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ORIGINAL ARTICLE

Formation of 3-nitrotyrosine by riboflavin photosensitized oxidation of tyrosine in the presence of nitrite

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Abstract The results of the present investigation show the susceptibility of tyrosine to undergo visible light-induced photomodification to 3-nitrotyrosine in the presence of nitrite and riboflavin, as sensitizer. By changing H_2O by D_2O, it could be established that singlet oxygen has a minor role in the reaction. The finding that nitration of tyrosine still occurred to a large extent under anaerobic conditions indicates that the process proceeds mainly through a type I mechanism, which involves the direct interaction of the excited state of riboflavin with tyrosine and nitrite to give tyrosyl radical and nitrogen dioxide radical, respectively. The tyrosyl radicals can either dimerize to yield 3,3′-dityrosine or combine with nitrogen dioxide radical to form 3-nitrotyrosine. The formation of 3-nitrotyrosine was found to increase with the concentration of nitrite added and was accompanied by a decrease in the recovery of 3,3′-dityrosine, suggesting that tyrosine nitration competes with dimerization reaction. The riboflavin photosensitizing reaction in the presence of nitrite was also able to induce nitration of tyrosine residues in proteins as revealed by the spectral changes at 430 nm, a characteristic absorbance of 3-nitrotyrosine, and by immunoreactivity using 3-nitrotyrosine antibodies. Since riboflavin and nitrite are both present endogenously in living organism, it is suggested that this pathway of tyrosine nitration may potentially occur in tissues and organs exposed to sunlight such as skin and eye.

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Abbreviations

NO_2Tyr	3-Nitrotyrosine
Dityr	3,3′-Dityrosine
RF	Riboflavin
DTPA	Diethylenetriaminepentaacetic acid
BSA	Bovine serum albumin

Introduction

The nitration of tyrosine residues to yield 3-nitrotyrosine derivatives is an in vivo post-translational protein modification, with potentially important consequences for cell function (for most recent reviews: Souza et al. 2008; Abello et al. 2009; Ischiropoulos 2009). Selective nitration of proteins, quite often nitration of a single tyrosine residue, may result in structural changes and could promote alteration (loss or gain) of functional activity (Ischiropoulos 2003; Bartesaghi et al. 2007). It has been suggested that tyrosine nitration, under certain physiological conditions, fulfills the criteria of a physiologically relevant signal with a regulatory role in the normal cell activity (Yacovlev and Mikkelsen 2010). The detection of 3-nitrotyrosine in a variety of different pathological states as well as during the normal aging process (Greenacre and Ischiropoulos 2001; Schopfer et al. 2003) was suggestive of the occurrence of nitrating pathways and has become an important marker for the production of nitric oxide-derived oxidants in vivo (van der Vliet et al. 1995; Eiserich et al. 1998a; Ischiropoulos 1998; Hurst 2002; Radi 2004). In particular, it has been

demonstrated that peroxynitrite ($ONOO^-$), a toxic species generated by reaction of nitric oxide (NO) with superoxide anion (Koppenol et al. 1992; Huie and Padmaja 1993; Pryor and Squadrito 1995), is able to convert free tyrosine and tyrosine protein residues into the corresponding nitrated molecules (Halliwell et al. 1999; Reiter et al. 2000; Alvarez and Radi 2003; Pacher et al. 2007). Although peroxynitrite has been the most widely studied nitrating species, several alternative routes for in vitro and in vivo tyrosine nitration have been steadily established. In this regard, free tyrosine and tyrosine protein residues nitration can also be achieved through mechanisms involving peroxidase/H_2O_2-dependent oxidation of nitrite to nitrogen dioxide ($^{\bullet}NO_2$) (van der Vliet et al. 1997; Sampson et al. 1998; Brennan et al. 2002). In inflammatory processes, this mechanism of tyrosine nitration is catalyzed by myeloperoxidase, an abundant enzyme secreted from activated phagocytes (Eiserich et al. 1998b; Burner et al. 2000; Gaut et al. 2002). In addition to peroxidases, nitrite/H_2O_2-mediated tyrosine nitration is efficiently catalyzed by free metals, heme and several heme proteins (Thomas et al. 2002; Bian et al. 2003; Castro et al. 2004; Herold 2004). Furthermore, in a previous study, we have shown that dye (methylene blue) sensitized photooxidation of tyrosine in the presence of nitrite produces 3-nitrotyrosine, through a process which involves singlet oxygen (Pecci et al. 2001). Since the production of oxygen reactive species such as singlet oxygen is associated with the activation of phagocytes, we suggested that the singlet oxygen-mediated oxidation of tyrosine in the presence of nitrite might represent an alternative or additional pathway of 3-nitrotyrosine formation during inflammatory processes.

Photooxidations sensitized by natural compounds are being increasingly investigated in relation to substrates of relevance in biology, pharmacology and medicine. A daylight-absorbing pigment of particular interest is the naturally occurring vitamin B2, riboflavin (RF). RF is an essential component of living organisms and is a well-known sensitizer for the light-promoted oxidations of different substrates such as amino acids, DNA, and fatty acids, among others (Chacon et al. 1988; Ito and Kawanishi 1997; Edwards and Silva 2001). To account for the oxidative processes in RF-sensitized events, type I (radical-mediated) and type II (singlet oxygen-mediated) mechanisms have been postulated (Heelis 1991). The type I mechanism involves the formation of free radicals or radical ions through the transfer of hydrogen atoms or electrons by interaction of the triplet excited state of the sensitizer with target molecules. The type II process involves the generation of singlet oxygen ($^1O_2$) by energy transfer from the excited triplet sensitizer to a ground state oxygen molecule. RF-sensitized photooxidation of tyrosine occurs preferentially through the type I mechanism with generation of tyrosyl radicals, which can dimerize to form 3,3′-dityrosine (Silva and Godoy 1994; Lu and Liu 2002).

In the present investigation, the nitration of free tyrosine and tyrosine residues in proteins by riboflavin photosensitizing reaction in the presence of nitrite has been studied. The results show the susceptibility of tyrosine to undergo visible light-induced photomodification to 3-nitrotyrosine in the presence of riboflavin and nitrite, both present endogenously in living organism. This pathway of tyrosine nitration may potentially occur in tissues and organs exposed to sunlight such as skin and eye.

