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## Abstract

It is well known that eosinophils are involved in tyrosine nitration. In this study, we evaluated tyrosine nitration by rat eosinophils isolated from peritoneal fl uid and constituent eosinophils in the stomach. Rat peritoneal eosinophils activated with 1 $\mu$M phorbol myristate acetate (PMA) and 50 $\mu$M NO2 ﾝ showed immunostaining for nitrotyrosine only in smaller cells, despite the fact that eosinophils are capable of producing superoxide (O2·ﾝ). Free tyrosine nitrating capacity after incubation with PMA and NO2 ﾝ was 4-fold higher in eosinophils than in neutrophils. Catalase and ｸ- and ｺ -tocopherol inhibited free tyrosine nitration by reactive nitrogen species from eosinophils but not that by peroxynitrite. Superoxide dismutase augmented free tyrosine nitration by activated eosinophils and peroxynitrite. The concentration of nitric oxide released from eosinophils was relatively low (0.32 $\mu$M/106 cells/h) and did not contribute to the formation of nitrotyrosine. On the other hand, most constituent eosinophils constituent in the rat stomach stimulated by PMA and NO2 ﾝ showed tyrosine nitration capacity. These results suggest that intact cells other than apoptotic-like eosinophils eluted in the intraperitoneal cavity could not generate reactive species responsible for nitration by a peroxidase-dependent mechanism. In contrast, normal eosinophils in the stomach were capable of nitration, suggesting that the characteristics of eosinophils in gastric mucosa are diff erent from those eluted in the peritoneal cavity.

**KEYWORDS:** eosinophil peroxidase, reactive nitrogen species, nitrotyrosine

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*Original Article*

# Biochemical Characterization of Reactive Nitrogen Species by Eosinophil Peroxidase in Tyrosine Nitration

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**It is well known that eosinophils are involved in tyrosine nitration. In this study, we evaluated tyrosine nitration by rat eosinophils isolated from peritoneal fluid and constituent eosinophils in the stomach. Rat peritoneal eosinophils activated with 1 μM phorbol myristate acetate (PMA) and 50 μM $NO_2^-$ showed immunostaining for nitrotyrosine only in smaller cells, despite the fact that eosinophils are capable of producing superoxide ($O_2^{\cdot-}$). Free tyrosine nitrating capacity after incubation with PMA and $NO_2^-$ was 4-fold higher in eosinophils than in neutrophils. Catalase and α- and γ-tocopherol inhibited free tyrosine nitration by reactive nitrogen species from eosinophils but not that by peroxynitrite. Superoxide dismutase augmented free tyrosine nitration by activated eosinophils and peroxynitrite. The concentration of nitric oxide released from eosinophils was relatively low (0.32 μM/$10^6$ cells/h) and did not contribute to the formation of nitrotyrosine. On the other hand, most constituent eosinophils constituent in the rat stomach stimulated by PMA and $NO_2^-$ showed tyrosine nitration capacity. These results suggest that intact cells other than apoptotic-like eosinophils eluted in the intraperitoneal cavity could not generate reactive species responsible for nitration by a peroxidase-dependent mechanism. In contrast, normal eosinophils in the stomach were capable of nitration, suggesting that the characteristics of eosinophils in gastric mucosa are different from those eluted in the peritoneal cavity.**

**Key words:** eosinophil peroxidase, reactive nitrogen species, nitrotyrosine

Eosinophils in biological systems have been suggested to be important in the defense against parasitic helminths [1, 2]. Eosinophil recruitment is a characteristic feature of asthma and other allergic inflammatory diseases [3-5]. Inappropriate accumulation of eosinophils and eosinophil-derived granules in the tissue and sputum are not only considered as biomarkers for monitoring the severity of asthmatic events [3-8] but also suggested to evoke cellular and tissue damage [3-6]. Eosinophils possess numerous highly basic and toxic granules that are released upon activation or during cell necrosis. They are also equipped with the enzymes that inflict oxidative damage upon biological targets [3, 6]. The respira-

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tory burst occurs in activated eosinophils where $O_2^{\cdot-}$ and its dismutation product, $H_2O_2$, are formed [1, 9].

The formation of peroxynitrite ($ONOO^-$) and peroxidase-catalyzed reactive nitrogen species is implicated in eosinophils of the lung in asthma patients [10, 11]. $ONOO^-$ is a potent oxidant formed by a rapid reaction of NO and $O_2^{\cdot-}$ [12]. Inducible nitric oxide synthase (iNOS), which generates NO from L-arginine, is expressed in human circulating eosinophils [13] and rat peritoneal eosinophils [14]. Simultaneous production of $O_2^{\cdot-}$ and NO in eosinophils enables the production of $ONOO^-$. Peroxynitrite can react with tyrosine residues at a physiologically relevant pH to yield nitrotyrosine [15 16]. On the basis of nitrotyrosine formation, $ONOO^-$ has been implicated in the pathogenesis of a variety of diseases including neurodegenerative disease [17], atherosclerosis [18], allergic asthma [10, 11], and inflammatory gastro-intestinal diseases [19, 20].

Peroxidase-catalyzed formation of reactive nitrogen species using $NO_2^-$ and $H_2O_2$ as substrates has been demonstrated in purified myeloperoxidase (MPO) [21, 22] and eosinophil peroxidase (EPO) [23]. In healthy human subjects, $NO_2^-$ can be detected at levels of 0.5–3.6 $\mu$M in plasma [24]. Increased levels of $NO_2^-$ have been reported in a variety of pathological conditions [25, 26]. It is likely that eosinophils generate reactive nitrogen species, which contributes to tyrosine nitration, if EPO utilizes $H_2O_2$ as a cosubstrate to oxidize $NO_2^-$. However, there is little evidence for the production of reactive nitrogen species from eosinophils other than $ONOO^-$ that contributes to tyrosine nitration. Therefore, in the present study we aimed at clarifying the precise mechanisms for generation of nitrotyrosine by rat eosinophils.

## Materials and Methods

***Animals.*** Male Wistar (Kwl) rats weighing 250–300 g were purchased from Kiwa Laboratory Animals. The animal experiments were carried out according to protocols approved by the Kanazawa University Committee on Laboratory Animals.

***Isolation of rat peritoneal eosinophils.*** To elicit an eosinophil rich exudate, we injected rats with 1 ml of horse serum i.p. 3 times at 2-day intervals and performed a peritoneal lavage 24 h after the final injection. After centrifugation at 200 × g for 5 min, cells were suspended in 3 ml of $Ca^{2+}$ $Mg^{2+}$-free Hanks' balanced salt solution (HBSS) containing 10 % rat plasma and 0.01% ethylenediamine tetraacetic acid (EDTA), and layered on a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient, consisting of 45%, 55%, 60%, 65% Percoll (based on 100% = isotonic Percoll, made up with 10 × concentrated HBSS), each layer containing 10% rat plasma and 0.01% EDTA. The gradient was centrifuged at 400 × g for 30 min. The purest eosinophil fraction was recovered at 65% Percoll interphase, yielding $2–4 \times 10^6$ eosinophils per rat. The purity of the cell population > 95% was confirmed by cytocentrifuge smears stained with hematoxylin and eosin and Luxol-fast blue (LFB) [27]. Contaminating cells were mainly macrophages with < 1% neutrophils. Viability of the cells by trypan blue exclusion was > 99%.

***Isolation of rat peritoneal neutrophils.*** Rat neutrophils were prepared from intraperitoneal fluid [28]. The rats were injected i.p. with 20 ml of sterile 12% sodium caseinate in physiological saline. Twelve h later, the peritoneal lavage was performed. After centrifugation at 200 × g for 5 min, cells were suspended in 1 ml of physiological saline, and we then induced hypotonic lysis of contaminated erythrocytes. The cell suspension in $Ca^{2+}$ $Mg^{2+}$-free HBSS was layered on sodium metrizoate Ficoll (d = 1.09) to separate neutrophils from macrophages and lymphocytes, and centrifuged at 1,000 × g for 30 min. The purity of the cell population > 99% was determined by Wright's staining of cytocentrifuge smears. Cell viability by Trypan blue exclusion was > 97%.

