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Myeloperoxidase has Directly-opposed Effects on Nitration Reaction—Study on Myeloperoxidase-deficient Patient and Myeloperoxidase-knockout Mice

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Myeloperoxidase (MPO) catalyzes a nitration reaction to form nitrotyrosine in the presence of high nitrite, the metabolite of NO. Human leukocyte was shown to cause phenolic nitration using released MPO as a catalyst in the presence of nitrite. It opposes our previous finding that inhibition of MPO was essential for phenol nitration in human leukocyte study. To clarify the role of MPO, we utilized MPO-deficient human leukocytes and MPO-knockout mice. Even in the absence of exogenously added nitrite, high nitration product was observed in MPO-deficient leukocytes. In liver subjected to ischemia/reperfusion injury, a significantly higher amount of nitrotyrosine was produced in MPO-knockout mice than in normal mice. These results clearly demonstrate that MPO inhibits the accumulation of nitration products *in vivo*. Further experiments showed that MPO could degrade nitrotyrosine in the presence of glutathione. Thus, MPO-induced degradation of nitration products may cause the underestimation of the nitration product generated *in vivo*. We conclude that MPO may act predominantly to scavenge nitrotyrosine under physiological nitrite condition, and protect against injurious effect of nitrotyrosine.

Keywords: MPO; Nitrotyrosine; Nitric oxide; Peroxynitrite; Neutrophil; Alanine aminotransferase

Abbreviations: ABAH, 4-aminobenzoic acid hydrazide; ALT, alanine aminotransferase; BSA, bovine serum albumin; HPA, 4-hydroxyphenylacetic acid; HRP, horseradish peroxidase; MPO, myeloperoxidase; NOC7, $CH_3N[N(O)NO](CH_2)_3NH_2^+CH_3$; NO_2HPA, 3-nitro-4-hydroxyphenylacetic acid; PMA, phorbol-12-myristate-13-acetate; SOD, superoxide dismutase

INTRODUCTION

Myeloperoxidase (MPO) is an enzyme abundant in neutrophils, and it plays an important role in neutrophil-dependent host defense.[1–3] When neutrophils are activated, they release MPO and produce superoxide ($O_2^{\bullet -}$) which immediately dismutates to hydrogen peroxide (H_2O_2). Then MPO catalyzes the reaction of H_2O_2 and chloride (Cl^-) to form a potent oxidant, hypochlorous acid (HOCl).[4,5] In addition to generating HOCl, MPO can utilize nitrite (NO_2^-), an end product of nitric oxide (NO), to catalyze the nitration reaction of phenols, including tyrosine, via the MPO/H_2O_2/NO_2 system.[6]

Interest in the nitration reaction in biology was initiated by the discovery that peroxynitrite, a reaction product of $O_2^{\bullet -}$ and NO, is a potent nitrating molecule and forms nitrotyrosine.[7,8] Since then, evidence supporting an involvement of peroxynitrite in various pathological conditions has rapidly accumulated, based on studies using nitrotyrosine as a footprint of peroxynitrite formation *in vivo*.[9–15] A role of MPO in nitrotyrosine formation was subsequently recognized.[16–20] Furthermore, it was shown that MPO is an essential player in the human polymorphonuclear

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leukocytes-induced nitration.[21] This was in conflict with our previous study,[22] in which we found that nitration of phenol in human leukocytes occurred only in the presence of an MPO inhibitor. We proposed the existence of a peroxynitrite-mediated nitration pathway in leukocytes, and MPO-driven interference with the nitration. The experimental methods used to examine the nitration reaction were identical in the two studies except for one point: Eiserich supplemented NO_2^-, up to 50 μM on the assumption that a high NO flux would be present in inflammatory foci when leukocytes accumulated. Our study was performed without additional NO_2^-, since leukocytes can generate NO simultaneously with $O_2^{\bullet -}$ upon activation.[23–26] Inspection of the literature revealed that all previous experiments that examined the role of MPO in nitration reaction had been performed in the presence of high NO_2^-.[27–29] This is a critical difference because the *in vivo* concentration of NO_2^- may not reach as high as the level of previous experiments (2–50 μM) and the peroxynitrite-mediated pathway is likely to predominate over the MPO/H_2O_2/NO_2^- system pathway.

Thus, it is very important to resolve the discrepancy concerning the role of MPO in leukocyte-catalyzed nitration, from both mechanistic and pathological points of view. This is because nitration of tyrosine residue of functionally crucial proteins, such as ribonucleotide reductase, cytochrome *c* and c-SRC, interferes with cellular and organ function[30–34] and identification of the predominant nitration pathway *in vivo* would allow the development of preventive and therapeutic strategies.