Materials and methods

Chemicals

Riboflavin, sodium nitrite, L-tyrosine, 3-nitrotyrosine, sodium azide (NaN_3), diethylenetriaminepentaacetic acid (DTPA), horseradish peroxidase (type VI), catalase (from bovine liver), α-crystallin (from bovine eye lens), elastin (soluble, from bovine neck ligament), and L-amino acid oxidase (from *Crotalus adamanteus*) were obtained from Sigma (St. Louis, MO). Bovine serum albumin (BSA), pyruvate kinase (from rabbit muscle) and lysozyme (from hen egg white) were purchased from Fluka Chemie AG (Buchs, CH). Rhodanese was a kind gift of C. Cannella, Sapienza University of Rome. Deuterium oxide (D_2O) was obtained from Aldrich (Milwaukee, WI). 3,3′-Dityrosine was synthesized by reaction of L-tyrosine with horseradish peroxidase and hydrogen peroxide as described (Malencik et al. 1996).

Nitration of free tyrosine

The reaction mixture contained tyrosine (200 μM), RF (10 μM) and potassium nitrite (0–1 mM) in 0.1 M phosphate buffer adjusted to the desired pH with 10% NaOH or with 5% H_3PO_4. DTPA (0.1 mM) was included in the reaction mixture to avoid interfering reactions with contaminating metal ions. The reaction was initiated by illumination with a 200-W tungsten halogen lamp at the distance of about 20 cm from the solution (9.96 J/m^{2}s) and allowed to proceed at 25°C for various periods of time, under stirring. Reaction mixtures were analyzed by HPLC, using a Waters Chromatograph equipped with a model 600 pump, and a model 600 gradient controller. The column was a Nova-pak C18 (3.9 mm × 150 mm), 4 μm (Waters). The mobile phase was: A, 50 mM K-phosphate/H_3PO_4, pH 3.0; B, acetonitrile:water (50:50, *v/v*). A linear gradient from A to 33% B for 10 min was used at a flow rate of 1 ml/min. 3-Nitrotyrosine was detected at 360 nm, using a Waters 996 photodiode array. 3,3′-Dityrosine was analyzed

fluorometrically ($\lambda_{ex} = 260$ nm and $\lambda_{em} = 410$), using a Waters 474 scanning fluorescence detector. Peaks were identified using external standards and quantified using Millenium 32 software (Waters). The elution times of 3,3′-dityrosine and 3-nitrotyrosine were 7.5 and 8.9 min, respectively. The detection limit for 3,3′-dityrosine and 3-nitrotyrosine was 1 and 20 pmol, respectively.

Nitration of tyrosine in proteins

3-Nitrotyrosine formation in proteins was assessed by measuring the increase in absorbance at 430 nm after alkalinization to pH ~ 10 ($\varepsilon_{430} = 4.4$ mM^{-1} cm^{-1}) (Giese and Riordan 1975). Briefly, the reaction mixture containing the protein (1 mM, unless otherwise stated), RF (10 μM), and nitrite (10 mM), in 0.1 M potassium phosphate buffer, pH 7.4, plus 0.1 mM DTPA, was exposed to light (200-W tungsten halogen lamp) at the distance of about 10 cm from the solution and allowed to proceed at 25°C for 90 min, under stirring. Then, the absorbance at 430 nm was determined after 1:1 dilution of the incubation mixture with 0.2 M bicarbonate buffer, pH 10.5 (final pH ~ 10). The experimental results were obtained by subtracting the absorbance of the corresponding system not exposed to light.

Western blot analysis

Nitrated BSA samples (70 μg) were subjected to SDS–PAGE on 10% acrylamide gels (Bio-Rad, Hercules, CA) and then transferred to PDVF membranes (Immobilon-P; Millipore, Billerica, MA) using a mini trans blot cell (Bio-Rad) for 1 h at 100 V. Blots were blocked in 3% BSA/Tris-buffered saline and probed with rabbit polyclonal 3-nitrotyrosine antibodies (1:200; Upstate Biotechnology) for 2 h. Bands were visualized with horseradish peroxidase-conjugated secondary antibodies (1:5,000; Sigma), and chemiluminescence substrate (Amersham, GE Healthcare, Fairfield, CT).

Statistics

Results are expressed as mean ± SEM for at least three separate experiments performed in duplicate. Graphics and data analysis were performed using GraphPad Prism 4 software.

Results

The riboflavin photosensitized oxidation of tyrosine in the presence of nitrite

As reported, RF-sensitized photooxidation of tyrosine leads, through a tyrosyl radical intermediate, to the production of 3,3′-dityrosine (Silva and Godoy 1994; Edwards and Silva 2001). Figure 1a shows that when the solution containing tyrosine (200 μM) and RF (10 μM) was exposed to visible light in the presence of 1 mM nitrite (hereafter referred to as nitrite/RF/light system), 3-nitrotyrosine (NO_2Tyr) was produced in the course of the photooxidative process. After 30-min irradiation, the yield of NO_2Tyr was 7.1 ± 0.3 and 16.7 ± 0.7 μM, at pH 7.4 and 5.9, respectively. No detectable tyrosine nitration was observed in dark controls or in irradiated controls lacking RF. Figure 1b shows the relative yields of NO_2Tyr and 3,3′-dityrosine (Dityr) produced by the photochemical system in the presence of increasing concentrations of nitrite. The 3,3′-dityrosine formation decreased in the presence of nitrite and was inversely related to tyrosine nitration, which was found to increase with the concentration of nitrite added.

