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Protein tyrosine nitration in hydrophilic and hydrophobic environments

Review Article

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Summary. In this review we address current concepts on the biological occurrence, levels and consequences of protein tyrosine nitration in biological systems. We focused on mechanistic aspects, emphasizing on the free radical mechanisms of protein 3-nitrotyrosine formation and critically analyzed the restrictions for obtaining large tyrosine nitration yields in vivo, mainly due to the presence of strong reducing systems (e.g. glutathione) that can potently inhibit at different levels the nitration process. Evidence is provided to show that the existence of metal-catalyzed processes, the assistance of nitric oxide-dependent nitration steps and the facilitation by hydrophobic environments, provide individually and/or in combination, feasible scenarios for nitration in complex biological milieux. Recent studies using hydrophobic tyrosine analogs and tyrosine-containing peptides have revealed that factors controlling nitration in hydrophobic environments such as biomembranes and lipoproteins can differ to those in aqueous compartments. In particular, exclusion of key soluble reductants from the lipid phase will more easily allow nitration and lipid-derived radicals are suggested as important mediators of the one-electron oxidation of tyrosine to tyrosyl radical in proteins associated to hydrophobic environments. Development and testing of hydrophilic and hydrophobic probes that can compete with endogenous constituents for the nitrating intermediates provide tools to unravel nitration mechanisms in vitro and in vivo; additionally, they could also serve to play cellular and tissue protective functions against the toxic effects of protein tyrosine nitration.

Keywords: Tyrosine nitration – Peroxynitrite – Nitrogen dioxide – Hemeperoxidases – Free radicals – Hydrophobic environments

Tyrosine nitration: definition, levels and biological significance

The nitration of protein tyrosine residues constitutes the substitution of hydrogen by a nitro group ($-NO_2$; +45 Da) in the *3*-position of the phenolic ring and represents a post-translational modification produced by nitric oxide ($^{\bullet}NO$)-derived oxidants such as peroxynitrite[1] ($ONOO^-$; ONOOH) and nitrogen dioxide radical ($^{\bullet}NO_2$). Early protein chemistry work (Sokolovsky et al., 1966) indicated that nitration by agents such as tetranitromethane could result in dramatic changes in protein structure and function; however, it was not until early in the nineties (Beckman et al., 1990; Ischiropoulos et al., 1992) when the potential biological significance of protein tyrosine nitration was appreciated after recognizing the formation of strong oxidizing and nitrating intermediates during the biological oxidation of $^{\bullet}NO$ (Beckman et al., 1990; Koppenol et al., 1992; Radi et al., 1991a, b). Since then, protein tyrosine nitration by a number of biologically-relevant nitrating intermediates has been steadily established to occur both in vitro and in vivo (Ischiropoulos, 2003; Radi, 2004).

Protein tyrosine nitration is, in general terms, a low yield process and while substantial progress has been made in detecting nitrated proteins in biological samples by immunochemical methods (i.e. using antibodies against protein 3-nitrotyrosine) (Radi et al., 2001; Ye et al., 1996) and in identifying individual nitrated proteins by proteomic-based strategies (Turko et al., 2003), precise quantitative determination of 3-nitrotyrosine levels in fluids and tissues represents a real challenge as it requires, develop-

[1] IUPAC recommended names for peroxynitrite anion ($ONOO^-$) and peroxynitrous acid (ONOOH) ($pK_a = 6.8$) are oxoperoxonitrate (1−) and hydrogen oxoperoxonitrate, respectively. The term peroxynitrite is used to refer to the sum of $ONOO^-$ and ONOOH.

ment of analytical methodologies that must both be highly specific and sensitive and avoid potential artifactual nitration which can easily occur during sample processing[2].

Indeed, reported levels of protein 3-nitrotyrosine in stressed tissues is in the range of 10–100 pmol/mg, corresponding to about 1–5 nitrated residues over 10,000 tyrosines (100–500 μmol/mol) (Radi, 2004; Zheng et al., 2005). The extent of tyrosine modification by nitration is comparable to that of other oxidative modifications including chlorination, bromination and hydroxylation to 3-chloro, 3-bromo and 3-hydroxy-tyrosine, respectively, modifications that may coexist with tyrosine nitration at variable ratios, depending on the dominant nitration mechanism (*vide infra*).

The increase of the basal levels of 3-nitrotyrosine found under normal conditions is established to serve as a footprint of nitro-oxidative damage in vivo both in animal models and humans diseases; moreover, it has been also revealed as a strong biomarker and predictor of disease progression and severity in conditions such as acute and chronic inflammatory processes, cardiovascular disease, neurodegeneration and diabetic complications, among others (Ceriello, 2002; Radi, 2004; Shishehbor et al., 2003; Zhang et al., 2001; Zheng et al., 2005). In addition, protein tyrosine nitration could contribute to alterations (loss or gain) of protein function in vivo; in this regard, however, few examples of a correlation between the extents of protein nitration and inactivation in vivo have been substantiated because for a significant loss-of-function, a large proportion of nitrated protein molecules are required. Moreover, many times, protein tyrosine nitration occurs concomitantly with other oxidative modifications which may also influence protein function and therefore a cause-consequence relationship is in many cases not possible to demonstrate. A remarkable and well-documented example is constituted by the in vivo nitration and inactivation of MnSOD (MacMillan-Crow et al., 1996; Quijano et al., 2005; Radi, 2004), a critical mitochondrial antioxidant enzyme, that becomes modified in animal and human inflammatory disease conditions. MnSOD nitration occurs site-specifically in Tyr-34 located at 5 Å of the active site, with the manganese atom playing a key role on the nitration process (Beckman et al., 1992; Quijano et al., 2001; Quint et al., 2006; Yamakura et al., 1998). Other reported proteins to which extents of nitration might cause significant decrease in activity in vivo in cell and animal disease models are actin (in sickle cell disease) (Aslan et al., 2003), prostacyclin synthase (in vascular dysfunction) (Zou and Ullrich, 1996; Zou et al., 1997), tyrosine hydroxylase (in Parkinson's disease) (Giasson et al., 2002; Ischiropoulos and Beckman, 2003), and prostaglandin endoperoxide synthase-2 (PHS-2) (in vascular inflammation) (Schildknecht et al., 2006). Alternatively, and as a more novel concept, tyrosine nitration may promote a gain-of-function, in which case modification of only a small protein fraction may trigger a substantive signal from a previously weak or non-existent function; this appears to be the case of cytochrome c (i.e. peroxidase activity) (Batthyany et al., 2005; Cassina et al., 2000), protein kinase Cε (i.e. translocation and interaction with RACK2) (Balafanova et al., 2002) and glutathione S-transferase (i.e. enzyme activation) (Ji et al., 2006). Similarly, small amounts of a nitrated protein can serve in nucleation steps in the process of making protein fibers (e.g. fibrinogen and pro-coagulant activity) (Vadseth et al., 2004) or protein aggregates (e.g. α-synuclein and Lewy bodies) (Giasson et al., 2000; Hodara et al., 2004). Thus, considering the low yield of tyrosine nitration for most proteins, evaluation of new activities or interactions by nitrated proteins becomes a relevant matter in the context of alterations of cell homeostasis by nitro-oxidative stress.