To investigate the role of MPO in the nitration reaction by leukocytes *in vitro* and in nitrotyrosine formation *in vivo*, we firstly examined leukocytes obtained from a patient with complete MPO deficiency [MPO(−)]. It was found that MPO(−) leukocytes were much more efficient than non-MPO(−) leukocytes in generating nitrated products. Secondly, we examined possible mechanisms by which MPO might interfere with the nitration. Thirdly, we examined nitrotyrosine formation in the liver of MPO-knockout ($MPO^{-/-}$) mice subjected to ischemia-reperfusion injury. We found that nitrotyrosine formation was higher in $MPO^{-/-}$ mice than in $MPO^{+/+}$ mice, and liver injury was also more severe in the $MPO^{-/-}$ mice than in the $MPO^{+/+}$ mice, supporting an association between the flux of nitrotyrosine and the extent of injury.

MATERIALS AND METHODS

Profile of the MPO-deficient Patient

The individual was a 39-year-old healthy man with no evidence of infectious disease. Diagnosis of complete MPO-deficiency was incidentally made when his differential leukocyte count was performed using the Technicon H (Bayer-Sankyo Company, Tokyo), since this method is to obtain differential leukocyte counts based on peroxidase activity. The prevalence of complete MPO deficiency in the Japanese population was reported to be one in 57,000.[35] The patient and his mother, who had partial MPO deficiency, gave informed consent for the study. Venous blood was obtained in a syringe with EDTA and automated flow cytometric analysis of peroxidase-stained samples was performed.

Isolation of Leukocytes and Measurement of Peroxidase Activity

Leukocytes from normal volunteers and the patient were isolated from heparinized venous blood by gradient centrifugation (400*g* for 30 min) using Mono-Poly resolving medium (Dainippon Pharmaceutical Company Ltd., Japan) at room temperature. The final cell suspension contained more than 95% leukocytes and was >95% viable as examined by trypan blue exclusion test. Peroxidase activity of polymorphoneucler cells was assessed spectrophotometrically based on oxidation of tetramethylbenzidine in the presence of H_2O_2, as reported elsewhere.[36]

Measurements of Superoxide, NO_2^- and NO_3^- Production by Leukocytes

Superoxide production was measured in terms of the SOD-inhibitable portion of cytochrome *c* reduction.[37] Total NO_2^- and NO_3^- production by leukocytes was measured by use of the Griess reaction in a flow-injection system with a Cd/Cu column for the reduction of NO_3^- to NO_2^-.[38]

Experimental Protocol of Nitration Reaction by Leukocytes

Nitration reaction by leukocytes obtained from the MPO(−) patient and normal volunteers [MPO(+)] was evaluated using 4-hydroxyphenylacetic acid (HPA) as a chemical probe.[22,39] Leukocytes (2×10^6 cells/ml), HPA (1 mM), and Cu, Zn-superoxide dismutase (SOD, 0.1 mg/ml) in Hanks' balanced salt solution buffered with 40 mM HEPES (HBSS-HEPES, pH 7.4) was placed on a 24-well plate at 37°C. SOD (0.1 mg/ml) is an established catalyst for the phenol nitration by peroxynitrite and does not inhibit peroxynitrite formation from fast reaction between NO and superoxide.[8,39] Activation of leukocytes was started by the addition of phorbol-12-myristate-13-acetate (PMA, 1 μM). After 3 h incubation, supernatants were collected through an Ultrafree C3 LBC00 filter (Millipore). They were acidified with 10% H_3PO_4 and 20% acetonitrile

(final concentration) was added immediately prior to analysis. HPA, nitrated HPA (NO_2HPA) in the supernatants (20 μl each) were measured using a 4.6 mmϕ × 150 mm C_{18} reverse-phase column and 20/80% acetonitrile/10% H_3PO_4 (pH 3.2) (v/v) as the mobile phase with an HPLC-UV detector system consisting of two pumps (PU-980, JASCO), a UV/visible absorbance detector (UV-970, JASCO), and an 807IT integrator (JASCO, Japan). The column was eluted with a linear gradient of 20–60% acetonitrile over 10 min at a flow rate of 1 ml/min. To confirm that leukocytes use endogenously produced NO for the nitration, leukocytes were pretreated with 100 μM L-monomethyl-L-arginine 30 min before PMA activation. 4-Amminobenzoic acid hydrazide (ABAH, 100 μM) was also added to inhibit MPO and catalase was added to eliminate the effect of H_2O_2 in some experiments. The effect of NO_2^- on NO_2HPA formation was evaluated by adding 50 μM NO_2^- in some experiments, as it was shown that NO_2^- increases nitration dose-dependently.[40]

Studies to Clarify the Mechanism Underlying MPO-driven Interference with the Nitration

Possible reasons why MPO interferes with nitration are either inhibition of the nitration reaction or enhancement of degradation of the nitrated product, or both. Thus, we first examined the effect of MPO on peroxynitrite-mediated nitration. Peroxynitrite was synthesized by means of a quenched-flow method and the concentration was determined based on a value of $\varepsilon_{302} = 1670\,M^{-1}\,cm^{-1}$.[41] Peroxynitrite (1 mM) was added dropwise to 1 mM HPA solution containing MPO (1 μM) and/or H_2O_2 (100 μM) if necessary in HBSS-HEPES with vigorous stirring. Aqueous NaOH (5 N, 1/50 volume) was added to the resulting solution and NO_2HPA was determined at 430 nm ($\varepsilon = 4.58\,mM^{-1}\,cm^{-1}$).