As shown in Fig. 2, the yield of NO_2Tyr is strongly dependent on the pH during irradiation, with a maximum in the pH 3.9–4.4. This finding is consistent with nitrite anion

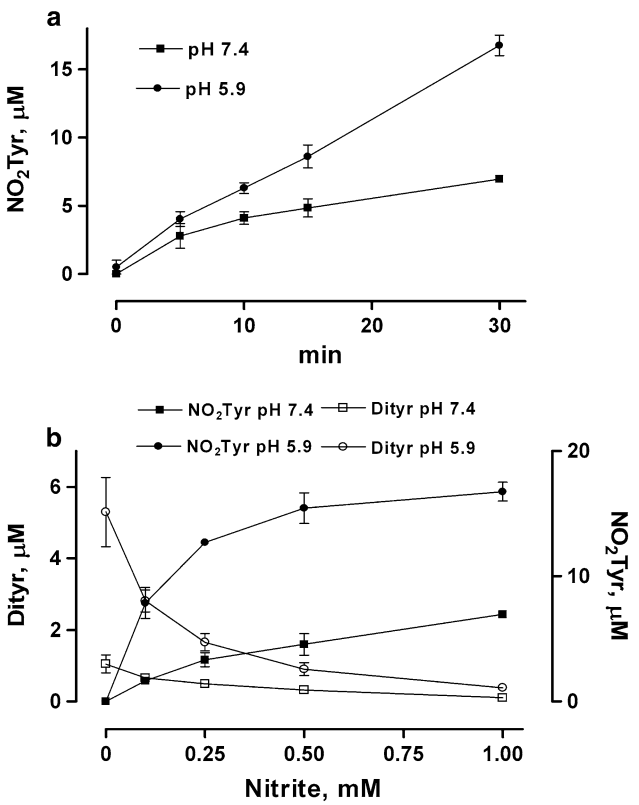

Fig. 1 Nitration of tyrosine by nitrite/RF/light. The reaction mixtures containing 200 μM tyrosine, 10 μM RF and nitrite in phosphate buffer (0.1 M, pH 7.4 or 5.9, DTPA 0.1 mM), were exposed to visible light at 25°C, under stirring. **a** Time course of 3-nitrotyrosine (NO_2Tyr) formation with 1 mM nitrite. **b** Yields of 3-nitrotyrosine and 3,3′-dityrosine (Dityr) with the indicated concentrations of nitrite, 30 min illumination. 3-Nitrotyrosine and 3,3′-dityrosine were measured by HPLC as described in "Materials and methods"

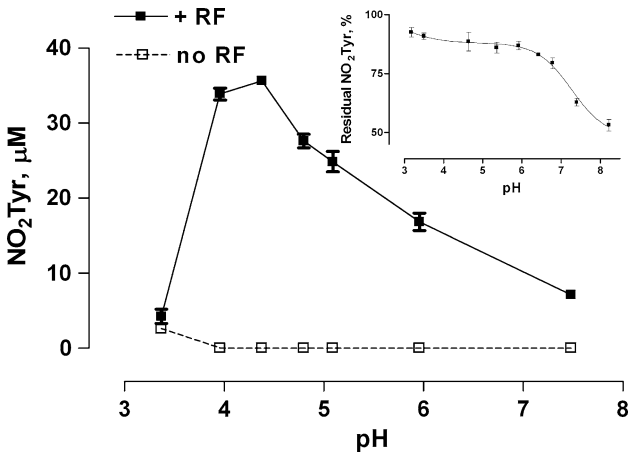

Fig. 2 Yields of 3-nitrotyrosine (NO_2Tyr) by nitrite/RF/light as a function of pH. The reaction mixtures containing 200 μM tyrosine, 10 μM RF and 1 mM nitrite in 0.1 M phosphate buffer plus 0.1 mM DTPA, at the indicated pH, were exposed to visible light for 30 min at 25°C. The *broken line* indicates the formation of 3-nitrotyrosine in control experiments lacking RF. *Inset* loss of 3-nitrotyrosine by the RF-photosensitized reaction as a function of pH at 25°C and 30 min illumination. Initial NO_2Tyr concentration 10 μM. 3-Nitrotyrosine was measured by HPLC as described in "Materials and methods"

as the reactive species (pK = 3.25). Control experiments using authentic 3-nitrotyrosine, showed that on exposure to visible light and RF, NO_2Tyr is gradually decomposed to undetectable products in a reaction, which increases with pH (Fig. 2, inset). This probably reflects the fact that the ionized phenolate form of NO_2Tyr is photodecomposed faster than the protonated form (pK = 7.2). It follows that the pH-profile of NO_2Tyr yields shown in Fig. 2 is the result of two concurring processes: the production of NO_2Tyr and its decomposition, both depending on pH.

It is known that nitrite, under acidic conditions, generates nitrating species, which, in the presence of tyrosine, lead to the formation of 3-nitrotyrosine (Oldreive et al. 1998). Control experiments, in which tyrosine and nitrite in the pH 3–8 were exposed to light in the absence of RF indicates that, under our experimental conditions, this reaction pathway may contribute to the observed production of NO_2Tyr only at pH lower than 4 (Fig. 2, broken line).

Additionally, it has been shown that exposure at mild acidic pH of tyrosine to nitrite plus hydrogen peroxide results in 3-nitrotyrosine formation, most likely through a reaction involving peroxynitrous acid (ONOOH), which is a well known nitrating agent (Oury et al. 1995). This mechanism of tyrosine nitration may also be operative under our experimental conditions since hydrogen peroxide can be generated during RF photooxidative reactions (Silva and Godoy 1994). To check this, tyrosine (200 μM) was incubated for 30 min, at pH 5.75, with nitrite (1 mM) in the presence of hydrogen peroxide at concentrations much higher than those possibly generated by the photooxidative process. No detectable NO_2Tyr was found even with 1 mM H_2O_2 added, indicating that the possible formation of hydrogen peroxide does not participate, to an appreciable extent, in the reaction leading to the nitration of tyrosine (not shown).

