraction that, in respect to preparations from mock infected animals, was significantly higher in the ileum of rats sacrificed at 1 W (+76%) and 6 W (+56%) postinfection (PI). Pre-treatment with verapamil significantly impaired the increase in muscle tension evoked by $CaCl_2$ in ileum preparations from mock as well as HSV-1 infected rats; 6 W after i.g. challenge the inhibitor even abolished the Ca^{2+}-sensitizing effect of HSV-1. Contractile responses to RPIA were increased 1 W PI throughout the tested concentration range, whereas reduction of ADO-induced contractions occurred at 1 and 6 W PI, and was limited to the highest concentrations of the nucleoside. Finally, the contractile response to ATP showed a clear concentration-dependency only in preparations from HSV-1 infected animals, at 1 W PI. In conclusion, following i.g. delivery, HSV-1 establishes a latent infection in the intestine that significantly affects Ca^{2+} sensitivity of ileum segments, pointing out the possible role of voltage-gated ion channels as targets of infection-induced alterations. HSV-1 latency in rat ENS appears to be associated also with a modified expression of purinergic receptors and/or adenosine deaminase, a key enzyme in regulating ADO levels for the activation of low and high affinity adenosine receptors.

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EFFECTS OF CHRONIC EXPOSURE TO PHENCYCLIDINE: RELATIONSHIP BETWEEN SCHIZOPHRENIA AND METABOLIC DISTURBANCES

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Schizophrenia is one of the most serious human brain diseases, yet its aetiology and pathophysiology remain largely unknown. Patients with schizophrenia appear to suffer from various aspects of the metabolic syndrome, as higher rates of obesity, impaired glucose tolerance, insulin resistance, type II diabetes mellitus, and cardiovascular disease (1). Several lines of evidence suggest that glutamatergic mechanisms play an important role in the pathophysiology of schizophrenia. Phencyclidine (PCP), a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) glutamate receptor, is a psychotomimetic agent that produces a psychotic syndrome very similar to human schizophrenia (2). The fact that acute PCP administration to rats lead to increased locomotor activity, ataxia, rearing, and stereotyped behaviour has rendered the “PCP model of schizophrenia” a valuable tool (3). PCP administration acutely in high doses or chronically in lower doses causes neurotoxicity (4). We have previously demonstrated that rats exposed to 6-8 weeks of social isolation rearing, an “environmental animal model” of schizophrenia, show increased visceral fat, impaired fasting glucose tolerance, and higher cortisol levels, which may be related to an overactivity of the hypothalamic-pituitary-adrenal (HPA) axis (5). In analogy with this model, in order to evaluate whether the pharmacological model of schizophrenia develops certain metabolic disturbances, the purpose of this study was to analyze the effect of a chronic PCP administration on glucose metabolism, food intake, hormonal variations, and accumulation of visceral fat. In agreement with data reported in the literature, our results show that PCP-treated rats exhibit hyperlocomotion and enhanced immobility in an open field test. Metabolic alteration is also observed after PCP administration with a loss of weight compared with control animals, and higher sensitivity to insulin, although fasting glucose concentrations do not differ from controls. In contrast with isolated rats, treatment with PCP does not result in a significant increase or reduction of cortisol or amylase concentrations. PCP does not induce accumulation of visceral fat at three weeks from treatment. Finally, the behavioural data strongly argue for validity of subchronic PCP administration as an animal model of schizophrenia but it is difficult to draw conclusions about its involvement in metabolic alterations and further investigations are needed to evaluate the mechanisms throughout PCP reduces the weight of rats and their sensitivity to insulin. PCP animal model of schizophrenia produces different effects in comparison to social isolation rearing and seems to be not related with abnormality of HPA axis, so this model could be less powerful in mimicking schizophrenia-like metabolic deficits.

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