Next, we examined the effect of MPO on the nitration under conditions allowing coproduction of NO and superoxide, which yields peroxynitrite. Xanthine oxidase (Boehringer) and NONOate NOC7 ($CH_3N[N(O)NO](CH_2)_3NH_2^+CH_3$, Dojin, Japan) were used as superoxide and NO sources, respectively.[42] L-Tyrosine (0.1 mM) and various amount of MPO were incubated at 25°C for 5 min in 0.2 ml of 0.1 ml of 0.1 M sodium phosphate buffer with 0.1 mM diethylenetriaminepentaacetic acid, pH 7.0, in the presence of NOC7 (10 μM), pterin (50 μM), and xanthine oxidase (40 mU/ml). The reaction was terminated by the addition of allopurinol (0.2 mM), an inhibitor of xanthine oxidase to the reaction mixture. Resulting 3-nitrotyrosine was analyzed by HPLC with a reverse-phase column (TSKgel ODS-80TS, 4.6 × 250 mm, Tosoh Company Ltd.) equipped with an electrochemical detector (12 channels, CoulArray, ESA, Incorporation, Chelmsfold, MA).[42] The sample was applied to the column eluted with 50 mM acetate buffer (pH 4.7) containing 5% methanol at a flow rate of 0.8 ml/min to separate 3-nitrotyrosine. The amount of 3-nitrotyrosine was quantified from data obtained at a channel with +750 mV potential.

Then, we examined whether MPO enhances the degradation of nitrated products. The effect of horseradish peroxidase (HRP), as another representative peroxidase, was also examined. The solution of 3-nitrotyrosine (5 μM) and MPO or HRP (2 or 10 μM) in 20 mM sodium phosphate buffer (pH 7.4) was incubated for 1–3 h at 37°C. Glutathione (10 mM) and peroxidase inhibitor (NaN_3 1 mM, or ABAH 100 μM) were added if necessary. The resulting solution was analyzed with an HPLC system equipped with an electrochemical detector[43] to determine the concentration of 3-nitrotyrosine. The effect of leukocyte homogenate on the degradation of 3-nitrotyrosine was also examined. Leukocytes (10^7 cells/ml) from healthy volunteers were sonicated in 20 mM phosphate buffer (pH 7.4) with 0.05% cetyltrimethylammonium bromide, soybean trypsin inhibitor (10 μg/ml), benzamidine (10 μg/ml), aprotinin (0.005 T.I.U./ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), antipain (5 μg/ml), phenylmethylsulfonyl fluoride (200 μM), and Na_2EDTA (100 μM). The leukocyte homogenate was incubated with 3-nitrotyrosine (5 μM) with or without ABAH (100 μM) at 37°C. The resulting mixture was filtered through a syringe filter unit (0.45 μm pore, DISMIC-3, Advantech, Tokyo) and applied to the HPLC system described above to determine 3-nitrotyrosine.

Experimental Protocol of Ischemia-reperfusion Injury in $MPO^{+/+}$ and $MPO^{-/-}$ Mice

The surgical and anesthetic procedures were approved by the Animal Experimentation Committee, School of Medicine, Tokai University. MPO-deficient mice were generated by disrupting exon 6 of the MPO gene using a gene targeting technique on ES cells of strain 120/Ola mice as previously described.[44] Briefly, male chimeric mice were mated with strain C57BL/6 female mice to produce heterozygous $MPO^{+/-}$ mice. Homozygous $MPO^{-/-}$ mice were produced by interbreeding of heterozygous offspring and backcrossed eight times with C57BL/6 mice. Northern blot analysis of mRNA isolated from the bone marrow was negative for MPO and peroxidase activity of polymorphonuclear cells was below the limit of detection.

