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Formation of Nitrotyrosine by Methylene Blue Photosensitized Oxidation of Tyrosine in the Presence of Nitrite

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Methylene blue photosensitized oxidation of tyrosine in the presence of nitrite produces 3-nitrotyrosine, with maximum yield at pH 6. The formation of 3-nitrotyrosine requires oxygen and increases using deuterium oxide as solvent, suggesting the involvement of singlet oxygen in the reaction. The detection of dityrosine as an additional reaction product suggests that the first step in the interaction of tyrosine with singlet oxygen generates tyrosyl radicals which can dimerize to form dityrosine or react with a nitrite-derived species to produce 3-nitrotyrosine. Although the chemical identity of the nitrating species has not been established, the possible generation of nitrogen dioxide ($^{\cdot}NO_2$) by indirect oxidation of nitrite by intermediately produced tyrosyl radical, via electron transfer, is proposed. One important implication of the results of this study is that the oxidation of tyrosine by singlet oxygen in the presence of nitrite may represent an alternative or additional pathway of 3-nitrotyrosine formation of potential importance in oxidative injures such as during inflammatory processes.

Key Words: **singlet oxygen; tyrosine nitration; 3-nitrotyrosine; nitrite.**

Nitration of tyrosine represents *in vivo* a mechanism of protein modification which can severely compromise the cell function (1). The detection of 3-nitrotyrosine (NO_2Tyr) in pathological tissues was suggestive of the occurrence of nitrating pathways and considered a possible diagnostic marker for reactive nitrogen species (RNS) production *in vivo* (2). In particular, it has been demonstrated that peroxynitrite, a toxic species generated by reaction of nitric oxide (NO) with superoxide anion (3), is an effective nitrating agent leading to the production of 3-nitrotyrosine (4, 5). However, nitration of tyrosine can occur also through mechanisms involving peroxidase-dependent oxidation of nitrite (NO_2^-), the major end-product of nitric oxide metabolism, forming a reactive nitrogen species, presumably nitrogen dioxide ($^{\cdot}NO_2$) (6–8). Recent studies demonstrated that in inflammatory processes, this mechanism of tyrosine nitration is catalyzed by myeloperoxidase, an abundant enzyme secreted from activated phagocytes (9). Another possible pathway for 3-nitrotyrosine formation at sites of inflammation, involves secondary oxidation of nitrite by myeloperoxidase-generated hypochlorous acid (HClO), forming an intermediate species, possibly nitryl chloride (Cl-NO_2), capable of nitrating tyrosine (9, 10). Thus nitrite, rather than being solely an end product of NO metabolism, may function as an additional source of reactive nitrogen species leading to 3-nitrotyrosine formation.

Singlet oxygen has been shown to be produced in biological systems (11) and to play an important role in phagocyte-mediated oxidative reactions at sites of inflammation where is generated by the myeloperoxidase/H_2O_2/Cl^- system (12, 13).

In this note we present evidences indicating the production of 3-nitrotyrosine by a pathway which involves singlet oxygen, tyrosine, and nitrite.

MATERIALS AND METHODS

Methylene blue (MB), sodium nitrite, L-tyrosine, 3-nitrotyrosine, deuterium oxide (D_2O), diethylenetriaminepentaacetic acid (DTPA), and horseradish peroxidase were obtained from Sigma. 3,3′-Dityrosine was synthesized by reaction of L-tyrosine with horseradish peroxidase and hydrogen peroxide as described (14). All other reagents were of the highest purity commercially available. The reaction mixture contained 10 μM MB, tyrosine (25–400 μM) and nitrite (0–1 mM) in 20 mM phosphate buffer adjusted to the desired pH

Abbreviations used: $^1O_2$, singlet oxygen; NO_2Tyr, 3-nitrotyrosine; NO, nitric oxide; $^{\cdot}NO_2$, nitrogen dioxide; NO_2^-, nitrite; DTPA, diethylenetriaminepentaacetic acid.

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with 10% NaOH or with 5% H_3PO_4. DTPA 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 10 cm from the solution 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. Tyrosine and 3-nitrotyrosine were analyzed at 274 and 360 nm respectively, using a Waters 996 photodiode array. Dityrosine was analyzed by fluorescence detection, using a Perkin–Elmer LS-1LC with a 260 nm excitation filter and 410 nm emission wavelength. Peaks were identified using external standards and quantified using Millenium 32 software (Waters). The elution times of tyrosine, dityrosine and 3-nitrotyrosine were 3.8, 5.9 and 8.2 min respectively. The detection limit for 3-nitrotyrosine and dityrosine was 10 pmoles and 1 pmole, respectively. Oxygen consumption was measured with a Clark type electrode in a water jacketed cell (1.9 ml, 25°C) connected to a Gilson 5/6 oxygen analyzer.