***Nitration of endogenous protein in eosinophils and neutrophils.*** We used both unfixed and fixed cells to detect nitrotyrosine formation by eosinophil peroxidase and myeloperoxidase. In the study of unfixed cells, eosinophils ($5 \times 10^5$) and neutrophils ($5 \times 10^5$) were incubated with 50 $\mu$M $NO_2^-$ and 1 $\mu$M phorbol myristate acetate (PMA) in 250 $\mu$l of HBSS containing 0.1 mM diethylenetriamine pentaacetic acid (DTPA) at 37 °C for 1 h. After incubation, cells were cytocentrifuged onto the slides and fixed in 10% neutral-buffered formalin for 20 min.

In the study of fixed cells, cells were cytocentrifuged onto the slides first and then fixed with 10% neutral-buffered formalin for 5 min and washed with PBS 3 times for 5 min. Next, slides were incubated

with the combination of $NO_2^-$ (1, 5, and 1,000 $\mu$M) and $H_2O_2$ (1, 5, and 1,000 $\mu$M) in 250 $\mu$l of phosphate buffered saline (PBS) commonly containing 0.1 mM DTPA, 1 mM phenylmethylsulfonyl fluoride (Wako Pure Chemical Industries, Osaka, Japan), 1 $\mu$g/ml leupeptine (Sigma-Aldrich, St. Louis, MO, USA), and 1 $\mu$g/ml aprotinin (Roche Diagnostics, Basel, Switzerland) at 37 °C for 30 min. After incubation, the slides were refixed in 10% neutral-buffered formalin for 20 min.

***Immunocytochemical study for nitrotyrosine.*** The slides of fixed eosinophils and neutrophils were treated with 5% $H_2O_2$ in methanol for 20 min to inactivate endogenous peroxidase. After washing with PBS for 10 min 3 times, slides were mounted with 2% normal goat serum for 1 h in PBS. Polyclonal anti-nitrotyrosine antibodies (rabbit immunoaffinity purified IgG, Upstate Biotechnology, Inc., Lake Placid, NY, USA) or monoclonal anti-nitrotyrosine antibodies (mouse IgG, Upstate Biotechnology) was diluted 800-or 100-fold with PBS, respectively, and layered on the slides overnight at 4 °C. Slides were washed 3 times with PBS and incubated with a second antibody, horseradish peroxidase polymer-conjugated Envision plus (DAKO, Denmark) for 30 min at room temperature. After washing 3 times with PBS, peroxidase activity was localized by 3,3'-diaminobenzidine tetrahydrochloride and 0.01% $H_2O_2$, and slides were counterstained with hematoxylin or LFB.

The specificity of immunocytochemistry for nitrotyrosine was confirmed by preincubation of antibodies with 10 mM 3-nitrotyrosine for 2 h or by pretreatment of the slides with 1 mg/ml dithionite in PBS for 1 h. A negative control study was performed using non-immune rabbit or mouse immunoglobulin. Light microscopic observations were carried out with an Olympus IX-70.

***Superoxide generation from eosinophils and neutrophils.*** Superoxide release was measured following the superoxide dismutase (SOD)-inhibitable reduction of ferricytochrome c at 550 nm in a single spectrophotometer by a modification of the procedure of Babior *et al.* [29]. Eosinophils or neutrophils ($1 \times 10^6$) were incubated at 37 °C for 1 h in 1 ml of HBSS with 0.1 mM ferricytochrome c, 1 $\mu$M PMA, and 50 $\mu$M $NO_2^-$. Before incubation, 50 $\mu$g/ml of SOD was added as a reference.

***Histochemical detection of superoxide generation from eosinophils.*** Superoxide generation was detected histochemically by a modification of the nitro blue tetrazolium (NBT) reduction method [30]. Eosinophils ($1 \times 10^6$) were incubated at 37 °C for 1 h in 1 ml of HBSS with 500 $\mu$g/ml NBT, and the addition of 1 $\mu$M PMA or 1 $\mu$M PMA and 50 $\mu$M $NO_2^-$. After incubation, cells were cytocentrifuged on slides and fixed with 10% neutral-buffered formalin, and we then performed an immunohistochemical study for nitrotyrosine.

***Western blot analysis of nitrated proteins.*** Eosinophils ($1 \times 10^6$) or neutrophils ($1 \times 10^6$) were incubated at 37 °C for 1 h in HBSS (500 $\mu$l) with PMA (1 $\mu$M), $NO_2^-$ (0–50 $\mu$M), $H_2O_2$ (50 $\mu$M), SOD (50 $\mu$g/ml), catalase (30 $\mu$g/ml), 1 mg/ml bovine serum albumin (BSA), or the combination of these reagents. After incubation, cells were immediately centrifuged and the pellets were solubilized by the addition of 50 $\mu$l non-reducing Laemmli sample buffer and then boiled for 5 min. Samples (40 $\mu$g protein) were subjected to 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes.

The membranes were blocked by overnight incubation at 4 °C with 3% (w/v) nonfat dried milk in 20 mM Tris-HCl, pH 7.7, containing 137 mM NaCl and 0.1% (w/v) Tween-20 (buffer A). The membranes were washed twice for 5 min with buffer A, following incubation for 2 h with a polyclonal anti-nitrotyrosine antibody (IgG, Upstate Biotechnology, Lake Placid, NY, USA) diluted 1,000-fold in buffer A containing 1.5% (w/v) nonfat dried milk. After washing 3 times for 20 min with buffer A, the membranes were incubated for 1 h with a horseradish peroxidase (HRP)-labeled anti-mouse IgG antibody, which was diluted 2,000-fold in buffer A containing 1.5% nonfat dried milk. The membranes were washed three times for 20 min with buffer A, and peroxidase activity was detected on X-ray film with enhanced chemiluminescence (NEN Life Science Products, Boston, MA, USA). The specificity of nitrated proteins was clarified by pre-incubation of anti-nitrotyrosine antibody with 10 mM 3-nitrotyrosine (Aldrich Chemical Co., Milwaukee, WI, USA) for 2 h.

***Nitration of free tyrosine.*** We investigated

free tyrosine nitration by eosinophils and neutrophils using the following incubation mixture. Eosinophils or neutrophils ($2.5 \times 10^5$) were incubated at 37 °C for 1 h in 250 $\mu$l of HBSS with 1 mM DL-tyrosine, and the addition of 1 $\mu$M PMA, 0–50 $\mu$M sodium nitrite ($NO_2^-$), 0–50 $\mu$M $H_2O_2$, or the combination of these reagents.

To clarify a characteristic of reactive nitrogen species concerning tyrosine nitration from eosinophils, we added one of various antioxidants, 50 $\mu$g/ml superoxide dismutase (SOD), 30 $\mu$g/ml catalase, 1 mM methionine, 50 mM mannitol, 50 mM dimethylsulfoxide (DMSO), 1 mM urate, 1 mM cysteine, 69 mM ethanol, 0.1 mM $\alpha$-tocopherol (in 69 mM ethanol), or 0.1 mM $\gamma$-tocopherol (in 69 mM ethanol), to the incubation mixtures described above. The concentrations of PMA and $NO_2^-$ in the mixture used for the antioxidative study were 1 $\mu$M and 50 $\mu$M, respectively. In some experiments, 0.1 mM peroxynitrite (synthesized from $NO_2^-$ and acidified $H_2O_2$ [31], Dojindo Laboratories, Kumamoto, Japan) was used instead of the combination of PMA and $NO_2^-$.

The incubation was terminated by centrifugation (5,000 rpm) on ULTRAFREE-MC (10 kDa molecular weight cut-off) (Millipore Co., Billerica, MA, USA) to remove proteins. Tyrosine and its nitration products in filtrates were analyzed by high-performance liquid chromatography (HPLC) using a 5-$\mu$m Sperisorb ODS-2RP-18 column, with 93% 50 mM potassium phosphate (pH 3.0), 7% methanol as the mobile phase at 1 ml/min, and UV detection at 274 nm [32]. Identification and quantitation of nitrotyrosine were performed with excess sodium hydrosulfite, which reduces nitrotyrosine to aminotyrosine [33], and by comparison with external standards.