In addition to functional changes, protein nitration may cause other biological effects: a) nitrated proteins may

[2] Techniques that are based on anti-protein 3-nitrotyrosine antibody (e.g. ELISA) should be considered semi-quantitative. At present, the gold standard analytical technique for the quantitation of 3-nitrotyrosine is either GC or LC coupled to MS/MS; recently, much improvement has been accomplished using LC/MS/MS due to the innovation on mild ionization sources such us electrospray ionization (ESI). The basal level of free 3-nitrotyrosine in human plasma is 1.5 ± 1.0 nM. Thus, analytical approaches to measure free 3-nitrotyrosine must have a limit of quantification (LOQ) below 0.5 nM. Care should be taken in sample preparation and processing since some of these techniques require derivatization reactions that can lead to artifactual acid-catalyzed nitration (for extensive reviews see (Duncan, 2003; Tsikas and Caidahl, 2005)). In this regard, isotope dilution methods using uniformly labeled tyrosine and 3-nitrotyrosine (e.g. ^{13}C-tyrosine) represent major advances for quantitation purposes (see for example Gaut et al., 2002; Nicholls et al., 2005). The source of free 3-nitrotyrosine is not fully established but it should mainly arise from the turnover of nitrated proteins as free 3-nitrotyrosine can not be incorporated in the de novo synthesis of proteins. Quantitation of protein-3-nitrotyrosine involves total hydrolysis of sample proteins to release 3-nitrotyrosine. Acid or alkaline hydrolysis and enzymatic digestion of proteins is commonly used. In spite of the facts that protein acid hydrolysis is a tedious process and meticulous controls should be taken to cope with artifactual nitration, it still represents the most accepted approach (for further considerations on the hydrolysis methods, see (Fountoulakis and Lahm, 1998). The reported basal concentration for human plasma protein 3-nitrotyrosine is in the range of 0.4–1.6 over 1×10^6 (3-nitro-Tyr/Tyr). However, more important discrepancies in values are found in the literature for protein-3-nitrotyrosine than for free 3-nitrotyrosine mainly because of the protein hydrolysis step required to perform this measurement (Tsikas and Caidahl, 2005). Other analytical techniques such as HPLC with electrochemical, fluorescence or even UV-Vis detection have been successfully applied for in vitro well-controlled biochemical experimental setups (Radi et al., 2001).

become auto-antigens and trigger immunological responses (Herzog et al., 2005; Radi, 2004; Whiteman et al., 2006); b) since nitrated tyrosines are uncapable to undergo phosphorylation in the phenolic −OH (Kong et al., 1996; Tien et al., 1999), tyrosine kinase-dependent signal transduction could be affected, and c) nitrated proteins may be more readily targeted for protein degradation. The presence of repair systems for tyrosine nitrated proteins (e.g. the existence of ''denitrase activity'') has been proposed (Irie et al., 2003; Kamisaki et al., 1998) but these pathways remain to be established.

Site- and protein-specificity in tyrosine nitration processes

Tyrosine in a protein constitutes in average 3–4 mol% of the amino acids and therefore proteins typically contain several tyrosine residues. However, peptide mapping studies have shown that within a protein typically one or two tyrosine residues become preferentially nitrated; the determinants of which are not fully established but depend on three main factors: a) protein structure, b) nitration mechanism, and c) environment where the protein is located. Regarding protein structure, the available information indicates that factors that favor nitration are the presence of an acidic amino acid close to tyrosine (Ischiropoulos, 2003; Souza et al., 1999), the localization of a tyrosine residue on a loop structure (Ischiropoulos, 2003; Souza et al., 1999; Sacksteder et al., 2006) and the nearby presence of transition metal centers and binding sites for hemeperoxidases (Shao et al., 2005). In particular, some transition metal centers site-specifically enhance peroxynitrite-dependent nitration (Quijano et al., 2001; Schmidt et al., 2003) and interactions of the positively charged myeloperoxidase with negatively charged clusters of a target protein can also focus protein nitration (Shao et al., 2005). The presence of amino acids such as cysteine, methionine and tryptophan that compete for the proximal nitrating species (e.g. $^{\bullet}NO_2$), might be inhibitory for tyrosine nitration, although this view has been recently challenged (Sacksteder et al., 2006); moreover, cysteine can also promote electron transfer through the protein backbone and reduce a tyrosyl radical to tyrosine with the consequent formation of a cysteinyl radical and the inhibition of nitration (Zhang et al., 2005; Sacksteder et al., 2006). The influence of the nitration mechanism and the protein environment (polar or nonpolar) on the selectivity of the nitration site is discussed elsewhere in the review and new data is rapidly emerging (Sacksteder et al., 2006; Heijnen et al., 2006).

Proteomic based approaches involving peptide mapping and sequencing are revealing that some proteins are preferentially nitrated in vivo, and that within those proteins one or at most a few tyrosine residues are nitrated (see for example Kanski et al., 2005; Zheng et al., 2005) Thus, there is an ongoing interest in the identification of nitrated proteins in disease states and since nitrating intermediates react in the close proximity of their site of formation, intra- or extracellular distribution of nitrated proteins also provide information regarding the compartments on which nitration events predominantly take place (Heijnen et al., 2006). For example, the significant nitration of mitochondrial proteins observed in a variety of disease states, strongly suggests the contribution of mitochondria in the formation of $^{\bullet}NO$-derived oxidants in conditions associated to mitochondrial dysfunction and signaling of apoptosis (Quijano et al., 2005; Sacksteder et al., 2006), where some nitrated proteins, in addition, can play a key contributory role.

Structural and functional studies with pure and well-characterized (i.e. with identified nitration site(s)) mononitrated species are required to unambiguously and precisely define the potential biological effects of tyrosine nitration in specific proteins; isolation and characterization of mono-, di- and polynitrated species can be accomplished by chromatographic separation and peptide mapping of fractions derived from the in vitro treatment of native protein with nitrating agents (e.g. peroxynitrite), which will initially yield a mixture containing a variety of modified species plus the remaining native protein (Batthyany et al., 2005); among this mixture, a percentage will be one or more mononitrated species, which in turn may be the species preferentially formed in vivo. Importantly, depending on the protein under study, the nitration system and the experimental conditions, is possible that in vitro nitration does not completely match the nitration sites identified in vivo, an aspect that warrants evaluation in each specific case.

The free radical mechanism of tyrosine nitration

There is now agreement on that tyrosine nitration can occur biologically by a variety of routes (e.g. peroxynitrite- or hemeperoxidase-dependent) but that are all based in free radical chemistry. Indeed, 3-nitrotyrosine as evidenced in vitro and most probably in vivo is the product of at least two consecutive reactions. Peroxynitrite-mediated nitration is well studied and will serve as a starting point. Subsequently we will broaden the view to consider alternative precursors for the mechanism of nitration. In

