Free Radical Research, Vol. 35, pp. 735–747
Reprints available directly from the publisher
Photocopying permitted by license only

Catalase Catalyzes Nitrotyrosine Formation from Sodium Azide and Hydrogen Peroxide

KEIKI OGINO[a,*], NORIO KODAMA[a], MADOKA NAKAJIMA[a], AKIHIRO YAMADA[a], HIROYUKI NAKAMURA[a], HIROHUMI NAGASE[a], DAIKAI SADAMITSU[b] and TAKESHI MAEKAWA[b]

[a]*Department of Environmental and Preventative Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, 920-8640 Kanazawa, Japan;* [b]*Department of Emergency and Critical Care, Yamaguchi University School of Medicine, Kogushi Ube, Japan*

Accepted by Professor B. Halliwell

(Received 23 November 2000; In revised form 24 April 2001)

Sodium azide (NaN_3) is known as an inhibitor of catalase, and a nitric oxide (NO) donor in the presence of catalase and H_2O_2. We showed here that catalase-catalyzed oxidation of NaN_3 can generate reactive nitrogen species which contribute to tyrosine nitration in the presence of H_2O_2. The formation of free-tyrosine nitration and protein-bound tyrosine nitration by the NaN_3/catalase/H_2O_2 system showed a maximum level at pH 6.0. Free-tyrosine nitration induced by peroxynitrite was inhibited by ethanol and dimethylsulfoxide (DMSO), and augmented by superoxide dismutase (SOD). However, free-tyrosine nitration induced by the NaN_3/catalase/H_2O_2 system was not affected by ethanol, DMSO and SOD. NO_2^- and NO donating agents did not affect free-tyrosine nitration by the NaN_3/catalase/H_2O_2 system. The reaction of NaN_3 with hydroxyl radical generating system showed free-tyrosine nitration, but no formation of nitrite and nitrate. The generation of nitrite (NO_2^-) and nitrate (NO_3^-) by the NaN_3/catalase/H_2O_2 system was maximal at pH 5.0. These results suggested that the oxidation of NaN_3 by the catalase/H_2O_2 system generates unknown peroxynitrite-like reactive nitrogen intermediates, which contribute to tyrosine nitration.

Keywords: Catalase; Sodium azide; Nitrotyrosine; Azidyl radical; Peroxynitrite

INTRODUCTION

Azide has been widely used as an inhibitor of metalloenzymes, such as catalase and various peroxidases in oxygen biochemistry.[1] In addition to inhibiting catalase action, azide shows extensive oxidation to several gaseous nitrogenous products such as nitrous oxide, nitric oxide (NO), and nitrogen in the presence of hydrogen peroxide.[2] Catalytically important intermediate forms of catalase, Compound I

*Corresponding author. Fax: +81-76-234-4233. E-mail: ogino@med.kanazawa-u.ac.jp

and II, which are formed upon reaction with H_2O_2, oxidize azide to an azidyl radical ($^{\cdot}N_3$) subsequent reaction of the azidyl radical with oxygen generates NO.[3] The formation of NO by such a mechanism occurs in human neutrophils stimulated by phorbol myristate acetate and supplemented with azide or azide plus catalase.[4] The NO produced is believed to account for the ability of azide to activate guanylate cyclase and promote vascular relaxation.[5] Moreover, azidyl radical formation was demonstrated in pulse radiolysis of azide oxidation,[6] in succinate-driven respiration of azide-inhibited rat brain submitochondrial particles,[7] inactivation of lignin peroxidase from *Phanerochaete chrysosporium* by azide,[8] and inhibition of cytochrome c oxidase by azide in the presence of H_2O_2.[9] Covalently, incorporation of an azidyl radical into a peroxidase prosthetic group was involved in its inactivation.[10] On the other hand, the azidyl radical generates tyrosine phenoxyl radicals[11] and simultaneously generates NO. Tyrosine phenoxyl radicals react with NO and subsequent formation of nitrotyrosine occurs under prostaglandin endoperoxide synthase turnover.[12] Nitrotyrosine is a biomarker of the peroxynitrite ($ONOO^-$) attack and the attacks of other reactive nitrogen species on tyrosine.[13] Peroxynitrite is formed by the reaction of O_2^- and NO,[14] and is emphasized as a toxic oxidant in inflammatory diseases.[15] Therefore, this study was designed to investigate whether or not reactive species responsible for tyrosine nitration are generated in the reaction of azide with catalase and H_2O_2.

MATERIALS AND METHODS

Chemicals

Sodium azide (NaN_3), luminol sodium salt, diethylenetriaminepentaacetic acid (DTPA), and superoxide dismutase (SOD) (bovine erythrocyte, 3500 U/mg), were purchased from Wako Pure Chemicals. 3-Nitrotyrosine was obtained from Alexis (Switzerland). Bovine serum albumin (essentially fatty acid-free, approx. 0.005%) (BSA), ovalbumin (#A 5253) and L-tyrosine were obtained from Sigma. Catalase (65,000 U/mg) was obtained from Boehringer Mannheim GmbH. A rabbit polyclonal anti-nitrotyrosine antibody was obtained from Upstate Biotechnology, and a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was obtained from Dako. Peroxynitrite, synthesized in a quenched flow reactor[16] and with contaminating H_2O_2 removed by manganase dioxide, was purchased from Dojindo. All other reagents were of the highest purity commercially available.

Chemiluminescence

Chemiluminescence was measured with a luminescence reader (Aloka, BLR102) for a single detection at 37°C as described previously.[17] The standard reaction mixtures for a single detection contained 0.07 mM luminol and 100 μM DTPA in 1 ml of continuously stirred 50 mM potassium phosphate buffer (pH 7.4). For the NaN_3/-catalase/H_2O_2 system, 0–1 mM NaN_3, 0–5 μg/ml catalase and 0–200 μM H_2O_2 were added at critical times to the standard reaction mixtures. The intensity of the peak chemiluminescence was expressed as counts per min.

Nitration of L-tyrosine

Nitration of free-tyrosine in the NaN_3/catalase/H_2O_2 system was measured by changing the concentrations of NaN_3, catalase, and H_2O_2. L-tyrosine (1 mM) was dissolved in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA, 10–500 μg/ml catalase, 1–10 mM NaN_3, and 1–10 mM H_2O_2. The reaction was initiated by addition of H_2O_2 and allowed to proceed at 37°C for 1 h. To test the effect of pH on the nitration of free-tyrosine, L-tyrosine (1 mM)