Mice ($MPO^{+/+}$ and $MPO^{-/-}$, $n = 4$ in each group) were anesthetized initially with 2.5% isoflurane in 30% oxygen and 70% nitrous oxide and maintained with 1.5% isoflurane. The abdomen was

opened through a median incision and the hepatic artery and portal vein were temporarily ligated at the portal triangle. Twenty minutes after ligation the ligature was removed to reperfuse the liver for 30 min. In the sham-operated group the abdomen was opened, the hepatic artery and vein were isolated without ligation. At the end of the experiment, blood was collected for the measurement of serum alanine aminotransferase (ALT) and the liver was excised, snap-frozen in liquid nitrogen and stored at −80°C until use. Serum ALT concentrations were measured using commercial laboratory kits as a marker of liver damage. Values are given in units per liter of serum. To validate that detected nitrotyrosine is derived from nitric oxide synthase, peritoneal injection of *N*-monomethyl-L-arginine (100 mg/kg) was performed 30 min before ischemic insult. The study was approved by the Animal Care Committee of Tokai University.

Measurement of 3-nitrotyrosine Concentration in the Liver by ELISA

3-Nitrotyrosine residues in proteins were quantified by a competitive ELISA[45] with some modifications. Briefly, the assay was performed in 96-well plates coated with 0.5 μg/ml nitro-BSA[46] (immobilized antigen) which had been blocked with 1% casein in PBS to prevent non-specific binding. Homogenized liver (50 μl) was added to each well and the plate was incubated with immunoaffinity-purified polyclonal anti-nitrotyrosine rabbit IgG (1:500) (100 μl) for 2 h at 37°C. It was washed with PBS containing 0.05% Tween 20 and sequentially incubated with donkey anti-rabbit IgG horseradish peroxidase complex. After further washing, color development was initiated by the addition of *o*-phenylenediamine-H_2O_2 mixture. Color was allowed to develop for up to 5 min at room temperature and the development was terminated by the addition of 2.5 M sulfuric acid. Antibody binding was determined by measuring the absorbance at 490 nm with a microplate reader (Spectra Max 250, Molecular Devices, USA).

The concentrations of nitrated proteins were estimated from a standard curve, constructed by using serial dilutions of nitro-BSA. This provides a semi-quantitative estimation owing to differences in the affinity of various proteins in tissues and that of nitro-BSA for the antibody. The results were expressed as nitro-BSA equivalent. Data for the standard curves were fitted to a logistic plot and IC_{50} values calculated on a Macintosh computer using SOFT max Pro software (Molecular Devices, USA).

Statistical Analysis

Results were expressed as the mean ± SEM. Statistical analyses were performed with the unpaired Student's *t* test for the difference between two groups and one-way ANOVA followed by Newman–Keuls multiple comparison test for three or more groups.

RESULTS

Figure 1 shows cytogram of leukocytes from the normal volunteer and the MPO-deficient patient [MPO(−)]. In the patient's cytogram, peroxidase-positive neutrophils are absent and there is no increase in eosinophils, which are located in the peroxidase-positive area due to the presence of eosinophil peroxidase. This cytogram contrasts with that of normal volunteer obtained, which shows peroxidase-positive neutrophils in the right upper area. The total peroxidase activity of leukocytes was 0.3 units/10^6 cells in the patient, 30.5 units/10^6 cells in the patients's mother and 47.9 units/10^6 cells in the normal volunteer. The residual activity in the patient's leukocytes is considered attributable to that in eosinophils.

Values of superoxide generation determined in terms of cytochrome *c* reduction were comparable in MPO(−) and MPO(+) leukocytes; 146 ± 20 and 150 ± 24 nmol/10^6 cells/h, respectively. Total NO_2^- and NO_3^- contents were also comparable; 83 ± 16 pmol/10^6 cells/h in MPO(−) and 94 ± 18 pmol/10^6 cells/h in MPO(+) leukocytes.

Figure 2 shows representative HPLC chromatograms obtained from MPO(−) and MPO(+) leukocytes. An NO_2HPA peak was not observed in the MPO(+) leukocytes (a), but in the presence of ABAH, an MPO inhibitor, a peak appeared (b). In contrast, MPO(−) leukocytes produced a distinct peak of NO_2HPA (c) and ABAH had no effect, as expected (data not shown). The emergence of the peak was inhibited by the pretreatment of MPO(−)

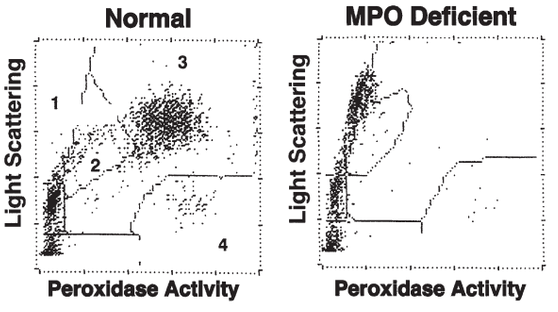

FIGURE 1 Cytograms of leukocytes from normal and MPO(−) individuals. Automated flow cytometric analysis of peroxidase-stained samples was performed with a Technikon H series. Peroxidase activity is plotted on the *x*-axis and light scattering on the *y*-axis. Cells with high peroxidase activity appear toward the right. Area 1; Lymphocytes, area 2; monocytes, area 3; neutrophils, area 4; eosinophils (A). No peroxidase-positive neutrophils were seen in MPO(−) leukocytes (B).

