# NITRATION OF INDUCIBLE NITRIC OXIDE SYNTHASE TYROSINE RESIDUES IN RAW 264.7 MACROPHAGES

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## ABSTRACT

In RAW 264.7 murine macrophages co-induced by LPS and IFN-γ, some bound tyrosine residues of purified nitric oxide synthase are nitrated *in vivo.* Aminoguanidine (3 mM) and *S*-methyl-iso-thiourea (0.1 mM), added before and concurrent to priming, protected from cytotoxicity while amount of nitration increased.

Key words: RAW 264.7 inducible nitric oxide synthase; tyrosine nitration; aminoguanidine; *S*-methyl-iso-thiourea.

## INTRODUCTION

Among the isoforms of nitric oxide synthase (NOS) purified [1, 2] and cloned [3-5] around a decade ago from different cell types, murine macrophage type 2 NOS, characterized as induced after Stuehr and Marletta [6, 7] by endotoxins, cytokines or interferon-γ and a variety of products related to inflammation, have been found in almost two-thirds soluble and one-third particulate fractions [8, 9]; these do not appear to be clearly differentiated in terms of co-factors, selective modulatory behaviors, and sensitivity to inhibitors. Structural post-translational modifications of the N-terminal sequence of the first 17 aminoacid residues, possibly by aminopeptidase activity or alternately spliced mRNA's, were immunologically proved only in membrane-associated iNOS [10]; by contrast, serine (positions 13 and 16), and tyrosine (position 14) residues were not analysed, even though they may contribute to regulating mechanisms such as protein folding and polymerization, translocation and related activities through their phosphorylative and possible nitrative acylations.

Our first aim was to study unidentified post-translational covalent tyrosine modifications, which may be associated with the well-known NO-self inhibition effect [11-16], which may disrupt the balanced phosphorylation vs nitration of these residues.

We report our preliminary data on iNOS tyrosine nitration *in vivo*, which may be submitted to phospho- and nitro-proteomic and comprehensive metabonomic kinetic investigation. In the only related nitroproteomic work [17], iNOS escaped identification, possibly due to the inadequacy of current techniques. By contrast, as for same products of the 518 identified genes of the human kinome [18], the hypothesis of a balance, interference and turnover of (selective) tyrosine nitrations vs phosphorylations has never previously been examined.

Here we discuss our observations in the context of the available information and of a wider integrative, holistic, and cellular bio-patho-physio-pharmacological perspective.

These data have been presented and discussed at internal Department Meetings and have partially been communicated in a Faculty review [19].

## MATERIAL AND METHODS

RAW 264.7 cells from American Type Culture Collection (Rockville, MD) were grown in 10 cm diameter culture disks at 37°C in a humidified water incubator. Sterile filtered RPMI-1640 medium (Mediatech, Inc. CELLGRO; 0.2 g/L L-arginine free base) was supplemented with 10% fetal bovine serum (prewarmed for 30 min at 56°C) and 1% penicillin/streptomicin Sigma mix.

### *Cell lysate preparation*

Control and primed cells [by scaled addition of 0.5 μg/ml lipopolysaccharide endotoxin (LPS; from *Escherichia coli* strain 055:B5, Sigma) plus 2.5 ng/ml recombinant *E. coli* murine interferon-γ (IFN-γ; Calbiochem, 50 units/ml) from 0 to 16 h] were observed under the microscope at each time point to detect damage or death (by Trypan blue incorporation). The nitrite produced, diffused and accumulated in the medium was measured in triplicate in 50 μl samples of supernatant centrifuged at 4°C for 5 min at 1,000 rpm using Griess reagent [20, 21]. Centrifuged 600 μl samples of cell suspension were resuspended in an equal volume of

sterilized Tris-HCl (TBS) or Dulbecco phosphate buffered saline (PBS) 1:10, pH 7.40 (0°C), rapidly washed two times by recentrifugation and resuspended in 50 mM hypotonic Tris, pH 7.40, added with 2 mM dithiothreitol and protein inhibitors [1 mM phenylmethylsulfonyl fluoride, N-alpha-p-tosyl-L-lysine and N-tosyl-L-phenylalanine chloromethyl ketone HCl; 1 M leupeptin HCl, pepstatin A, trypsin inhibitor (type I-S from soybean), aprotinin and antipain; 2 μM benzamidine; 1 μM aprotinin and antipapain, all from Sigma], sonicated carefully at 0°C by repeated short pulses until complete lysis, verified by observation, and finally recentrifuged at 0°C for 60 min at 110,000 x g using a standard table microfuge.

The precipitated lysates and supernatants were prepared at least three times and submitted to protein and enzyme activity measurements, immunoprecipitation and Western blotting as specified below.