was dissolved in 50 mM glycine–HCl buffer (pH 3.0) or 50 mM acetate buffer (pH 4.0, 5.0) or 50 mM potassium phosphate buffer (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate buffer (pH 9.0), containing 100 μM DTPA, 500 μg/ml catalase, 5 mM NaN_3 and 5 mM H_2O_2. Nitrous acid can nitrate tyrosine in the presence of H_2O_2. L-Tyrosine (1 mM) was dissolved in 50 mM glycine–HCl buffer (pH 3.0) or 50 mM acetate buffer (pH 6.0 or 7.0) containing 100 μM DTPA, 100 μM NO_2^- and 5 mM H_2O_2. To evaluate the formation of NO_3^- from the oxidation of NO_2^- by catalase, 1 mM NO_2^- was added in place of NaN_3 in the reaction of NaN_3/catalase/H_2O_2. Boiled catalase or catalase dialyzed overnight with 50 mM potassium phosphate buffer (pH 7.4), was also added in place of catalase in the reaction with the NaN_3/catalase/H_2O_2 system, to block the function of catalase as a protein and the possible role of any contaminating low molecular weight materials. The contribution of nitrous acid to tyrosine nitration in the NaN_3/catalase/H_2O_2 system was evaluated by incubation of 1 mM L-tyrosine with 100 μM DTPA, 100 μM NO_2^- and 5 mM H_2O_2 in 50 mM glycine–HCl buffer (pH 3.0) or 50 mM acetate buffer (pH 6.0, 7.0) for 1 h at 37°C. To evaluate the nitrogen species from the NaN_3/catalase/H_2O_2 system, various antioxidants were added to the NaN_3/catalase/H_2O_2 system containing 1 mM L-tyrosine, 100 μM DTPA, 500 μg/ml catalase, 5 mM NaN_3 and 5 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.4), and then compared with tyrosine nitration by the reaction of 100 μM peroxynitrite with 1.0 mM tyrosine in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA. For the contribution of NO_2^- or NO to tyrosine nitration by the NaN_3/catalase/H_2O_2 system, NO_2^- (10–100 μM) or 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5) (10–100 μM) and 4-methyl-2-[hydroxyimino]-5-nitro-6-methoxy-3-hexenamide (NOR-1) (100 μM), a NO donor, was added to the NaN_3/catalase/H_2O_2 system. The participation of azidyl radicals in tyrosine nitration was elucidated by incubation of 17 mM NaN_3 with 1 mM L-tyrosine, 300 μM Fe(II) and 2 mM H_2O_2 in 20 mM phosphate buffer (pH 7.4) containing 150 mM KCl.[7] The Fe(II) was added last. All reactions containing catalase were terminated by centrifugation (7000 rpm) with ULTRAFREE-MC (10 kDa molecular weight cutoff) (Millipore) to remove proteins. Tyrosine and its nitration products were analyzed by HPLC using a 5 μM Spherisorb ODS-2RP-18 column, with 93% of 50 mM potassium phosphate (pH 3.0), and 7% methanol as the mobile phase at 1 ml/min, and UV detection at 274 nm.[18] Identification and quantification of nitrotyrosine were performed by treatment with excess sodium dithionite ($Na_2S_2O_4$), which reduces nitrotyrosine to aminotyrosine,[19] and comparison with external standards.

Immunoblot Analysis of Protein-bound Nitrotyrosine

BSA (1 mg/ml) in 50 mM glycine–HCl buffer (pH 3.0), 50 mM acetate buffer (pH 4.0 or 5.0 or 6.0), potassium phosphate buffer (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate buffer (pH 9.0) was incubated with 100 μM DTPA, 500 μg/ml catalase, 5 mM NaN_3, and 5 mM H_2O_2 for 1 h at 37°C. After incubation, a part of reaction mixture of pH 6.0 containing the NaN_3/catalase/H_2O_2 system was added with 1 mg/ml sodium dithionite to change nitrotyrosine to aminotyrosine. Then, 50 μl aliquots of incubation mixtures were suspended in 50 μl of a sample loading buffer (125 mM Tris–HCl, pH 6.8/30% glycerol/10% β-mercaptoethanol/2% SDS/0.01% bromophenol blue), and heated for 5 min at 95°C. Next, 10 μg/ml of samples were loaded on 5–20% SDS-polyacrylamide gels and electrophoresed. After electrophoresis, the separated proteins were transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 3% (w/v) ovalbumin in 20 mM Tris–HCl (pH 7.7)

containing 137 mM NaCl and 0.1% (w/v) Tween 20 (buffer A). After blocking, the membranes were washed twice for 5 min with buffer A, then incubated for 2 h with a rabbit polyclonal anti-nitrotyrosine antibody (1:800 dilution) or with a rabbit polyclonal anti-nitrotyrosine antibody preincubated for 1 h with 10 mM 3-nitrotyrosine. After three 10 min washes in buffer A, the immunocomplexed membranes were probed for 1 h with a goat anti-rabbit (1:1000 dilution) horseradish peroxidase-conjugated secondary antibody. Probed membranes were washed three times for 10 min with buffer A and immunoreactive proteins were detected using enhanced chemiluminescence (NEN™ Life Science Products). After chemiluminescence, the membranes were stained with Coomassie blue.

Nitrotyrosine from Protein Hydrolysis

Formation of protein-bound nitrotyrosine was detected as 3-nitrotyrosine by protein hydrolysis. BSA (1 mg/ml) was incubated with 100 μM DTPA, 500 μg/ml catalase, 10 mM NaN_3 and 5 mM H_2O_2 in 50 mM glycine–HCl buffer (pH 3.0), potassium phosphate buffer (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate buffer pH 9.0 for 1 h at 37°C. Control studies were performed by omitting NaN_3 or H_2O_2 from incubation mixtures of 50 mM potassium phosphate buffer (pH 6.0). After incubation, trichroloacetic acid was added to the samples (5% final concentration) and they were centrifuged at 12,000 rpm for 5 min. The precipitated proteins were hydrolyzed with 6M HCl overnight at 110°C under a vacuum, and analyzed by HPLC using a TSK-Gel ODS-80TS column (25 cm × 4.5 cm), with 90% of 50 mM potassium phosphate (pH 3.0) 10% methanol as the mobile phase at a flow rate of 1 ml/min.[20] The concentrations of 3-nitrotyrosine and tyrosine were determined by UV at 274 nm.

Nitrite NO_2^- and Nitrate NO_3^- Assays

Formation of NO_2^- and NO_2^- by the NaN_3/catalase/H_2O_2 system was determined by capillary zone electrophoresis,[21] because NaN_3 inhibits nitrate reductase in spectrophotometric assays of NO_2^- and NO_2^- with Greiss reagents. The incubation mixtures contained 100 μM DTPA in 1 ml of 50 mM potassium phosphate buffer (pH 7.4), and where indicated, 500 μg/ml catalase, 10 mM NaN_3, 5 mM H_2O_2, 30 μM NO_2^-, 50 μg/ml SOD, 300 μM ferrous sulfate or combinations of these reagents. To observe the effect of pH on the production of NO_2^- and NO_3^- via the NaN_3/catalase/H_2O_2 system, the reaction mixtures containing 100 μM DTPA, 500 μg/ml catalase, 10 mM NaN_3 and 5 mM H_2O_2 were incubated in 1 ml of 50 mM glycine–HCl buffer (pH 3.0) 50 mM potassium phosphate buffer (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate buffer (pH 9.0). All incubations were performed at 37°C for 1 h.

Statistical Analysis

All data are expressed as mean ± SD of several experiments. The significance of the differences between groups was analyzed by one-way analysis of variance, and $P < 0.05$ was considered significant.