***Gastric mucosal slice preparation and incubation.*** Rat gastric mucosal slices were prepared and incubated by a modification of the method described previously [34]. Briefly, rats were killed by cervical dislocation after a slight ether anesthesia. The stomach was then removed and placed in ice-cold oxygenated (95% $O_2$/5% $CO_2$) Krebs-Henseleit buffer. Gastric mucosal slices of about 0.3-mm in thickness were cut with a microslicer and put into 5-ml vials containing 3 ml of Waymouth's culture medium (pH 7.4) supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 1 $\mu$g insulin/ml, 84 $\mu$g gentamycin/ml, and 25 mM glucose. The vials were closed with caps and gassed with a mixture of 95% $O_2$/5% $CO_2$. The slices were incubated at 37 °C with shaking (80 strokes/min) for 1 h prior to experiments. Then, sections were incubated with or without 1 $\mu$M of PMA and 1 $\mu$M $NO_2^-$ in Krebs-Henseleit buffer at 37 °C for 1 h. The resulting sections were fixed with 10% neutral buffered formalin and then embedded in paraffin, and serial sections were retrieved to investigate the localization of eosinophils and nitrotyrosine.

***Nitric oxide production from eosinophils and neutrophils.*** Nitric oxide (NO) was measured as $NO_2^-$ by the reaction of $NO_2^-$ and 2,3-diaminonaphthalene [35]. Eosinophils or neutrophils ($1 \times 10^6$) were incubated with 0.5 mM L-arginine, 12 $\mu$M S-methylisothiourea, or a combination of each reagent at 37 °C for 3 h in 500 $\mu$l of HBSS. After incubation, cells were centrifuged at 3,000 rpm for 5 min, and then the supernatants were incubated with 50 $\mu$g 2,3-diaminonaphthalene dissolved in 1 ml of 0.62 N hydrochloric acid for 10 min, followed by the addition of 5 ml of 2.8 N sodium hydroxide. Incubation mixtures (100 $\mu$l) were dissolved with 4 ml of distilled water and measured for fluorescence detection (excitation, 365 nm and emission, 450 nm).

***Immunocytochemical study for iNOS.*** The slides of cytocentrifuged eosinophils and neutrophils were fixed with ice-cold methanol for 5 min and incubated with non-immune mouse IgG2a to prevent non-specific binding for 20 min, and then incubated with fluorescein isothiocyanate (FITC)-conjugated mouse anti- iNOS monoclonal antibodies (IgG2a, Transduction Laboratories, Lexington, KY, USA) dilated 25-fold with PBS for 2 h. After 3 times washing with PBS for 5 min, slides were mounted in Immunon™ (Shandon, Pittsburgh, PA, USA) and sealed with clear nail varnish. A control study was performed using FITC-conjugated non-immune IgG2a (DAKO). Fluorescence microscopic observations were analyzed using an Olympus IX-70 equipped with an IX-FLA fluorescence observation attachment.

***Reverse transcription polymerase chain reaction (RT-PCR).*** Total RNA was isolated from eosinophils or neutrophils ($1 \times 10^6$) using an Isogen RNA reagent (Wako), and 0.5 $\mu$g of the resulting RNA was treated with DNase I (Takara, Kyoto, Japan) for 1 h at 37 °C. DNase I was inactivated for 5 min at 90 °C. First-strand cDNA synthe-

sis was performed using 0.5 $\mu$g of each RNA sample, 0.125 $\mu$M random primers, and 0.25 U avian myeloblastosis viral reverse transcriptase (RT) (Takara) in a 20-$\mu$l reaction volume. Contamination with DNA was excluded by performing polymerase chain reaction (PCR) from templates incubated without RT. The PCR primers used were as follows: iNOS (5'-GATCAATAACCTGAACCCG-3', and antisense primer 5'-GCCCTTTTTTGCTCCATAGG-3', 578-bp fragment), glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (5'-ACCACAGTCCATGCCATCAC-3', and antisense primer 5'-TCCACCACCCTGTTGCTGTA-3', 452-bp fragment).

The PCR reaction mixture (100 $\mu$l) contained 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1 $\mu$M primers, and 2.5 U Taq DNA polymerase. Samples were amplified for 30 or 40 cycles for iNOS and 30 cycles for G3PDH by 30 sec denaturation at 94 °C, 30 sec annealing at 56 °C, and 1.5 min elongation at 72 °C in a Takara PCR thermal cycler (Takara). The PCR reactions were then visualized on a 2% agarose gel (Takara) containing 5 $\mu$g/ml of ethidium bromide. Steady-state expression of G3PDH mRNA was used to control for equal product loading.

***Statistical analysis.*** All values are expressed as means ± SD of several experiments. The significance of the difference among groups was analyzed by one-way analysis of variance following *post hoc* multiple-comparisons, and $p < 0.05$ was considered significant.

## Results

***Immunocytochemistry for nitrotyrosine in peritoneal eosinophils and neutrophils.*** The specificity of eosinophils was confirmed by staining with hematoxylin and eosin (Fig. 1A). Incubation of eosinophils with 1 $\mu$M of PMA and 50 $\mu$M of $NO_2^-$ showed prominent immunostaining for nitrotyrosine in a few smaller cells or cells containing a round nucleus (Fig. 1B). Almost all eosinophils changed the morphology to small nuclei within 5 h of being isolated (data not shown). Incubation with 5 $\mu$M $NO_2^-$ and 5 $\mu$M $H_2O_2$ showed diffuse cytoplasmic staining (Fig. 1C), while incubation with 1 $\mu$M $NO_2^-$ and 1 $\mu$M $H_2O_2$ showed focal staining in the site of exocytosis (Fig. 1D, indicated as arrows). Significant disruption of eosinophils was observed following exposure to 1 mM of $NO_2^-$ and 1 mM of $H_2O_2$ (Fig. 1E). Pretreatment of anti-nitrotyrosine antibody with 10 mM of nitrotyrosine abolished the immunostaining for nitrotyrosine by PMA and $NO_2^-$ (data not shown). Incubation of neutrophils with 1–5 $\mu$M $NO_2^-$ and 1–5 $\mu$M $H_2O_2$ did not show immunoreactivity for nitrotyrosine (Fig. 1F).

***Superoxide generation from activated eosinophils and neutrophils.*** Superoxide generation from eosinophils or neutrophils activated by PMA was $60.4 \pm 1.3$ or $57.4 \pm 1.3$ nmol/$1 \times 10^6$/h, respectively.

***Histochemical detection of superoxide generation.*** Superoxide generation from activated eosinophils by PMA was detected using the histochemical NBT reduction method. After incubation of eosinophils with 1 $\mu$M of PMA, blue granules were observed in the eosinophil fraction (Fig. 2A). A phase-contrast study of the same slides revealed that NBT-reducible granules were located in most eosinophils (Fig. 2B). The staining of NBT in eosinophils was not changed by the incubation of cells with 1 $\mu$M PMA and 50 $\mu$M $NO_2^-$.

***Western blot analysis.*** Protein tyrosine nitration in eosinophils and neutrophils was analyzed by SDS-PAGE and Western blotting after incubation of cells with a combination of agents (Fig. 3A). Incubation of eosinophils with PMA and 50 $\mu$M $NO_2^-$ or 50 $\mu$M $NO_2^-$ and 50 $\mu$M $H_2O_2$ showed many nitrated proteins (lane 3, lane 7). The staining intensity of nitrated protein bands was augmented by SOD (lane 5) and diminished by catalase (lane 6). Bovine serum albumin in the incubation of eosinophils with PMA and 50 $\mu$M $NO_2^-$ did not show the staining band for nitrotyrosine (lane 8). Neutrophils incubated with PMA and 50 $\mu$M $NO_2^-$ or 50 $\mu$M $NO_2^-$ and 50 $\mu$M $H_2O_2$ did not show marked bands of nitration proteins (lane 9, lane 10). The nitrated proteins were abolished by pretreatment of antibodies with 3-nitrotyrosine (Fig. 3B).