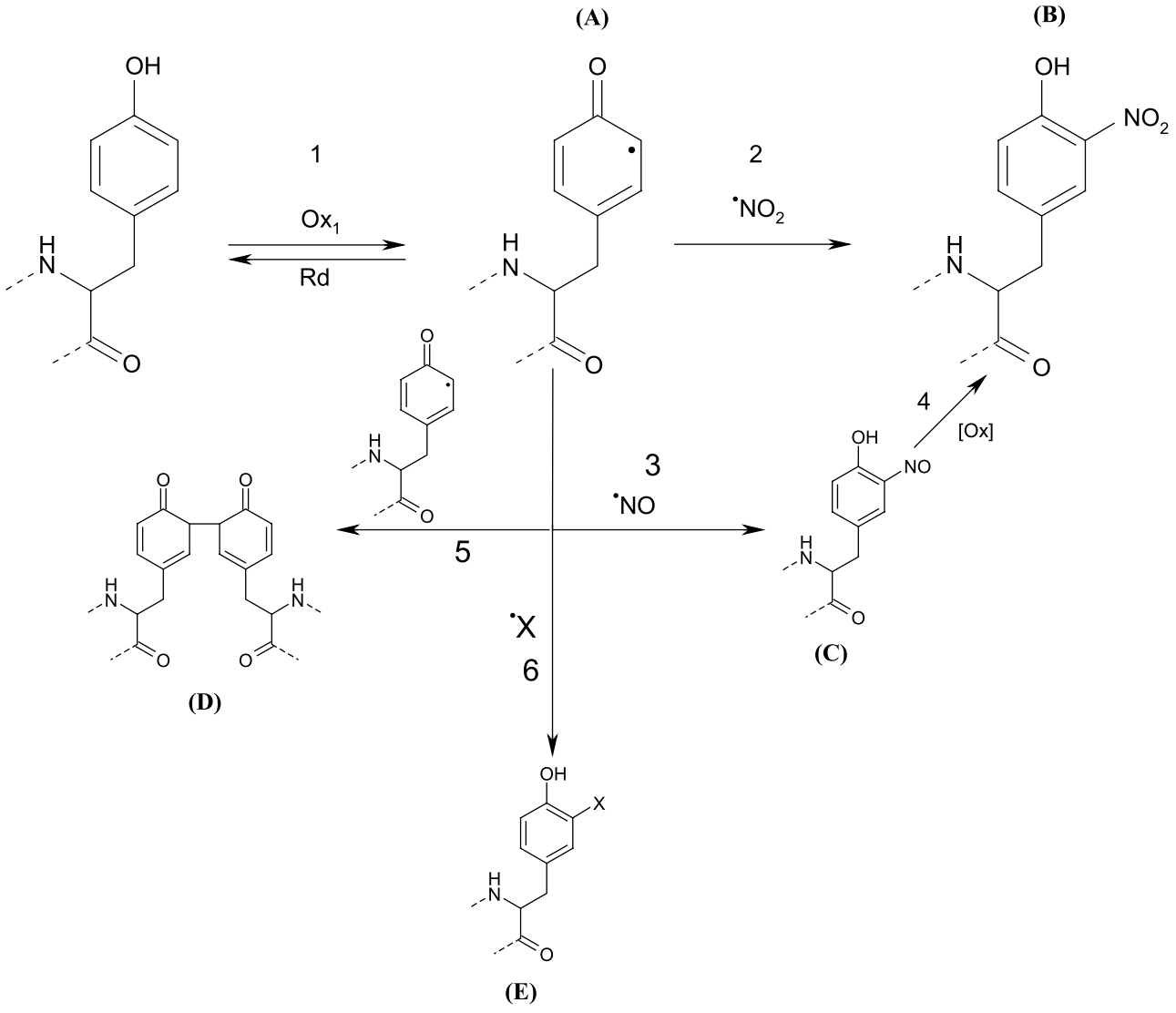

Fig. 1. Reactions leading to nitration or away from it. Reaction 1 is a one-electron oxidation that yields the intermediate tyrosine phenoxyl radical (A) this reaction reverts to tyrosine in the presence of numerous reductants. If (A) adds a nitrogen dioxide radical (reaction 2) the main product (B) is 3-nitrotyrosine. In reaction 3, the radical added to tyrosyl radical is nitric oxide and the new intermediate (C) is 3-nitrosotyrosine, which in turn can be oxidized by two one-electron steps (reaction 4) to yield (B). Intermediate (A) can dimerize (reaction 5) to yield 3,3′-dityrosine (D) or can add radicals other than $^{\bullet}NO_2$ in reaction 6, for instance, if $^{\bullet}X = {}^{\bullet}OH$, then (E) is 3,4-dihydroxy-phenylalanine (DOPA). Reaction 3 constitutes a particular case of reaction 6. The sequence of reactions 1, 3 and 4 has been observed in systems containing tyrosine, H_2O_2 and a hemeperoxidase, see Sturgeon et al. (2001). Reactions and products shown in gray represent those diverting the (A) from nitration pathways

a chemical system consisting in tyrosine, peroxynitrite (added as bolus) and an inert buffer at neutral pH (such as 50 mM phosphate), tyrosine is first oxidized by either of the radicals arising from homolysis of ONOOH ($^{\bullet}OH$ or $^{\bullet}NO_2$ radicals (Radi et al., 2001)) (see Fig. 1) yielding the tyrosine phenoxyl radical (**A**) which in turn couples with $^{\bullet}NO_2$ to produce 3-nitrotyrosine (**B**). Briefly, an oxidation step (Fig. 1, reaction 1) followed by an addition step (Fig. 1, reaction 2).

The identity of the first oxidant (Ox_1) is not critical and one-electron oxidants can produce **A**. In the original example, $^{\bullet}OH$ does it very fast ($1.3 \times 10^{10}\,M^{-1}\,s^{-1}$) but in a low yield (5%, which can be increased by the base-catalyzed dehydration of the main product) (Solar et al., 1984), whereas $^{\bullet}NO_2$ is much slower ($3.2 \times 10^{5}\,M^{-1}\,s^{-1}$, pH 7.5) (Prutz et al., 1985). This example is of limited biological relevance (as ONOOH homolysis is a minor route of peroxynitrite decay in vivo (Radi et al., 2001)) but serves as a starting point to understand the reaction mechanism and its details. The next step in complexity involves the addition of a nitration promoter. Carbon dioxide, for instance, reacts with $ONOO^-$ before homolysis

Table 1. Promoters of peroxynitrite-mediated tyrosine nitration: yields in in vitro systems for bolus addition

Promoter	Ox_1	Ox_1 yield (%)	k for reaction 1 ($M^{-1}\,s^{-1}$)	NO_2-Y yield (%)	Ref
H^+	$^{\bullet}OH$	33	1.3×10^{10}	6–10	Beckman et al. (1992)
CO_2	$CO_3^{\bullet-}$	35	3.0×10^{7}	35	Denicola et al. (1996)
MPO	compound II	100	1.6×10^{4}		Marquez and Dunford (1995)
MnTM-2-PyP	$O=Mn^{IV}$TM-2-PyP	100	4.9×10^{3}	30	Ferrer-Sueta et al. (2003)
a	$^{\bullet}NO_2$		3.2×10^{5}		

[a] $^{\bullet}NO_2$ can perform as Ox_1 and is produced during the reaction of peroxynitrite with all the promoters listed

to produce carbonate radical ($CO_3^{\bullet-}$), which will be Ox_1 in this case (Radi et al., 2001). $CO_3^{\bullet-}$ excels in oxidizing tyrosine ($3 \times 10^7\,M^{-1}\,s^{-1}$) with quantitative yield. Another possible promoter would be myeloperoxidase (MPO), that is oxidized by peroxynitrite to yield compound II, which in turn reacts with tyrosine with a rate constant of $1.57 \times 10^4\,M^{-1}\,s^{-1}$ (Marquez and Dunford, 1995). Finally, low molecular weight transition metal complexes such as MnPorphyrins, would yield a high oxidation state oxo-metal complex (e.g. $O=Mn^{IV}$) that can act as Ox_1 in Fig. 1, reaction 1 (in the case of MnTM-2-PyP with a rate constant of $4.9 \times 10^3\,M^{-1}\,s^{-1}$ (Ferrer-Sueta et al., 1999)).

There is an apparent lack of correlation between the rate constant of reaction 1 and the maximum nitration yield achieved (see Table 1), even in relatively simple systems like those mentioned. One factor influencing the yield of nitration is the yield of formation of Ox_1 when $ONOO^-$ is the precursor. Other important factors would be the relative stability of Ox_1 (Ferrer-Sueta et al., 2003) and its possible involvement in oxidation reactions not yielding 3-nitrotyrosine.

