RESEARCH COMMUNICATION

Inducible nitric oxide synthase (NOS2) expressed in septic patients is nitrated on selected tyrosine residues: implications for enzymic activity

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Tyrosine nitration is a post-translational protein modification with potentially significant biological implications. In the present study we demonstrate, for the first time, that tyrosine residues of human inducible nitric oxide synthase (NOS2) can be nitrated by peroxynitrite *in vitro*, leading to a decreased activity. Moreover, we show that NOS2 expressed in a skeletal muscle from septic patients is nitrated on selective tyrosine residues belonging to a canonic sequence. This phenomenon could be an endogenous mechanism of *in vivo* modulation of NOS2 enzymic activity.

Key words: peroxynitrite, sepsis, tyrosine nitration.

INTRODUCTION

Inducible nitric oxide synthase (NOS2) has been described for several years in rats and humans, where it is expressed in several inflammatory states, including sepsis [1–3]. NOS2 produces much larger quantities of NO than the constitutive isoforms NOS1 (neuronal NOS) and NOS3 (endothelial NOS), and is found in multiple cell types of multiple organs. Several effects of NOS2 are related to oxidative stress, such as the generation of peroxynitrite ($ONOO^-$) [4], a highly reactive oxidant formed by the reaction of NO with superoxide anion (O_2^-) [5]. Peroxynitrite can oxidate different amino acids, such as cysteine, tryptophan and methionine. Furthermore, it can nitrate cysteine and tyrosine residues. In this last-mentioned case it leads to the formation of 3-nitrotyrosine, which can be detected globally by anti-nitrotyrosine antibodies or, more specifically, by MS analysis [6–8]. Such peroxynitrite-induced protein post-translational modifications have been described as reducing, *in vitro*, the activity of several enzymes such as manganese-superoxide dismutase (Mn-SOD), tyrosine hydroxylase, glutathione reductase and NOS3 [6–9].

Recently we found by immunohistochemistry that NOS2 expression was co-localized with nitrotyrosine residues in skeletal muscles from septic patients [10]. Similar co-localization was reported by Ichinose and co-workers in alveolar macrophages from chronic-obstructive-pulmonary-disease (COPD) patients [11]. These co-localizations were suggested to be related to the tyrosine nitration of proteins belonging to NOS2 neighbourhood [10,11]. Alternatively, they could also be related to nitration of tyrosine residues on the NOS2 molecule itself. Occurrence of this phenomenon has potentially important functional consequences, since it could be an endogenous mechanism of enzymic activity modulation. However, to the best of our knowledge, no data are available in the current literature regarding whether NOS2 can be nitrated on tyrosine residues *in vitro* and/or *in vivo*.

The present study has therefore been designed to evaluate (1) whether human recombinant NOS2 could be nitrated on its tyrosine residues *in vitro*, and the functional consequences of such a phenomenon, and (2) whether human NOS2 is nitrated on its tyrosine residues *in vivo*. This last point was evaluated by analysing NOS2 expressed in skeletal muscles from septic patients.

MATERIALS AND METHODS

Tyrosine nitration of human recombinant NOS2

Tyrosine nitration of human recombinant NOS2 [hrNOS2, EC 1.14.13.39, expressed in baculovirus-infected Sf9 (*Spodoptera frugiperda*) insect cells] was performed by incubation of the protein with peroxynitrite. To do so, 5 μg of hrNOS2 was dissolved in 100 mM potassium phosphate/25 mM $NaHCO_3$ buffer, pH 7.4 (buffer solution). The nitrating reaction was initiated by the addition of 1 mM peroxynitrite to the protein solution and immediate vortex mixing, as peroxynitrite is stable at alkaline pH, but has a half-life of only 1 s at pH 7.4 [10]. For control experiments, peroxynitrite was added to the buffer solution, pH 7.4, 5 min before adding hrNOS2, thus leading to its pH-inactivation.