RESULTS AND DISCUSSION

The Photosensitized Oxidation of Tyrosine

In this study, methylene blue (MB) was used as sensitizer for the production of singlet oxygen ($^1O_2$). It has been reported that the interaction of singlet oxygen with tyrosine results in a chemical reaction which leads, through a tyrosyl radical intermediate, to the oxidative cleavage of the aromatic ring but the final products are not well defined (15, 16). The reaction rate is strongly dependent on pH, being faster in alkaline solutions where the phenolate ion is present (17). Moreover it has been demonstrated that photosensitized oxidation of tyrosine involves both type I (electron transfer or hydrogen atom abstraction mediated by the photoexcited triplet state of the sensitizer) and type II (singlet oxygen-mediated) mechanisms. The relative contributions of the two mechanisms appears to be dependent on the pH, being type I mechanism predominant at pH values higher than 8 (18).

Figure 1A shows that exposure to light at pH 5.75 of the reaction mixture containing 200 μM tyrosine and 10 μM MB, resulted in the loss of tyrosine and in the formation of dityrosine. This indicates that the first step in the photooxidation of tyrosine generates tyrosyl radicals followed by dimerization to form dityrosine. Dityrosine levels reached a maximum (13.2 μM) after 30–60 min illumination, then decrease possibly because dityrosine is oxidized further to other undetectable products.

The Photosensitized Oxidation of Tyrosine in the Presence of Nitrite

When the photooxidation of tyrosine was performed in the presence of 1 mM nitrite, 3-nitrotyrosine (NO_2Tyr) was found as an additional product (Fig. 1B). No detectable tyrosine oxidation or nitration was observed in dark controls or in illuminated controls lacking MB. Nitrite could not be replaced by nitrate. In the presence of nitrite the yield of dityrosine was decreased (8.8 μM after 30 min illumination) and NO_2Tyr was found to accumulate during the exposure to light (up to 36.8 μM after 3 h illumination), suggesting that tyrosine nitration competes with dityrosine formation. This competition would imply that both products are formed by a related mechanism, via intermediate formation of tyrosyl radicals. Control experiments using authentic 3-nitrotyrosine showed that the compound is slowly decomposed when exposed to light in the presence of MB at pH 5.75. The recovery of NO_2Tyr was close to 90% after 30 min illumination and decreased to about 70% after 3 h illumination. Therefore, in further experiments, the production of 3-nitrotyrosine was determined after 30 min reaction time.

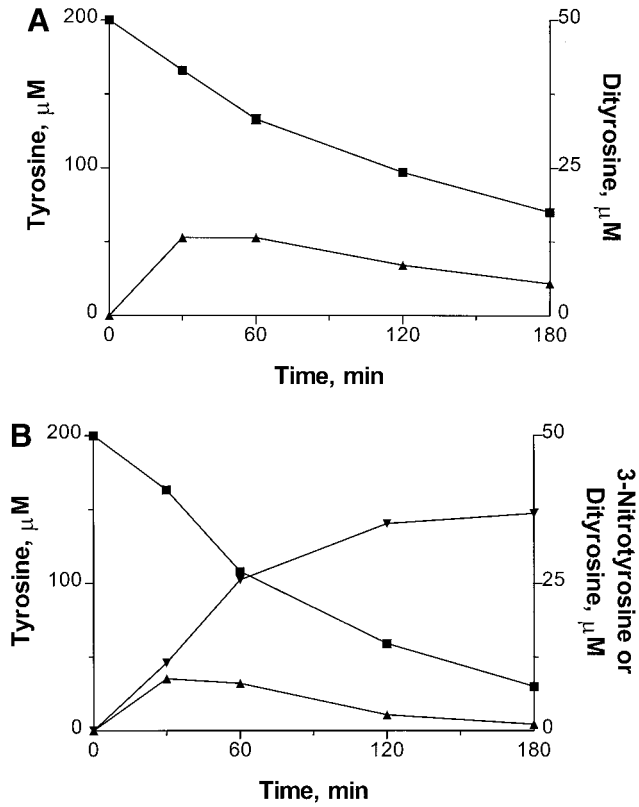

FIG. 1. Time course of the MB photooxidation of tyrosine in the absence (A) or presence (B) of nitrite. The reaction mixture contained 200 μM tyrosine, 10 μM MB and 1 mM nitrite (when present) in 20 mM phosphate buffer, including 100 μM DTPA, pH 5.75. The reaction was started by illumination and allowed to occur at 25°C under stirring. At the indicated time intervals, aliquots were withdrawn and analyzed for tyrosine (■), dityrosine (▲), or 3-nitrotyrosine (▼) by HPLC as described under Materials and Methods. Results are the mean ± SEM of three separate experiments.

