Nitrosation is an important reaction elicited by nitric oxide (NO). To better understand how nitrosation occurs in biological systems, we assessed the effect of myeloperoxidase (MPO), a mediator of inflammation, on nitrosation observed during NO autoxidation. Nitrosation of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ; 10 μM) to 2-nitroso-3-methylimidazo[4,5-f]quinoline (N-NO-IQ) was monitored by HPLC. Using the NO donor spermine NONOate at pH 7.4, MPO potentiated N-NO-IQ formation. The minimum effective quantity of necessary components was 8.5 nM MPO, 0.25 μM H₂O₂/min, and 0.024 μM NO/min. Autoxidation was only detected at ≥12 μM NO/min. MPO potentiation was not affected by a 40-fold excess flux of H₂O₂ over NO or less than a 2.4-fold excess flux of NO over H₂O₂. Potentiation was due to an 8.8-fold increased affinity of MPO-derived nitrosating species for IQ. Autoxidation was inhibited by azide, suggesting involvement of the nitrosium ion, NO⁺. MPO potentiation was inhibited by NADH, but not azide, suggesting oxidative nitrosylation with NO₂⁻ or an NO₂⁻ like species. MPO nonnitrosative oxidation of IQ with 0.3 mM NO₂⁻ at pH 5.5 was inhibited by azide, but not NADH, demonstrating differences between MPO oxidation of IQ with NO compared with NO₂⁻. Using phorbol ester-stimulated human neutrophils, N-NO-IQ formation was increased with superoxide dismutase and inhibited by catalase and NADH, but not NaN₃. This is consistent with nitrosation potentiation by MPO, not peroxynitrite. Increased N-NO-IQ formation was not detected with polymorphonuclear neutrophils from two unrelated MPO-deficient patients. Results suggest that the highly diffusible stable gas NO could initiate nitrosation at sites of neutrophil infiltration.

Nitric oxide (NO)³ is an essential regulator for a variety of processes critical to normal functions in the cardiovascular, nervous, and immune systems (1). Impaired responses are observed with excessive production of NO in pathological conditions associated with chronic inflammation. Effects of NO can be divided into direct and indirect (2). Direct effects of NO are mediated by low nanomolar concentrations of NO and are illustrated by its binding to guanylate cyclase and eliciting numerous effects, including smooth muscle relaxation. In contrast, indirect effects occur at higher concentrations of NO and result from the reaction of NO with either oxygen (autoxidation) or superoxide to produce reactive nitrogen oxygen species (RNOS).

Indirect effects of NO elicited by RNOS include nitrosation, oxidation, and nitration reactions with numerous biological targets representing lipids, proteins, and DNA (2). This can cause lipid peroxidation, inhibition of enzymes, and deamination of DNA. Autoxidation of NO results in the formation of N₂O₃, which yields the nitrosium ion, NO⁺ (3, 4). The latter is a potent nitrosating agent. Autoxidation is slow and considered unlikely to occur in biological systems because NO can be rapidly inactivated. For example, NO is removed from the vascular compartment by near diffusion-limited interaction with erythrocyte oxyhemoglobin, yielding ferric hemoglobin and nitrate (5). However, significant amounts of S-, N-, and heme-nitrosylation are detected in vivo, suggesting that modes of nitrosation other than by autoxidation must exist (6, 7).

NO is a physiologic substrate for several mammalian peroxidases, including myeloperoxidase (MPO) (8). Direct spectroscopic and rapid kinetic studies support a facile reaction between peroxidases and NO (8, 9). Peroxidases may play an important role in attenuating direct effects of NO. Organ chamber studies with preconstricted vascular and tracheal rings have demonstrated that catalytic amounts of peroxidase hydrolyzed NO (10). This prevented smooth muscle relaxation and NO-mediated ring dilation. A subsequent study further emphasized the role of MPO as a leukocyte-derived NO oxidase (11). The product of MPO metabolism of NO is thought to be NO⁺ (8). Surprisingly, little consideration has been given to the possible role of MPO-derived NO⁺ in biologically important nitrosations.