Reaction 2 is evidently much less ambiguous; the only possibility is $^{\bullet}NO_2$ reacting with the TyrO$^{\bullet}$ formed in reaction 1. Nitrogen dioxide can be formed in the same reaction that produces Ox_1, as is the case of all the promoters of Table 1 reacting with $ONOO^-$. It can also be formed independently, for instance, by oxidation of nitrite. In regard to this, some years ago a series of works disputed the prevalence of $ONOO^-$ as being the only ultimate causative agent of tyrosine nitration in vivo (van der Vliet et al., 1997). The main alternative proposed the combined presence of H_2O_2 as the oxidant taking an electron each from nitrite and tyrosine in a reaction catalyzed by MPO (and potentially several other hemeperoxidases). In fact, this MPO-mediated tyrosine nitration also fits well in our general scheme, namely, the reaction of H_2O_2 with MPO produces compound I which can oxidize nitrite and tyrosine by one electron each yielding the TyrO$^{\bullet}$ and $^{\bullet}NO_2$ necessary for reaction 2. In this case either compound I or compound II can act as Ox_1. The rate constants indicate that nitrite is oxidized faster than tyrosine by compound I and the reverse is true for compound II (Burner et al., 2000; Marquez and Dunford, 1995).

An alternative mechanism to the addition of $^{\bullet}NO_2$ for the second step in nitration is the reaction with nitric oxide (Fig. 1, reaction 3) which can readily react with TyrO$^{\bullet}$ radicals. Initially, reaction 3 was studied to understand the effect of $^{\bullet}NO$ on catalytically-active TyrO$^{\bullet}$ radicals (Eiserich et al., 1995) but then it was discovered that the nitroso intermediate (C) was further oxidized to form an iminoxyl radical and finally the nitro compound (Gunther et al., 1997; Sanakis et al., 1997). This reaction was initially identified in tyrosine residues of prostaglandin H synthase-2 (PHS-2) and photosystem II, but later on it was shown to take place with free tyrosine in solution in the presence of hydrogen peroxide, a peroxidase and $^{\bullet}NO$ (Sturgeon et al., 2001). This alternate route to 3-nitrotyrosine has not been studied in detail as reactions 1 and 2, partly because of the elusive nature of intermediate (**C**), but offers an interesting bypass to the need of $^{\bullet}NO_2$ to reach the nitrated product (Chen et al., 2004).

In addition to the nitration of tyrosine, side reactions can divert TyrO$^{\bullet}$ from adding to $^{\bullet}NO_2$ and result in the formation of secondary products. The most studied of these reactions is the dimerization to yield 3,3′-dityrosine (Product **D**, reaction 5). No other reactants are needed for this reaction, in fact even under strict stoichiometric conditions relating Ox_1 and $^{\bullet}NO_2$ concentrations, 3,3′-dityrosine is a readily observable product of the reaction and its proportion to the overall oxidation depends mainly on the relative concentrations of Tyr, TyrO$^{\bullet}$ and $^{\bullet}NO_2$ during the reaction. Also the freedom of movement can govern the probability of the encounter of two TyrO$^{\bullet}$ radicals, thus this reaction would be more important with free tyrosine in non-viscous solutions (*vide infra*). Excess radicals other than $^{\bullet}NO_2$ ($^{\bullet}X$, Fig. 1, reaction 5) can convert TyrO$^{\bullet}$ to a 3-substituted tyrosine derivative (E). For instance, during Fenton oxidation of tyrosine derivatives, excess

$^{\bullet}$OH leads to the formation of 3-hydroxylated derivatives (Bartesaghi et al., 2006). The colocalization of 3-hydroxytyrosine and 3-nitrotyrosine was once considered to be a useful tool to discriminate between peroxynitrite and other nitrating species (Santos et al., 2000), but it was later on discovered that 3-hydroxytyrosine formation can also be inferred from the observed tyrosine semiquinone radical in the reaction of cytochrome c with excess hydrogen peroxide (Chen et al., 2004). On the other hand, the concomitant presence of 3-chloro or 3-bromotyrosine with 3-nitrotyrosine, is indicative of the participation of MPO and eosinophil peroxidase (EPO), respectively in the nitration process (Brennan et al., 2002; Radi, 2004).

Tyrosine nitration in soluble proteins and proteins associated to hydrophobic phases

The hydrophathy of tyrosine (relative preference for aqueous and nonpolar environments) is in a midway value respect to highly hydrophilic (e.g. arginine, aspartate) and hydrophobic (e.g. phenylalanine, tryptophan) amino acids. The relatively hydrophilicity and hydrophobicity of tyrosine measured as free energy values derived from the partition coefficients in vapor/water and water/cyclohexane, respectively, are of -8.5 kcal/mol and -2.3 kcal/mol (considering a zero value for glycine), which can be compared, for example, to -22.31 and $+3.0$ kcal/mol for arginine and -3.15 and -2.5 kcal/mol for phenylalanine (Creighton, 1992). The free energy of transfer from water to nonpolar solvents of the side chain of tyrosine is close to zero kcal/mol, but reported values vary from slightly negative (ethanol and dioxane) to slightly positive (in cyclohexane), depending on the organic solvent used for the measurement. For comparative purposes, in the case of the more hydrophobic tryptophan and phenylalanine, the value is ~ -1.5 to 2 kcal/mol regardless of the organic solvent used.

The $-$OH group in tyrosine tends to interact with water while the aromatic ring with other non-polar groups. Thus, it is difficult to *a priori* classify tyrosine as simply solvent-exposed or buried amino acid; in average only 15% of the residues are at least 95% buried and therefore inaccessible to the solvent (Creighton, 1992). In the case of mammalian cytochrome c, for example, which is highly soluble in aqueous environments[3] and contains four highly conserved tyrosine residues, two tyrosines are close to the protein surface (Tyr97; Tyr74), while one is in an intermediate position (Tyr67) and one protected from the solvent (Tyr48). Cytochrome c nitration by peroxynitrite-derived radicals ($^{\bullet}$OH, $CO_3^{\bullet-}$ and $^{\bullet}NO_2$) results in the preferential formation of mono-nitrated species in either Tyr97 or Tyr74 (the most solvent-exposed); importantly, di-nitrated species involve either 3-nitro-Tyr97 or 3-nitro-Tyr74 plus 3-nitro-Tyr67 since nitration of the first tyrosine residue (Tyr97 or 74) promotes a conformational change in cytochrome c which results in a easier accessibility for peroxides to the 6th coordination position of the heme-Fe (Batthyany et al., 2005), which would promote the direct reaction of peroxynitrite with the Fe^{III} and the site-specific nitration of the adjacent Tyr67. Interestingly, cytochrome c association to cardiolipin, the major phospholipid of the inner mitochondrial membrane, also promotes conformational changes in cytochrome c (Kagan et al., 2005) which may favor nitration of Tyr67, assuring that nonpolar environments can differentially modulate nitration sites and yields in proteins.