As shown in Fig. 2A, the formation of NO_2Tyr by the photochemical system at pH 5.75 was found to increase with the concentration of nitrite added. The yield of NO_2Tyr as a function of tyrosine concentration is re-

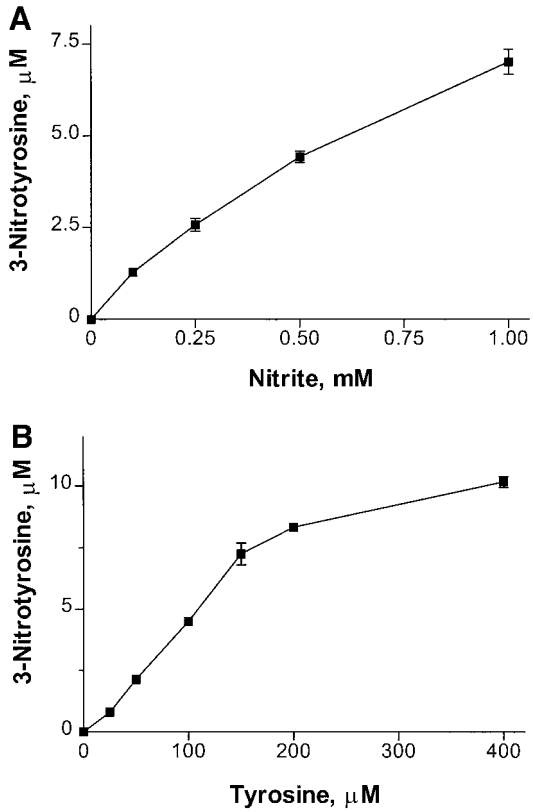

FIG. 2. Nitration of tyrosine by the MB photochemical system and nitrite as a function of nitrite concentration and tyrosine concentration. The reaction mixtures containing tyrosine, nitrite, and 10 μM MB, in 20 mM phosphate buffer, including 100 μM DTPA, pH 5.75, were exposed to light for 30 min at 25°C and 3-nitrotyrosine formation was measured by HPLC as described. (A) 100 μM tyrosine in the presence of the indicated concentrations of nitrite. (B) 500 μM nitrite in the presence of the indicated concentrations of tyrosine. Results are the mean ± SEM of three separate experiments.

ported in Fig. 2B: tyrosine nitration in the presence of 500 μM nitrite appeared to increase linearly up to 150 μM tyrosine; at higher tyrosine concentrations smaller increases of NO_2Tyr were observed. These findings most likely reflect the competitive formation of dityrosine, being more significant with increasing levels of tyrosine.

As shown in Fig. 3, the yield of NO_2Tyr is strongly dependent on pH, with a maximum at approximately pH 6. At lower pH, smaller yields of NO_2Tyr were observed; at higher pH, the amount of NO_2Tyr fell sharply with no detectable 3-nitrotyrosine above the neutrality. Control experiments using authentic 3-nitrotyrosine, showed that on exposure to light and MB, NO_2Tyr is gradually decomposed to undetectable products in a reaction which increases with pH (Fig. 3, insert). This probably reflects the fact that the ionized phenolate form of NO_2Tyr is photooxidized faster than the protonated form (pK = 7.2). Therefore it is conceivable that the pH-profile of NO_2Tyr yields is the result of two concurring processes: the production of NO_2Tyr and its decomposition, both increasing with 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 (19). Control experiments, in which tyrosine and nitrite in the pH range 4–6 were exposed to light in the absence of MB indicates that the contribution to nitration due to this reaction pathway appears significant only at pH lower than 5 (Fig. 3, 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 (20). This mechanism of tyrosine nitration may also be operative under our experimental conditions since MB photosensitized reactions can produce hydrogen peroxide with a stoichiometry of 1 mol of H_2O_2 formed per mol of substrate oxidized (21). To check this, tyrosine (100 μM) was incubated for 30 min, at pH 5.75, with nitrite (500 μM) in the presence of hydrogen peroxide at concentrations much higher than those possibly contributed by the photooxidation of tyrosine. No detectable 3-nitrotyrosine was found even with 500 μM H_2O_2 added (not shown), indicating that in the photochemical system under study, the possible formation of hydrogen peroxide does not participate, to an appreciable extent, in the nitration reaction.