Cellular nitrosation has been demonstrated to be distinct from that catalyzed by NO autoxidation in aqueous solution. Experiments, using an oxidant to convert NO directly to NO₂⁻, demonstrated that this RNOS facilitates nitrosation by combining with NO to form N₂O₃ (12). NO₂⁻-mediated nitrosation

**This work was supported by the Department of Veterans Affairs (to T. V. Z. and W. M. N) and NCI, National Institutes of Health (NIH), Grant CA72613 (to T. V. Z.). Mass spectrometry was performed at the Iowa City, Iowa 52241.

* The abbreviations used are: NO, nitric oxide; 2-Cl-IQ, 2-chloro-3-methylimidazo[4,5-f]quinoline; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; IQ dimer, 2-(5'-amino)-3,3’-dimethylimidazo[4,5-f]quinoline; MPO, myeloperoxidase; 2-N₂-IQ, 2-azido-3-methylimidazo[4,5-f]quinoline; N-NO-IQ, 2-nitroso-3-methylimidazo[4,5-f]quinoline; NO₂⁻IQ1, 2-amino-5-nitro-3-methylimidazo[4,5-f]quinoline; NO₂⁻IQ2, 2-nitroso-3-methylimidazo[4,5-f]quinoline; PMA, β-phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear neutrophils; RNOS, reactive nitrogen oxygen species; SpN, spermine NONOate; SOD, superoxide dismutase; HPLC, high pressure liquid chromatography; DETAPAC, diethylenetriamine-pentaacetic acid.

**To whom correspondence should be addressed: VA Medical Center (11G-JB), St. Louis, MO 63125. Tel.: 314-894-6510; Fax: 314-894-6614; E-mail: zenser@slu.edu.

** The abbreviations used are: NO, nitric oxide; 2-CI-IQ, 2-chloro-3-methylimidazo[4,5-f]quinoline; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; IQ, 2-amino-3-methylimidazo[4,5-f]quinoline; IQ dimer, 2-(5'-amino)-3,3’-dimethylimidazo[4,5-f]quinoline; MPO, myeloperoxidase; 2-N₂-IQ, 2-azido-3-methylimidazo[4,5-f]quinoline; N-NO-IQ, 2-nitroso-3-methylimidazo[4,5-f]quinoline; NO₂⁻IQ1, 2-amino-5-nitro-3-methylimidazo[4,5-f]quinoline; NO₂⁻IQ2, 2-nitroso-3-methylimidazo[4,5-f]quinoline; PMA, β-phorbol 12-myristate 13-acetate; PMNs, polymorphonuclear neutrophils; RNOS, reactive nitrogen oxygen species; SpN, spermine NONOate; SOD, superoxide dismutase; HPLC, high pressure liquid chromatography; DETAPAC, diethylenetriamine-pentaacetic acid.

Published, JBC Papers in Press, November 5, 2004, DOI 10.1074/jbc.M411263200

Received for publication, October 1, 2004

Published, JBC Papers in Press, November 5, 2004, DOI 10.1074/jbc.M411263200

Vijaya M. Lakshmi‡‡, William M. Nauseef§§, and Terry V. Zenser††∥∥

From the ¶Veterans Administration Medical Center and |Edward A. Doisy Department of Biochemistry and Molecular Biology and ¶|Division of Geriatric Medicine, St. Louis University School of Medicine, St. Louis, Missouri 63125 and the "Inflammation Program and Department of Medicine, University of Iowa and Veterans Administration Medical Center, Iowa City, Iowa 52241
was shown to be similar to that observed in cells. We have recently demonstrated RNOS nitration of the colon carcinogen 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), forming 2-nitroso-3-methylimidazo[4,5-f]quinoline (N-OIQ) (13). The latter binds DNA and is mutagenic. In this study, we have used IQ as a target for evaluating MPO potentiation of NO-mediated nitrosylation. In view of the potential close temporal and spatial association between NO formation (inducible nitric-oxide synthase), hydrogen peroxide (H2O2) formation, and MPO during an inflammatory response, a better understanding of NO-mediated nitrosylation may improve treatment and aid in prevention of inflammation-related diseases (i.e., certain cancers, atherosclerosis, and asthma).