*iNOS purification*

The medium was changed every 48 h; cells were scraped, resuspended as above in sterile TBS or PBS medium (pH 7.40, 37°C), centrifuged at the same temperature for 5 min at 1,000 rpm, and placed 1:3, 3:9, 9:27 in fresh medium. Then 270 ml of cell suspension was collected and added to 1.5 l of freshly prepared medium, counted (average $1.56 \times 10^{-6}$ cells/ml), repeatedly verified by observation of Trypan blue exclusion, and subdivided in three 500 ml batches: a), b), and c). Batches b) and c) were added with 0.1 mM aminoguanidine (AG; hemisulfate salt, Sigma) and 3 mM *S*-methyl-iso-thiourea (SMITU; sulfate salt, Calbiochem), respectively. After 30 min the inducers LPS and IFN-γ were added to all three batches at same concentrations as detailed above, and incubation continued at 37°C under gentle shaking until 12 h. Cells (on ice) were then centrifuged at 0°C for 5 min at 1,000 rpm, suspended 1:10 in an equal volume of TBS or Dulbecco PBS, pH 7.40 (0°C), rapidly washed two times by recentrifugation, resuspended in 5 ml + 5 ml 50 mM hypotonic Tris, pH 7.40, added with 2 mM dithiothreitol and protein inhibitors, sonicated, and finally recentrifuged for 60 min at 110,000 x g at 0°C. Pellets were used as membrane fraction (after adding 1 M KCl for 5 min at 0°C and reprecipitation), and the soluble preparations were immediately passed through a preswollen 2'-5'-bisphosphate coupled agarose resin (2',5'-ADP-Sepharose Pharmacia) affinity column, followed by gel filtration chromatography on a 6 or 12 Superose column. All procedures were carried out in the cold room of a Pharmacia FPLC instrument by adding substrates, reducing agents and co-factors as needed [2, 22-24]. Determinations were repeated three times, and eluted proteins concentrated using Centricon 100 (Amicon) filters.

Protein and enzyme activity measurements, immunoprecipitations and Western blotting procedures were immediately repeated in purified preparations.

*Assay of NOS activity*

NOS activity was determined by the nitrite-nitrate method [20, 21] adapted from Stuehr et al [22] and by measuring the conversion of L-[$^3$H]arginine to L-[$^3$H]citrulline, as described by Bredt and Snyder [25], following standard laboratory procedures.

*Immunoprecipitation*

Immunoprecipitation was quantitatively performed in the supernatants of the culture disk cell lysates centrifuged at 110,000 x g, and in purified, concentrated most specifically active fractions centrifuged at 110,000 x g. Controls using nonimmune rabbit serum vs primary polyclonal rabbit purified anti-iNOS, polyclonal rabbit anti-nitrotyrosine, and monoclonal mouse anti-phosphotyrosine antibodies were added with appropriate Protein A/G Plus Sepharose (Santa Cruz Biotech Inc) bead suspensions and incubated overnight in the cold

room under continuous smooth oscillation. Spinnings at 5,000 rpm were repeated after resuspension of the pellets and washing in cold TBS or PBS, respectively. The preparations were warmed for 30 min at 37°C, 15 min at 65°C or 5 min at 100°C, after addition of sodium dodecylsulfate (SDS), 4% 2-mercaptoethanol reducing loading buffer. They were fractionated being the 1$^{st}$ 150 V, room temperature electrophoresis extended up to 4.5 h to achieve separation of interfering heavy and light chains of denatured antibody traces.

Titrations of final iNOS protein and of nitro- and phosphotyrosines, using the appropriate anti-nitrotyrosine and anti-phosphotyrosine secondary antibodies, were repeated at least three times as detailed below.