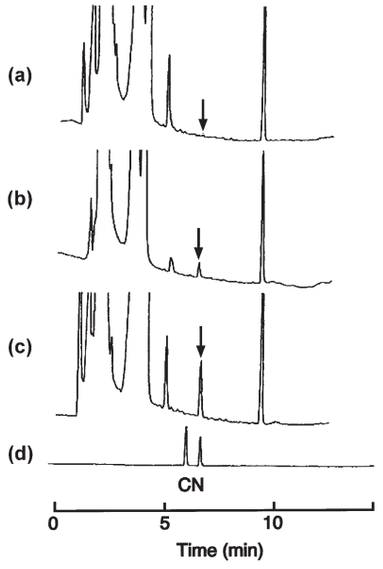

FIGURE 2 Generation of NO_2HPA peak in MPO(−) leukocytes is HPLC chromatogram. Chromatograms were obtained from the supernatant of the incubation medium (500 μl) containing leukocytes (2×10^6 cells/ml), HPA (1 mM), Cu, Zn-superoxide dismutase (0.1 mg/ml), and 40 mM HEPES (pH 7.4) in Hank's balanced salt solution. After 3 h incubation on a 24-well plate at 37°C initiated with the addition of PMA (1 μM), the supernatant was collected through an Ultrafree C3 LBC00 filter (0.45 μm pore size) and subjected to HPLC. (A) MPO(+) leukocytes, (B) MPO(+) leukocytes with ABAH (100 μM), (C) MPO(−) leukocytes, (D) Standard solution containing 3-chloro-4-hydroxyphenylacetic acid (Cl-HPA) for peak C and 3-nitro-4-hydroxyphenylacetic acid (NO_2HPA) for peak N. ↓ indicates NO_2HPA.

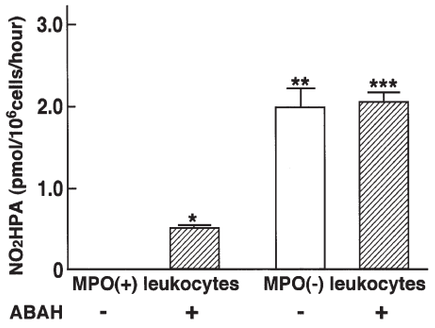

FIGURE 3 NO_2HPA peaks of MPO(+) and MPO(−) leukocytes obtained from 3 or 4 separate measurements. *$P < 0.05$; MPO(+) ABAH(−) vs. MPO(+) ABAH (+), **$P < 0.05$; MPO(+) ABAH(+) vs. MPO(−) ABAH(−); ***$P < 0.05$; MPO(+) ABAH(+) vs. MPO(−) ABAH(+).

leukocytes with 100 μM L-monomethyl-L-arginine, an inhibitor of NO generation. Unstimulated leukocytes did not produce any NO_2HPA peak in either case. Figure 3 summarizes the results obtained from 3 or 4 separate measurements. NO_2HPA were 0.51 ± 0.03 pmol/10^6 cells/h in MPO(+) leukocytes with ABAH, 2.02 ± 0.20 pmol/10^6 cells/h in MPO(−) leukocytes without ABAH, and 2.08 ± 0.11 pmol/10^6 cells/h in MPO(−) leukocytes with ABAH. Even in the absence of ABAH, NO_2HPA was detectable by exogenous addition of NO_2^- (50 μM) in MPO(+) leukocytes (0.47 ± 0.06 pmol/10^6 cells/h). This is consistent with the results of previous studies.[6,16,21] Since H_2O_2 is a known substrate for MPO-catalyzed nitration and may accumulate more in MPO(−) leukocyte media, catalase was added to eliminate the influence of H_2O_2. Addition of catalase did not decrease NO_2HPA formation in MPO(−) leukocytes, indicating that accumulated H_2O_2 was not the reason for the enhancement of NO_2HPA in MPO(−) leukocytes.

The above results show that leukocytes possess a peroxynitrite-mediated nitration pathway, but it remains to be established why the presence of MPO decreases the NO_2HPA peak, i.e. what is the mechanism of the MPO-driven interference with NO_2HPA formation.

In order to clarify the mechanism underlying MPO-driven interference with the nitration, the interaction of MPO with the peroxynitrite-mediated nitration system and with the NO and superoxide co-generation-mediated nitration system was examined MPO (1 μM) enhanced then nitration of HPA (1 mM) in the peroxynitrite-mediated nitration system by *ca* 2.5-fold, which is consistent with the previous report demonstrating that MPO catalyzes this type of nitration.[40] H_2O_2(100 μM), which activates MPO to Compound I or II, did not suppress the MPO-driven enhancement of nitration. Thus, this effect of MPO, whether activated or not, cannot explain its interference in the reaction of peroxynitrite-mediated nitration.