Riboflavin-photosensitized reactions are known to involve both type I (electron transfer from the substrate to the triplet state of RF) and type II (singlet oxygen-mediated) mechanisms (Heelis 1991). In order to investigate the role of singlet oxygen ($^1O_2$) in the reaction under study, the yields of 3-nitrotyrosine in H_2O and D_2O as solvents were compared. Replacement of H_2O by D_2O increases the lifetime of $^1O_2$ and is expected to stimulate singlet oxygen-mediated reactions (Foote and Clennan 1995). As shown in Fig. 3, the production of NO_2Tyr was greater by a factor of about 1.5 in D_2O. This effect, although not large, suggests at least some singlet oxygen intermediacy in the reaction. Furthermore, the nitration of tyrosine also occurred rather efficiently when the reaction mixture containing tyrosine, nitrite and RF was irradiated under nitrogen bubbling (Fig. 3, anaerobiosis). This result indicates that type I mechanism, which does not require oxygen to proceed, has a predominant role in the production of NO_2Tyr. In Fig. 3 is also shown that the yield of NO_2Tyr was greatly reduced when the reaction mixture was irradiated in the presence of 1 mM sodium azide (NaN_3), a quencher of both singlet oxygen (Foote 1979), and of the excited triplet state of RF (Foote 1976).

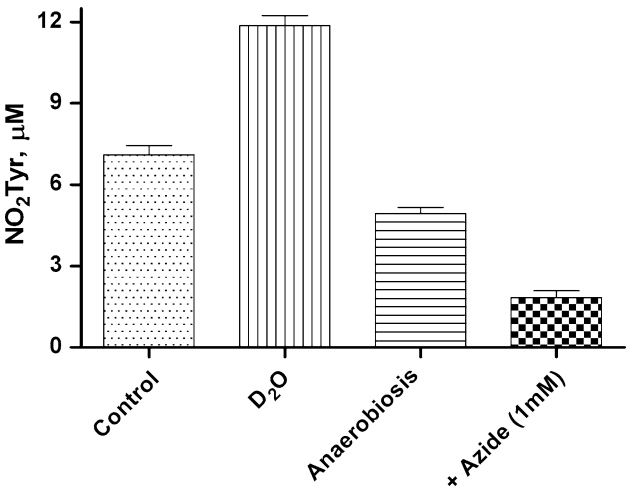

Fig. 3 3-Nitrotyrosine (NO_2Tyr) formation by nitrite/RF/light in different conditions. The reaction mixtures containing 200 μM tyrosine, 10 μM RF and 1 mM nitrite in phosphate buffer (0.1 M, pH 7.4, DTPA 0.1 mM) were exposed to visible light for 30 min at 25°C. *Control* air-saturated solutions. Anaerobiosis: nitrogen-saturated solutions. In D_2O, pD = 7.4 was taken as pH measured +0.4. 3-Nitrotyrosine was measured by HPLC as described in "Materials and methods"

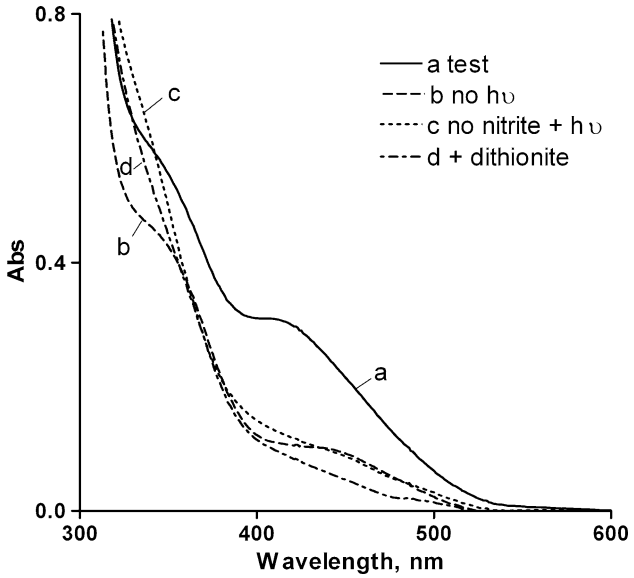

Fig. 4 Spectral changes obtained for the reaction of BSA with nitrite/RF/light. Bovine serum albumin (1 mM) in phosphate buffer (0.1 M, pH 7.4, DTPA 0.1 mM) was exposed to visible light in the presence of nitrite (10 mM) and RF (10 μM) for 90 min at 25°C (*a*). BSA in the presence of nitrite and RF not exposed to light (*b*). *c*, as in *a*, but no nitrite. *d*, as in *a*, and then treated with dithionite (10 mM in 100 mM Na borate, pH 9, for 40 min). The spectra were recorded after 1:1 dilution of the incubation mixtures with 0.2 M bicarbonate buffer, pH 10.5 (final pH ~ 10)

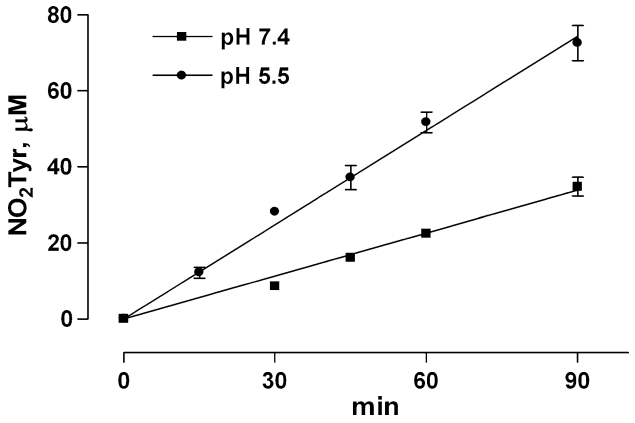

Fig. 5 Time course of BSA nitration by nitrite/RF/light. Bovine serum albumin (1 mM) in phosphate buffer (0.1 M, pH 7.4 or 5.5, DTPA 0.1 mM) was exposed to light in the presence of nitrite (10 mM) and RF (10 μM) for the indicated times at 25°C. Nitration was quantified by the increase in the absorbance at 430 nm after alkalinization to pH ~ 10 of the incubation mixtures