*Immunoblotting and miscellaneous methods*

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5, 10, or 12%, 1.5 mm slab depth gels, pH 8.8, using 4% stacking gel, pH 6.80, in a double Bio-Rad mini-gel apparatus according to Laemmli [26]. Proteins were mixed with standard 4 x loading buffer (25 mM Tris-HCl, pH 6.80; 16% glycerol, 8% SDS, 0.4% bromophenol blue) containing (or not) 20% 2-mercaptoethanol, normally heated to 65°C for 15 min or boiled for 5 min, as specified. Molecular weight references were taken from Bio-Rad Kaleidoscope Prestained or Precision Protein Standards. The first electrophoresis run was 100-150 min, 100-150 V at room temperature; proteins were then transferred (second run) for 30 to 90 min, 100 V, on ice. Membranes were nitrocellulose (Protran Schleicher & Schuell # BA85) treated with Ponceau S and washed during the procedures, or methanol activated polyvinylidene difluoride (Bio-Rad, cat 162-0177). Gels were finally treated with Coomassie Blue, properly washed, and stored at 0°C. Blocking was for 1 h at room temperature with milk (boiled or not) and/or 5% bovine serum albumin (BSA; Sigma) in standard TBS or PBS, containing 0.1% Tween (TBS-T, or PBS-T). Primary anti-iNOS (rabbit, 1: 2.000) antibodies were produced in the lab and named fractions G7 or G8; anti-nitrotyrosine antibodies (1: 2.000) were mouse monoclonal (1: 500; Upstate Biotech or Zymed), and rabbit polyclonal (1: 2.000; Upstate Biotech). Anti-phosphotyrosine monoclonal mouse antibodies (1: 750; Calbiochem) were only used diluted in TBS-T, 5% BSA, after inhibition of cell protein phosphotyrosyl-phosphatase activity prior to lysis with 100 µM sodium orthovanadate for 30 min at 0°C, activated as recommended by Upstate Biotech. Incubations were performed overnight in the cold room. Secondary antibodies were used as appropriate, diluted up to 1: 15.000, in the cold room, 30 min for BSA containing buffers, or 1 h. Controls and blanks were systematically repeated. Detection was by means of ECL or ECL+Plus Amersham Pharmacia Biotech; quantitations were made throughout using Kodak 1D Image Analysis Software. Stripping and reprobing of membranes were repeated as needed.

Protein content was determined according to Bradford [27] using the Bio-Rad assay solution and BSA as standard.

## RESULTS

*Cell preservation*

While cells in batches b) and c) grew normally, growth in batch a) and in the corresponding plates progressively deteriorated, with a reduction in the number of viable cells to 75% and 63% at 8 and 16 h, respectively. At 48 h, in a few samples from batch a), all cells were dead, whereas cell growth in batches b) and c) continued.

*Kinetics of NOS induction*

Table I reports the kinetics of NO produced and measured in the medium of cultured RAW cells, and the protein content and NOS activity quantitatively assayed in supernatants at different times of incubation after addition of LPS and IFN-γ. Increase of NO synthesis, diffused and accumulated as $NO_2^-$ (258% and 479% at 5 h and 8 h, respectively), and its inhibition (71% at 16 h) were confirmed (see [13]). Total protein, which increased especially between 3 h and 5 h, decreased between 8 h and 16 h of incubation. NOS activity increased 47.8 and 17 times, respectively, at the former time points, and decreased 1.4 times thereafter. Proteins and iNOS kinetics were titrated by Western immunoblotting both in the supernatant and in the membrane fractions. Nitrotyrosine and phosphotyrosine were not detected in the unprimed samples, but their presence, which appeared at 8 h and 16 h of priming, showed no differences thereafter, calling for immunoprecipitation and enzyme purification procedures.

*Nitration of iNOS tyrosine residues*

Figure 1 reports the data on bound nitrotyrosine found in a purified enzyme preparation incubated 8 h after the double activation described above. This observation was made many times in the last two years at different priming time points in different soluble and membrane preparations [19].

Nitration, confirmed at the molecular weight of dimeric iNOS (around 135 KDa), sometimes appeared as two or three major distinct bands, more bands resulting in the same range, and at lower weights (about 65-70 and 80 KDa), in the limits that have been reported for identified monomer(s) [5, 28]. It was never detected with separations by primary monoclonal antibodies, and, for the primary polyclonals, when blocking and following in TBS or PBS, 2-mercaptoethanol present or not, warming 15 min at 65°C or 5 min at 100°C, except with 5% BSA (Sigma, or Interagen Bovimunar Cohn fraction V, pH 7.0). It has never been confirmed in the commonly used 5% milk solutions (boiled or not) [ie: 17], nor when the proteins were incubated with myeloperoxidase, flavins, tetrahydrobiopterin ($BH_4$), and glucose plus NADPH before Western blotting.

With reference to a widely used experiment, nitration disappeared after overnight incubation in the cold room, or 30 min at 37°C) with 10 mM free 3-nitrotyrosine, pH 7.35, and under freshly prepared 1.0 M $Na_2S_20_4$ in 0.1 M PBS, pH 9.0 [29] (30 min, 0°C, under nitrogen; no reducing agents added) (data not shown).