NOS2 activity was then evaluated by the conversion of L-[^{3}H]arginine into L-[^{3}H]citrulline, as previously described [11], accordingly to a method initially described by Bredt and Snyder [12]. Briefly, 5 μg of recombinant protein was incubated for 20 min at 37 °C in the presence of cofactors and substrate [50 mM Hepes, pH 7.4, 0.5 mM NADPH, 5 μM FAD, 5 μM FMN, 1 mM $MgCl_2$, 5 mM tetrahydrobiopterin and 50 nM of L-[^{3}H]arginine (specific radioactivity 35.7 Ci/mmol)]. The enzymic reaction was stopped by addition of 2 ml of ice-cold 20 mM Hepes (pH 5.5)/2 mM EDTA, and the total volume applied to a Dowex-50W X8 column (Bio-Rad Laboratories), pre-equilibrated with the same buffer. L-[^{3}H]Arginine was retained on the column, whereas L-[^{3}H]citrulline was eluted with 2 ml of deionized water and its concentration determined by

Abbreviations used: COPD, chronic obstructive pulmonary disease; hr, human recombinant; IFN-γ, interferon-γ; LPS, lipopolysaccharide; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS; Mn-SOD, manganese superoxide dismutase; NOS, nitric oxide synthase; NOS1, neuronal NOS; NOS2, inducible NOS; NOS3, endothelial NOS; PDB, Protein Data Bank.

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liquid-scintillation counting. Initial experiments showed that the reaction was inhibited by addition of 1 mM N^G-monomethyl-L-arginine to the reaction buffer.

Tyrosine nitration was evaluated by Western blot (see the next subsection).

Detection and characterization of nitrated tyrosine residues in NOS2 from rectus abdominis muscle of septic patients

Rectus abdominis muscle was obtained from the same control ($n = 21$) and septic patients ($n = 16$) previously described [11,13]. Briefly, sepsis was defined based on the criteria of the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference [14]. The source of sepsis was either intra-abdominal or chest infection and required surgical treatment. Control patients consisted of patients undergoing elective laparotomy ($n = 10$) for various non-septic conditions or elective cardiac surgery ($n = 11$). No patient in the study received inhaled NO. This part of the study was performed with the approvals of the local board governing research on human subjects at our institution (Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale, Hôpital Saint-Louis, Paris, France). All patients gave informed written consent.

A biopsy specimen (weighing ≈ 500 mg) was obtained from the rectus abdominis muscle during the initial phase of the operation, either for elective surgery in the control group or surgery for infection in the septic group. Tissue samples were quickly frozen in liquid nitrogen and stored at -80 °C.

NOS2 and nitrotyrosine immunoblotting

Samples were analysed by SDS/PAGE, followed by transfer on to PVDF membranes. Immunoblotting was performed using an anti-NOS2 antibody directed against C-terminal amino acids 961–1144 (Transduction Laboratories) or an anti-nitrotyrosine antibody (Upstate Biotechnology).

In order to verify the specificity of the anti-nitrotyrosine antibody, the following experiments were performed.

Competition experiments

In order to compete with the nitrotyrosine residues present in the samples, 10 mM free nitrotyrosine, pH 7.5, was added to the anti-nitrotyrosine antibody solution. In addition, to test whether this competition was specific for nitrotyrosine, we also combined 10 mM L-tyrosine (not nitrated), pH 7.5, with the anti-nitrotyrosine antibody.

Reduction of nitrotyrosine residues

Specificity of nitrotyrosine antibody was also tested by the reduction of nitrotyrosine to aminotyrosine. Samples were incubated for 30 min before SDS/PAGE with 100 mM sodium hydrosulphite (dithionite) under alkaline conditions (100 mM $NaHCO_3$, pH 9–10). In the control experiment, incubation of samples was in 100 mM $NaHCO_3$, pH 9–10, alone. Reduction of nitrotyrosine residues was also performed directly on the membrane, prior to incubation with the first antibody.

MS

Proteins from muscular samples were first resolved by SDS/PAGE (50 μg/lane). The protein band corresponding to 130 kDa was excised and in-gel-digested with modified trypsin (Promega) without reduction/alkylation. The digested peptides were first analysed by matrix-assisted laser-desorption ionization time-of-flight MS (MALDI-TOF MS) as previously described [15].