The Involvement of Singlet Oxygen

To investigate the role of singlet oxygen ($^1O_2$) in the mechanism of the reaction under study, the yields of nitrotyrosine in H_2O and D_2O as solvents were compared. The substitution of D_2O for H_2O increases the lifetime of ($^1O_2$) and generally stimulates singlet oxygen-dependent reactions. As seen in Table I, line 2, the production of NO_2Tyr was greater by a factor of about 1.5 in D_2O. This effect, although not large, is indicative of the involvement of singlet oxygen in the reaction. Moreover to establish whether type I reaction, i.e., the electron transfer with excited triplet MB, was also involved in the nitration of tyrosine, the reaction mixture containing tyrosine, nitrite, and MB (up to 100 μM) was exposed to light under strict anaerobiosis. In these conditions, where only type I mechanism is operative, no NO_2Tyr was detected (Table I, line 3), indicating that, under our experimental conditions, this reaction does not participate in the process leading to the nitration of tyrosine.

The Nitrating Species

Collectively the results reported above indicate that, under our experimental conditions, the MB photosensitized oxidation of tyrosine is mediated by singlet oxygen with generation of intermediate tyrosyl radicals ($Tyr^{\bullet}$) which can dimerize to form dityrosine or react

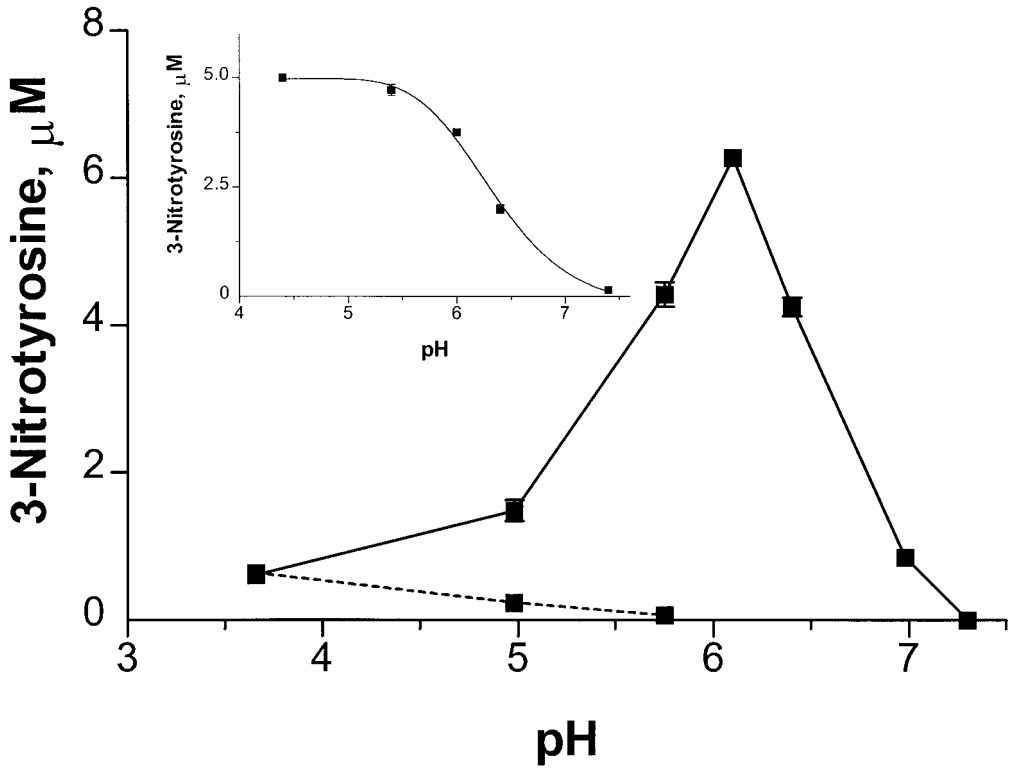

FIG. 3. Yields of 3-nitrotyrosine by the MB photochemical system and nitrite as a function of pH. The reaction mixtures containing 100 μM tyrosine, 500 μM nitrite and 10 μM MB in 20 mM phosphate buffer plus 100 μM DTPA, at the indicated pH, were exposed to light for 30 min at 25°C. The broken line indicates the formation of 3-nitrotyrosine in control experiments lacking MB. Insert: loss of 3-nitrotyrosine by the MB photochemical system as a function of pH at 25°C and 30 min illumination. Initial NO_2Tyr concentration 5 μM. Results are the mean ± SEM of three separate experiments.