RESULTS

Chemiluminescence

The addition of 4 μg/ml catalase and 25 μM NaN_3 to luminol caused a barely detectable level of chemiluminescence, but the further addition of H_2O_2, ranging from 6 to 60 μM, increased chemiluminescence in a dose-dependent manner (Fig. 1). When the concentrations of NaN_3 and H_2O_2 were fixed at 5 and 10 μM, respectively, luminol-dependent chemiluminescence increased in parallel with an increase in the

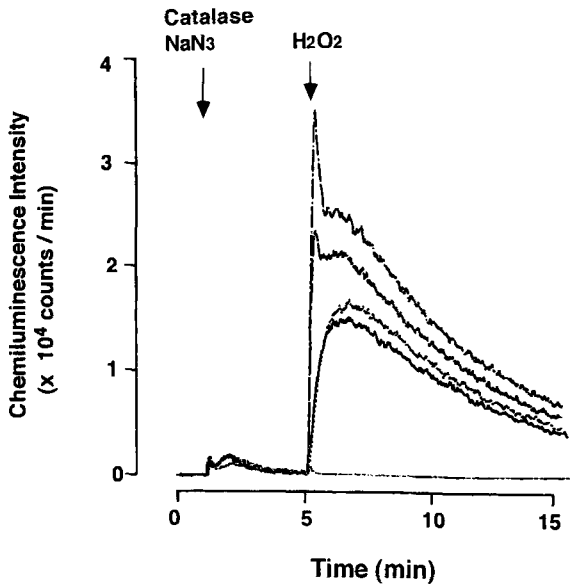

FIGURE 1 Luminol-dependent chemiluminescence produced by NaN_3/catalase/H_2O_2. The incubation of reaction mixtures containing 0.07 mM luminol, 100 μM DTPA, and 50 mM potassium phosphate buffer, pH 7.4, in a final volume of 1 ml was started at 37°C, and 1 min later, 4 μg/ml catalase and 25 μM NaN_3 were added. Five min later, H_2O_2 (0, 6, 12, 30, or 60 μM) was added. The tracings are representative of five experiments on different sample preparations.

concentration of catalase from 0.5 to 5 μg/ml. When 1 μg/ml catalase and 6.0 μM H_2O_2 were added to the incubation mixture, chemiluminescence was augmented by an increase in the concentration of NaN_3 from 5 to 50 μM. The lower limits of the concentration of NaN_3, catalase, and H_2O_2 required in the reaction mixtures in order to detect an increase in chemiluminescence were 5 μM, 2 μg/ml and 6 μM, respectively. No apparent increase in chemiluminescence was detected when any of the reagents were absent from the NaN_3/catalase/H_2O_2 mixture.

Nitration of L-tyrosine

The formation of nitrotyrosine with the reaction of 100 μg/ml catalase, 1 mM NaN_3, and various concentrations of H_2O_2 is shown in Fig. 2A. Nitrotyrosine formation was maximal at 5 mM H_2O_2. The formation of nitrotyrosine by the combination of 100 μg/ml catalase, 5 mM H_2O_2, and various concentrations of NaN_3 showed a dose-dependency on NaN_3 from 0.1 to 10 mM (Fig. 2B). When the concentrations of NaN_3 and H_2O_2 were fixed at 1 and 5 mM, respectively, the concentration of nitrotyrosine formed by the addition of 20 μg/ml catalase was 0.034 ± 0.005 μM. However, the formation of nitrotyrosine was not detected with 10 μg/ml catalase (Fig. 2C). The detection limit of authentic nitrotyrosine was 0.03 μM.

The formation of nitrotyrosine by the NaN_3/catalase/H_2O_2 system was demonstrated from pH 3.0 to 9.0 (Fig. 3). Maximal formation of nitrotyrosine was observed at pH 6.0. However, nitrotyrosine formation by the reaction of NO_2^- and H_2O_2 was observed at pH 3.0, but not at pH 6.0 or 7.0 (Fig. 3). Nitrotyrosine formation was not observed due to the oxidation of NO_2^- or by using boiled catalase in the NaN_3/catalase/H_2O_2 system (Fig. 3).

Nitrotyrosine formation was observed by the reaction of azide with H_2O_2 and Fe^{2+}, but was inhibited by the addition of DTPA and was not observed in the absence of Fe^{2+} (Fig. 4).

The effect of various scavengers on tyrosine nitration by both the NaN_3/catalase/H_2O_2 system and peroxynitrite are compared in Fig. 5. Nitrotyrosine formation in control studies by the NaN_3 (5 mM)/catalase (500 μg/ml)/H_2O_2 (5 mM) system and 100 μM peroxynitrite was 1.25 ± 0.27 and 6.62 ± 0.16 μM, respectively. Tyrosine nitration by peroxynitrite was augmented by SOD, but significantly inhibited by mannitol, ethanol and dimethylsulfoxide (DMSO). On the contrary, tyrosine nitration by the NaN_3/catalase/H_2O_2 system was not affected by SOD, and was not inhibited by mannitol or DMSO. Methionine, cysteine and uric acid showed inhibitory effects on tyrosine nitration by both the NaN_3/catalase/H_2O_2 system and peroxynitrite.