Molecular modelling

In order to visualize the localization of nitrated tyrosine residues in the structure of human NOS2, and to appreciate the impact of its nitration on the molecule's structural integrity and biological function, we used molecular-modelling techniques. Two crystal structures are available in the Protein Data Bank (PDB) (mouse, PDB code 2NOD [16]; human, PDB code 4NOS [17]; amino acid identity for NOS2 is 94% among mouse, rat and human). We choose the human NOS2 crystal structure (PDB code 4NOS) as the more pertinent crystal for our study. A simulation protocol was used to relax and to analyse the three-dimensional model. Graphical interface was performed with the INSIGHTII package (Accelrys Inc, San Diego, CA, U.S.A.; in part this company was formerly the Genetics Computer Group) and all molecular-modelling calculations were performed with the CHARMM program [18].

Statistical analysis

Values are given as means ± S.E.M. The difference between means were analysed using the Mann–Whitney U-test. Significance was accepted at $P < 0.05$.

Reagents

Peroxynitrite was from Upstate Biotechnology, and hrNOS2 was from Alexis (San Diego, CA, U.S.A.). L-[^{3}H]Arginine was from NEN/DuPont. All other reagents were from Sigma.

RESULTS

Nitration of hrNOS2

As shown in Figure 1, incubation of hrNOS2 with 1 mM peroxynitrite resulted in tyrosine nitration and a significant decrease in enzymic activity (Figures 1A and 1B respectively). No such phenomena were observed when peroxynitrite was added to the buffer 5 min before the addition of the protein (untreated samples).

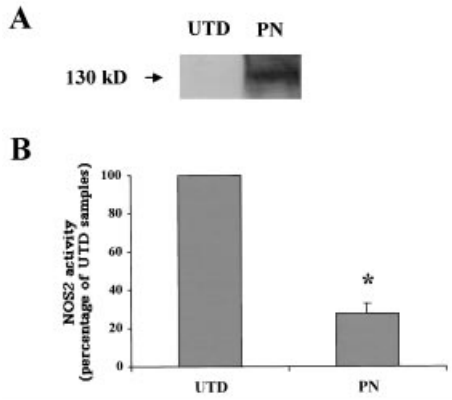

Figure 1 (A) Representative Western-blot analysis of nitrotyrosine residues detected on hrNOS2 protein after incubation with 1 mM peroxynitrite (PN) and (B) hrNOS2 activity after incubation with 1 mM peroxynitrite

(**A**) UTD, untreated sample (sample in which peroxynitrite was added to the buffer solution 5 min before adding hrNOS2). (**B**) NOS2 activity was expressed as a percentage of NOS2 activity obtained for untreated samples. Each bar represents the mean ± S.E.M. for three different experiments. *$P < 0.05$ versus untreated.

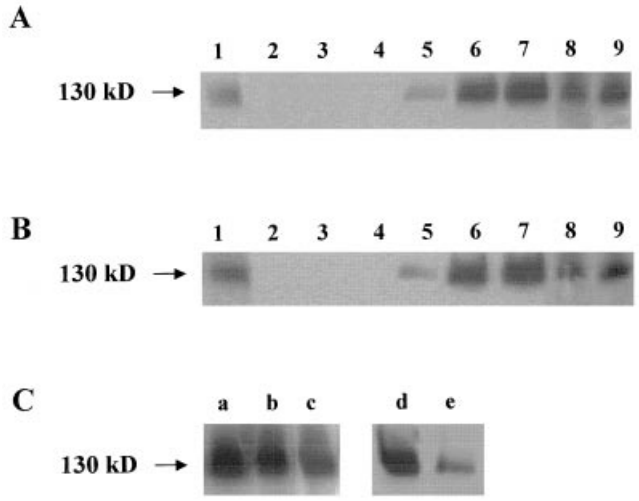

Figure 2 Representative Western-blot analysis of NOS2 protein (A) and nitrotyrosine residues (B) in the rectus abdominis muscle of control patients (lanes 2–4) and those with sepsis (lanes 5–9) and (C) representative Western-blot analysis of nitrotyrosine residues after the different control experiments