with a nitrite-derived species (NO_x) to produce 3-nitrotyrosine (Reactions 1–3).

$$^1O_2 + \text{Tyr} \rightarrow \text{Tyr}^{\bullet} + O_2^{\bullet -} \quad [1]$$

$$\text{Tyr}^{\bullet} + \text{Tyr}^{\bullet} \rightarrow \text{Dityrosine} \quad [2]$$

$$\text{Tyr}^{\bullet} + NO_x \rightarrow NO_2\text{Tyr} \quad [3]$$

As first hypothesis, it appeared plausible that the photochemical system could also oxidize nitrite to form a reactive species able to accomplish the nitration reaction. To check this, the MB sensitized photooxidation of nitrite was evaluated by oxygen consumption experiments. However, the results indicated no reactivity, i.e., no O_2 consumption, even during prolonged illumination, using nitrite (up to 100 mM) over the pH range 4.5–7 (not shown). This finding led to exclude that the mechanism of generation of the nitrating species involves a direct oxidation of nitrite by the photochemical system. As the interaction of tyrosine with singlet oxygen produces intermediate tyrosyl radicals, it is possible (but remains to be proved) that indirect oxidation, via electron transfer, of nitrite by these radicals may generate nitrogen dioxide ($^{\bullet}NO_2$) (Reaction 4).

$$\text{Tyr}^{\bullet} + NO_2^- \rightarrow \text{Tyr} + {}^{\bullet}NO_2 \quad [4]$$

Nitrogen dioxide then combines with another tyrosyl radical leading to formation of 3-nitrotyrosine (Reaction 5), through a diffusion limited reaction ($k = 3 \times 10^9\ M^{-1}s^{-1}$) (23).

TABLE I

3-Nitrotyrosine Formation by MB Photooxidation of Tyrosine and Nitrite in Different Conditions

Condition[a]	3-Nitrotyrosine (μM)
MB photooxidation of tyrosine + nitrite	4.43 ± 0.17
MB photooxidation of tyrosine + nitrite in D_2O (pD = 5.75)[b]	6.35 ± 0.15
MB photooxidation of tyrosine + nitrite under anaerobiosis[c]	n.d.[d]

[a] The reaction mixtures containing 100 μM tyrosine, 500 μM nitrite, and 10 μM MB in 20 mM phosphate buffer plus 100 μM DTPA, pH 5.75, were illuminated for 30 min at 25°C.

[b] pD was taken as pH measured + 0.4 (22).

[c] The solution, in a cuvette sealed to a Thumberg tube, was deareated and purged with Nitrogen; this operation was repeated three times.

[d] Not detected also with MB increased to 100 μM.

$$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow NO_2Tyr \quad [5]$$

In the proposed mechanism, two tyrosyl radicals are needed for the nitration of one tyrosine.

CONCLUDING REMARKS

The results presented herein show that the singlet oxygen-mediated oxidation of tyrosine in the presence of nitrite produces 3-nitrotyrosine, with maximum yield at pH 6. Interestingly, in many of the pathologies where NO_2Tyr is detected, tyrosine nitration appears to be associated with the activation of phagocytes (2). A key feature of these cells is the production of reactive oxygen species (ROS) such as singlet oxygen, formed by the interaction of H_2O_2 with myeloperoxidase-generated HClO (12, 13), as well as an increased level of nitrite as consequence of stimulated generation of nitric oxide (24, 25). In addition, the pH in the phagosome falls to levels (pH 5.8–6.1) (26) where the yield of NO_2Tyr by the tyrosine/nitrite/singlet oxygen system is maximal. Hence, one important implication of the results reported herein is that oxidation of tyrosine by singlet oxygen in the presence of nitrite may represent an alternative pathway of 3-nitrotyrosine formation at sites of inflammation, to be added to those previously reported which involve myeloperoxidase, hydrogen peroxide and nitrite (7–10).

This potential contributing mechanism requires further studies to evaluate its physiological and/or pathological importance.

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