***Free tyrosine nitration.*** We investigated free tyrosine nitration by eosinophils or neutrophils by incubating cells with a combination of PMA and various concentrations of $NO_2^-$. Nitration of free tyrosine by eosinophils was 3.5-, 4.8-, and 7.3-fold higher than that by neutrophils when these cells were incubated with PMA and 5 $\mu$M $NO_2^-$, PMA and 50 $\mu$M $NO_2^-$, or 50 $\mu$M $NO_2^-$ and 50 $\mu$M $H_2O_2$,

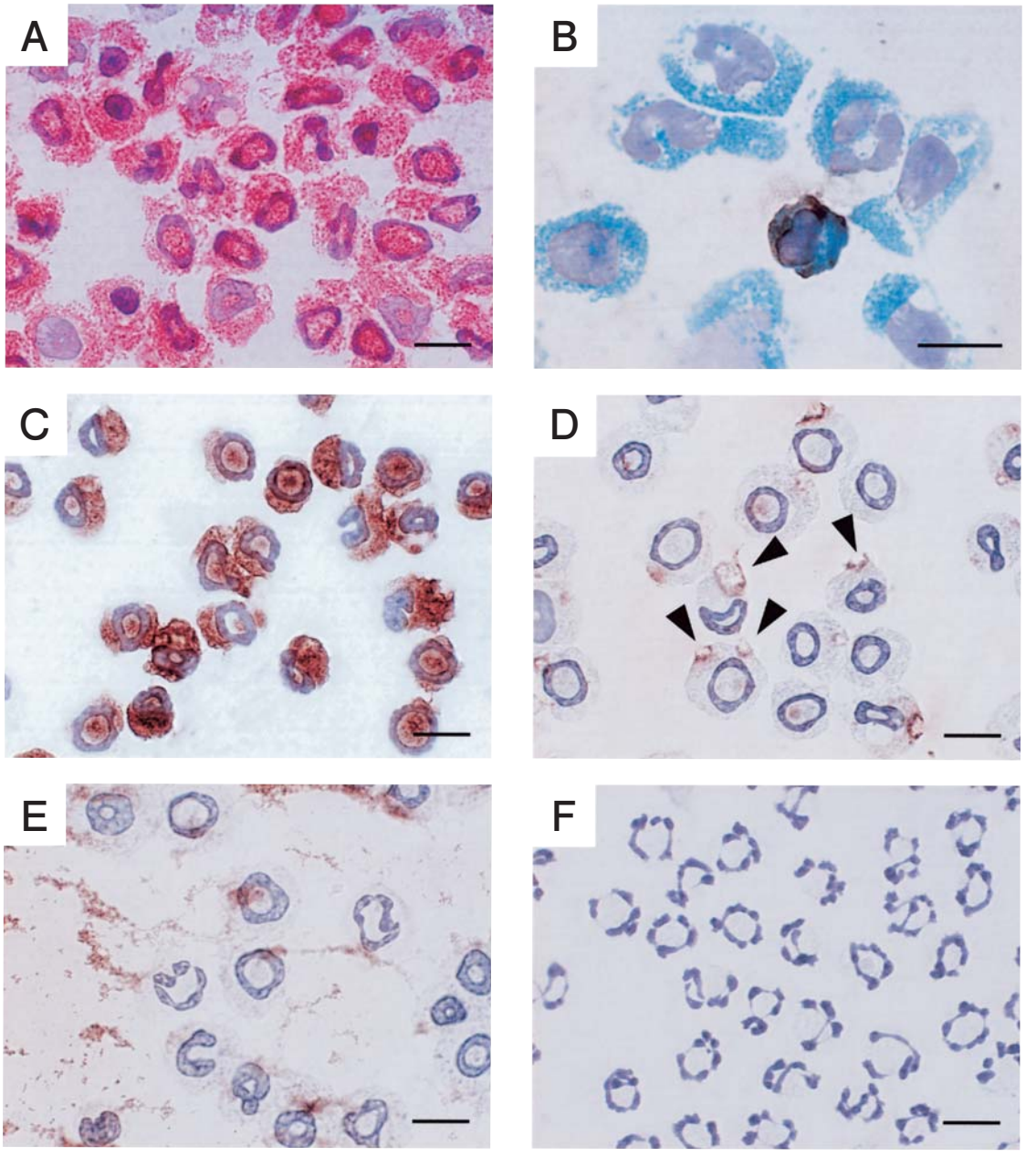


**Fig. 1** Histocytochemical purity of rat peritoneal eosinophils and immunocytochemistry of eosinophils for nitrotyrosine. The purity of peritoneal eosinophils is demonstrated with hematoxylin and eosin staining (**A**) and Luxol-fast blue staining (**B**). Staining for nitrotyrosine was detected in small-sized eosinophils incubated with 1 $\mu$M PMA and 50 $\mu$M $NO_2^-$ (**B**). Incubation of fixed eosinophils with 5 $\mu$M $H_2O_2$ and 5 $\mu$M $NO_2^-$ shows immunostaining of the entire cytoplasm of almost all eosinophils (**C**). When the slide was incubated with 1 $\mu$M $H_2O_2$ and 1 $\mu$M $NO_2^-$ after fixation with neutral-buffered formalin, focal immunoperoxidase staining was detected in the marginal space of almost all eosinophils (**D**). Eosinophils were damaged by a higher concentration (1 mM) of $H_2O_2$ and $NO_2^-$ (**E**). Under this condition, almost no neutrophils were stained (**F**). Bars indicate 10 $\mu$m.

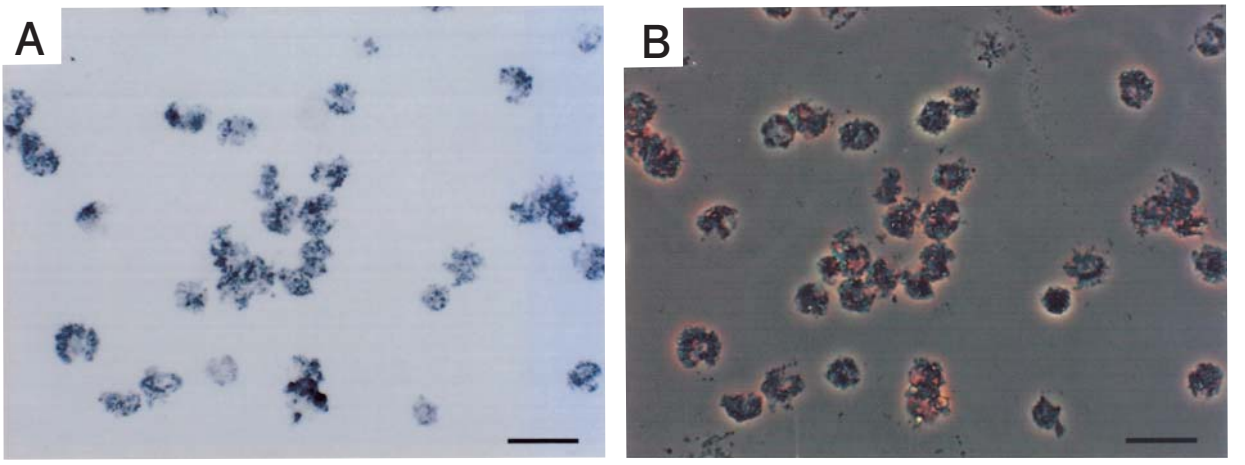


Fig. 2 Histocytochemical detection of superoxide generation by nitroblue tetrazolium (NBT) in activated rat peritoneal eosinophils. Eosinophils were incubated with 1 $\mu$M of PMA (**A**). A phase contrast of the same slide (**B**).