*iNOS inhibitors*

After initial assays in which AG and SMITU products were titrated around 50% inhibition of the purified preparations, the 3 incubations described above were worked out by exposing the 500 ml cell batches 30 min before standard co-induction with 3 mM AG, and 0.1 mM SMITU. Orthovanadate (100 μM) was added for 30 min at 37°C (or not) to a parallel series of (not induced) controls and samples primed for 12 h, before protein inhibitors and immediate sonication at 0°C; all steps were performed on Tris-HCl, without adding phosphate buffers. It should be stressed that, during purification, both NOS inhibitors were thoroughly washed, so that in the 110.000 x g centrifuged lysate supernatant and in the eluted fractions with highest NOS activity products were diluted down to practically inactive concentrations.

Whereas after vanadate treatment and before sonication cells disruption was almost 100%, after the 12.5 h incubation both AG and SMITU apparently protected cell viability, as mentioned above, and protein content and NOS activities were higher compared to those of control cells, activities being 3.3 and 4.4 times higher, respectively, for lysates, and up to 8.1 and 13.5 fold in final fractions after the treatments with the inhibitors.

As shown in Fig. 2, nitration was present in the control samples, and values increased after both AG and SMITU treatments. Notably, the titrations of NOS activities, normalized for unit protein of the respective controls, were higher in iNOS purified repeated preparations of the treated batches for both products.

Preparations and assays were repeated three times and the standard error never exceeded 10%.

*Phosphorylation of iNOS tyrosine residues*

In the membranes of Fig. 2, reprobed after 30 min, 50°C stripping with phosphotyrosine secondary mouse monoclonal antibodies in filtered boiled milk – or BSA – 5% TBS-T, the anti-mouse Ig-horseradish peroxidase linked whole antibody (Amersham Life Sci, from sheep, 1:10.000) yielded any positive finding.

Activated 100 µM orthovanadate, incubated 30 min before, present along lysis and remaining in the purification or immunoprecipitation steps, was then used; nevertheless, the product was reduced to almost 50% viability as assayed by Trypan Blue exclusion. The $NO_2^-$ produced and accumulated was increased by almost 25% over the highest value reached with the externally added cytokine-endotoxin mixture priming, and the same activity of the LPS + IFN-γ co-induction resulted much more elevated, while proteins decreased and SMITU inhibition almost irreversibly present after the actually adopted 8 h plate incubations.

Figure 3 shows some of the Westerns obtained with the immunoprecipitation protocol adopted for the supernatant and beads, washed two times and resuspended 1:5 in TBS buffer, with orthovanadate where it had been added. The basic separations were here performed using anti-iNOS primary antibodies, protein A/G plus agarose-enriched incubations, and confirmed with appropriate secondary antibodies. As expected, immunoabsorptions and bead precipitations performed with anti-nitrotyrosine or anti-phosphotyrosine primary antibodies, being the enzyme protein characterized by the anti-iNOS secondary probe, gave qualitatively similar results (not shown).

The only general comment on these separations, more than the confirmed increase of nitrations, iNOS phosphorylation resulted possibly seen even in the absence of the phosphatase blocker, being its presence quantitatively evaluated with the treatment of the inhibitors. This topic warrants further studies on both clearance and accumulation of the specific/selective reactive tyrosine sites, nitrated or phosphorylated, and in similar or different kinome protein domains.

## DISCUSSION

This short presentation of the original observations prompts some considerations on the experimental approaches reported previously (Rossini *et al* [30]), here restricted to some unresolved problems on the dynamics of *in vivo* protein tyrosine nitration.

With regard to general NOS activity, the $BH_4$ co-factor couples L-arginine oxidation to NADPH consumption and its concentration may regulate the ratio of superoxide ($O_2^{\cdot-}$) to nitric oxide ($^{\cdot}NO$) generated, while flavins enhance superoxide synthesis from the oxygen domain [31, 32]. Different signaling pathways mediate opposite effects on endogenous vs exogenous NO [33] and its mieloperoxydase-derived inflammatory oxidants, including nitrite [34-37]. Peroxynitrite ($ONOO^-$) from NO and $O_2^{\cdot-}$ can mediate DNA strand breakage and deplete NADH, decreasing mitochondrial respiration through activation, also in macrophages [38], of poly-adenosine-diphosphate ribosyl synthetase - the futile repair cycle leading to the PARS

suicide hypothesis. The association between mitochondrial dysfunction and severity and outcome of septic shock has recently been debated [39]: “demand management” in cells [40] and modulation of mito-respiration by endogenous NO show a revival [41-45], which can be confirmed by integrated genomic and proteomic/metabonomic analyses through a systematically perturbed metabolic modeling [46, 47]. NO and $O_2^{\cdot-}$ might be generated independently, but released simultaneously in intra- and/or extracellular environments, where they could form $ONOO^-$ by a reaction which would occur away from mitochondria; in the mito-compartments this could occur only if iso-superoxide-dismutase (SOD) activity were saturated or impaired [42]. In cultured ovine pulmonary arterial endothelial cells, the 4.5-fold increase in superoxide contributes to the inhibition of NOS activity, though not of gene expression [48]: non-transcriptional activation of eNOS has been documented, for example for estrogens, as have protective corticosteroid effects [49, 50]. Peroxynitrite from macrophage-derived NO has long been claimed to contribute to oxidative stress and cytoprotection [51], as shown through its decomposition catalysts [52]. It does not decompose to nitroxyl anion [53], which exerts redox-sensitive positive effects [54], and apparently it does not directly nitrate tyrosine residues [55-61], a reaction that is not always enhanced by carbon dioxide/bicarbonate [62, 63].