In the system where NO (NOC7 10 μM) and superoxide (pterin, 50 μM−xanthine oxidase, 40 mU) were individually generated, MPO-driven interference was not observed and MPO rather enhanced the nitration reaction dose-dependently. A 1 μM of MPO accelerated the nitration of tyrosine (0.1 mM) to produce 4.97 μM of nitrotyrosine, which was 8.6 fold increase compared to the value without MPO.

Finally, in an experiment to examine whether MPO enhances the degradation of nitrated phenol derivatives, we found that both MPO (2 μM) and HRP (10 μM) enhanced degradation of 3-nitrotyrosine in the presence of glutathione (10 mM) (Fig. 4). The effect was inhibited by peroxidase inhibitor (ABAH 100 μM or NaN_3 1 mM). The decrease was time-dependent and after incubation for 1 h, MPO (1 μM) and HRP (10 μM) degraded 3-nitrotyrosine to the extent of 63 and 50%, respectively. When leukocyte homogenate was added to 3-nitrotyrosine instead of MPO or HRP, 3-nitrotyrosine also decreased time-dependently (Fig. 5). The decrease was blocked by addition of a selective MPO inhibitor, ABAH.

As shown in Fig. 6a, 3-nitrotyrosine content in the liver remained low; 1.5 ± 0.6 and 2.1 ± 0.7 ng/g protein in sham-operated $MPO^{+/+}$ and $MPO^{-/-}$-mice. There was a markedly increased

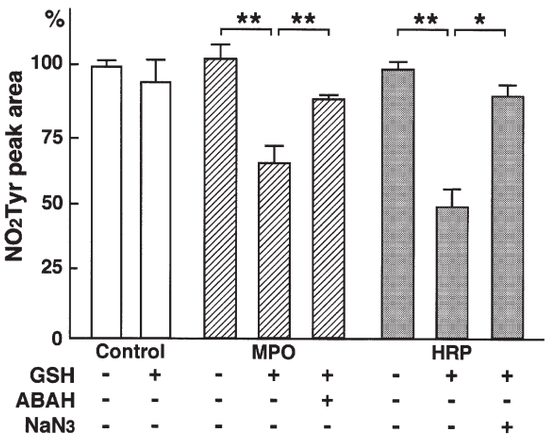

FIGURE 4 The effects of MPO and HRP on degradation of 3-nitrotyrosine. A solution of 3-nitrotyrosine (5 μM) and MPO (2 μM) or HRP (10 μM) was incubated for 1–3 h at 37°C with/without glutathione (10 mM). The effect of inhibitors of these peroxidases (ABAH 100 μM for MPO and NaN_3 1 mM for HRP) was also examined.

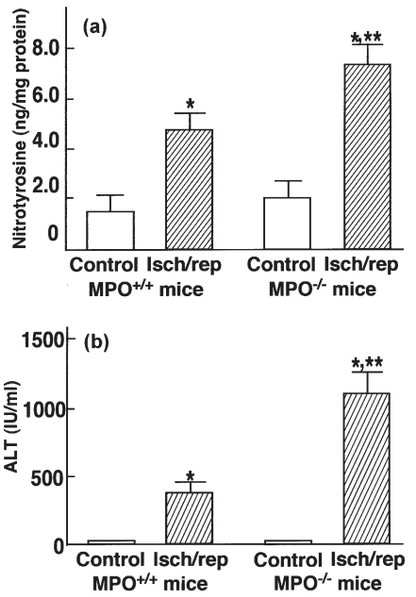

FIGURE 6 (a) Generation of 3-nitrotyrosine in $MPO^{-/-}$ mice subjected to ischemia/reperfusion injury. The hepatic artery and portal vein were occluded for 20 min and reopened. At 30 min after the opening of vessels, the liver was excised and 3-nitrotyrosine was measured by competitive ELISA. 3-Nitrotyrosine increased in the liver after ischemia/reperfusion and was higher in $MPO^{-/-}$ mice than in $MPO^{+/+}$ mice. Isch/rep indicates ischemia/reperfusion. $^{*}P < 0.05$; control vs. ischemia/reperfusion, $^{**}P < 0.05$; $MPO^{+/+}$ vs. $MPO^{-/-}$. (b) Release of ALT occurred in both strains after ischemia/reperfusion injury. Isch/rep indicates ischemia/reperfusion. $^{**}P < 0.05$; control vs. ischemia/reperfusion, $^{**}P < 0.05$; $MPO^{+/+}$ vs. $MPO^{-/-}$.