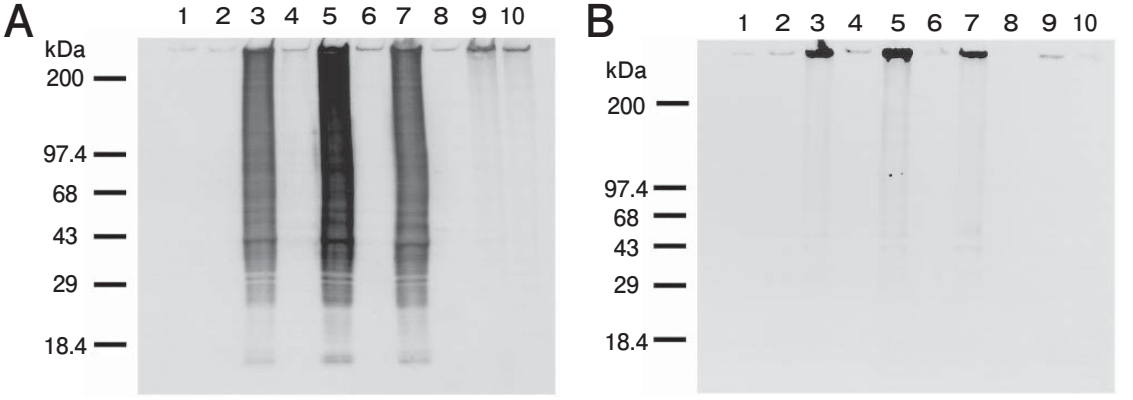


Fig. 3 Immunoblot analysis of protein nitration by rat peritoneal eosinophils and neutrophils. Samples of eosinophils are shown in lanes 1–8, and those of neutrophils are shown in lanes 9–10. Lane 1, cell (eosinophils); lane 2, cell + 1 $\mu$M PMA; lane 3, cell + 1 $\mu$M PMA + 50 $\mu$M $NO_2^-$; lane 4, cell + 50 $\mu$M $NO_2^-$; lane 5, cell + 1 $\mu$M PMA + 50 $\mu$M $NO_2^-$ + 50 $\mu$g/ml SOD; lane 6, cell + 1 $\mu$M PMA + 50 $\mu$M $NO_2^-$ + 30 $\mu$g/ml catalase; lane 7, cell + 50 $\mu$M $H_2O_2$ + 50 $\mu$M $NO_2^-$; lane 8, supernatant of cell + 1 $\mu$M PMA + 50 $\mu$M $NO_2^-$ + 1 mg/ml bovine serum albumin; lane 9; cell (neutrophils) + 1 $\mu$M PMA + 50 $\mu$M $NO_2^-$; lane 10, cell + 50 $\mu$M $H_2O_2$ + 50 $\mu$M $NO_2^-$. Tyrosine-nitrated proteins immunostained with anti-nitrotyrosine antibodies (**A**) and immunostained with anti-nitrotyrosine antibodies preincubated with 10 mM 3-nitrotyrosine for 2 h (**B**).

respectively (Table 1). Nitration of free tyrosine by eosinophils after incubation with PMA and 50 $\mu$M $NO_2^-$ was significantly augmented by SOD, methionine, and mannitol but inhibited by catalase, DMSO, uric acid, cysteine, $\alpha$-tocopherol, and $\gamma$-tocopherol. Tyrosine nitration by $ONOO^-$ was augmented by SOD but inhibited by methionine, mannitol, DMSO, uric acid, cysteine, and ethanol (Fig. 4).

***Nitrotyrosine formation in eosinophils in gastric mucosa.*** Immunostaining for nitrotyrosine was seen on infiltrating multinucleate cells (Fig. 5A), while the control study using non-immune rabbit IgG showed negative staining for nitrotyrosine (Fig. 5B). Counterstaining with LFB revealed concordance between the immunoreactive cells and LFB-positive cells (Fig. 5C). Pre-treatment of antibodies with 3-nitrotyrosine abolished the immunoreactivity (Fig. 5D).

Serial sections were recruited to investigate the status and localization of eosinophils further.

**Table 1** Free-tyrosine nitration by rat eosinophils and neutrophils

| Chemicals | Nitrotyrosine ($\mu$M) | |
|---|---|---|
| | Eosinophils | Neutrophils |
| Control | ND | ND |
| PMA | ND | ND |
| PMA + 1 $\mu$M $NO_2^-$ | ND | ND |
| PMA + 5 $\mu$M $NO_2^-$ | 0.25 ± 0.08 ** | 0.06 ± 0.02 |
| PMA + 50 $\mu$M $NO_2^-$ | 1.15 ± 0.10 ** | 0.24 ± 0.01 |
| 1 $\mu$M $H_2O_2$ + 1 $\mu$M $NO_2^-$ | 0.03 ± 0.03 | ND |
| 5 $\mu$M $H_2O_2$ + 5 $\mu$M $NO_2^-$ | 0.10 ± 0.03 | ND |
| 50 $\mu$M $H_2O_2$ + 50 $\mu$M $NO_2^-$ | 2.18 ± 0.12 ** | 0.46 ± 0.02 |
| 50 $\mu$M $H_2O_2$ | ND | ND |
| 50 $\mu$M $NO_2^-$ | ND | ND |

Eosinophils or neutrophils ($2.5 \times 10^5$ cells/250 $\mu$l) were incubated in HBSS at 37 °C with 1 mM DL-tyrosine, with the addition of 1 $\mu$M PMA, 1–50 $\mu$M $NO_2^-$, 1–50 $\mu$M $H_2O_2$ or a combination of these reagents. After incubation for 1 h, the reactions were stopped by pelleting the cells. Concentrations of nitrated tyrosine were determined by HPLC as described in Materials and Methods. Values are expressed as the mean ± SD of 5 experiments. ND = not detected. **$p < 0.01$ versus neutrophils (unpaired t-test).