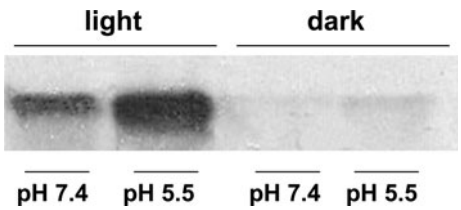

Fig. 6 Immunoblot of BSA nitrated by nitrite/RF/light. Bovine serum albumin (1 mM) was exposed to light in the presence of nitrite (10 mM) and RF (10 μM) at pH 7.4 or 5.5 for 90 min at 25°C. After dilution (1:10) with H_2O, aliquots of the reaction mixture containing 70 μg BSA were subjected to SDS–PAGE and examined by immunoblot analysis as described in "Materials and methods"

Nitration of proteins

Bovine serum albumin was used as model for probing nitration of tyrosine residues in proteins by the nitrite/RF/light system. Spectrum in alkaline solution of the incubation mixture containing BSA (1 mM), nitrite (10 mM) and RF (10 μM), at pH 7.4, exposed to visible light for 90 min, shows an increase of absorbance in the 400–450 nm range (Fig. 4, curve a) suggesting the formation of 3-nitrotyrosine, which exhibits a characteristic absorbance at 430 nm (Giese and Riordan 1975). Accordingly, this absorbance was decreased by dithionite (10 mM in 100 mM Na borate, pH 9, for 40 min), which chemically reduces 3-nitrotyrosine to 3-aminotyrosine (Fig. 4, curve d). No increase in absorbance at 430 nm was observed in dark controls or in illuminated controls lacking nitrite (Fig. 4, curve b and c, respectively). Therefore, in further experiments, the production of 3-nitrotyrosine in proteins was determined from the increase in absorbances at 430 nm after alkalinization of the samples to pH $\geq$ 10 (see "Materials and methods").

As shown in Fig. 5, 3-nitrotyrosine formation in BSA increases linearly over time of light exposure and strongly depends on the pH of the incubation mixture being about two times higher at pH 5.5 than at 7.4.

The nitration of BSA was further examined by western blot analysis using 3-nitrotyrosine antibodies. According to the results reported in Fig. 5, this analysis reveals immunoreactivity for 3-nitrotyrosine on BSA only in irradiated samples with enhanced signal at acidic pH (Fig. 6).

Table 1 shows that the nitrite/RF/light system resulted to induce tyrosine nitration of several proteins including α-crystallin and elastin, present in eye and skin, respectively. These tissues are permanently exposed to visible light and contain riboflavin that can act as sensitizer (Batey and Eckehert 1991).

Riboflavin is present as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) in several redox enzymes (flavoproteins). Therefore, it is possible that the photochemical process leading to tyrosine nitration could be also induced by replacement of RF with a flavoprotein. To check this, a reaction mixture containing tyrosine (200 μM) and nitrite (10 mM), in phosphate buffer pH 7.4, was exposed to light for 90 min at 25°C, in the presence of 50 μM L-amino acid oxidase, a FAD-containing enzyme. No detectable amount of 3-nitrotyrosine was found,

Table 1 Nitration of proteins by nitrite/RF/light system

	Mol 3-nitrotyrosine/ mol protein	mMol 3-nitrotyrosine/ mol tyrosine
BSA (1 mM)	0.066 ± 0.003	3.3
Lysozyme (1 mM)	0.024 ± 0.002	8
Pyruvate kinase (170 μM)	0.24 ± 0.01	26.6
Rhodanese (100 μM)	0.24 ± 0.01	24
Catalase (50 μM)	2.06 ± 0.034	103
α-Crystallin (1 mM)	0.065 ± 0.003	16.2
Elastin (400 μM)	0.073 ± 0.01	10.4

Proteins, at the indicated concentrations, were exposed to visible light in the presence of nitrite (10 mM) and RF (10 μM) at pH 7.4 for 90 min at 25°C. 3-Nitrotyrosine was estimated by the increase of the absorbance at 430 nm after alkalinization to pH ~ 10 of the incubation mixture

indicating that, under our experimental conditions, FAD bound to protein does not act as photosensitizer.

Discussion

Photooxidation of tyrosine sensitized by RF occurs predominantly through a type I mechanism (electron transfer from tyrosine to the triplet state of RF) with generation of tyrosyl radicals, which can dimerize to form 3,3′-dityrosine (Silva and Godoy 1994; Edwards and Silva 2001; Lu and Liu 2002).

$$\mathrm{Tyr} + \mathrm{RF(triplet)} \rightarrow \mathrm{Tyr}^{\bullet} + \mathrm{RF}^{\bullet -}\mathrm{(RF\ anion\ radical)} \quad (1)$$

$$\mathrm{Tyr}^{\bullet} + \mathrm{Tyr}^{\bullet} \rightarrow 3,3'\text{-dityrosine}. \quad (2)$$

The results presented herein show that when RF-photooxidation of tyrosine is performed in the presence of nitrite, 3-nitrotyrosine is generated as an additional product (Fig. 1). The mechanism underlying the formation of 3-nitrotyrosine likely involves the combination of tyrosyl radical with nitrogen dioxide radical ($^{\bullet}NO_2$), which may be generated by RF-photooxidation of nitrite via type I mechanism (direct interaction of nitrite with the triplet state of RF; Lu et al. 1999). Tyrosine nitration would thus proceed through the following reactions:

$$NO_2^- + \mathrm{RF(triplet)} \rightarrow^{\bullet} NO_2 + \mathrm{RF}^{\bullet -}\mathrm{(RF\ anion\ radical)} \quad (3)$$

$$\mathrm{Tyr}^{\bullet} +^{\bullet} NO_2 \rightarrow \text{3-nitrotyrosine}. \quad (4)$$

The concomitant formation of tyrosyl radicals and $^{\bullet}NO_2$ by the RF-photochemical system (reactions 1 and 3) implies that the dimerization of tyrosyl radicals to 3,3′-dityrosine (reaction 2, $k = 2.25 \times 10^8\ M^{-1}\ s^{-1}$, Hunter et al. 1989) would compete with the formation of 3-nitrotyrosine (reaction 4, $k = 3 \times 10^9\ M^{-1}\ s^{-1}$, Prütz et al. 1985). According to this, we found that the yield of 3,3′-dityrosine, the only product in the absence of nitrite, decreases with the concentration of nitrite added, whereas nitration becomes the predominant reaction at high nitrite (and thus $^{\bullet}NO_2$) concentrations (Fig. 1b).