(**A** and **B**) Lane 1, human alveolar macrophages *in vitro* stimulated with LPS + IFN-γ. (**C**) Lane a, muscle from patients with sepsis without any treatment; lanes b and c, same sample as in lane a, but after incubation with 10 mM L-tyrosine (lane b) or with 10 mM free nitrotyrosine (lane c). Lanes d and e, muscle from patient with sepsis with (lane e) or without (lane d) incubation with 100 mM dithionite.

Detection of NOS2 and nitrotyrosine residues in human muscular samples

Figure 2(A) shows a representative Western blot for NOS2 protein in five patients with sepsis and three controls. As previously described in the same samples [11], whole muscular homogenates from most of the septic patients (12/16), but none of the controls, expressed NOS2 protein (as detected by Western blot) with a molecular mass identical with NOS2 protein expressed by *in vitro* lipopolysaccharide (LPS) + interferon (IFN)-γ-stimulated human alveolar macrophages (Figure 2A). No band was detected in cells transfected with the empty vector or in non-stimulated alveolar macrophages (results not shown).

Immunoblotting with an anti-nitrotyrosine antibody revealed a band similar in shape and molecular mass to the one observed when using anti-NOS2 antibody (Figure 2B). All the patients expressing NOS2 protein showed this band, whereas it was absent from all of the controls. Figure 2 also showed that nitrotyrosine antibodies are specific to nitrotyrosine and not to tyrosine (Figure 2C). Indeed, addition of 10 mM unlabelled free nitrotyrosine to the first antibody solution reduced the nitrotyrosine labelling, while addition of free L-tyrosine did not have any effect on nitrotyrosine immunostaining. In addition, dithionite, which reduced nitrotyrosine to aminotyrosine, dramatically decreased nitrotyrosine labelling (Figure 2C).

Localization of nitrotyrosine residues in human muscular samples

MS analysis was performed in muscular samples from a subset of three patients with sepsis and three controls. All of the chosen samples from patients with sepsis expressed NOS2 and did show the 130 kDa nitrotyrosine band on immunoblotting. MS analysis demonstrated nitration of tyrosine residues at only four sites among the 31 tyrosine residues present in human NOS2 (Figure 3, left-hand page). Nitrated tyrosine was located at positions 299 and 336 in the three patients examined. In addition, Tyr^{446} was nitrated in one patient, whereas Tyr^{698} was nitrated in another patient. Those four tyrosine residues (located at positions 299, 336, 446 and 698) were also nitrated when hrNOS2 was incubated with peroxynitrite *in vitro*.

The analysis of the four nitrated sites revealed remarkable conservation in the physical nature of the residues that frame the central nitrated tyrosine residue and allow us to define a new canonic sequence motif. A lysine residue is always present one or two residues before the central nitrated tyrosine residue. A negatively charged acidic amino acid (aspartic acid or glutamic acid) or polar (glutamine) is distant by three to six residues from the latter lysine residue, at the N-terminal side. An indeterminate (likely hydrophobic or polar) amino acid sequence generally separates the lysine residue and the N-terminal acidic site. A glutamine residue is observed in the three first N-terminal canonic motifs. However, the spatial arrangement of this glutamine side chain within the motif sequence pharmacophore is similar to the acidic one found in the other canonic motif. The glutamine side chain has also a hydrogen-bond-accepting group such as glutamic acid or aspartic acid. An acidic residue (aspartic acid or glutamic acid) is found at either four or seven residues at the C-terminal side of the tyrosine and is always separated from central tyrosine residue by a indeterminate chain. The global sequence of the canonic motif is Asp/Glu/Gln-Xaa_{3-6}-Lys-Xaa_{0-1}-Tyr-Xaa_{4-7}-Asp/Glu, where Xaa is an indeterminate amino acid residue.