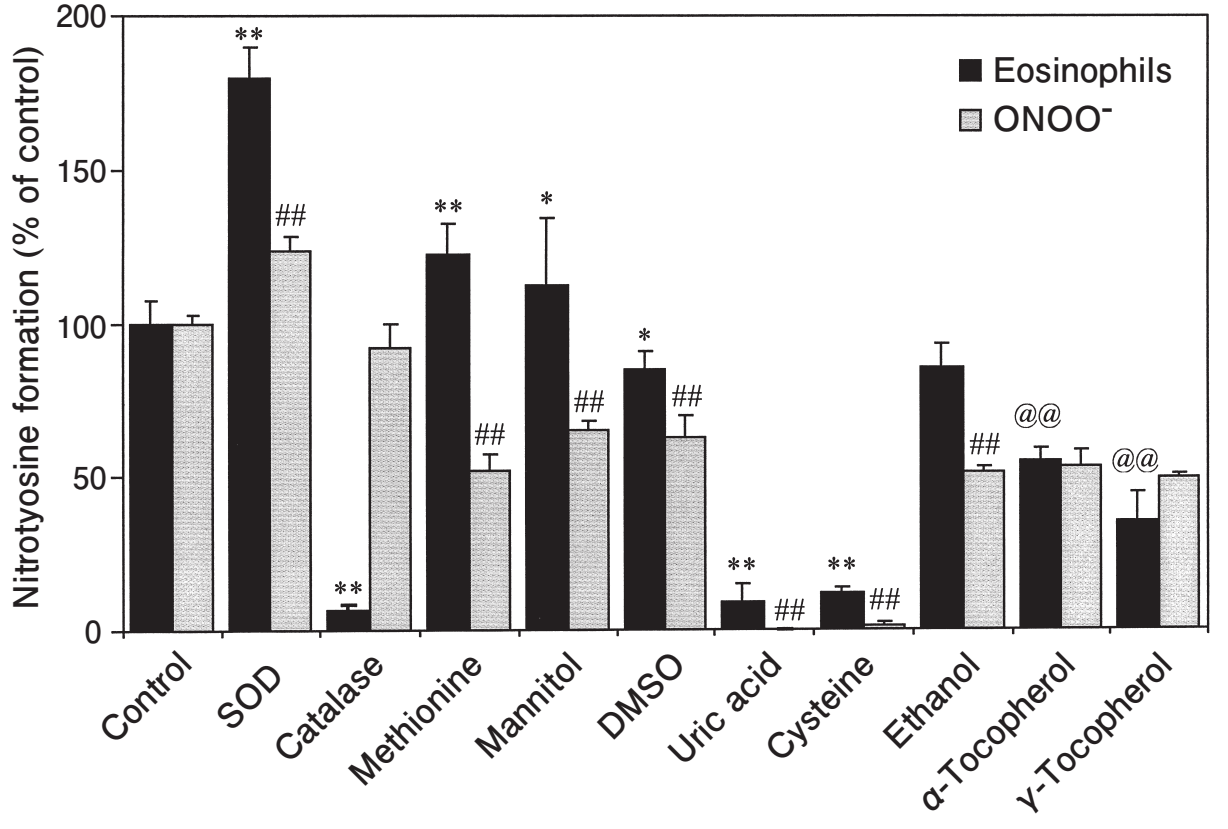


**Fig. 4** Effect of various scavengers on tyrosine nitration by rat peritoneal eosinophils and peroxynitrite. SOD (50 $\mu$g/ml), 30 $\mu$g/ml catalase, 1 mM methionine, 50 mM mannitol, 50 mM DMSO, 1 mM urate, 1 mM cysteine, 69 mM ethanol, 0.1 mM $\alpha$-tocopherol (in 69 mM ethanol), and 0.1 mM $\gamma$-tocopherol (in 69 mM ethanol) were added to the incubation mixtures containing eosinophils ($2.5 \times 10^5$), 1 mM DL-tyrosine, 1 $\mu$M PMA, and 50 $\mu$M $NO_2^-$ in 250 $\mu$l of HBSS or 0.1 mM $ONOO^-$ and 1 mM DL-tyrosine in 250 $\mu$l of HBSS. Each value represents the mean ± SD of 5 determinations. **$p < 0.01$, *$p < 0.05$ versus eosinophil control; ##$p < 0.01$ versus $ONOO^-$ control; @@$p < 0.01$ versus ethanol control.

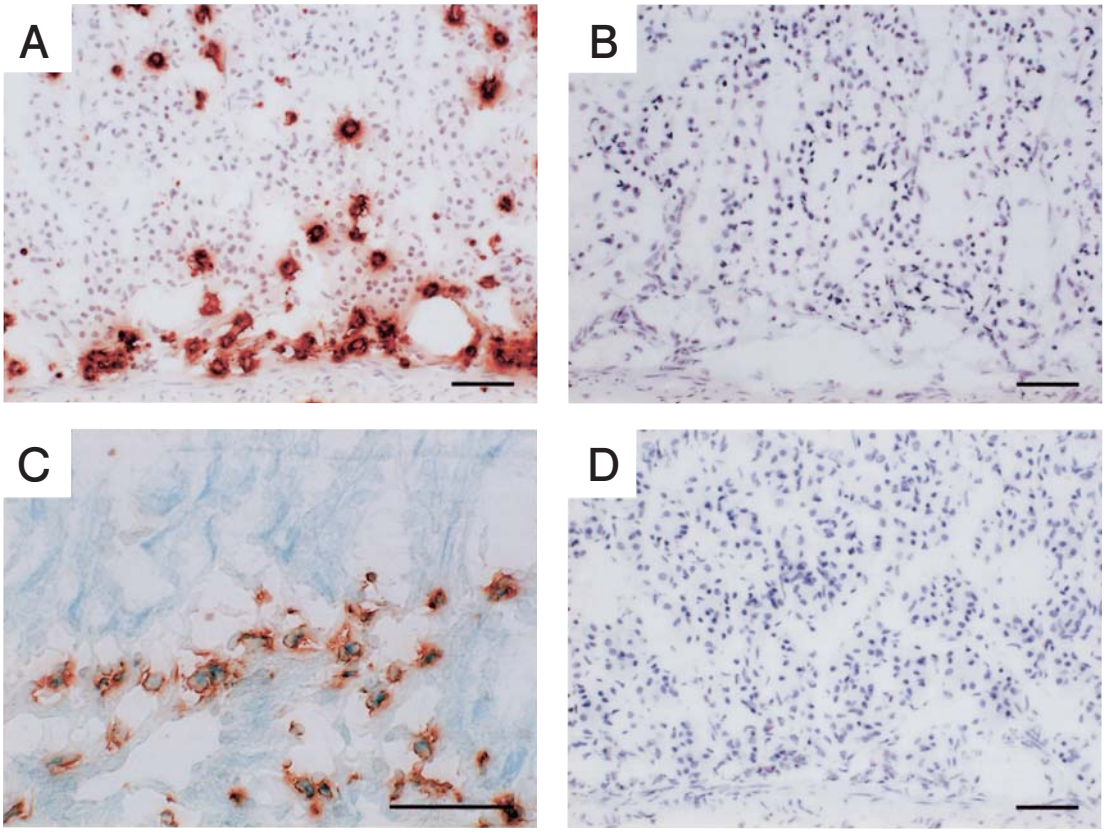


**Fig. 5** The presence of eosinophils and immunohistochemistry for nitrotyrosine in rat gastric wall. Gastric wall was incubated with 1 $\mu$M of $H_2O_2$ and 1 $\mu$M of PMA at 37 °C for 1 h. Paraffin-embedded sections were immunostained for nitrotyrosine. We used 10 mM of nitrotyrosine to block the reaction of anti-nitrotyrosine antibody (**D**). Counterstaining was held with hematoxylin (**A**, **B**, **D**) or LFB (**C**). Bars indicate 50 $\mu$m.

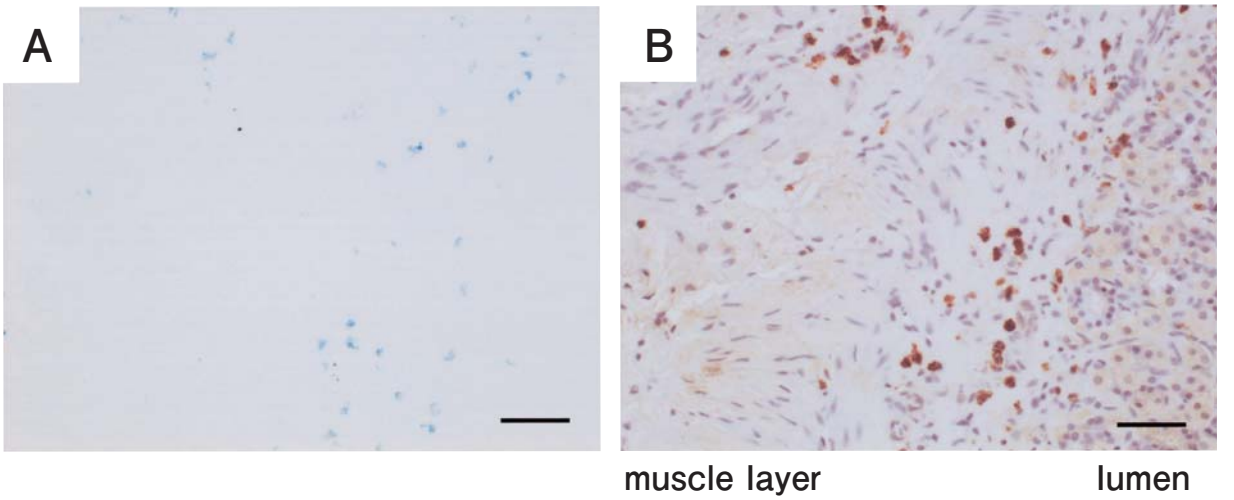


**Fig. 6** Co-localization between nitrotyrosine and eosinophil granules in rat gastric wall. Gastric wall slices were incubated with 1 $\mu$M of $H_2O_2$ and 1 $\mu$M of PMA at 37 °C for 1 h before being embedded in paraffin. Serial sections were stained with LFB (**A**), or immunostained for nitrotyrosine with hematoxylin counterstaining (**B**). Bars indicate 50 $\mu$m.

Staining with LFB revealed that eosinophils were localized in lamina muscularis mucosa in the gastric mucosa (Fig. 6A). Almost all of the eosinophils were 3-nitrotyrosine-positive (Fig. 6B).

***Nitric oxide production from eosinophils and neutrophils.*** The release of NO from neutrophils was elevated by the addition of L-arginine and suppressed by an inhibitor of iNOS, S-methylisothiourea. The basal level of NO produced from eosinophils was not changed by the addition of L-arginine and S-methylisothiourea (Fig. 7).