Consistent with a predominant role for type I mechanism in the process leading to the formation of NO_2Tyr (reactions 1 and 3), is the finding that nitration of tyrosine still occurred efficiently when going from air-saturated to deareated solution (about 70% of the yield obtained under aerobic conditions) (Fig. 3). Additionally, the low effect of D_2O, an enhancer of singlet oxygen ($^1O_2$) lifetime, in the production of NO_2Tyr supports a minor role of $^1O_2$ (type II mechanism) within the whole process (Fig. 3). In the $^1O_2$-mediated mechanism, the interaction of singlet oxygen with tyrosine would produce tyrosyl radical and superoxide anion radical:

$$\mathrm{Tyr} +^1 O_2 \rightarrow \mathrm{Tyr}^{\bullet} + O_2^{\bullet -}. \quad (5)$$

Tyrosyl radicals can either dimerize or react with a nitrite-derived species to form 3-nitrotyrosine. To our knowledge, the production of nitrating species by direct interaction of nitrite with singlet oxygen has not been reported. It is possible that indirect oxidation of nitrite by the radicals produced in reaction 5 may lead to the generation of reactive nitrogen species, such as nitrogen dioxide.

On the basis of these findings, the inhibitory effect exerted by sodium azide possibly obeys to two different reasons: the first one is the known quenching of singlet oxygen (Foote 1979) and the second is due to a quenching of the excited triplet state of RF (Foote 1976).

In a previous work on the nitration of tyrosine by nitrite and methylene blue as photosensitizer, we found that singlet oxygen was the main active species involved in the reaction (Pecci et al. 2001). It is known that the mechanism of photosensitized processes has a strong dependence on the type of sensitizer and methylene blue is reported to cause almost exclusively $^1O_2$-mediated oxidations (Tanielian et al. 1984).

In the presence of nitrite, the RF-photochemical reaction was also found capable of nitrating tyrosine residues in BSA as revealed by the spectral changes at 430 nm, a characteristic absorbance of 3-nitrotyrosine (Figs. 4, 5) and by immunoreactivity using 3-nitrotyrosine antibodies (Fig. 6). The nitrite/RF/light system also resulted to modify tyrosine residues in several other proteins to form 3-nitrotyrosine (Table 1). The different extent of protein nitration is indicative that tyrosine nitration induced by the RF-photochemical system is a selective process. As reported, protein tyrosine nitration may depend on several factors such as the surface exposure of the tyrosine residue, and the local environment where the tyrosine is located

(hydrophobicity of the surrounding amino acids, proximity to acidic residues and to sulfur-containing residues) (Ischiropoulos 2003; Bartesaghi et al. 2007). From our results, the number of tyrosine residues in the protein does not seem to influence the degree of nitration. As can be seen in Table 1, lysozyme, with three tyrosine residues, is more susceptible to nitration than BSA, which contains 20 tyrosine residues. Furthermore, the high yield of 3-nitrotyrosine on catalase may be explained by the occurrence of other mechanisms of tyrosine nitration, which can operate concurrently with the RF-mediated pathway. As catalase is capable of oxidizing nitrite through a peroxidase-like reaction (Chance 1950), it is possible (but remains to be proved) that the one electron oxidation of nitrite by catalase generates nitrogen dioxide, that may then trigger nitration of tyrosine residues of the protein.

Of interest, the nitration of α-crystallin and elastin, present in eye lens and skin, respectively, suggesting that this pathway of tyrosine nitration may potentially occur in these tissues, which are permanently exposed to the action of light. It has been already reported that visible light irradiation of α-crystallin in the presence of riboflavin produces several changes indicative of oxidative protein damage (Viteri et al. 2003). Here, we demonstrate that during RF-photooxidation of α-crystallin, nitrite promotes, as a further modification of the protein, nitration of tyrosine residues to yield 3-nitrotyrosine.

Nitrite is present in blood as a stable oxidation product of NO synthesis or as a consequence of intake of nitrite-containing food (Rhodes et al. 1995; Walker 1990). Another important source of nitrite comes from cigarette smoke that contains large amounts of NO and nitrogen dioxide (Borland and Higenbottam 1987). Although serum concentrations of nitrite are on the order of micromolar concentrations (Leone et al. 1994), during chronic inflammation, tissues may be exposed to elevated nitrite levels for prolonged periods (Padgett and Pruett 1992). In the eye, nitrite accumulation occurs during ocular inflammation such as NO-mediated experimental allergic conjunctivitis (Meijer et al. 1996). Nitrite is also present on or in human skin as a constituent of sweat (Weller et al. 1996). Under normal sun- and heat-exposed conditions, the sweat surface layer will undergo rapid concentration by fluid evaporation, and thereby local concentration of nitrite may increase many times. Hence, the presence of nitrite in ocular lens and skin, might exert toxic effects inducing protein tyrosine nitration by riboflavin and light. It is also worth to mention that α-crystallin and elastin are structural proteins known to have very slow turnover, so that the nitrative/oxidative damage induced by nitrite/RF/light may be accumulated over years.

Moreover, the skin has long been known to have an acidic pH that contributes to the optimal barrier function of this tissue (Marchionini and Hausknecht 1938). The average surface pH of the forearm of a healthy man is around 5.4–5.9 (Braun-Falco and Korting 1986). Skin pH variations have been clearly documented in various skin diseases (Anderson 1951), reporting an overall elevation of about 0.5 units of the pH values. Thus, on account of the pH dependence of the 3-nitrotyrosine formation observed in this study, the photonitrative modification of tyrosine in the skin can be relevant physiologically as well as in skin diseases.

It has been reported that UV photolysis of 3-nitrotyrosine generates highly oxidizing species, of which one shows the characteristics of hydroxyl radical (Nauser et al. 2004). Hence, nitration of tyrosine residues in proteins not only may lead to conformational and functional changes of the affected protein, but also could represent and additional source of photooxidative damage.