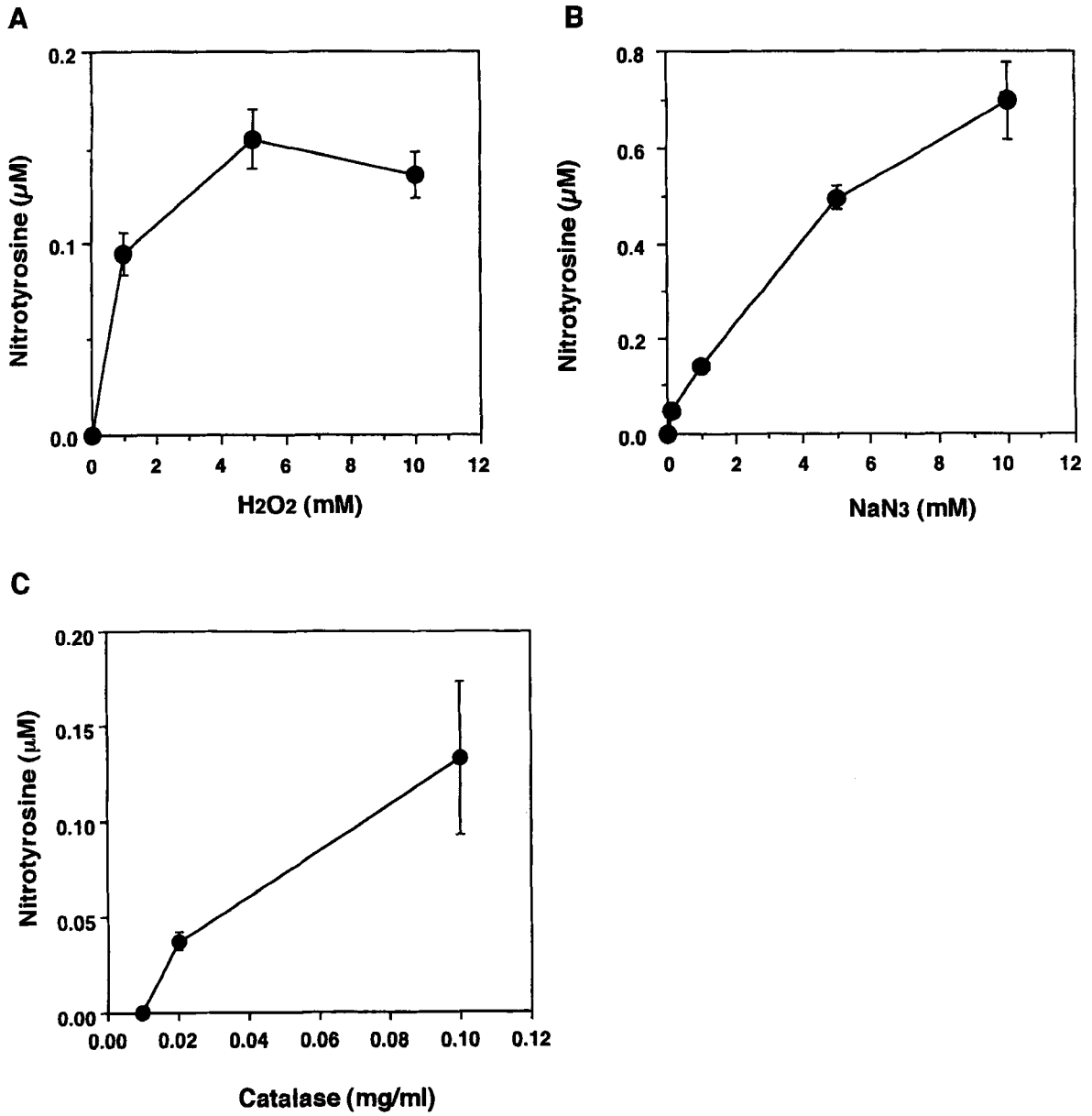

FIGURE 2 Tyrosine nitration by NaN_3/catalase/H_2O_2. (A) Tyrosine (1 in 50 mM potassium phosphate buffer containing 100 μM DTPA (pH 7.4)) was incubated with 400 μg/ml catalase, 1 mM NaN_3, and the indicated concentrations of H_2O_2. (B) Tyrosine (1 mM mM) dissolved in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA was incubated with 400 μg/ml/ml catalase, 5 mM H_2O_2, and the indicated concentrations of NaN_3. (C) Tyrosine (1 mM) dissolved in 50 mM potassium phosphate buffer (pH 7.4) containing 100 μM DTPA was incubated in the presence of 1 mM NaN_3, 5 mM H_2O_2, and the indicated concentrations of catalase. Each value represents the mean ± SD of five determinations.

The addition of NO_2^- (10–100 μM) or NOC-5 (10–100 μM) and NOR-1 (100 μM), a NO donor, to the NaN_3/catalase/H_2O_2 system, and the use of dialyzed catalase in the NaN_3/catalase/H_2O_2 system did not alter the nitration of tyrosine (data not shown).

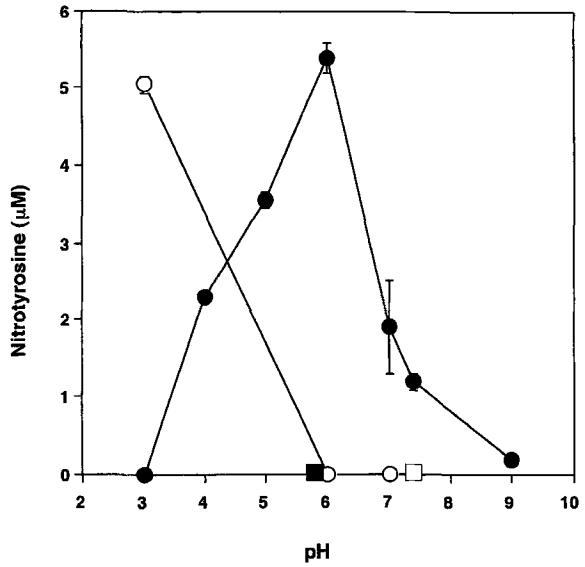

FIGURE 3 Effect of pH on tyrosine nitration by the NaN_3/catalase/H_2O_2 system and by the reaction with NO_2^- and H_2O_2. Tyrosine (1 mM) was reacted with 100 μM DTPA, 10 mM NaN_3, 500 μg/ml catalase and 5 mM H_2O_2 in each pH buffer (closed circles). Tyrosine (1 mM) was reacted with 100 μM NO_2^- with 5 mM H_2O_2 at pH 3.0, 6.0 or 7.0 (open circles). NO_2^- (1 mM) was added in place of NaN_3 in the NaN_3/catalase/H_2O_2 system at pH 6.0 (a closed square). Boiled catalase was added in place of intact catalse in the reaction mixtures at pH 7.4 (an open square). Each value represents the mean ± SD of five determinations.

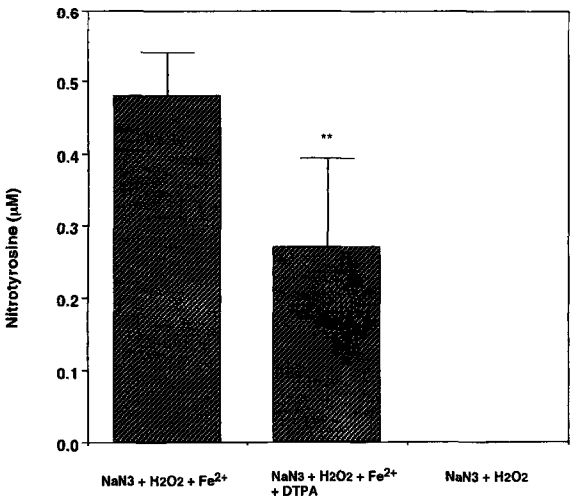

FIGURE 4 Tyrosine nitration bin the reaction of NaN_3 with H_2O_2 and Fe^{2+}, a hydroxyl radical generating system. Tyrosine (1 mM) was reacted with 17 mM NaN_3, 2 mM H_2O_2, 300 μM Fe^{2+},100 μM DTPA or combinations of these reagents at room temperature for 1 h. Each value represents the mean ± SD of five determinations. $^{**}P < 0.01$ versus $NaN_3 + H_2O_2 + Fe^{2+}$.

Immunoblot Analysis of Protein-bound Nitrotyrosine

The effect of pH on the nitration of BSA was examined by SDS–PAGE with Coomassie blue staining and Western blotting (Fig. 6A, B). A nitrated protein band corresponding to BSA was seen from pH 4.0 to 7.4. The maximal intensity of nitrated proteins was seen at pH 6.0 (lane 3, 4). Reaction mixtures of pH 3.0 (lane7) and 9.0 (lane 1) did not show nitrated protein band, although a band of Coomassie blue staining was shown. A nitrated protein band was abolished by pretreatment of sample in pH 6.0 with 5 mg/ml dithionite (lane 8). Pretreatment of the anti-nitrotyrosine antibody with 10 mM 3-nitrotyrosine for 2 h completely inhibited the staining (Fig. 6C).

Nitrotyrosine from Protein Hydrolysis

Protein-bound nitrotyrosine formation was elevated to maximal levels at pH 6.0 compared to the levels of pH 3.0 and 9.0 (Fig. 7). The reaction of 100 μM NO_2^- and 5 mM H_2O_2 did not generate protein-bound nitrotyrosine.