***Immunocytochemical detection of iNOS.*** Eosinophils did not show immunofluorescence for iNOS (Fig. 8A), but neutrophils and contaminating mononuclear cells did show such immunofluorescence (Fig. 8B). FITC-conjugated non-immune mouse IgG2a did not show the specific fluorescence for nitrotyrosine.

***Expression of iNOS mRNA in eosinophils and neutrophils.*** We evaluated the iNOS mRNA expression in rat eosinophils. iNOS mRNA expression in eosinophils was not detected by 30 PCR cycles, but it was faintly detectable by 40 PCR cycles. iNOS mRNA in neutrophils was detected by 30 PCR cycles (Fig. 9).

## Disscussion

Eosinophils are considered to be an important source of protein nitration [11], but details of eosinophils involving nitration have not been investigated. In the present study we demonstrated the nitration of protein-bound or free tyrosine by the peroxidase system of eosinophils. We found a marked difference in the immunocytochemical appearance of nitrotyrosine between eosinophils incubated with PMA in the presence of $NO_2^-$ and eosinophils incubated with $NO_2^-$ and $H_2O_2$. The NBT test for activated eosinophils showed that almost all eosinophils generated $O_2^{\cdot -}$ by PMA. Therefore, theoretically, $O_2^{\cdot -}$ generated in the outside of eosinophils should be converted to $H_2O_2$. Hydrogen peroxide easily penetrates the cell wall and acts on eosinophil peroxidase to produce immunostainable nitrotyrosine in the presence of 50 $\mu$M $NO_2^-$. Incubation with $H_2O_2$ and $NO_2^-$ indicated that all eosinophils possess the capacity for nitration (Fig. 1C, D). However, it seems only eosinophils with a small round nucleus (Fig. 1B) contributed to tyrosine nitration when stimulated with PMA and 50 $\mu$M $NO_2^-$.

Generally, eosinophils collected from blood easily fall into apoptosis [36]. We observed that almost all eosinophils had small nuclei within 5 h of being isolated (data not shown). Thus, small eosinophils, probably apoptotic-like cells, and eosinophilic granules released from these cells are concerned in local nitration of eosinophils. Since most intact eosinophils were not concerned in nitration, we can say that tyrosine nitration couldn't be raised without apoptotic-like cells in peritoneal fluid. Further investigation is needed to clarify whether small nuclei cells are apoptotic.

Why were the intact cells prevented from nitration? The higher extracellular excretion potential of $NO_2^-$ and enzymatic activity of catalase in intact cells might be a cause. Further investigation to elucidate the nitration mechanism by peripheral eosinophils will be important in clarifying the cause.

Cytocentrifuged eosinophils showed immunostaining for nitrotyrosine when they were incubated with 1 $\mu$M $H_2O_2$ and 1 $\mu$M $NO_2^-$ (Fig. 1D), implying that fixation before the stimulation may cause the disruption of the cytoplasmic membrane and lead to remarkable nitration. Moreover, freezing for storage and

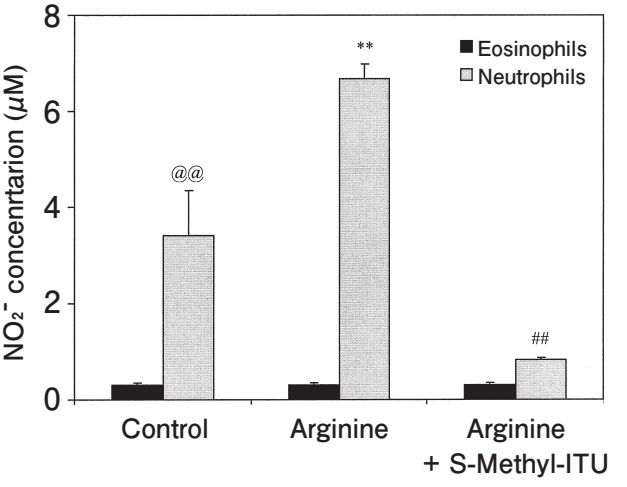


Fig. 7 Nitric oxide (NO) production from rat peritoneal eosinophils and neutrophils. NO production from eosinophils and neutrophils was measured after incubation with 0.5 mM L-arginine or 0.5 mM L-arginine and 12 $\mu$M S-methylisothiourea (S-Methyl-ITU). Each value represents the mean ± SD of 5 determinations. @@$p < 0.01$ versus eosinophil control, **$p < 0.01$ versus neutrophil control, and ##$p < 0.01$ versus arginine.

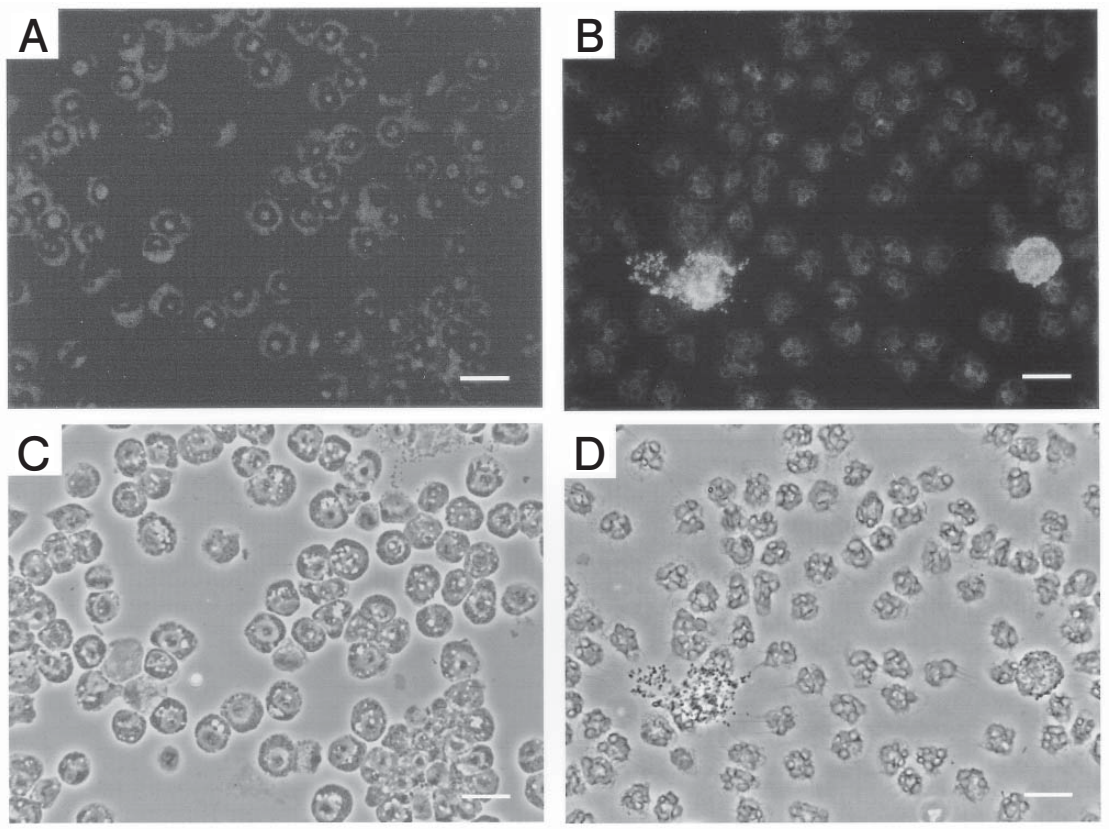


Fig. 8 Immunofluorescence for iNOS in rat peritoneal eosinophils and neutrophils. Immunofluorescence for iNOS was not detected in eosinophils (A). A phase contrast of the slide shown in (A) (C). Immunofluorescence was detected in neutrophils and contaminating mononuclear cells (B). A phase contrast of the same slide shown in (B) (D).