In conclusion, this study confirms and extends our previous observations that nitrite can induce nitration of tyrosine during photosensitized processes. Since exposure to sunlight has long been recognized as a source of aging of the eye lens proteins and skin elastin (West et al. 1998; Suwabe et al. 1999), the present study may provide insights into the biochemical events responsible for protein structural and functional modifications induced by solar radiations.

Conflict of interest The authors declare that they have no conflict of interest.

References

Abello N, Kerstjens HAM, Postma DS, Bischoff R (2009) Protein tyrosine nitration: selectivity, physicochemical and biological consequences, denitration, and proteomics methods for the identification of tyrosine-nitrated proteins. J Proteome Res 8:3222–3238

Alvarez B, Radi R (2003) Peroxynitrite reactivity with amino acids and proteins. Amino Acids 25:295–311

Anderson DS (1951) The acid-base balance of the skin. Br J Dermatol 63:283–296

Bartesaghi S, Ferrer-Sueta G, Peluffo G, Valez V, Zhang H, Kalyanaraman B, Radi R (2007) Protein tyrosine nitration in hydrophilic and hydrophobic environments. Amino Acids 32:501–515

Batey DW, Eckehert CD (1991) Analysis of flavins in ocular tissues of the rabbit. Investig Ophtalmol Vis Sci 32:1981–1985

Bian K, Gao Z, Weisbrodt N, Murad F (2003) The nature of heme/iron-induced protein tyrosine nitration. Proc Natl Acad Sci USA 100:5712–5717

Borland C, Higenbottam T (1987) Nitric oxide yields of contemporary UK, US and French cigarettes. Int J Epid 16:31–34

Braun-Falco O, Korting HC (1986) Der normale pH-Wert der Haut. Hautarzt 3:126–129

Brennan ML, Wu W, Fu X, Shen Z, Song W, Frost H, Vadseth C, Narine L, Lenkiewicz E, Borchers MT, Lusis AJ, Lee JJ, Lee NA, Abu-Soud HM, Ischiropoulos H, Hazen SL (2002) A tale of two controversies. Defining both the role of peroxidases in nitrotyrosine formation in vivo using eosinophil peroxidase and

myeloperoxidase-deficient mice, and the nature of peroxidase-generated reactive nitrogen species. J Biol Chem 277:17415–17427

Burner U, Furtmüller PG, Kettle AJ, Koppenol WH, Obinger C (2000) Mechanism of reaction of myeloperoxidase with nitrite. J Biol Chem 275:20597–20601

Castro L, Eiserich JP, Sweeney S, Radi R, Freeman BA (2004) Cytochrome c: a catalyst and target of nitrite-hydrogen peroxide-dependent protein nitration. Arch Biochem Biophys 421:99–107

Chacon JN, McLearie J, Sinclair RS (1988) Singlet oxygen yields and radical contributions in the dye-sensitised photo-oxidation in methanol of esters of polyunsaturated fatty acids (oleic, linoleic, linolenic and arachidonic). Photochem Photobiol 47:647–656

Chance B (1950) On the reaction of catalase peroxides with acceptors. J Biol Chem 182:649–658

Edwards AM, Silva E (2001) Effect of visible light on selected enzymes, vitamins and amino acids. J Photochem Photobiol B 63:126–131

Eiserich JP, Patel RP, O'Donnell VB (1998a) Pathophysiology of nitric oxide and related species: free radical reactions and modification of biomolecules. Mol Aspects Med 19:221–357

Eiserich JP, Hristova M, Cross CE, Jones AD, Freeman BA, Halliwell B, van der Vliet A (1998b) Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. Nature 391:393–397

Foote CS (1976) Photosensitised oxidation and singlet oxygen: consequences in biological systems. In: Pryor WH (ed) Free radicals in biology, vol II. Academic Press, New York, pp 85–133

Foote SC (1979) Quenching of singlet oxygen. In: Wasserman HH, Murray RW (eds) Singlet oxygen. Academic Press, New York, pp 139–171

Foote CS, Clennan EL (1995) Properties and reactions of singlet oxygen. In: Foote CS, Valentine JS, Greenberg A, Liebman JF (eds) Active oxygen in chemistry. Blackie Academic and Professional, London, pp 105–141

Gaut JP, Byun J, Tran HD, Lauber WM, Carrol JA, Hotchkiss RS, Belaaouaj A, Heinecke JW (2002) Mieloperoxidase produces nitrating oxidants in vivo. J Clin Invest 109:2311–2319

Giese RW, Riordan JF (1975) Nitrotyrosine internal standard for amino acid analysis. Anal Biochem 64:588–592

Greenacre SA, Ischiropoulos H (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. Free Radic Res 34:541–581

Halliwell B, Zaho K, Whitheman M (1999) Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good. Free Radic Res 31:651–669

Heelis PF (1991) The photochemistry of flavins. In: Muller F (ed) Chemistry and biochemistry of flavoenzymes. CRC Press, Boca Raton, pp 171–193

Herold S (2004) Nitrotyrosine, dityrosine, and nitrotryptophan formation from metmyoglobin, hydrogen peroxide, and nitrite. Free Radic Biol Med 36:565–579

Huie RE, Padmaja S (1993) The reaction of NO with superoxide. Free Radic Res Commun 18:195–199

Hunter EP, Desrosiers MF, Simic MG (1989) The effect of oxygen, antioxidants, and superoxide radicals on tyrosine phenoxyl radical dimerization. Free Radic Biol Med 6:581–585

Hurst JK (2002) Whence nitrotyrosine? J Clin Invest 109:1287–1289

Ischiropoulos H (1998) Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. Arch Biochem Biophys 356:1–11

Ischiropoulos H (2003) Biological selectivity and functional aspects of protein tyrosine nitration. Biochem Biophys Res Commun 305:776–783

Ischiropoulos H (2009) Protein tyrosine nitration—an update. Arch Biochem Biophys 484:117–121

Ito K, Kawanishi S (1997) Site-specific DNA damage induced by UVA radiation in the presence of endogenous photosensitizer. Biol Chem 378:1307–1312

Koppenol WH, Moreno JJ, Prior WA, Ischiropoulos H, Beckman JS (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol 5:834–842