Our three-dimensional model of human NOS2 allowed us to analyse only the localization of the first three canonic sequences, the fourth being outside of the X-ray structure (Figure 3, right-hand page). All the canonic sequences are well exposed to the bulked solvent at the surface of the three-dimensional model. The other tyrosine residues, present in the oxygenase domain, are all accessible to nitration, as seen in the model (Figure 3, right-hand page), but are not localized inside a canonic sequence. In our model, Tyr^{336} could trigger a local conformational change of the swapping domain and thus could alter the association of the two NOS2 monomers. This local spatial modification is promoted by the repulsion between the two nitrated tyrosine residues that tightly interact with Pro^{273} and Pro^{334}.

DISCUSSION

The present results demonstrated that (i) *in vitro* peroxynitrite-induced tyrosine nitration of hrNOS2 and reduced NO production and (ii) *in vivo* nitration of NOS2 in skeletal muscles from patients with sepsis occurred on four tyrosine residues exclusively belonging to a canonic sequence. To the best of our knowledge, this is the first biochemical and molecular demonstration of an *in vivo* selective tyrosine nitration of human NOS2 with potential consequences on its activity.

All nitrated tyrosines present in the crystals (Tyr^{299}, Tyr^{336} and Tyr^{446}; Figure 3B) were exposed to the surface of the protein and thus potentially available for nitration. However, these three nitrated residues represent a minority of the 28 exposed tyrosine residues. These data indicate that exposure of the aromatic ring to the surface of the protein is not the only requirement for targeting a tyrosine residue to nitration. This is in agreement with results published by Souza and co-workers [19], showing that only part of the exposed tyrosine residues were nitrated, *in vitro*, in RNase A, lysozyme and phospholipase A_2.

All of the nitrated tyrosine residues were part of a canonic sequence that has never been identified for human NOS2. Whole-genome database screening did not reveal any similarities with

existing motif sequences. Thus this sequence is not similar to those found −5 to +5 residues around the *in vitro*-nitrated tyrosine in RNase A or lysozyme [19]. The mechanism(s) explaining how the negative charge directs nitration to a neighbouring tyrosine residue is (are) still a matter of controversy. On one hand, Crow and associates [20] suggested that the carboxy group of acidic residues facilitates the nitration of nearby tyrosine residues by hydrogen-bonding with one of the two equivalent hydrogen atoms at the *ortho* position of tyrosine. On the other hand, Souza and colleagues [19] postulated that the electrostatic repulsion of negatively charged nitrating agents by the carboxy group directs the nitrating agent toward the aromatic ring of the neighbouring tyrosine. In spite of these discrepancies, these results show that the local environment of the tyrosine residue appears to be critical in determining the site of nitration. In addition, according to data concerning metalloproteins (SOD)