Nitrite NO_2^- and Nitrate NO_3^- Assays

The production of NO_2^- and NO_3^- in the NaN_3/catalase/H_2O_2 system, the effect of NO_2^- and SOD on the system, and the production of NO_2^- and NO_3^- in the azidyl radical producing system are shown in Table I. NO_2^- and NO_3^- were present in trace amounts in the solutions of 500 μg/ml catalase. The incubation of mixtures containing 500 μg/ml catalase, 1 mM NaN_3, and 5 mM H_2O_2 produced significantly increased amounts of NO_2^- and NO_3^-. The concentration of NO_3^- formed was 2.2-fold higher than that of NO_2^-. Oxidation of NO_2^- to NO_3^- was not observed by

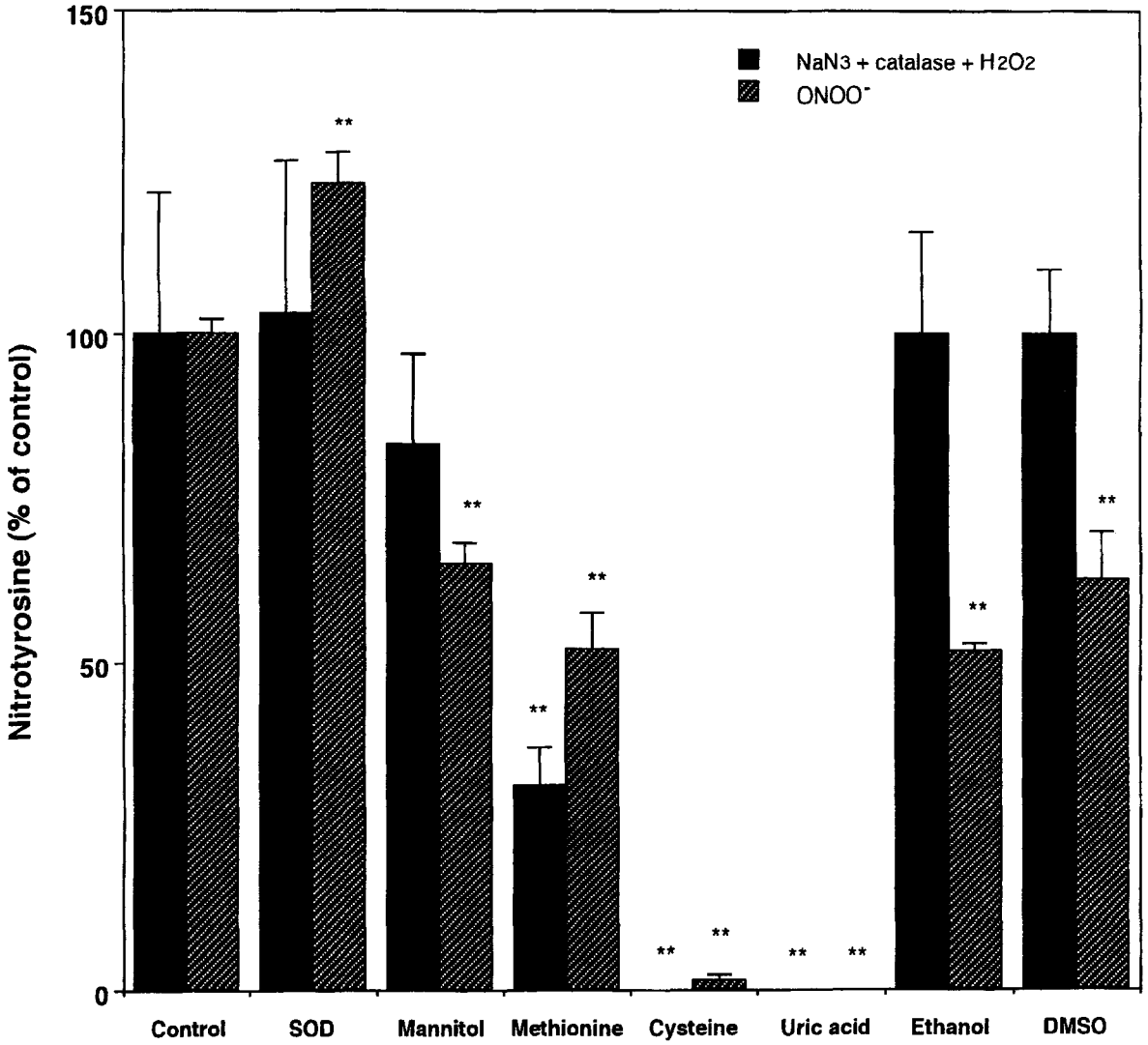

FIGURE 5 Effects of various scavengers on tyrosine nitration by the NaN_3/catalase/H_2O_2 system and peroxynitrite. Incubation mixtures of 100 μM DTPA, 1 mM tyrosine with 5 mM NaN_3, 500 μg/ml catalase and 5 mM H_2O_2, or with 100 μM peroxynitrite were added to 50 μg/ml SOD, 50 mM mannitol, 1 mM methionine, 1 mM cysteine, 1 mM uric acid, 80 mM ethanol or 50 mM dimethylsulfoxide (DMSO). Each value represents the mean ± SD of five determinations. $^{**}P < 0.01$ versus control.

addition of 30 μM NO_2^- and 50 μg/ml SOD, respectively, in the NaN_3/catalase/H_2O_2 system. Addition of Fe^{2+} to the reaction mixtures of 10 mM NaN_3 and 5 mM H_2O_2 did not show any elevation of NO_2^- and NO_3^-.

The effect of pH on the production of NO_2^- and NO_3^- via the NaN_3/catalase/H_2O_2 system is shown in Fig. 8. From neutral to acidic pH, the production of NO_3^- was predominant.

DISCUSSION

NaN_3 has been used as an NO-donating agent in biological research in studies on the increased formation of NO by neutrophils,[4] the activation of guanylate cyclase,[22], and the relaxation of isolated rings of the rat aorta.[5] These reports showed NO formation resulting from the reaction of NaN_3 with endogenous catalase and

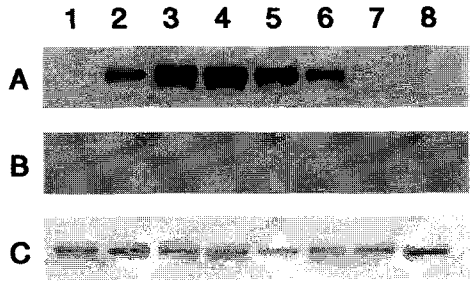

FIGURE 6 Immunoblot analysis of protein nitration by NaN_3/catalase/H_2O_2. Bovine serum albumin (BSA) (1 mg/ml) in 50 mM glycine–HCl buffer (pH 3.0), 50 mM acetate buffer (pH 4.0, 5.0 or 6.0) 50 mM potassium phosphate (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate (pH 9.0)) was incubated in the presence of 100 μM DTPA, 10 mM NaN_3, 500 μg/ml catalase, and 5 mM H_2O_2. (A) After incubation for 1 h, 5.5 μg/ml of protein was loaded onto 5–20% SDS–polyacrylamide gels for electrophoresis, then transferred to membranes, and immunostained with polyclonal antibodies against nitrotyrosine or (B) immunostained with antibodies preincubated with 10 mM 3-nitro-L-tyrosine for 2 h and (C) stained with Coomassie blue. Lane 1, pH 9.0; lane 2, pH7.4; lanes 3, 4 pH6.0; lane 5, pH 5.0; lane 6, pH 4.0; lane 7, pH 3.0. An incubation mixture containing 1 mg/ml BSA, 100 μM DTPA, 10 mM NaN_3, 500 μg/ml catalase, and 5 mM H_2O_2 was treated with 1 mg/ml sodium dithionite for 1 h at room temperature (lane 8).