Most of the mechanistic studies of tyrosine nitration for free tyrosine or tyrosine analogs (e.g. p-hydroxyphenyl acetic acid), tyrosine-containing peptides or proteins have been performed in aqueous solution (Beckman et al., 1992; Cassina et al., 2000; Kong et al., 1996; Quijano et al., 2001; Radi, 2004; Tien et al., 1999). However, in many cases, tyrosine nitration can occur in vitro and in vivo in proteins associated to non-polar or hydrophobic compartments as indicated by early work in lipoproteins (Beckman et al., 1994) and biomembranes (Velsor et al., 2003). Indeed, the first in vivo report detecting nitrated protein in a human disease conditions, was performed in an atherosclerotic human coronary artery, where a significant portion of the nitrated protein, as detected by immunohistochemistry, was associated to the lipid-rich material of the atheroma plaque. Later studies involving isolation of lipoproteins and analytical detection of 3-nitrotyrosine revealed that both Apo A (Shao et al., 2005) and to a lesser extent Apo B (Hazen et al., 1999) are nitrated in vitro and in vivo and that apolipoprotein nitration increases in cardiovascular patients, correlating well with the severity of the disease and effectiveness of the treatment (Zhang et al., 2001; Zheng et al., 2004, 2005). In biomembranes, protein tyrosine nitration in specific proteins has been revealed at the plasma (e.g. erythrocyte membrane band 3 (Mallozzi et al., 1997), mitochondrial (e.g. complex I of the inner membrane) (Murray et al., 2003), sarcoplasmic reticulum (e.g. Ca^{2+}-ATPase, SERCA) (Viner et al., 1999; Xu et al., 2006) and microsomal (e.g. glutathione-S-transferase) membranes

[3] Cytochrome c is a mitochondrial protein that interacts with the external leaflet of the inner mitochondrial membrane shuttling electrons from cytochrome b to cytochrome a; at least two different populations of cytochrome c exist at any time, one membrane-bound (through both electrostatic and hydrophobic interactions) and one soluble in the intermembrane space.

(Ji et al., 2006). In proteins associated to hydrophobic structures, nitration could occur in tyrosines located in hydrophobic domains but also in solvent-exposed tyrosines. Although with the structural data available sometimes is difficult to assign the position of a tyrosine within a protein because not in all cases the native three-dimensional structure is available, in some this information exists or can be inferred. For example, in case of erythrocyte membrane band 3, tyrosine nitration occurs in the cytosolic, solvent exposed, domain but not in the transmembrane domain (Mallozzi et al., 1997). In the case of SERCA, Tyr294 and 295, located on a transmembrane domain are nitrated, both in vitro and in vivo (Viner et al., 1999; Xu et al., 2006); indeed, the specific nitration of SERCA at tyrosines 294, 295, has been just reported in arteries and skeletal muscle during vascular degeneration and aging in both animals and human patients (Xu et al., 2006). For microsomal glutathione S-transferase, Tyr92 close to the active site and on a transmembrane domain becomes nitrated; nitration may be catalyzed by the heme center and would be responsible for the gain-of-function reported (Ji et al., 2006). Finally, in ApoA the relatively hydrophilic Tyr-192 results nitrated but becomes resistant to myeloperoxidase-dependent nitration once located more hydrophobically secondary to association to HDL (Shao et al., 2005).

Probes for studying tyrosine nitration in hydrophilic and hydrophobic environments

Most mechanistic studies of tyrosine nitration have been performed in aqueous environments. L-Tyrosine has low solubility in water (0.045 g/100 ml at 25 °C) (Dawson et al., 1986) and therefore its final concentration in buffers at physiological pH and temperature can rarely go above 2.0 mM. Thus, when higher concentrations are required for mechanistic studies, hydrophilic analogs such as p-hydroxyphenyl acetic acid (pHPA) have been extensively used (Beckman et al., 1992; Mani et al., 2003; Moore and Mani, 2002; Quijano et al., 2001). In addition, the first report on the formation of peroxynitrite by human cells was performed by following the nitration of pHPA added extracellularly to activated iNOS-containing rat alveolar macrophages, which evolved to 3-nitro-pHPA. Alternative probes have been represented by tyrosine-containing peptides (Eiserich et al., 1999; Kong et al., 1996; Zhang et al., 2003) and mutated forms of CuZn-SOD containing a tyrosine residue adjacent to the copper center that becomes prone to nitration (Macfadyen et al., 1999).

On the other hand, factors that influence tyrosine nitration in hydrophobic environments are just starting to be defined (Bartesaghi et al., 2006; Trujillo et al., 2005; Zhang et al., 2001, 2003). To this aim, there has been a recent appreciation of the need to develop hydrophobic probes which should be a) incorporated and retained into a lipid phase, b) stable before and after nitration, and c) separated from the lipid phase components for quantitation purposes. In this regard, two types of hydrophobic probes have been recently generated and tested. On the first place, different hydrophobic tyrosine analogs have been evaluated, in particular tyrosine esters in liposomes. Of all the potential compounds studied, *N-t*-BOC L-tyrosine *tert* butyl ester (BTBE) resulted to be a valuable hydrophobic tyrosyl probe for investigating nitration and other oxidation reactions (i.e. dimerization, hydroxylation) in membranes. Indeed, BTBE was incorporated in high yields (>98%) into the lipid phase of phosphatidylcholine (PC) liposomes and turned to be an extremely stable compound being resistant to hydrolysis for at least 40 h. Spin-label data indicated that the highest concentration of BTBE was present near the glycerol backbone (Zhang et al., 2001) and kinetic data supported that the phenolic hydroxyl group was located towards the lipid-water interphase where it could dissociate with an apparent pKa ~10 (Bartesaghi et al., 2006), in agreement with the structural properties of tyrosine (Fig. 2). The *tert*-butyl moieties of BTBE serve to "anchor" it in the lipid bilayer and to minimize its diffusion back to the aqueous phase. Other tested compounds such as tyrosine methyl and ethyl esters were not suitable since they underwent slow hydrolysis to form tyrosine, except for the case of tyrosine butyl ester which was resistant to hydrolysis but its level of incorporation into liposomes was only 53% of the initial amount added. Exposure of multi- and unilamellar liposomes to nitrating systems caused the formation of both 3-nitro-BTBE and 3,3′-di-BTBE, with the yield of the nitro-derivative much higher than that of the corresponding dimer (Bartesaghi et al., 2006; Zhang et al., 2001). More recently, we have also detected the transient formation of the corresponding BTBE-derived phenoxyl radical by spin trapping EPR, the one-electron oxidation product of BTBE, as well as small amounts of a hydroxylated derivative of BTBE, assigned as 3-hydroxy-BTBE, as determined by HPLC separation, fluorimetric detection and electrospray ionization mass spectrometry (ESI-MS/MS) analysis of parent and daughter ions of products arising from treatment of BTBE-containing PC liposomes with peroxynitrite (Bartesaghi et al., 2006).

Secondly, membrane spanning peptides containing 23 amino acids with a single tyrosine residue at position 4, 8 or 12 have been synthesized and incorporated success-