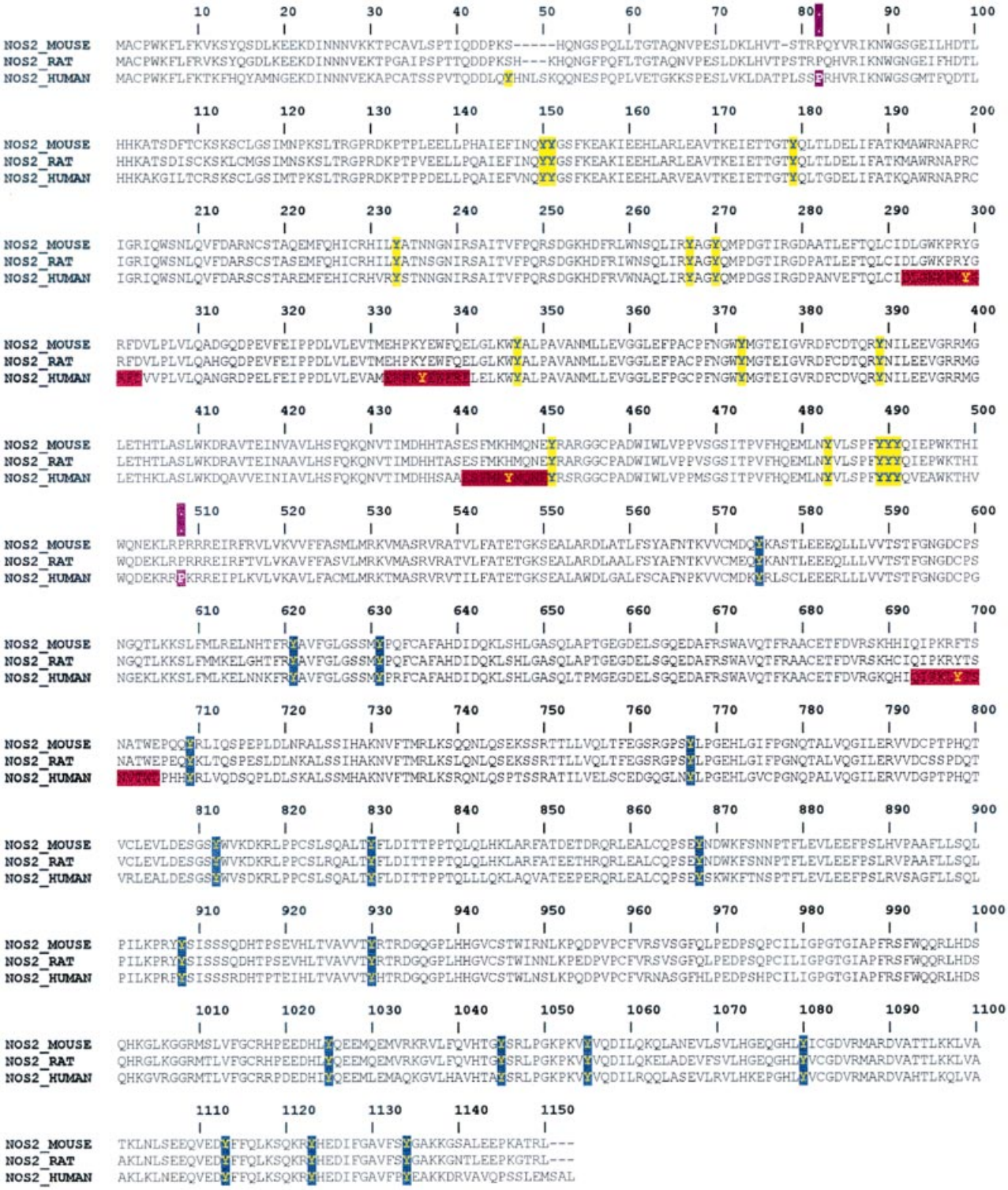

Figure 3 For legend see facing page.