Nevertheless, activation of the L-arginine/NO pathway results in pronounced protein tyrosine nitration in primary peritoneal macrophages isolated from thioglicollate-treated mice via a mechanism that does not appear to involve $ONOO^-$, as also demonstrated *in vivo* with LPS (0.5 µg/ml) and IFN-y (100 units/ml) -activated cells. Data obtained *in vitro* were quantitatively similar to those reported by the same researchers for cultured RAW 264.7 macrophages, activated with LPS (0.5 µg/ml) and IFN-y (50 units/ml), upon addition of 1 mM exogenous L-arginine to cell suspensions. A striking mismatch in the time course of iNOS induction, resembling protein-bound tyrosine nitration kinetics (determined after derivatization to N-acetyl 3-amino-tyrosine by HPLC with electrochemical detection, on cell lysates, after pronase digestion and removal of nitrite), vs the burst at much earlier times of $O_2^{\cdot-}$ and $H_2O_2$ productions has been definitely shown in both models, as well as in animals *in vivo*. In fact, release of NO, accompanied by the accumulation of nitrite in the cell cultures, was detected at 7 h, as confirmed, and nitration occurred with a pronounced lag phase to 18 h, reaching a max at 24 h post-stimulation and slowly declining during the next 24 h, while $O_2^{\cdot-}$ (and $H_2O_2$) maxima were reached at 2 h, declining to basal rates 4 h after stimulation [55-61]. For cytokine-activated macrophages these authors support the alternative mieloperoxydase or another yet unidentified heme-peroxidase tyrosine nitration, and for $H_2O_2$ the oxidation of nitrite to $NO_2$ radical, another potent nitrating species. Alternative nitrating pathways are not excluded, and they may be specifically active in the different cells and tissue patho-physiological regulations of pharmacotoxicological interest.

Peroxynitrite-dependent nitrosylations and nitrations can be modified by cell/tissue nitratases and nonenzymatic factors [64-70] and may induce protein modifications in tyrosine phosphorylation and degradation [71-73]. Some factors determining the selectivity of protein tyrosine nitration have been described in some substrates [73-79]: redox compartmentalization and proteosomal dysfunction may be unambiguously significant also in the interplay between NO, reactive nitrogen oxide-oxygen species, and $O_3$ [80-85]. Diversity of LPS-induced nitrotyrosine formation in endothelium-macrophage-rich organs [86], and constitutive and differential expressions of endothelial and inducible NOS mRNAs and proteins, also described in normal and pathological human tissues [87], take account of the species differences among macrophage NO productions, turnovers, and concentration-dependent protective or toxic effects [88, 89].

As regards (murine) macrophage inducible NO synthase, tyrosine residue phosphorylation, described since 1996, has been associated with increased activity [90]; nevertheless, potential post-translational regulation has not yet been discussed as balanced with unclarified nitrosative $N_2O_3$ production capacity, which also appears dependent on induction signals [91, 92].