3-nitrotyrosine formation in the liver after ischemia/reperfusion insult in both mice; 4.7 ± 0.6 and 7.3 ± 0.9 ng/g protein, respectively. The level of 3-nitrotyrosine was higher in the $MPO^{-/-}$-mice than in $MPO^{+/+}$-mice. *N*-monomethyl-L-arginine (100 mg/kg) completely abolished the formation of 3-nitrotyrosine. Marked elevation of serum ALT concentration was observed in $MPO^{+/+}$- and $MPO^{-/-}$-mice after ischemia/reperfusion insult (Fig. 6b), while serum ALT remained low in sham-operated mice of both strains (data not shown). The level of ALT was significantly higher in $MPO^{-/-}$-mice (1145 ± 170 IU/ml) than in $MPO^{+/+}$-mice (391 ± 90 IU/ml).

DISCUSSION

Based on previous reports of nitration reactions in biological systems,[6,8,16,21,27,29,40,47,48] pathways for the formation of nitrotyrosine *in vivo* is summarized in Fig. 7. Pathway (1): Peroxynitrite ($ONOO^-$) generated by the reaction between superoxide and NO reacts with tyrosine to produce nitrotyrosine. Carbon dioxide and transition metal ion complexes such as FeEDTA, SOD, and heme peroxidases may catalyze this process.[8,40] Pathway (2): MPO is

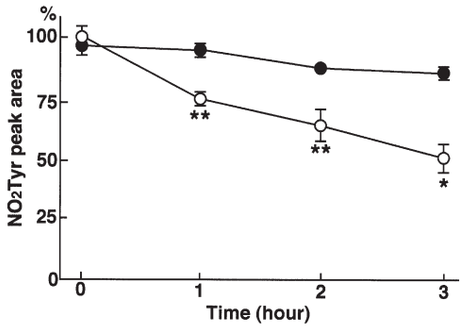

FIGURE 5 Time course of the degradation of 3-nitrotyrosine (5 μM) by the homogenate of leukocytes (10^7 cells/ml). ●; with ABAH (100 μM), ○; without ABAH. $^{*}P < 0.01$, $^{**}P < 0.05$.

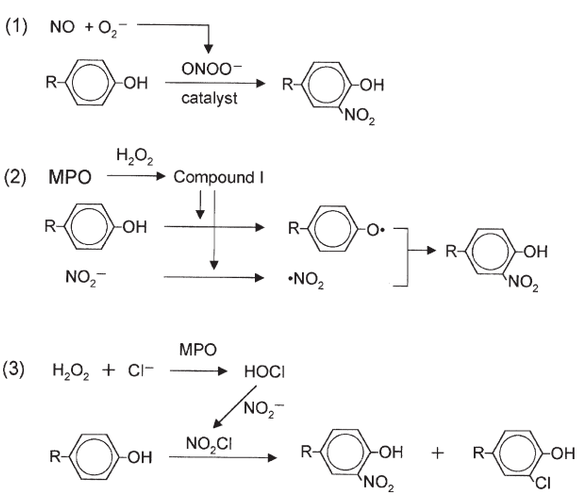

FIGURE 7 Schema of reactions related to myeloperoxydase (MPO) peroxynitrite ($ONOO^-$).

activated by H_2O_2 to form Compound I, which reacts with both tyrosine and NO_2^- to produce corresponding radicals which combine to form nitrotyrosine.[47,48] Pathway (3). Hypochlorous acid (HOCl) generated by MPO reacts with NO_2^- to produce NO_2Cl, which nitrates or chlorinates tyrosine.[21] The first pathway (peroxynitrite pathway) does not require MPO and NO_2^-, but both are essential in the other two pathways.

We previously reported that leukocyte-mediated nitration could be observed only in the presence of an MPO inhibitor[22] and the present study confirmed a distinct peak of phenol nitration in leukocytes obtained from an MPO(−) patient. Both results indicate that MPO is not necessary for nitration and peroxynitrite-mediated nitration (pathway 1) is the most important route of leukocyte-mediated nitration. One must consider the possibility that the eosinophil peroxidase/H_2O_2/NO_2 system may have mediated nitrotyrosine formation in MPO(−) leukocytes. However, this is unlikely, because total peroxidase activity, including eosinophil peroxidase, was only 2% that of the normal control in MPO(−) leukocytes, and the nitrating activity of eosinophil peroxidase was reported to be only four times that of MPO.[49] Pathway 3 involves the generation of chlorinated phenol (Cl-HPA) and nitrated phenol (NO_2HPA) at the same time, and it was shown that the peak of Cl-HPA was higher than that of NO_2HPA.[18,20,21] In our experiments with leukocytes, C1-HPA is hardly detectable because 40 mM HEPES can scavenge HOCl effectively. The addition of NO_2^- enhanced the generation of nitrated product, which is consistent with previous studies,[6,16,21] and we do not rule out a significant role of the MPO/H_2O_2/NO_2 pathway in the presence of a high nitrite concentration.