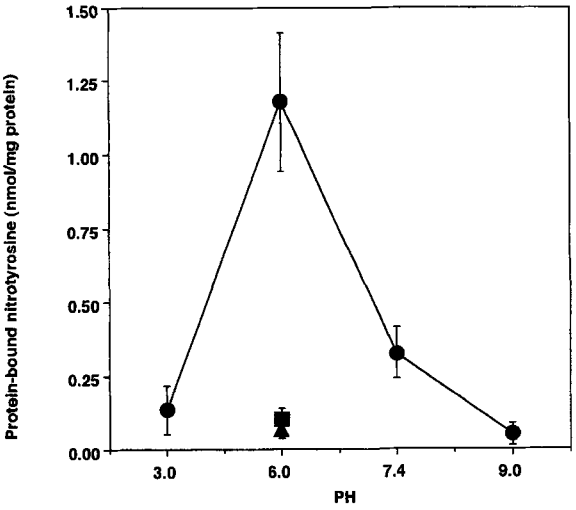

FIGURE 7 Effects of pH on protein-bound nitrotyrosine formation by the NaN_3 catalase/H_2O_2 system. BSA (1 mg/ml) incubated with 100 μM DTPA, 10 mM NaN_3, 500 μg/ml catalase, and 5 mM H_2O_2 at pH 3.7, 6.0, 7.4, or 9.0 (closed circle), hydrolyzed to free amino acid by 6N HCl, and nitrotyrosine formed was analyzed by HPLC. NaN_3 or H_2O_2 was omitted in the reaction mixtures at pH 6.0, shown by a closed triangle or a closed square.

endogeneously generated H_2O_2. In several *in vitro* studies, NO formation by the oxidation of NaN_3 with the catalase/H_2O_2 system was conclusively shown.[2,23,24] Azidyl radicals have been detected by ESR spin-trapping analysis as a result of the oxidation of NaN_3 with the catalase/H_2O_2 system[3] and various peroxidase[7–9] dependent on H_2O_2 or ferry iron. One electron oxidation of N_3^- by catalase Compound I produces Compound II and $\cdot N_3$, and then Compound II oxidizes N_3^- to $\cdot N_3$.[3] $\cdot N_3$ may react with oxygen to form N_2O and NO.[3] However, NO itself does not show luminol-dependent chemiluminescence.[25,26] Peroxynitrite ($ONOO^-$) revealed chemiluminescence for luminol.[26] However, it is not clear whether $\cdot N_3$ shows luminol-dependent chemiluminescence. Although luminol-dependent chemiluminescence provides less specificity for the detection of radicals.[27] it is suggested that oxidants besides NO may be associated with luminol-dependent chemiluminescence in the NaN_3/catalase/H_2O_2 system.

TABLE I Nitrite (NO_2^-) and nitrate (NO_3^-) production via NaN_3/catalase/H_2O_2 system. Nitrite (NO_2^-) and nitrate (NO_3^-) was determined by capillary electrophoresis after incubation of 500 μM catalase, 1 mM NaN_3, 5 mM H_2O_2, 30 μM NO_2^-, 50 μg/ml SOD, 30 μg/ml ferrous sulfate or a combination of each reagents in 50 mM pottasium phosphate buffer (pH 7.4) for 1 h at 37°C.

Chemicals	NO_2^- (μM) (mean±SD)	NO_3^- (μM) (mean±SD)
Catalase	2.1±0.8	4.1±1.0
Catalase+NaN_3	2.0±0.3	3.9±1.3
Catalase+H_2O_2	1.9±1.2	3.4±1.2
Catalase+NaN_3+H_2O_2	7.4±0.5	16.6±2.1
Catalase+NaN_3+H_2O_2+NO_2^-	38.2±1.7	16.6±2.2
Catalase+NaN_3+H_2O_2+SOD	8.4±1.2	15.6±0.9
NaN_3+H_2O_2	1.0±0.3	0.5±0.1
NaN_3+H_2O_2+Fe^{2+}	0.8±0.2	0.3±0.2

The nitration of amino acid tyrosine and protein tyrosine at physiological pH is caused by peroxynitrite itself,[28] nitronium ions (formed by metal-catalyzed heterolytic cleavage of peroxynitrite,[29] nitrogen dioxide ($\cdot NO_2$),[30] peroxidase-catalyzed oxidation of NO_2^-,[18] and the reaction of $\cdot NO_2$ or NO with tyrosine radicals.[12,30] Besides

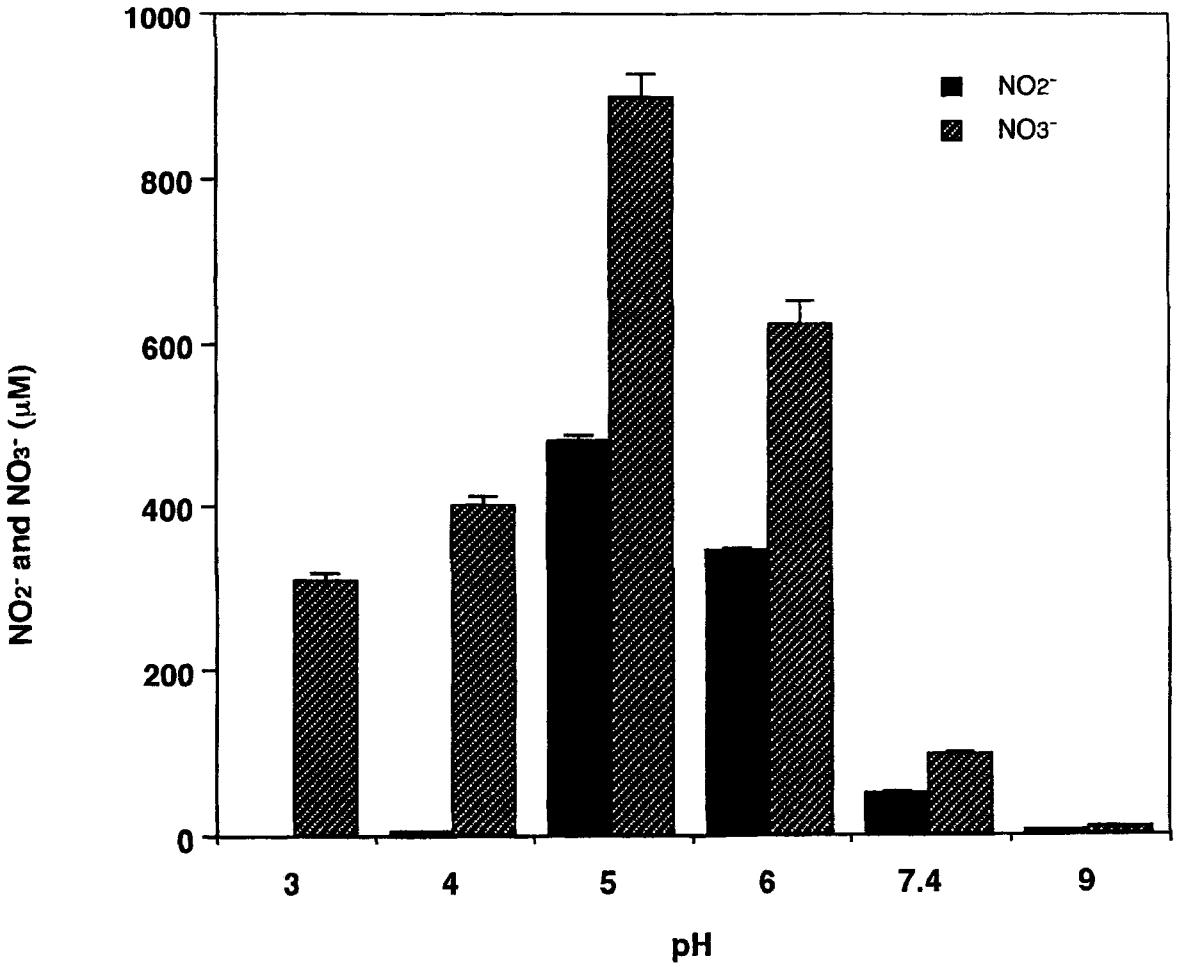

FIGURE 8 Effects of pH on nitrite (NO_2^-) and nitrate (NO_3^-) formation by NaN_3/catalase/H_2O_2. The reaction mixtures contained 100 μM DTPA, 10 mM NaN_3, 500 μg/ml catalase, and 5 mM H_2O_2 in 50 mM glycine–HCl buffer (pH 3.0), 50 mM potassium phosphate (pH 6.0 or 7.4) or 50 mM potassium pyrophosphate buffer (pH 9.0) NO_2^- and NO_3^- were analyzed by capillary zone electrophoresis. Each value represents the mean ± SD of three determinations.