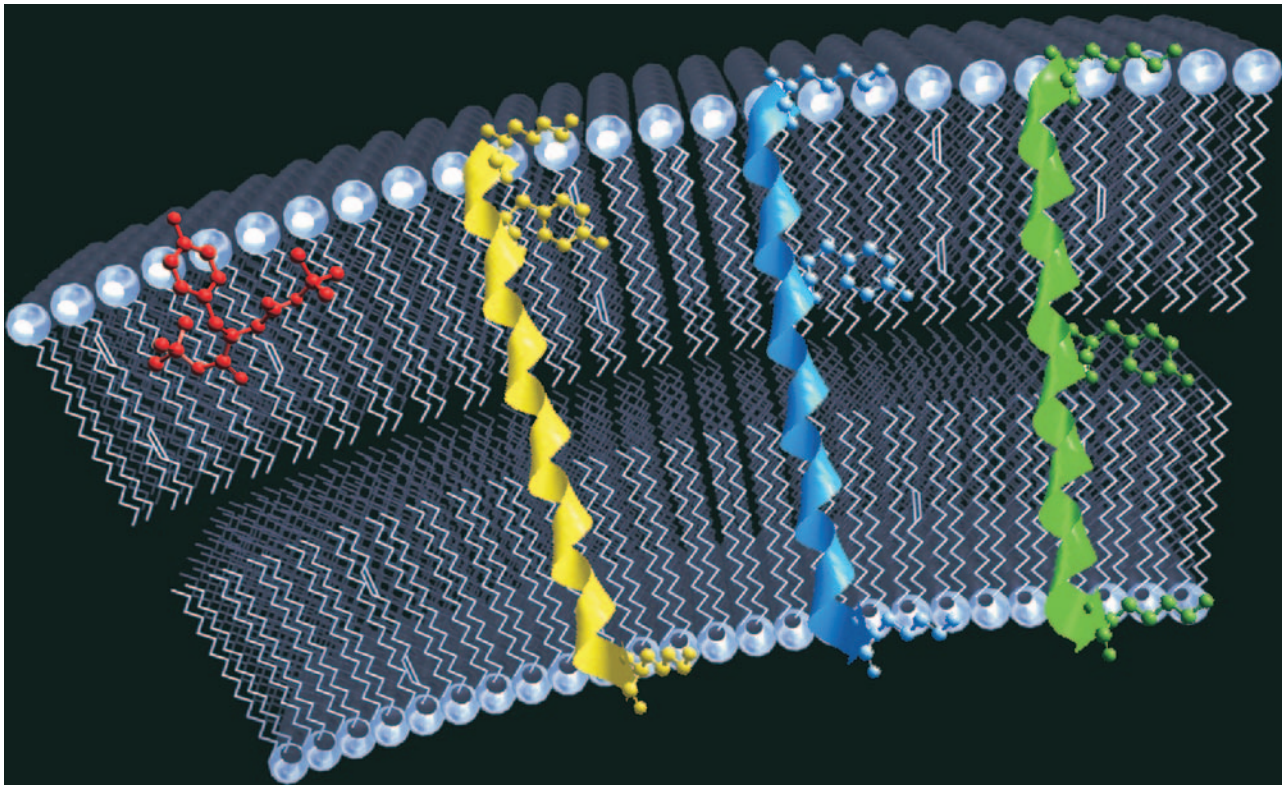

Fig. 2. Tyrosine analog, BTBE, and tyrosine-containing transmembrane peptides as hydrophobic probes to study tyrosine nitration in biomembranes. From left to right, the structure of BTBE (in red) and of transmembrane peptides are shown. BTBE is anchored to the bilayer by the hydrophobic *tert*-butyl moieties; while the aromatic ring is immersed in the bilayer, the phenolic −OH group emerges at the lipid-water interphase. The 23-amino acid transmembrane peptides (in yellow, blue and green) contain tyrosines at position 4-, 8- and 12, respectively which are located in the hydrophobic region of the bilayer; the side chains of the N- and C-terminal lysines are shown and, being highly hydrophilic, interact with the phospholipid polar head groups and water

fully to PC liposomes to study tyrosine nitration in biomembranes (Zhang et al., 2003) (Fig. 2). Indeed, these transmembrane peptides are formed by two hydrophilic and charged residues (e.g. K; Lys) both at the N- and C-terminus, and a hydrophobic sequence (repeats of AL; Ala-Leu) in the peptide central region containing the tyrosine residue at different positions. Prototypical peptides have the following general structure (in this example tyrosine is located in position 4 from the N-terminus):

Y-4 Ac-NH-KKAYALALALALALALALALAKK
-$CONH_2$ 2350 Da

These peptides, designed as hydrophobic probes, resemble more closely the structure of a protein transmembrane domain of a protein and allow to study the influence of the intramembrane location of tyrosine and the role of neighboring amino acids on nitration yields. For instance, we have observed that peroxynitrite-dependent tyrosine nitration is favored for tyrosines located deeper in the membrane (e.g. Tyr12) while myeloperoxidase/hydrogen peroxide/nitrite-dependent nitration is favored in the tyrosine closer to the membrane surface (e.g. Tyr4). The explanation for this different distribution is still not clear, but may be due to the fact that peroxynitrite can undergo homolysis inside the membrane to $^{\bullet}OH$ and $^{\bullet}NO_2$, while myeloperoxidase-derived oxidants will be only formed outside the liposome and will have to diffuse to reach a tyrosine residue, thus minimizing nitration of more distant tyrosines (e.g. Tyr12). In the case of peroxynitrite, in spite that ONOOH homolysis could occur throughout the membrane, tyrosyl radical formation from $^{\bullet}OH$ requires the initial tyrosine-hydroxyl radical adduct to be dehydrated. The transient formation of peptide-derived tyrosyl radical has been detected by spin trapping-EPR, confirming that tyrosine nitration in transmembrane peptides, as in the case of BTBE, is a free radical-dependent mechanism. In the case of the transmembrane peptides, no dimer product from peroxynitrite-dependent oxidations was observed, and tyrosine hydroxylation has not been explored yet.

The data obtained with transmembrane peptides may be more easily extrapolable to biological systems as compared to BTBE that is an isolated amino acid analog in-

corporated into a membrane. However, some advantages for BTBE also exist. Its synthesis is less costly and tedious, which allows to have larger quantities of the probe to perform a large number of experiments testing different variables that include alternative nitration systems and influence of pH, scavengers, catalysts and membrane composition on nitration yields (Bartesaghi et al., 2006). Also, incorporation of the transmembrane peptides to preformed hydrophobic structures such as lipoproteins is expected to be, at least, very difficult. On the other hand, we have successfully incorporated BTBE to erythrocyte membranes and lipoproteins (unpubl. data). BTBE and transmembrane peptides can both provide useful information and represent complementary probes to study physicochemical and biochemical factors that control tyrosine nitration and other oxidation processes in biologically-relevant hydrophobic environments.

The gathered data utilizing the available hydrophobic tyrosyl probes (Fig. 2), (Bartesaghi et al., 2006; Zhang et al., 2001, 2003) indicate that many assumptions valid for the aqueous phase are not readily applicable or not applicable at all in lipid phases due to the different polarity of the environment which will influence protein conformation, limit acid-base chemistry, cause spatial restrictions and diffusional constraints of both reactants and target molecules, and result in an unique distribution of free radical scavengers and nitration catalysts, among other factors.

Nitration, dimerization and hydroxylation in membranes

Tyrosine nitration processes usually occur concomitantly with other oxidative modifications, namely dimerization (to 3,3′-dityrosine) and hydroxylation (to 3-hydroxytyrosine) due to the nature of the nitration process and the chemical properties of the reactive species (e.g. $^{\bullet}OH$, $^{\bullet}NO_2$) (Fig. 1, reactions 5 and 6). In analogy to tyrosine in solution, BTBE nitration in PC liposomes occurs through free radical mechanisms and a pH-dependent distribution of the different oxidation products have been established[4]. Typically, at physiological pH nitration yields are higher than that of dimerization and hydroxylation. The relative yields of tyrosine dimerization are diminished in biomembranes[5], which can be fully explained by the diffusional constraints. Indeed, the kinetic competition between tyrosine nitration and dimerization in biomembranes will largely favor the first process due to a) the facile diffusion of $^{\bullet}NO_2$ in hydrophobic environments which achieves efficient trapping of tyrosyl radicals and b) the low probability of reaction between two tyrosyl radicals within the organized structure of membranes, where lateral diffusion is at least two orders of magnitude slower relative to the aqueous phase (Vanderkooi and Callis, 1974). The D of molecules such as $^{\bullet}NO$ (and $^{\bullet}NO_2$) is also lowered once in liposomes, but to a much less extent (e.g. $D_{NO} = 4500$ vs $310\,\mu m^2\,s^{-1}$ for buffer and egg PC liposomes, respectively (Moller et al., 2005), since they do not follow the Stokes-Einstein law, i.e. their diffusion is not inversely related to the viscosity of the solvent (Moller et al., 2005). As $^{\bullet}NO$ and $^{\bullet}NO_2$ concentrate 4–5 fold in hydrophobic environments (Liu et al., 1998; Moller et al., 2005), the apparent D value ($D' = 1500\,\mu m^2\,s^{-1}$) (Denicola et al., 1996) results to be very close to that of the aqueous phase ($4500\,\mu m^2\,s^{-1}$) (Moller et al., 2005). On the other hand, the dimerization reaction constant between two BTBE-derived phenoxyl radicals has been estimated $\sim 10^6\,M^{-1}\,s^{-1}$, 100 times less than that of tyrosyl radicals in aqueous solution ($k = 2.25 \times 10^8\,M^{-1}\,s^{-1}$). Indeed, while the diffusion coefficient (D) value of amino acids such as tyrosine in the aqueous phase is in the order of $800–1000\,\mu m^2\,s^{-1}$ (Lide, 1990), the estimated D for BTBE in PC liposomes can be safely assumed as $\sim 5\,\mu m^2\,s^{-1}$ as extrapolated from data obtained with the hydrophobic fluorescence aromatic probe pyrene (Vanderkooi and Callis, 1974), implying a 100–200 fold decrease in tyrosyl radical diffusion in the membrane. Intermolecular tyrosine dimerization will be even less likely in integral peptides and proteins as D values become $>10^3–10^4$ times smaller than in solution (Sackmann et al., 1973; Vanderkooi and Callis, 1974) and on line the lack of tyrosine dimerization in peroxynitrite-treated transmembrane peptides (Zhang et al., 2003).