In our culture medium L-arginine was never limiting, so the novel $O_2^{\cdot -}$ and $ONOO^-$ generation pathway, which is controlled by cytosolic L-arginine depletion after the exclusion of the aminoacid in the medium [93], is apparently not effective. In fact, after blocking stimulation by cytokine $O_2^{\cdot -}$ generation from NADPH oxidases, *when and only L-arginine is depleted*, the induced RAW 264.7 mouse macrophage cell line iNOS [by 24 h treatment with 2 µg/ml LPS + 100 units/ml IFN-γ (9)], produced both NO and $O_2^{\cdot -}$, which would rapidly dismutate either spontaneously or by the action of SOD, triggering the production of $ONOO^-$ [93-95]. Indeed, in the absence of L-arginine and the presence of NADPH and FAD, FMN and $BH_4$ co-factors, NOS does not catalyze $O_2^{\cdot -}$ [94], and the co-enzyme and co-factors induce and co-accelerate $O_2^{\cdot -}$ dependent $OH^{\cdot}$ radical generation, abolished by catalase, suggesting that $H_2O_2$ may be involved and that nonenzymatic dismutation of $O_2^{\cdot -}$ may be one of the sources of $H_2O_2$ formation in the reaction mixture, while NO scavenges the $^{\cdot}OH$ radical, protecting the enzyme [96]. Thus, the often reported [11-16] feedback self-inhibition of iNOS may be due to the accumulating nitrite, seemingly coincident with verified bound tyrosine nitration. After the work of Mitchell *et al* on the endothelial form, by oxygen derived radicals [94], murine macrophage iNOS feedback inhibition, suggested to be irreversible [13], has been simultaneously described *in vitro* as well *in vivo* for different NO synthases, as mentioned above [11, 12, 14, 16]. More recently, peroxynitrite added *in vitro* to murine lung epithelial cells showed inhibition of iNOS-induced expression by α-TNF, IL-I and IFN-γ (all 10 ng/ml), while unidentified tyrosine residues proved to be nitrated [97]. Anyway, the options [98, 99] remain open, supporting the still not fully established regulation of NOS isoforms, which should be identified in terms of selective residues covalently modified by phosphorylation acylation and nitrosation/nitrosilative (poly)nitration.

The subject brings to mind our observation of 1975 on purified glycogen-synthase, where the mostly phosphorylated *D* form presented greater tetranitromethane *in vitro* tyrosine reactivity: the identified 3-nitrotyrosine residue resulted to be essential for catalysis, not contributing to allosteric G6P modulation [100]. Now, to quote an example related to the integrated system, the (cardio)protection through glycogen-synthase kinase-3-β inactivation [by phosphatydilinositol-3-kinase (P13Kinase) and Akt-kinase serine-9-phosphorylation], - eventually an expression of ischemic preconditioning [101] -, appears counteracted while phosphorylation of tyrosine-216 (by a distinct kinase) compounds the ischemic damage [102]. The inherently obvious conclusion is then reached that microscopic, selective residue-sites patterns in the locally proper redox, phosphate and eventually nitrosative and nitrating potentials environment, need to be fully described before any pharmacotoxicological native, integer, receptorial useful definition can be formulated by protein expression and post-translational modification functionally associated [30, 103, 104].

In the present instance, a few more comments can finally be made in relation to the specific NOS type 2 inhibitors assayed, whose many therapeutic targets and potential pitfalls have been widely discussed [ie: 105].

Aminoguanidine (AG), the bifunctional reagent containing the guanidino group of L-arginine linked to hydralazine, had been found to be almost equipotent to $N^G$-monomethyl-l-

arginine as an inhibitor of the LPS 9 h induced RAW 264.7 isoform, and 10 to 100 times less potent than the constitutive endothelial and brain forms [106]; *S*-methyl-iso-thiourea (SMITU), one of the competitive inhibitor of LPS + IFN-γ mouse macrophage RAW 264.7-induced iNOS (2 to 30 times more potent)[107, 108], has recently been described to block NF-$_k$B activation and tissue iNOS itself in adenosine $A_3$ receptor of Langendorff-perfused mouse heart subjected to late preconditioning-induced ischemia/reperfusion [109]. In the same activated mouse macrophages, AG has been shown to reduce the expression of iNOS protein [110]; nevertheless, a similar S-substituted-iso-thiourea failed to influence transcription of iNOS mRNA, translocation of iNOS protein or degradation of translated iNOS protein [111].

Here, the documented two steps LPS triggering and associated, synergistic potentiated priming by IFN-γ enhanced transcription of macrophage NOS mRNA, which requires ongoing protein synthesis [112-114], shown increased in the control observations, were not analyzed further following the verification of the increase of cytotoxicity, protected by the two inhibitors. After the stimulation by the prototypical highly acylated *E coli* LPS in murine macrophages, a signaling complex of clustered receptors is formed, including, among others, heat shock proteins 70 and 90, the chemokine receptor 4 and growth differentiation factor 5, and the Toll-Like Receptor TLR4, with phosphorylation of the signal transducers and transcription activators STAT I α and β, TIRAP (Toll-Interleukin I Receptor domain containing Adaptor Protein) -dependent, whose induction kinetics have proved different from that of IFN-γ [115-117]. The protection against TNF-α, LPS-induced lethal shock, requires functional iNOS [118, 119], and, mediated by CO, heme-oxygenase-1 [120, 121]. In the LPS-treated rat polymorphonuclear leukocyte, AG significantly inhibited free radical generation [122]. In the present context, concurrent phosphorylation potential was not analyzed, nevertheless, the confirmed protection from cytotoxicity given by AG and SMITU products added before, and present during priming, appears valuable and interesting at the time iNOS activity - increased by the co-induction process -, found much higher after exposure to concentrations of the products that would produce almost 50% inhibition. Anyway, in this respect we are aware of the opposite hormetic multifunctional effects (see [123]) and of the biphasic trends that have been demonstrated to be relevant in S-nitrosylation in different experimental conditions (see [124, 125]). Equally interesting was the confirmed nitration in the purified control induced preparations, increased following treatment with the two different classes of inhibitors. This observation was beyond the scope of this study, that is to investigate the underlying molecular basis, whose mechanisms may nevertheless be connected with the differences of the referred specific/selective bound tyrosine nitrating pathways.