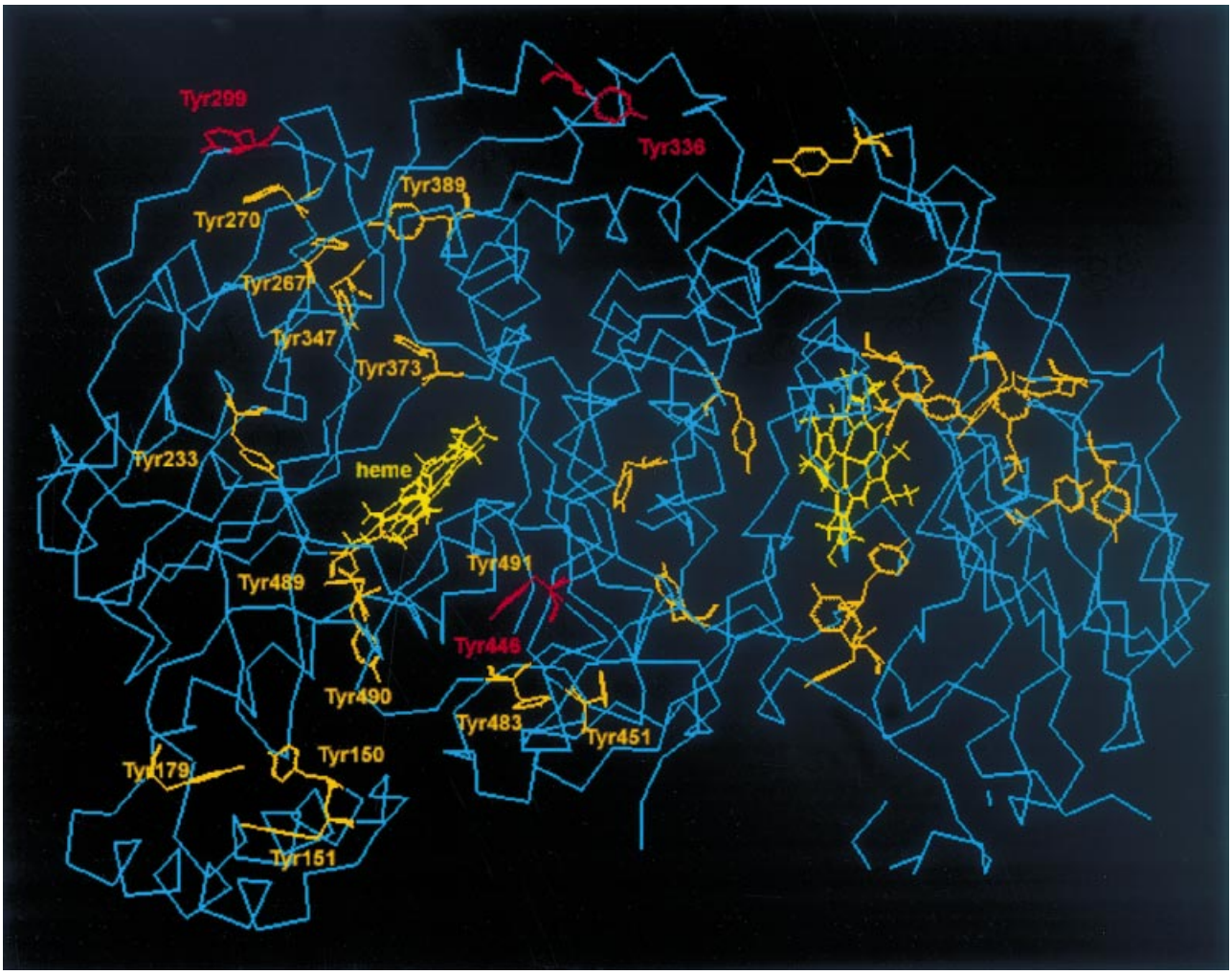

Figure 3 Multiple sequence alignment of mouse, rat and human NOS2 (corresponding SWISSPROT registration codes are respectively P29477, Q06518 and P35228) (facing page) and view of the human NOS2 three-dimensional model based on crystal 4NOS (this page)

Facing page: four tyrosine (Y) residues were found nitrated in human NOS2 (Y^{299}, Y^{336}, Y^{446} and Y^{698}). Proportions of nitrated Y were as follows: Y^{299} and Y^{336}, 79% and 90% respectively (mean for three patients examined); Y^{446} (50%) in one patient and Y^{698} (55%) in another patient. Canonic sequences corresponding to the tyrosine nitration site are shaded in red, the central nitrated tyrosine residue is coloured yellow. N- and C-terminal ends of NOS2 crystal (PDB code 4NOS) are framed by purple shading. Tyrosine residues that do not belong to nitration site are coloured in blue, shaded in yellow within the crystal sequence (oxygenase domain) and coloured in yellow, shaded in blue within the resting part of the NOS2 sequence. This page: solvent-accessible tyrosine residues are coloured orange. Nitrated tyrosine residues belonging to the canonic sequence are drawn in lines and coloured red. The prosthetic group is coloured yellow.

in which metal ions (such as Zn^{2+} and Cu^{2+}) are critical for the nitration mechanism, the lysine residue in the canonic sequence could facilitate formation of transition state before nitration.