Another puzzling result in this study, as well as in our previous study, is that MPO interferes with the nitration of phenols. This is completely contrary to a series of reports describing the enhancement of nitration by MPO.[40,48] To resolve the apparent contradiction, we first examined the effect of MPO on the peroxynitrite-mediated phenol nitration system (pathway 1). An MPO-driven decrease in nitration did not occur, and MPO rather enhanced the nitration. Similarly, the activated form of MPO (Compound I) did not interfere with the nitration, indicating that the role of MPO is an enhancing one. Since MPO Compound I or II can scavenge NO,[50] it is possible that MPO inhibits peroxynitrite formation, thereby depressing phenol nitration. In an experiment using an NO and superoxide co-generating system to test this hypothesis, MPO dose-dependently enhanced the tyrosine nitration. Since peroxynitrite formation is almost diffusion-limited[51] and the second-order rate constant between MPO Compound II and NO is $8 \times 10^3\ M^{-1}\ s^{-1}$.[50] MPO at physiological concentration would not be able to suppress peroxynitrite formation by scavenging NO. Thus, we conclude that MPO accelerates the formation of nitrated phenol under all circumstances, which does not explain the MPO-driven interference with nitration.

Taking account of work by Balabanli *et al.*[52] who demonstrated the existence of non-enzymatic degradation of 3-nitrotyrosine by hemoglobin in the presence of thiols, we performed experiments to examine whether MPO catalyzes degradation of 3-nitrotyrosine in the presence of thiols. As we had anticipated, MPO (2 μM) with glutathione (10 mM) decreased nitrotyrosine to 63% on incubation for 1 h at 37°C. The degradation activity is not specific to MPO, but is due to peroxidase activity as HRP, a representative peroxidase, worked in the same manner and the degradation activity was blocked by the inhibitors of peroxidase activity for MPO or HRP. The reported absence of nitration product in the phagosomes of leukocytes despite marked elevation of nitration product in the extracellular milieu[53] is consistent with the notion of MPO-driven inhibition since the concentration of MPO reaches as high as millimolar level in the phagosome.[2,4] Although the peroxynitrite pathway is less productive than the MPO/H_2O_2/NO_2^- pathway, it appears that the effect of MPO-driven degradation predominates over MPO-driven enhancement of nitration reaction In the presence of sufficient NO_2^-, the MPO/H_2O_2/NO_2^- system may keep producing nitrotyrosine, thereby masking the MPO-driven degradation of nitrotyrosine. Although the role of peroxidases in nitrotyrosine degradation is not clear, it seems plausible that heme in peroxidase coordinates nitrated compounds and facilitates electron transfer from sulfhydryl compounds to make amino compounds since the product of this heme-supported degradation from free nitrotyrosine was aminotyrosine.[52]

Regarding the role of MPO *in vivo*, we used ELISA in this study to quantify nitrotyrosine in tissue to eliminate any uncertainty involving the HPLC method.[54–56] The formation of nitrotyrosine was observed in the liver of $MPO^{-/-}$ mice subjected to ischemia/reperfusion injury, indicating that the nitration occurs via the peroxynitrite pathway. The higher nitration level in $MPO^{-/-}$ mice than in $MPO^{+/+}$ mice again supports the idea that MPO degrades nitrotyrosine. The unexpected protective role of MPO against injury, manifested by the higher ALT release in $MPO^{-/-}$ mice than in $MPO^{+/+}$ mice, is not consistent with the well-accepted injurious role of MPO on hosts, apart from its bactericidal effect.[1,4,57] However, a recent study found a 50% increase in atherosclerotic lesions in $MPO^{-/-}$ mice as compared with $MPO^{+/+}$ mice,[58] which supports

the present results. A higher concentration of nitrotyrosine does not explain the more severe damage in the $MPO^{-/-}$ mice, since the rate of its formation does not increase in $MPO^{-/-}$ mice. One possible explanation is that the formed nitrotyrosine itself has a cytotoxic effect, considering recent studies which showed that nitrotyrosine caused microtubule and endothelial dysfunctions.[33,34] This apparent direct effect of nitrotyrosine remains to be examined in detail.

In summary, nitrotyrosine was formed in the leukocyte-catalyzed reaction of an MPO-deficient patient without exogenous addition of NO_2^-, possibly via the peroxynitrite pathway. MPO enhances the rate of nitration reaction and also accelerates the degradation of nitrated phenols. MPO-independent nitration pathway, where the action of peroxynitrite is plausible, is relevant *in vivo*, since nitrotyrosine was formed in the liver of $MPO^{-/-}$ mice subjected to ischemia/reperfusion injury. Furthermore, $MPO^{-/-}$ mice showed more severe liver injury than $MPO^{+/+}$ mice after ischemia-reperfusion, suggesting a direct injurious effect of nitrotyrosine.

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