**EXPERIMENTAL PROCEDURES**

**Materials—**[2-14C]IQ (10 mCi/mmol, >98% radiochemical purity) was purchased from Toronto Research Chemicals (Toronto, ON). Hydrogen peroxide (H2O2), catalase (bovine liver), ascorbic acid, diethyl-aminepentaacetic acid (DETAPAC), β-phorbol 12-myristate 13-acetate (PMA), NaCl, NaN3, ADH, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), N-glucose oxidase, cytochrome c, and NADH, 5,5-dimethyl-1-pyrroline N-oxide, spermine NONOate (SpN), a NO donor, and MPO from human polymorphonuclear leukocytes. Prior to its use, IQ was kept as a stock solution of 100 mM in methanol/dimethylformamide (8:2) and analyzed by HPLC with mobile phase 1: 20 mM ammonium formate buffer (pH 3.1; mobile phase 2) in 0.1% TFA. Later supernatants were pooled after HPLC analysis, evaporated to dryness, and redissolved in 1N HCl was added for 30 min at 37 °C and then neutralized with 1 ml of 100 mM NaOH. Supernatants were incubated with 0.01 mM [14C]IQ in 12 × 75-mm polypropylene tubes at 37 °C for 36 min in Hanks’ balanced salt solution containing calcium, magnesium, and bicarbonate. All protocols were in accordance with institutional guidelines on research involving human subjects of either the St. Louis VA Medical Center or the University of Iowa. All participants gave written informed consent.

For experiments with MPO-deficient patients, blood was collected in lithium heparin tubes and shipped overnight for analysis the next day. A normal healthy control sample of blood was drawn and included in the shipment. Neutrophils were isolated by a two-step procedure. First red blood cells were removed by dextran sedimentation, M, 200,000–300,000 (MP-Biomedicals, Irvine, CA). Neutrophils were isolated from the supernatant following Ficoll-Hypaque Plus (Amer sham Biosciences) gradient centrifugation. Neutrophils were resuspended in Hanks’ balanced salt solution containing calcium, magnesium, and bicarbonate. Molecular characteristics of the two MPO-deficient patients have been reported (17, 18).

**Metabolism of IQ by Neutrophils—**Neutrophils (0.3 × 10^6 cells in 0.3 ml) were incubated with 0.01 mM [14C]IQ in 12 × 75-mm polypropylene tubes at 37 °C for 36 min in Hanks’ balanced salt solution containing calcium, magnesium, and bicarbonate without phenol red. Where indicated, 0.054 mM PMA, 1.2 mM NO/min (0.05 mM SpN), 1 mM NaN3, and 0.1 mM NADH were added at 5, 6, 8, and 8 min, respectively, whereas 66 μg/ml catalase and 66 μg/ml SOD were present at zero time. Blank values were obtained in the absence of SpN. The reaction was stopped by placing on ice and freezing at −70 °C. Samples were sonicated for 15 ± 3 times, and 0.3 ml of dimethylformamide containing 2 mM ascorbic acid was added. Samples were spun at 1,500 × g for 10 min. The supernatant was evaporated and residue was dissolved in 1 ml of methanol/dimethylformamide (8:2) and analyzed by HPLC with mobile phase 1.

The oxidant burst response was measured for each PMN preparation (19). Superoxide generation was activated by the addition of PMN. Superoxide-specific reduction of cytochrome c was determined spectrophotometrically (ε_m = 21.1 mM cm⁻¹) and was inhibited by superoxide dismutase (10 μM/ml). Values observed in cells in the absence of PMN were considered as blanks.

To assess the authenticity of neutrophil-derived NO-IQ, selected supernatants were pooled after HPLC analysis, evaporated to dryness, and converted to either 2-azido-3-methylimidazo[4,5-f]quinoline (2-N-IQ) or 2-chloro-3-methylimidazo[4,5-f]quinoline (2-Cl IQ) derivatives. For 2-N-IQ, pH 2.0, ammonium formate was added along with 10 mM NaN3. For 2-Cl-IQ, the residue was dissolved in 0.18 ml of acetonitrile, and 0.02 ml of 1N HCl was added for 30 min at 37 °C and then neutralized with 1 N NH4OH. Authentic N-NO-IQ (98% pure) was treated in a similar manner to prepare standards. A recent study has verified the structure of these compounds (20). Samples were analyzed by HPLC on mobile phase 1 and 2.