the reaction of azide with peroxidases in the presence of H_2O_2, azidyl radicals are generated by the reaction of azide with hydroxyl radicals.[7,31] Azidyl radicals attack tyrosine, and subsequently tyrosine radicals are generated.[11] Simultaneous generation of NO and azidyl radicals tends to form nitrotyrosine. Our result that azidyl radicals, generated by the reaction of azide with hydroxyl radicals, could nitrate tyrosine but could not form nitrite or nitrate, suggests that azidyl radicals may be related to the formation of nitrotyrosine, but not to the formation of NO or $\cdot NO_2$. The tyrosine nitration in the reaction of NaN_3 with the hydroxyl radical generating system may have contributed to the oxidation of contaminating NO_2^- to $\cdot NO_2$ by hydroxyl radicals because addition of NO_2^- augmented the formation of nitrotyrosine (data not shown). Tyrosine nitration via the reaction of $\cdot NO_2$ and tyrosine radicals is less efficient in acidic solutions due to the natural disproportionation of $\cdot NO_2$.[30] The addition of NO_2^- and NO donating agents to the NaN_3/catalase/H_2O_2 system could

not promote tyrosine nitration. Therefore, azidyl radicals are not likely to be associated with the formation of NO and nitrotyrosine in the NaN_3/-catalase/H_2O_2 system.

The high concentrations of NO_3^- formed by the reaction of NaN_3 with catalase and H_2O_2 suggest the formation of peroxynitrite as a reactive nitrogen species. Peroxynitrite is stable in alkaline solutions, but at pH 7.4, it undergoes protonation to give peroxynitrous acid (ONOOH) (pKa 6.8), which rapidly decomposes to yield NO_3^-.[15] However, the difference in the inhibition of free-tyrosine nitration by both NaN_3/catalase/H_2O_2 and peroxynitrite with SOD, ethanol and DMSO suggests that tyrosine-nitrating species contributing to the nitration of tyrosine in the NaN_3/catalase/H_2O_2 system may be different from peroxynitrite. At acidic pH, peroxynitrous acid oxidizes DMSO to formaldehyde.[32] The promotion of peroxynitrite-dependent tyrosine nitration by SOD was explained by the formation of nitronium ions (NO_2^+).[29] However, the ineffectiveness of SOD in tyrosine nitration by NaN_3/catalase/H_2O_2 system may be associated with the inactivation of superoxide dismuatse by added H_2O_2.[33]

Inactivation of catalase in the presence of azide and H_2O_2 is associated with the formation of Compound III from the reaction of Compound II with H_2O_2.[34] Compound III has properties similar to the oxy-ferrous compounds of myoglobin and hemoglobin.[35] Nitrate and methemoglobin are formed by the reaction of oxy-ferrous compounds with NO. The formation of nitroxyl anions (NO^-) is suggested from the ferrous–NO complex generated by the reaction of Compound I with N_3^- in the inactivation of lignin peroxidase by azide.[36] The formation of the ferrous–NO complex has been detected in the NaN_3/catalase/H_2O_2 system.[2] However, the contribution of Compound III, ferrous–NO complex and NO^- in the tyrosine nitration by NaN_3/catalase/H_2O_2 system is not clear.

It was reported that NO_2^- can be oxidized to NO_3^- by the catalase–H_2O_2 complex II (Compound II).[37] The oxidation of NO_2^- to NO_3^- was not demonstrated in the NaN_3/-catalase/H_2O_2 system. It is not clear why a discrepancy between our results and a previous report exist. It is possible that Compound II may not be a major form in the NaN_3/-catalase/H_2O_2 system. Moreover, the discrepancy may be due to the method used, because our method is a newly developed and reliable direct method to measure nitrate, whereas the spectrophotometric assay, most widely used, requires the treatment of samples with nitrate reductase or reductants, which reduce nitrate to nitrite.[21]

The inhibition of endogenous peroxidase is a prerequisite for immunohistochemical study. A commercial kit is available for the inhibition of endogeneous peroxidase using high concentrations of azide and hydrogen peroxide. Therefore, it is possible that there is some peroxidase in tissue other than catalase, which produces tyrosine-nitrating species in the presence of azide and hydrogen peroxide. If some proteins in tissue are nitrated after treatment with inhibitors containing azide and hydrogen peroxide, the immunoreactivity of proteins against specific antibodies may change because tertiary conformational enzyme changes occur after tyrosine nitration.[38]

Azidyl radicals are likely to contribute little to tyrosine nitration in the NaN_3/catalase/H_2O_2 system. NO_3^- formation and inhibition studies on tyrosine nitration with scavengers resembles the properties of peroxynitrite. Therefore, it is concluded that unknown reactive nitrogen species, clearly different from, but similar to peroxynitrite, may be generated in the NaN_3/catalase/H_2O_2 system. However, the contribution of Compound III, the ferrous–NO complex and NO^- in tyrosine nitration by the NaN_3/catalase/H_2O_2 system and the possibility of tyrosine nitration after treatment of tissue with inhibitors containing azide and hydrogen peroxide should be clarified in the future.

Acknowledgements

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture. We express many thanks to Mrs Hitomi Ikemoto of Yamaguchi University School of Medicine for her helpful assistance.

References

[1] Smith, R.P. and Wilcox, D.E. (1994) "Toxicology of selected nitric oxide-donating xenotibotics, with particular reference to azide", *Crittical Reviews in Toxicology* **24**, 355–377.

[2] Nicholls, P. (1964) "The reactions of azide with catalase and their significance", *Biochemical Journal* **90**, 331–343.

[3] Kalyanaraman, B., Janzen, E.G. and Mason, R.P. (1985) "Spin trapping of the azidyl radical in azide/catalase/H_2O_2 and various azide/peroxidase/H_2O_2 peroxidizing systems", *Journal of Biological Chemistry* **260**, 4003–4006.

[4] Klebanoff, S.J. and Nathan, C.F. (1993) "Nitrite production by stimulated human polymorphonuclear leukocytes supplemented with azide and catalase", *Biochemical Biophysical Research Communications* **197**, 192–196.

[5] Mian, K.B. and Martin, W. (1995) "The inhibitory effect of 3-amino-1,2,4-triazole on relaxation induced by hydroxylamine and sodium azide but not hydrogen peroxide or glyceryl trinitrate in rat aorta", *British Journal of Pharmacology* **110**, 3302–3308.

[6] Eriken, J.E., Lind, J. and Merenyi, G. (1981) "The reactivity of the azide radical/$N_3^{\bullet}$/towards dioxygen species", *Radiochemical Radioanalytical Letters* **48**, 405–410.