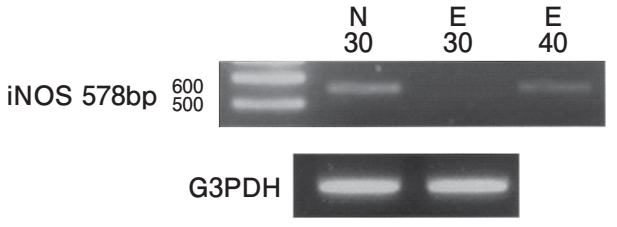


Fig. 9 Expression of iNOS mRNA in rat peritoneal eosinophils (E) and neutrophils (N). "30" or "40" indicate the PCR cycles run.

drying after cytocentrifugation may inactivate catalase and other enzymes that suppress nitration.

We also investigated the status of constituent eosinophils in the stomach and found many eosinophils existed in lamina muscularis mucosa, which was consistent with a previous report [37]. More importantly, very high potency of nitration was observed in almost all cells. This suggests that the characteristics of eosinophils in gastric mucosa are different from those eluted in the peritoneal cavity. Eosinophils in the tissue are resistant to apoptosis, and interleukin-5 may be associated with this phenomenon [38]. However, the mechanisms of expression of nitration capacity without apoptosis are not yet known.

It is unclear what reactive nitrogen species contribute to the nitration of tyrosine by EPO-catalyzed oxidation of $NO_2^-$. There is no direct evidence that a potential nitrogen species contributes to tyrosine nitration by peroxidase-catalyzed oxidation of $NO_2^-$. However, 2 reactive nitrogen species from the results of MPO are postulated. One is nitrogen dioxide ($NO_2$) formed by one-electron oxidation [20], and the other is peroxynitrous acid (ONOOH) formed by two-electron oxidation [39]. The difference in the inhibition of free-tyrosine nitration by both EPO-catalyzed oxidation of $NO_2^-$ and $ONOO^-$ with catalase, methionine, mannitol, ethanol, $\alpha$-tocopherol,

and $\gamma$-tocopherol suggests that reactive nitrogen species contributing to the nitration of tyrosine in EPO-catalyzed oxidation of $NO_2^-$ may be different from $ONOO^-$ (Fig. 4). The inhibitory effect of catalase and the augmented effect of SOD (Fig. 3) on the generation of nitrotyrosine support this idea. $\alpha$-Tocopherol and $\gamma$-tocopherol reacted with $ONOO^-$ to become $\alpha$-tocopheryl quinone and 5-nitro-$\gamma$-tocopherol, respectively [40]. SOD1/$H_2O_2$-dependent oxidation of $NO_2^-$ depleted $\alpha$- and $\gamma$-tocopherol [41]. NO inhibition of $\alpha$- and $\gamma$-tocopherol in $ONOO^-$-dependent tyrosine nitration may have contributed to the high reaction rate constants of ethanol with hydroxyl radical-like oxidants from $ONOO^-$ [42] because ethanol was used as a solvent of $\alpha$- and $\gamma$- tocopherol. However, although oxidation and nitration products from the reaction of $\alpha$- and $\gamma$-tocopherol with reactive nitrogen species from EPO-catalyzed oxidation of $NO_2^-$ were not presented, tyrosine-nitrating species from EPO-catalyzed oxidation of $NO_2^-$ may be similar to that from SOD1/$H_2O_2$-dependent oxidation of $NO_2^-$ [41].

A previous report indicated that the tyrosine-nitrating capacity of porcine EPO exceeded that of MPO by at least 4-fold at physiological concentrations of halides, and every concentration of $NO_2^-$ examined [23]. In this study, similar results were obtained in the difference of the tyrosine-nitrating capacity between rat eosinophils and neutrophils activated by PMA and $NO_2^-$. The enzymological difference in the capacity of tyrosine nitration between EPO and MPO and the difference in the total contents of EPO in eosinophils and MPO in neutrophils may have contributed to the difference in tyrosine-nitrating activity between eosinophils and neutrophils [23, 43, 44].

Previous reports have described another nitrogen species generated from the reaction of $O_2^-$ with $NO_2$, namely peroxynitrate ($O_2NOO^-$) (Fig. 10) [45, 46]. $O_2NOO^-$ is reported to be unreactive to tyrosine and to act as an inactivator of $NO_2$ [47], and it theoretically forms nitrite and singlet oxygen ($^1O_2$) [48]. A recent report suggests that singlet oxygen is capable of forming nitrotyrosine under photosensitization [49]. In this study, we examined free tyrosine nitration by exposing eosinophils to PMA and $H_2O_2$. By our calculation, $10^6$ eosinophils can produce 30.2 nmol/ml/hr of $H_2O_2$ when stimulated with 1 $\mu$M PMA. The capacity for tyrosine nitration of eosinophils stimulated with 1 $\mu$M PMA (= 30.2 $\mu$M $H_2O_2$) and 50 $\mu$M $NO_2^-$ was proportional to that of 50 $\mu$M $H_2O_2$ and 50 $\mu$M $NO_2^-$, as indicated in Table 1. Therefore, the inhibitory effect of $O_2NOO^-$ on tyrosine nitration may be very limited, but such an effect cannot be ruled out because we did not investigate the formation of $O_2NOO^-$ and singlet oxygen in this study. Further investigation is needed to reveal the contribution of $O_2NOO^-$ toward nitration.

In conclusion, the present study demonstrated that smaller, possibly apoptotic eosinophils have a potent capacity to generate reactive nitrogen species

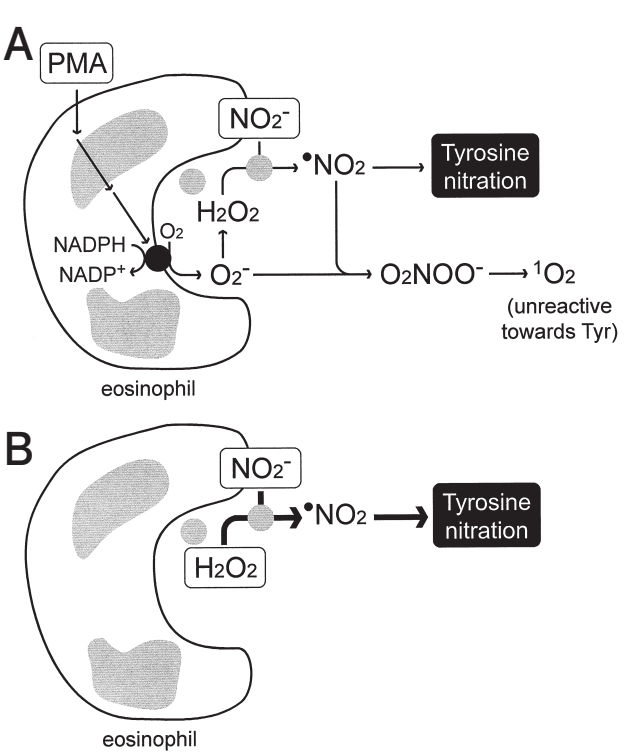


Fig. 10 Mechanisms of different levels of nitration efficiency. When exposed to PMA and $NO_2^-$, NADPH oxidase (closed circle) is activated by PMA stimulation to form $O_2^{\cdot-}$. Then, formation of $NO_2$ was catalyzed by secreted EPO (gray circle) with external $NO_2^-$ and $H_2O_2$ generated from $O_2^{\cdot-}$. Tyrosine nitration is facilitated by $NO_2$. However, PMA stimulates NADPH oxidase continuously; $O_2NOO^-$ can be formed from existing $NO_2$ and $O_2^{\cdot-}$, and it rapidly degrades to singlet oxygen molecule ($^1O_2$). This molecule is not reactive with tyrosine residue, and the formation of nitrotyrosine was suppressed as a result (**A**). However, when exposed to nitrite and $H_2O_2$, the amount of $NO_2$ production catalyzed by EPO would increase, because $H_2O_2$ is not supplied via $O_2^{\cdot-}$ and then the formation of $O_2NOO^-$ is lacking (**B**).

responsible for free or protein-bound tyrosine nitration via a peroxidase-catalyzed system. Moreover, we found a difference in the nitration capacity of eosinophils between eosinophils in the gastric mucosa and those in the peritoneal cavity. However, the mechanisms of heterogeneity in nitration are unknown and should be clarified in future studies.

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