**Statistical Analysis—**Data are expressed as a mean ± S.E., and significant differences were evaluated using Student’s paired t test with p < 0.05.

**RESULTS**

**MPO Potentiation of NO-mediated Nitrosation—**MPO was evaluated to determine its effect on NO-mediated nitrosation of IQ (Fig. 1). Previous studies have demonstrated significant time-dependent oxidation of IQ to a stable N-nitroso product, N-OIQ, during autoxidation of NO, using a high flux of NO (~30 μM NO/min, 0.1 mM diethyamine NONOate) (13). However, using a flux of NO more likely to occur during inflammation (2.4 μM/min, 0.1 mM SpN), N-OIQ formation due to autoxidation (0.2 μM) was near the limit of detection, 1.6% of the total radioactivity recovered by HPLC (Fig. 1, top). In the absence of NO, MPO (85 μM) and H2O2 (10 μM/min H2O2) did not catalyze N-OIQ formation or metabolize IQ (Fig. 1, middle). When incubation mixtures contained NO, MPO, and H2O2, significant N-OIQ formation (3.1 μM) was detected, 31% of the total radioactivity recovered by HPLC (Fig. 1, bottom). N-OIQ formation during the autoxidation of NO was not affected by 0.1 μM H2O2/min. However, H2O2 is required for the potentiation of N-nitroso formation observed with NO/MPO/H2O2. Using conditions illustrated in the bottom panel, N-OIQ formation was linear for at least 60 min. Thus, catalytic amounts of MPO
potentiate NO-mediated IQ nitrosation.

Effect of MPO Concentration, Fluxes of H2O2 and NO, and Concentrations of IQ on NO Nitrosation—MPO concentration dependent potentiation of NO-mediated IQ nitrosation was evaluated with a constant flux of NO (2.4 μM/min) and H2O2 (10 μM/min) (Fig. 2). Using the conditions illustrated in Fig. 1 (bottom), a range of MPO concentrations from 0 to 170 nM was evaluated. N-NO-IQ formation by autoxidation was significantly increased from 16 ± 2 to 53 ± 5 pmol (p < 0.05) with 8.5 nM MPO. A linear increase in N-NO-IQ formation was observed from 2.8 to 170 nM MPO. Thus, in the presence of a low flux of NO, MPO elicits a concentration-dependent increase in IQ nitrosation.

To investigate MPO potentiation of NO-mediated IQ nitrosation in more detail, N-NO-IQ formation was evaluated with increasing fluxes of H2O2, 0–20 μM/min, in the presence of a constant flux of NO (0.48 μM/min) and H2O2 (10 μM/min) (Fig. 3). A linear increase in N-NO-IQ formation was observed from 2.8 to 170 nM MPO. Thus, in the presence of a low flux of NO, MPO elicits a concentration-dependent increase in IQ nitrosation.

MPO potentiation of nitrosation was observed at high as well as low fluxes of H2O2.

IQ nitrosation was also assessed at varied fluxes of NO (0–0.48 μM/min) in the presence of constant H2O2 production (10 μM/min) (Fig. 4). With MPO and H2O2, N-NO-IQ formation was not detected in the absence of NO or with 0.012 μM NO/min. N-NO-IQ formation was detected with 0.024 μM NO/min and continued to increase from 0.012 to 0.48 μM NO/min. Formulation of N-NO-IQ was similar, with fluxes of NO from 0.48 to 2.4 μM/min (not shown). At 0.24 μM NO/min, the efficiency of N-NO-IQ formation (0.038 μM/min) was 16%. Nitrosation of IQ elicited by NO autoxidation was not detected with the fluxes of NO evaluated. Thus, with MPO and H2O2, low fluxes of NO exhibited a dose-responsive increase in IQ nitrosation.