The presence of CO_2 limits peroxynitrite-dependent tyrosine oxidation (nitration, dimerization and hydroxylation) in hydrophobic phases because formation and decay of $ONOOCO_2^-$ will occur exclusively in the aqueous phase, and the resulting negatively charged carbonate radical ($CO_3^{\bullet -}$; $pKa < 0$) (Czapski, 1999) is uncapable to permeate

[4] The yields of 3-nitrotyrosine, 3,3′-dityrosine and 3,4-di-hydroxy-phenylalanine vary rather differently as a function of pH. Indeed, 3-nitrotyrosine yields are bell-shaped with a maximum around pH 7.4 (Ischiropoulos et al., 1992); on the other hand, 3,3′-dityrosine yields are minimal at low pH (van der Vliet et al., 1995) and increase towards alkaline pH and 3,4-di-hydroxy-phenylalanine yields are high at acidic pH and become null at pH >7.4 (Santos et al., 2000).

[5] For example, for 250 μM peroxynitrite and pH 7.4, the ratio for [3-nitro-BTBE]/[3,3′-di-BTBE] and [3-nitro-tyrosine]/[3,3′-di-tyrosine] is ∼200 and 70, respectively (Bartesaghi et al., 2006; Zhang et al., 2001).

lipid bilayers (Bartesaghi et al., 2006; Khairutdinov et al., 2000) to promote the one-electron oxidation of tyrosine. The limited diffusion of anionic radical anions on membranes has been also well established for other species such as $Br_2^{\bullet-}$ and $Cl_2^{\bullet-}$ (Barber and Thomas, 1978).

In regard to tyrosine hydroxylation in hydrophobic phases concomitant to the process on nitration, it has been reported the hydroxylation of BTBE by peroxynitrite in PC liposomes; $^{\bullet}OH$, formed from ONOOH in the aqueous phase is capable to penetrate PC vesicles and react with aromatic probe molecules incorporated in the membrane interior as long as it is formed in the immediacy of the bilayer and as previously shown in water radiolysis studies for pyrene hydroxylation (Barber and Thomas, 1978); importantly, $^{\bullet}OH$ could be also formed inside the liposomes as ONOOH permeates the lipid bilayer (Denicola et al., 1998; Marla et al., 1997) and undergo homolysis of ONOOH in aprotic solvents (Zhang et al., 2001). Studies of tyrosine hydroxylation in peptides containing residues at different depths in the membrane will assist in defining the relevance of ONOOH homolysis in the aqueous vs lipid phase, as $^{\bullet}OH$ diffusing from the bulk solution will mainly react with residues located near the liposome surface. On the other hand, during heme-dependent nitration, hydroxylation would be expected only at tyrosines located near the membrane surface in the case of soluble hemeperoxidases but could potentially occur in tyrosines located deep in the membrane both in the case of hemin that readily intercalates and undergoes redox chemistry in the phospholipid bilayer (Bartesaghi et al., 2006) and of integral membrane hemeperoxidases.

Mechanistic considerations for protein tyrosine nitration in vivo: limitations and alternatives

Although in controlled-biochemical systems, the free radical mechanisms of tyrosine nitration are now well established, we are confronted with a more complex problem in vivo where 1) oxidants are formed as a flux (instead of the usual chemical experiments using bolus additions) and 2) antioxidant and radical repair mechanisms largely limit nitration reactions.

Tyrosine nitration initiated by a flux of the precursors of peroxynitrite ($^{\bullet}NO$ and $O_2^{\bullet-}$) in the presence of CO_2 has been studied by gamma radiolysis (Goldstein et al., 2000). The results of this study suggest that an optimum yield is achieved whenever the fluxes are equal and declines when either reactant is in excess, and this is explained by the excess precursor radicals ($^{\bullet}NO$ and $O_2^{\bullet-}$) reacting with the nitration intermediates ($CO_3^{\bullet-}$, $^{\bullet}NO_2$ and $TyrO^{\bullet}$) and diverting them from the nitration pathway. More recently, our group (Quijano et al., 2005) has shown, through numerical simulation of the reaction kinetics, that the maximum yield related to a 1:1 stoichiometric ratio is most probably unimportant in vivo. Provided that alternative consumption routes exist for $^{\bullet}NO$ (diffusion to other compartments where it is consumed) and $O_2^{\bullet-}$ (SOD-catalyzed dismutation) the yield of peroxynitrite (and concomitantly of nitration) is only limited by the availability of the precursor radicals, irrespective of the ratio of their transient concentrations. In any case 3-nitrotyrosine is a very minor end product of the reaction of $^{\bullet}NO$ with $O_2^{\bullet-}$; with relatively high fluxes (>1 μM/min) of the radicals only about one in 10^9 $^{\bullet}NO$ molecules ends up in 3-nitrotyrosine in a system with 10 μM SOD and a cell membrane permeable to $^{\bullet}NO$ but not to $O_2^{\bullet-}$.

The next and fundamental obstacle to nitration in vivo is the inclusion of reductants; as an example, 10 mM glutathione (GSH) in the aforementioned simulation (Quijano et al., 2005) reduces the expected nitration yield by more than nine orders of magnitude. GSH can act in the system shown in Fig. 1 by intercepting a number of reactants and intermediaries. Besides reacting directly with ONOOH, GSH can react with $TyrO^{\bullet}$ in the reverse of reaction 1, a reaction that has a relatively low rate constant (*ca.* $10^5\ M^{-1}\ s^{-1}$) but becomes significant in view of the elevated GSH concentration. Additionally, GSH is an excellent scavenger of $^{\bullet}NO_2$ ($2 \times 10^7\ M^{-1}\ s^{-1}$). Thus, the formation of 3-nitrotyrosine tends to be inhibited in cellular systems with plenty of antioxidant compounds prone to intercept any oxidant functioning as Ox_1, capable of pushing reaction 1 to the left, and proficient in scavenging any significant amount of $^{\bullet}NO_2$ formed, and therefore it is difficult to imagine 3-nitrotyrosine being formed at all under these conditions. Nevertheless 3-nitrotyrosine has been shown to be formed in vivo beyond doubt and to correlate well with the increased tissue levels of $^{\bullet}NO$. We will provide three possible explanations to account for presence of 3-nitrotyrosine in vivo.