Several mechanisms are known to lead to tyrosine nitration and could be implicated in the nitration of NOS2 observed in the present study. A first mechanism is a peroxidase-dependent nitrite oxidation [21,22]. Indeed, since sepsis is associated with ongoing inflammation, neutrophil myeloperoxidase could participate to nitrotyrosine formation via the oxidation of nitrite to form $NO_2^{\bullet}$, a strong oxidant that is capable of nitrating tyrosine [21,23]. However, as described previously [11], no inflammatory cell infiltration was observed in tissue sections of the analysed muscles. Consequently, a neutrophil peroxidase mechanism seems not to be involved in the nitration of NOS2 tyrosine residues in our muscular samples. A second mechanism leading to selective NOS2 tyrosine nitration could be the production of peroxynitrite by NOS2 itself. Indeed, it is now known that, under certain particular conditions of L-arginine depletion, NOS2 can generate both NO and O_2^-, leading to peroxynitrite generation [24]. We hypothesized that this NOS2-derived peroxynitrite formation might occur in our patients with sepsis, since sepsis is characterized by profound alterations in amino acid metabolism in skeletal muscle [25]. However, although we can not completely rule out this possibility, this mechanism seems unlikely, since no data concerning depletion of muscular L-arginine during sepsis are available in the literature. Finally, peroxynitrite generated by neighbouring cells or inside the skeletal myocytes could be responsible for nitration of NOS2. However, this hypothesis deserves further investigation.

Whatever the mechanism(s) of tyrosine nitration, it is highly likely that this phenomenon negatively modulated NOS2 activity *in vivo*. Indeed, proportions of nitration of the two tyrosine residues found nitrated in all patients examined (Tyr^{299} and Tyr^{336}), and likely involved in NOS2 activity (see below), were high, namely 79 and 90% respectively. In addition, using rhNOS2 we showed that incubation with peroxynitrite *in vitro* (1) led to nitration of the same four tyrosine residues that were nitrated in NOS2 from samples from patients with

sepsis, and (2) was associated with a significant decrease in NO synthesis. It must be noted that peroxynitrite could also inactivate NOS2 by its oxidative properties, as shown previously by Huhmer and colleagues [26]. However, the experimental conditions used in the present study (25 mM $NaHCO_3$ buffer, pH 7.4) favoured tyrosine nitration instead of oxidation [26], thus making an oxidative effect of peroxynitrite unlikely. These *in vitro* results are in accordance with data from several groups showing that different enzymes (namely NOS3, Mn-SOD, tyrosine hydroxylase and glutathione reductase) have a decreased activity following tyrosine nitration by peroxynitrite [6–9]. The exact mechanism of this decreased activity is yet not understood, but it could be related to structural alterations in NOS2. The impact of nitration on the dimerization and the catalytic function of NOS2 can be differently appreciated depending on the three-dimensional model, owing to a different contact surface between the two monomers. As stated in the Results section, nitration of Tyr^{299}, as in the first model, and Tyr^{336}, as in the second one, could trigger a local conformational change of NOS2 swapping domain and thus could alter the association of the two NOS2 monomers. Indeed, there is a repulsion between the two nitrated tyrosine residues that tightly interact with Pro^{273} and Pro^{297} in the first model and Pro^{334} in the second one. The motion of those proline residues could induce the modification of the spatial position of the swapping-domain loops, since proline residues can confer flexibility to protein segments. This conformational change may then alter the enzymic activity of NOS2. Alternatively, selective tyrosine nitration of NOS2 could decrease enzymic activity by interfering with post-translational modifications of tyrosine residues, i.e. tyrosine phosphorylation or sulphation. Indeed, protein phosphorylation or sulphation plays a role in the regulation of protein activity and function. No data concerning NOS sulphation is available in the literature. By contrast, tyrosine phosphorylation has been reported to increase the activity of murine NOS2 [27]. Occurrence of a similar phenomenon in human NOS2 and its interference with nitration could explain the decreased NOS2 activity induced by tyrosine nitration.

Overall, the present findings demonstrated the selective *in vivo* tyrosine nitration of NOS2 in patients with sepsis. This phenomenon seems to be not only a marker of the action of NOS2-derived oxidants, but, more importantly, an endogenous mechanism for the modulation of NOS2 enzymic activity.

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