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**Table I**
**Kinetics of $NO_2^-$ produced in the medium [22] by RAW 264.7 cells, lysate protein content [27] and NOS activity [25], during 0.5 µg/ml LPS plus 2.5 ng/ml (50 units/ml) recombinant murine IFN-γ.**

| *Hours of incubation (after addition of LPS and IFN-γ)* | *$NO_2^-$ ($\Delta \mu M$/min)* | *Protein (µg/ml)* | *NOS activity (% Δ cpm/mg protein)* |
|---|---|---|---|
| 0 | 0.00 | 1682.4 | 0.00 |
| 3 | 0.33 | 642.0 | 0.05 |
| 5 | 0.85 | 1427.3 | 2.44 |
| 8 | 4.07 | 2597.5 | 41.41 |
| 16 | 2.91 | 1720.0 | 29.94 |

All measurements made in triplicate; for all means standard deviations do not exceed 10%; $P<0.05$. (Exp. 11.21.00).

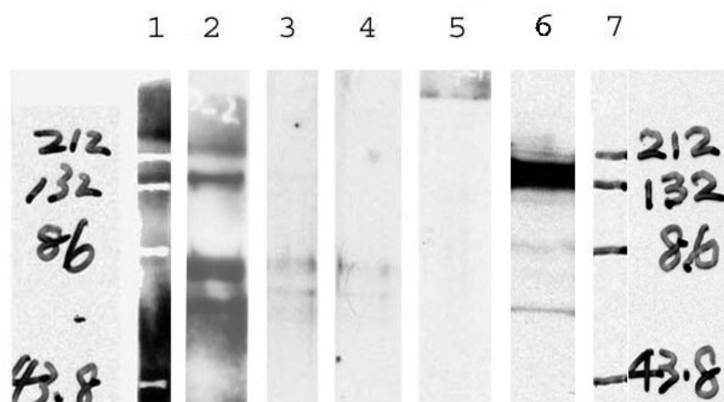


Fig. 1.

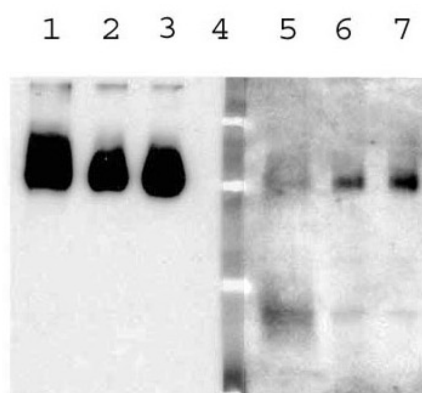


Fig. 2.

**Fig. 1.** Examples of Western blots showing at the level of purified iNOS, immunoprecipitated with the fraction G7 of the rabbit anti-iNOS primary antibodies (lane 6; PBS-T, boiled milk 5%), the corresponding nitrotyrosine band, by rabbit polyclonal primary antibodies (lane 2; PBS-T, BSA 5%). Relevant bands around 135 KDa according to the standards (lanes 1 and 7; Bio-Rad Kaleidoiscope, cat n. 161-0324, prestained, cont 90485, 10.11.00). Incubation with 10 mM 3-nitrotyrosine (lane 3) makes the nitrated band disappear. The nitrotyrosine band cannot be seen if rabbit polyclonals diluted in 5% milk (lane 4; boiled,) and/or (lane 5) mouse monoclonals are used, in 5% BSA, or, in 5% milk, boiled or not (not shown)

iNOS titrated in all lanes on 15 μg protein purified from cultured murine macrophages, RAW 264.7 line, using the Bio-Rad mini-apparatus. Acrylamide gel 10%; $1^{st}$ electrophoresis run, running buffer, 100 V, 100 min, *room t*; $2^{nd}$ electrophoresis run, transfer on nitrocellulose membrane, 100 V, 100 min, *on ice*. For each lane, 30 μl of the purified preparation + 14 μl 4 x loading buffer, without 2-mercaptoethanol; warming 15 min at 65°C.

All other conditions as detailed in Material and Methods. (Exps. 10.30.00 and 10.24.01).