Leone AM, Francio PL, Rhodes P, Moncada S (1994) A rapid and simple method for the measurement of nitrite and nitrate in plasma by high performance capillary electrophoresis. Biochem Biophys Res Commun 200:951–957

Lu CY, Liu YY (2002) Electron transfer oxidation of tryptophan and tyrosine by triplet states and oxidized radicals of flavin sensitizers: a laser flash photolysis study. Biochim Biophys Acta 1571:71–76

Lu CY, Wang WF, Lin WZ, Han ZH, Yao SD, Lin NY (1999) Generation and photosensitization properties of the oxidized radical of riboflavin: a laser flash photolysis study. J Photochem Photobiol B Biol 52:111–116

Malencik DA, Sprouse JF, Swanson CA, Anderson SR (1996) Dityrosine: preparation, isolation, and analysis. Anal Biochem 242:202–213

Marchionini A, Hausknecht W (1938) Säuremantel der Haut und Bakterienabwher. Klin Wochenschr 17:663–666

Meijer F, Van Delft JL, Garrelds IM, Van Haeringen NJ, Kijlstra A (1996) Nitric oxide plays a role as a mediator of conjunctival edema in experimental allergic conjunctivitis. Exp Eye Res 62:359–365

Nauser T, Koppenol WH, Pelling J, Schöneich C (2004) UV photolysis of 3-nitrotyrosine generates highly oxidizing species: a potential source of photooxidative stress. Chem Res Toxicol 17:1227–1235

Oldreive C, Zhao K, Paganga G, Halliwell B, Rice-Evans C (1998) Inhibition of nitrous acid-dependent tyrosine nitration and DNA base deamination by flavonoids and other phenolic compounds. Chem Res Toxicol 11:211–214

Oury TD, Tatro L, Ghio AJ, Piantadosi CA (1995) Nitration of tyrosine by hydrogen peroxide and nitrite. Free Radic Res 23:537–547

Pacher P, Beckman JS, Liaudet L (2007) Nitric Oxide and Peroxynitrite in Health and Disease. Physiol Rev 87:315–424

Padgett EL, Pruett SB (1992) Evaluation of nitrite production by human monocyte-derived macrophages. Biochem Biophys Res Commun 186:775–781

Pecci L, Montefoschi G, Antonucci A, Costa M, Fontana M, Cavallini D (2001) Formation of nitrotyrosine by methylene blue photosensitized oxidation of tyrosine in the presence of nitrite. Biochem Biophys Res Commun 289:305–309

Prütz WA, Mönig H, Butler J, Land EJ (1985) Reactions of nitrogen dioxide in aqueous model systems: oxidation of tyrosine units in peptides and proteins. Arch Biochem Biophys 243:125–134

Pryor WA, Squadrito GL (1995) The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. Am J Physiol 268:L699–L722

Radi R (2004) Nitric oxide, oxidants, and protein nitration. Proc Natl Acad Sci USA 101:4003–4008

Reiter CD, Teng RJ, Beckman JS (2000) Superoxide reacts with nitric oxide to nitrate tyrosine at physiological pH via peroxynitrite. J Biol Chem 275:32460–32466

Rhodes PM, Leone AM, Francis PL, Struthers AD, Moncada S (1995) The L-arginine: nitric oxide pathway is the major source of plasma nitrite in fasted humans. Biochem Biophys Res Commun 209:590–596

Sampson JB, Ye YZ, Rosen H, Beckman JS (1998) Myeloperoxidase and horseradish peroxidase catalyze tyrosine nitration in proteins

from nitrite and hydrogen peroxide. Arch Biochem Biophys 356:207–213

Schopfer FJ, Baker PR, Freeman BA (2003) NO-dependent protein nitration: a cell signaling event or an oxidative inflammatory response? Trends Biochem Sci 28:646–654

Silva E, Godoy J (1994) Riboflavin sensitised photooxidation of tyrosine. Int J Vitam Nutr Res 64:253–256

Souza JM, Peluffo G, Radi R (2008) Protein tyrosine nitration—functional alteration or just a biomarker? Free Radic Biol Med 45:357–366

Suwabe H, Serizawa A, Kajiwara H, Ohkido M, Tsutsumi Y (1999) Degenerative processes of elastic fibers in sun-protected and sun-exposed skin: immunoelectron microscopic observation of elastin, fibrillin-1, amyloid P component, lysozyme and α_1-antitrypsin. Pathol Int 49:391–402

Tanielian C, Golder L, Wolff C (1984) Production and quenching of singlet oxygen by the photosensitizer in dye-sensitized photooxigenations. J Photochem 25:117–125

Thomas DD, Espey MG, Vitek MP, Miranda KM, Wink DA (2002) Protein nitration is mediated by heme and free metals through Fenton-type chemistry: an alternative to the NO/O_2^- reaction. Proc Natl Acad Sci USA 99:12691–12696

van der Vliet A, Eiserich JP, O'Neill CA, Halliwell B, Cross CE (1995) Tyrosine modification by reactive nitrogen species: a closer look. Arch Biochem Biophys 319:341–349

van der Vliet A, Eiserich JP, Halliwell B, Cross CE (1997) Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. J Biol Chem 272:7617–7625

Viteri G, Edwards AM, De la Fuente J, Silva E (2003) Study of the interaction between triplet riboflavin and the alfa-, betaH- and betaL-crystallins of the eye lens. Photochem Photobiol 77:535–540

Walker R (1990) Nitrates, nitrites and *N*-nitrosocompounds: a review of the occurrence in food and diet and toxicological implications. Food Addit Contam 7:717–768

Weller R, Pattullo S, Smith L, Golden M, Ormerod A, Benjamin N (1996) Nitric oxide is generated on the skin surface by reduction of sweat nitrate. J Invest Dermatol 107:327–331

West SK, Duncan DD, Munoz B, Rubin GS, Fried LP, Bandeen-Roche K, Shein OD (1998) Sunlight exposure and risk of lens opacities in a population-based study. JAMA 280:714–718

Yacovlev VA, Mikkelsen RB (2010) Protein tyrosine nitration in cellular signal transduction pathways. J Recept Signal Transduct Res 6:420–429