MPO potentiation was evaluated with NO fluxes in excess of H2O2 (Fig. 5). N-NO-IQ formation was assessed with increasing fluxes of NO (0.6 to 9.6 μM/min) in the presence and absence of MPO and H2O2 (1 μM/min). From 0.6 and 9.6 μM NO/min, a general increase in N-NO-IQ formation was observed with MPO. N-NO-IQ formation due to autoxidation was observed at fluxes of NO ≥1.2 μM. MPO potentiation is expressed in Fig. 5 as the difference between values observed with MPO minus values observed in its absence (control). Potentiation gradually decreased over the range of NO fluxes assessed with significant decreases observed at all fluxes of NO ≥2.4 μM/min. Thus, MPO potentiation is sensitive to fluxes of NO greater than H2O2 with a ≥2.4-fold excess inhibitory.
Nitrosation of IQ illustrated in Fig. 1 (top and bottom) was evaluated kinetically over a range of IQ concentrations (0.005–0.08 mM) (Fig. 6). Keeping the flux of NO constant (2.4 μM/min), N-NO-IQ formation was evaluated in the presence and absence of MPO (85 nM) and H₂O₂ (10 μM/min). The affinities of nitrosating species produced by NO for IQ were greater in the presence of MPO/H₂O₂ (1/ιΝ0 = 21 + 2 μM) than with NO alone by autoxidation (1/ιΝ0 = 189 + 3 μM). By contrast at infinite concentrations of IQ, the maximal rates of N-NO-IQ formation were similar with MPO/H₂O₂ (1/ιΝ0 = 24 + 3 pmol/min) and autoxidation (1/ιΝ0 = 28 + 1 pmol/min). Thus, potentiation of NO-mediated IQ nitrosation is due to increased affinity of MPO-derived RNOS for IQ.

Inhibition of NO and MPO Potentiation of NO Nitrosation—To assess N-NO-IQ formation mediated by NO autoxidation, a high NO flux (9.6 μM/min) was necessary to test different agents (Table I). Autoxidation of IQ was not significantly altered by 100 mM NaCl, 33 μg/ml catalase, or 33 μg/ml SOD. Complete inhibition of N-NO-IQ formation was observed with 0.3 mM ascorbic acid, NaN₃ (1 mM), NADH (0.1 mM), and DMPO (30 mM) decreased N-NO-IQ formation 70, 73, and 56%, respectively. With 0.48 μM NO/min and 10 μM/min H₂O₂, these same agents characterized MPO potentiation of N-NO-IQ formation. MPO potentiation was not significantly altered by 1 mM NaN₃, 100 mM NaCl, or 33 μg/ml SOD. Complete inhibition was observed with 0.1 mM NADH, 0.3 mM ascorbic acid, and 33 μg/ml catalase. DMPO (30 mM) decreased N-NO-IQ formation 78%. Test agents did not affect the half-life of SpN. Thus, N-NO-IQ formation mediated by NO and potentiated by MPO have different sensitivities to the selected test agents, suggesting different mechanisms of N-NO-IQ formation.

Inhibition of NO Nonnitrosative Oxidation—To help interpret the effects of test agents in Table I, the same test agents were used to evaluate MPO nonnitrosative oxidation of IQ (Table II). This oxidation of IQ at pH 5.5 requires not only H₂O₂, but also NO₂⁻ (13). A previous study identified these
products of IQ oxidation by MPO as NO2-IQ1, NO2-IQ2, and IQ dimer. NaN3 (0.3 mM) and ascorbic acid (0.3 mM) completely inhibited the formation of all three products, whereas DMPO (30 mM) significantly reduced their formation. With NADH (0.1 mM), the formation of NO2-IQ1 and NO2-IQ2 was reduced, but not significantly. NaCl (100 mM) reduced the formation of all three products, but only NO2-IQ1 and NO2-IQ2 were significantly reduced. Catalase elicted complete inhibition of all products (not shown). These test agents reveal important differences in the mechanism by which MPO oxidizes IQ with NO (Table I) and oxidizes IQ with NO2, an end product of NO metabolism (Table II).