**Fig. 2.** iNOS purified by affinity chromatography ($1^{st}$ elution with NADH 0.5 mM; $2^{nd}$ by NADPH and 8 mM $NADP^+$; ionic exclusion to separate coenzymes) on the 110.000 x g supernatant of the cultured RAW 264.7 murine macrophages. Cells lysates after 12 h co-induction by 0.5 ηg/ml LPS + 2.5 ng/ml IFN-γ.

Bio-Rad Kaleidoscope Prestained Standards at 212, 132 and 86 KDa (lane 4; from above). Lanes 1, 5: protein contents 8 x all the others, 15 ηg purified proteins each; lanes 1, 2 and 3: iNOS protein identified by anti-iNOS fraction G8 rabbit polyclonals (PBS-T, 5% boiled milk); lanes 5, 6 and 7: same protein contents as 1, 2 and 3, titrated by anti-nitrotyrosine rabbit polyclonals (PBS-T, 5% BSA).

Lanes 1, 5: controls, induced; lanes 2, 6, and 3, 7: treatments with 3 mM AG, and 0.1 mM SMITU, respectively.

All other conditions as detailed in Material and Methods. (Exps. 11.14-20.01).

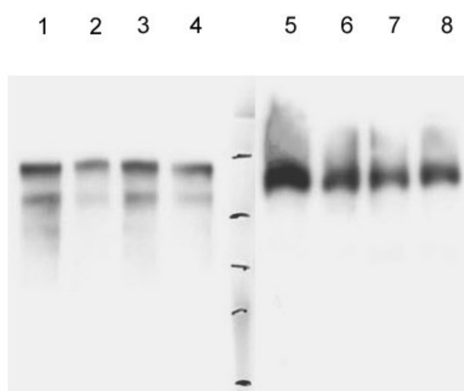


Fig. 3 (above).

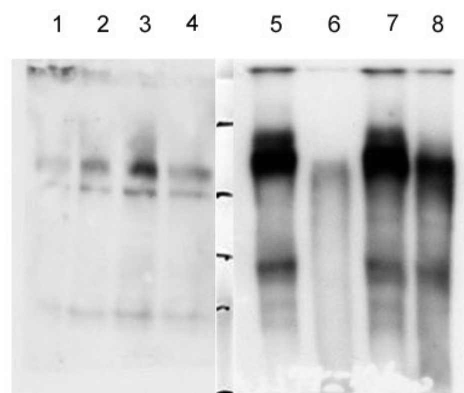


Fig. 3 (middle).

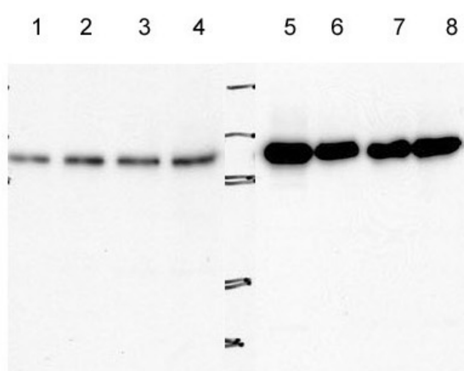


Fig. 3 (below).

**Fig. 3.** Examples of Western blots showing the separations by immunoabsorption obtained from RAW 264.7 cultured murine macrophages after 8 h LPS and IFN-γ co-induction at the concentrations indicated above.

Left: supernatant of the immunoabsorbed, precipitated beads; right: agarose beads washed two times and resuspended 1:5 in TBS before the addition of the 4 x loading buffer, resulting in 4% 2-mercaptoethanol, 2 min boiling before centrifugation and distribution to the blots.

Lanes 1, 5: induced controls; 2, 6: same controls added with 100 μM activated orthovanadate 30 min before sonication; 3, 7: lysates from cells treated for 30 min with 100 μM SMITU before priming; 4, 8: as for 3, 7, but 100 μM orthovanadate added as for lanes 2, 6.

Panel above: immunoabsorptions by primary rabbit anti-iNOS antibodies, fraction G8, and detections by same polyclonals (filtered, 5% boiled milk, TBS-T); middle panel: immunoabsorption as for the panel above, but using secondary rabbit polyclonal anti-nitrotyrosine antibodies for probing (5% BSA, TBS-T); panel below: immunoabsorption as for the first panel, but mouse monoclonal anti-phosphotyrosine as probing secondary antibodies (5%BSA, TBS-T).

Protein standard KDalton reference: Bio-Rad Precision Prestained Standards, broad range cat 161-0372, cont 90904, 12.6.01.

All other conditions as detailed in Material and Methods. (Exps. 12.6-15.01).

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