Site-specific nitration

The cases of MnSOD (Quijano et al., 2001), cytochrome $P450_{CAM}$ (Daiber et al., 2000) and prostaglandin H synthase-2 (Gunther et al., 1997) are often cited examples where the nitration of a specific tyrosine occurs via catalysis by an adjacent metal center of the enzyme. In site-specific nitration reaction intermediates may not be readily accessible to reductants and competitors and therefore nitration could not be abolished as easily. However, per-

oxynitrite-mediated MnSOD nitration is prevented by reductants and scavengers as shown in (Quijano et al., 2001) much in the same way as non-catalyzed nitration. Data on cytochrome $P450_{CAM}$ are less clear cut as the authors have not shown the effect of reductants and competitors on the nitration yield, nevertheless the catalysis of phenol nitration and oxidation (Daiber et al., 2000) performed by this enzyme suggests that nitration intermediates are susceptible to interception by external scavengers. Other redox active metalloenzymes promote nitration, for example Cu/Zn-SOD (Crow et al., 1997) and the aforementioned hemeperoxidases, by stabilizing strong oxidants but in no case it seems that this site-specific nitration effectively isolates the protein self-catalyzed nitration from outside reductants. There is still one possibility by which metalloenzymes[6] in vitro can promote site-specific nitration in vivo; this involves the selective formation of a protein TyrO$^{\bullet}$ in a site not easily accessible for being repaired. As $^{\bullet}NO_2$ seems to be scavengeable in all cases studied so far, nitration should be explained by an alternative mechanism, such as the one involving $^{\bullet}NO$.

Additional mechanisms

Both PHS (Goodwin et al., 1998; Sturgeon et al., 2001) and cytochrome c (Chen et al., 2004) are nitrated via 3-nitrosotyrosine and 3-iminoxyl tyrosine radical intermediates. This alternative mechanism may be common in proteins capable of stabilizing a TyrO$^{\bullet}$ followed by the addition of $^{\bullet}NO$ and subsequent two steps of one-electron oxidation (most likely mediated by the protein oxo-metal centers) to yield 3-nitrotyrosine (Fig. 1, reaction 3) and bypasses the need of $^{\bullet}NO_2$ which is efficiently scavenged by GSH. The conjunction of a site-specific formation (catalyzed by a metal center), of a TyrO$^{\bullet}$ protected from interaction with external reductants and a nitration pathway not involving $^{\bullet}NO_2$ (for instance, reactions 1, 3 and 4) appears as a kinetically sound candidate for rationalizing protein nitration in cellular milieu where reductants are abundant.

Compartmentalization

Nitration in hydrophobic environments such as membranes could be enhanced by a number of factors. First and foremost the great majority of antioxidants are excluded from the hydrophobic interior of a membrane, this includes low molecular weight reductants such as glutathione, ascorbate and urate, and also enzymatic antioxidants such as peroxiredoxins any one of which could abolish tyrosine nitration in aqueous solution. The reductants present in lipidic environments, such as tocopherols and carotenes, are much less efficient in scavenging $^{\bullet}NO_2$ and TyrO$^{\bullet}$ than their water-soluble counterparts. Some oxidants capable of performing as Ox_1 precursors, (for instance $^{\bullet}OH$ and $^{\bullet}NO_2$ from ONOOH homolysis) can freely diffuse in and out of membranes, others such as the polar and charged $CO_3^{\bullet-}$ and MPO compounds I and II can be excluded by partition. Additional oxidants can be formed locally as intermediates in lipid peroxidation chain reactions, of these, alkoxyl and alkylperoxyl radicals are strong oxidants ($E^{\circ\prime} = +1.76$ V and $+1.02$ V, respectively (Jonsson, 1996; Merenyi et al., 2002)) that could contingently act as Ox_1. This provides an additional advantage because the original strong oxidants are not really scavenged by lipids (which are in large abundance in the membranes or lipoproteins and react fast with $^{\bullet}OH$ ($k = \sim 10^9\ M^{-1}\ s^{-1}$ for PUFA) (Barber and Thomas, 1978)) and would outcompete protein tyrosine for the initial oxidants but transformed to species potentially performing as Ox_1 and even enhanced through the propagation phase of lipid peroxidation.

Thus, in spite of the chemical restrictions for obtaining tyrosine nitration in vivo, the combination of site-specificity, assistance of alternative mechanisms and favored nitration in hydrophobic compartments, can add up to provide feasible mechanisms of nitration in complex biological milieux. Indeed, recent studies using high-resolution immuno-electron microscopy have established that the subcellular distribution of tyrosine-nitrated proteins in different cells includes a substantial amount associated to the endoplasmic reticulum and mitochondrial membranes, consistent with an effect of the membrane environment in the facilitation of nitration (Heijnen et al., 2006).

Conclusions and perspectives

Tyrosine nitration has been revealed as a relevant post-translational modification linked to nitro-oxidative stress conditions and pathophysiology. Usually, the level of nitrated proteins in tissues and fluids correlate well with the severity of the disease and can be considered a useful biomarker and footprint of oxidative reactions mediated by $^{\bullet}NO$-derived oxidants. A number of biochemical precursor pathways of tyrosine nitration exist in vivo but current evidence supports free radical mechanisms with

[6] We should make a caution note here to make clear that various metal centers in proteins actually protect from peroxynitrite-mediated nitration by avoiding the oxidants to reach the tyrosine or diminishing the actual yield of oxidation. One example is the oxyhemoglobin-catalyzed isomerization of peroxynitrite to nitrate (Romero et al., 2003).

the transient formation of tyrosine-derived phenoxyl radicals. Nitration in vivo is limited by the presence of strong reductants such as glutathione, but metal-catalyzed site-specific nitration, transient formation and oxidation of 3-nitrosotyrosine and protein association to hydrophobic environments, among other factors, can partially circumvent antioxidant processes. While immunochemical-based methods have unambiguously revealed the presence of nitrated protein in a wide variety of disease states, analytical and proteomic-based methodologies are laboriously assisting on defining the levels of protein-3-nitrotyrosine and the preferential nitrated proteins (and within those proteins which tyrosine residues), while functional studies are revealing the cases under which tyrosine nitration triggers changes in biological function. Recent studies utilizing hydrophobic probes, including the tyrosine analog ester BTBE (Bartesaghi et al., 2006; Zhang et al., 2001) or tyrosine-containing transmembrane peptides (Zhang et al., 2001) have focused in defining nitration mechanisms in tyrosines located in liposomal membranes and revealing particular characteristics on nitration processes that sometimes are markedly different to those previously shown to occur in aqueous phases. These studies should be expanded to other biologically-relevant hydrophobic phases, in particular, biomembranes and lipoproteins. Hydrophobic and hydrophilic tyrosine probes can be also potentially utilized to follow nitration processes in vivo. Moreover, in conditions under which nitration processes play a contributory role to cell dysfunction and tissue injury, tyrosine analogs and/or tyrosine-containing peptides may serve as nitration targets and spare critical tyrosines in proteins, therefore potentially serving protective roles. Finally, nitration processes are not exclusive to proteins and can also occur in other biomolecules such as DNA bases (Niles et al., 2006; Sawa and Ohshima, 2006; Sawa et al., 2006) and lipids (Baker et al., 2004; Schopfer et al., 2005). In the latter case, a relationship between protein and lipid nitration in biomembranes and lipoproteins should be explored in detail in future studies as both polyunsaturated fatty acids and tyrosine residues will compete for the same nitrating intermediates. Importantly, lipid peroxidation processes are being revealed as a key contributory factor to promote protein tyrosine nitration in hydrophobic environments (Bartesaghi et al., 2006).

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