[7] Partridge, R.S., Monroe, S.M., Parks, J.K., Johnson, K., Parker, Jr, W.D., Eaton, Jr, G.R. and Eaton, Jr, S.S. (1994) "Spin trapping of azidyl and hydroxyl radicals in azide-inhibited rat brain submitochondrial particles", *Archives of Biochemistry and Biophysics* **310**, 210–217.

[8] Tuisel, H., Grover, T.A., Lancaster, Jr., J.R., Bumpus, Jr., J.A. and Aust, Jr., S.D. (1991) "Inhibition of lignin peroxidase H2 by sodium azide", *Archives of Biochemistry and Biophysics* **288**, 456–462.

[9] Chen, Y-R., Sturgeon, B.E., Guntter, M.R. and Mason, R.P. (1999) "Electron spin resonance investigation of the cyanyl and azidyl radical formation by cytochrome c oxidase", *Journal of Biological Chemistry* **274**, 24611–24616.

[10] Samokyszn, V.M. and Oritiz de Montellano, P.R. (1991) "Toplogy of the chloroperoxidase active site: regiospecificity of heme modification by phenylhydrazine and sodium azide", *Biochemistry* **30**, 11646–11653.

[11] Prutz, W., Butler, J. and Land, E.J. (1983) "Phenol coupling initiated by one-electron oxidation by tyrosine units in peptides and histone", *International Journal of Radiation Biology* **44**, 183–196.

[12] Goodwin, D.C., Gunther, M.R., Hsi, L.C., Crews, B.C., Eling, T.E., Mason, R.P. and Marnett, L.J. (1998) "Nitric oxide trapping of tyrosyl radicals generated during prostaglandin endoperoxide synthase turnover", *Journal of Biological Chemistry* **273**, 8903–8909.

[13] Halliwell, B., Zhao, K. and Whiteman, M. (1999) "Nitric oxide and peroxynitrite. The ugly, the uglier and the not so good. A personal view of recent controversies", *Free Radical Research* **31**, 651–669.

[14] Beckman, J.S. and Koppenol, W.H. (1996) "Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly", *American Journal of Physiology* **271**, C1424–C1435.

[15] Pryor, W.A. and Squadrito, G.L. (1995) "The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide", *American Journal of Physiology* **268**, L699–L722.

[16] Beckman, J.S., CHen, J., Ischiropoulos, H. and Crow, J.P. (1994) "Oxidative chemistry of peroxynitrite", *Methods in Enzymology* **233**, 229–240.

[17] Ogino, K., Ishiyama, H., Murata, T., Kobayashi, H. and Houbara, T. (1992) "Zinc hydroxide induced respiratory burst in rat neutrophils", *Biochemical and Biophysical Reasearch Communications* **185**, 1115–1121.

[18] Van der Vliet, A., Eiserich, J.P., Halliwell, B. and Cross, C.E. (1997) "Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite", *Journal of Biological Chemistry* **272**, 7617–7625.

[19] Sokalovsky, M., Riordan, J.F. and Vallee, B.L. (1967) "Conversion of 3-nitrotyrosine to 3-aminotyrosine in peptides and proteins", *Biochemical and Biophysical Research Communications* **27**, 20–25.

[20] Salman-Tabcheh, S., Guerin, M.C. and Torrelle, J. (1995) "Nitration of tyrosyl-residues from extra- and intacelluar proteins in human whole blood", *Free Radical Biology and Medicine* **19**, 695–698.

[21] Ueda, T., Maekawa, T., Sadamitsu, D., Oshita, S., Ogino, K. and Nakamura, K. (1995) "The determination of nitrite and nitrate in human plasma by capillary zone electrophoresis", *Electrophoresis* **16**, 1002–1004.

[22] Craven, P.A., DeRubertis, F.R. and Pratt, D.W. (1979) "Electron spin resonance study of the role of NO·catalase in the activation of guanylate cyclase by NaN_3 and NH_2OH. Modulation of enzyme responses by heme proteins and their nitrosyl derivatives", *Journal of Biological Chemistry* **254**, 8213–8222.

[23] Theorell, H. and Ehrenberg, A. (1952) "The reaction between catalase, azide and hydrogen peroxide", *Archives of Biochemistry and Biophysics* **4**, 462–474.

[24] Keilin, D. and Hatree, E.F. (1954) "Reactions of methaemoglobin and catalase with peroxides and hydrogen donors", *Nature* **173**, 720–723.

[25] Radi, R., Cosgrone, T.P., Beckman, J.S. and Freeman, B.A. (1993) "Peroxynitrite-induced luminol chemiluminescence", *Biochemical Journal* **290**, 51–57.

[26] Kooy, N.W. and Royall, J.A. (1994) "Agonist-induced peroxynitrite production from endothelial cells", *Archives of Biochemistry and Biophysics* **310**, 51–57.

[27] Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine* (Oxford University Press, New York).

[28] Crow, J.P. and Beckman, J.S. (1995) "The role of peroxynitrite in nitric oxide-mediated toxicity", *Current Topics in Microbiology and Immunology* **196**, 57–73.

[29] Ischropoulos, H., Zhu, L., Chen, J., Tsai, H.M., Martin, J.C., Smith, C.D. and Beckman, J.S. (1992) "Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide

dismutase", *Archives of Biochemistry and Biophysics* **298**, 431–437.
[30] Prutz, W.A., Moning, H., Butler, J. and Land, E.J. (1985) "Reactions of nitrogen dioxide in aqueous model systems: oxidation of tyrosine units in peptides and proteins", *Archives of Biochemistry and Biophysics* **234**, 125–134.
[31] Raw, J.M. and McCay, P.B. (1994) "Oxygen radical formation in well-washed rat liver microsomes: spin trapping studies", *Free Radical Research* **20**, 51–60.
[32] Crow, J.P., Spruell, C., Chen, J., Gunn, C., Ischiropoulos, H., Tsai, M., Smith, G.D., Radi, R., Koppenol, W.H. and Beckman, J.S. (1994) "On the -pH dependent yield of hydroxyl radical products from peroxynitrite", *Free Radical Biology and Medicine* **16**, 331–338.
[33] Uchida, K. and Kawasaki, S. (1994) "Identification of oxidized histidine generated at the active site of Cu, Zn-superoxide dismutase exposed to H_2O_2. Selective generation of 2-oxo-histidine at the histidine 118", *Journal of Biological Chemistry* **269**, 2405–2410.
[34] Lardinois, O.M. and Rouxhet, P.G. (1996) "Peroxidative degradation of azide by catalase and irreversible enzyme inactivation", *Biochimica et Biophysics Acta* **1298**, 180–190.
[35] Chance, B., Powers, L., Ching, Y., Poulos, T., Schonbaum, T.G.R., Yamazaki, I. and Paul, K.G. (1984) "X-ray absorption studies of intermediates in peroxidase activity", *Archives of Biochemistry and Biophysics* **235**, 596–611.
[36] Matthew, T. and Bumpus, J.A. (1997) "Further studies on the inactivation by sodium azide of lignin peroxidase from *Phanerochaete chrysosporium*", *Archives of Biochemistry and Biophysics* **339**, 200–209.
[37] Keilim, D. and Nicholls, P. (1958) "Reaction of catalase with hydrogen peroxide and hydrogen donors", *Biochim et Biophys Acta* **29**, 302–307.
[38] Francescutti, D., Baldwin, J., Lee, L. and Mutus, B. (1996) "Peroxynitrite modification of glutathione reductase: modeling studies and kinetic evidence suggest the modification of tyrosines at the glutathione disulfide site", *Protein Engineering* **9**, 189–194.