**NO-mediated Nitrosation with Human PMNs**—To assess the significance of MPO potentiation of NO-mediated IQ nitrosation, N-NO-IQ formation by human PMNs was assessed (Table III). Cells were incubated with 0.01 mM IQ and 1.2 µM NO/min (Fig. 7, top). In the absence of PMA, the amount of N-NO-IQ formed was similar to that observed in the absence of cells (not shown). PMA increased N-NO-IQ formation 2.7-fold. This increase was prevented by 66 µM catalase. Values observed with PMA were further increased by the presence of 66 µM SOD (2-fold) (Fig. 7, middle). Catalase and NADH, but not azide, suppressed the increase observed with PMA and SOD. To assess the authenticity of N-NO-IQ, neutrophil-derived N-NO-IQ was converted to 2-N3-IQ by pH 2.0/10 mM NaN3 treatment. As illustrated in Fig. 7 (bottom), this treatment resulted in the disappearance of the N-NO-IQ peak observed in Fig. 7 (middle panel) at 10.2 min and the appearance of a new peak at 15.5 min, 2-N3-IQ. Synthetic 2-N3-IQ standard also eluted at 15.5 min. The yield of 2-N3-IQ was 52%. The elution time of neutrophil-derived and synthetic 2-N3-IQ was identical on a different HPLC solvent system. In addition, treatment of neutrophil-derived N-NO-IQ with 1 N HCl produced 2-Cl-IQ. Neutrophil-derived and synthetic 2-Cl-IQ were shown to have identical elution times on two different HPLC solvent systems. This confirms the formation of N-NO-IQ by PMA-stimulated neutrophils exposed to a constant low flux of NO. Results with human neutrophils are consistent with MPO potentiation of NO-mediated IQ nitrosation.

To more directly demonstrate a catalytic role for MPO in IQ nitrosation by PMNs, two unrelated patients with MPO-deficient PMNs were evaluated. Normal PMNs incubated with 0.01 mM IQ and 1.2 µM NO/min for 36 min produced 26 ± 3 pmol of N-NO-IQ. In maximally stimulated neutrophils, PMA + SOD produced significantly more N-NO-IQ, 42 ± 3 pmol (p < 0.01). This increase in N-NO-IQ formation was completely inhibited by catalase. As demonstrated in Fig. 7 (bottom), the N-NO-IQ HPLC peak was converted to 2-N3-IQ by incubation of PMN-derived material under acidic conditions with 10 mM NaN3. In the MPO-deficient PMNs, N-NO-IQ production observed for one patient was 27 ± 1 and 26 ± 2 pmol for neutrophils and neutrophils with PMA + SOD, respectively. For the second patient, N-NO-IQ production was 26 ± 2 and 30 ± 2 pmol for neutrophils and neutrophils with PMA + SOD, respectively. The oxidant bursts observed with PMNs from the normal control and MPO-deficient patients were similar. The inability of MPO-deficient PMNs to increase N-NO-IQ formation in response to PMA + SOD treatment is consistent with a role for MPO in PMN nitrosation of IQ.

**DISCUSSION**

This is the first study to demonstrate MPO potentiation of NO-mediated nitrosation. MPO did not metabolize IQ in the absence of NO. With NO, MPO potentiates nitrosation of IQ observed with the NO donor SpN, forming N-NO-IQ. The latter is the only product observed. To simulate the in vivo situation, H2O2 was generated in situ. Potentiation of N-NO-IQ formation was observed over a range of MPO, NO, and H2O2 concentrations likely to occur during an inflammatory response. MPO altered the kinetics of N-NO-IQ formation by producing nitrosating species with an 8.8-fold higher affinity for IQ than those produced by autoxidation. Potentiation by MPO was not affected by a 40-fold excess flux of H2O2 over NO or less than a 2.4-fold excess flux of NO over H2O2. To further relate these observations to the in vivo situation, stimulated human PMNs demonstrated increased N-NO-IQ formation. This increase was not detected with PMNs from MPO-deficient patients, consistent with a role for MPO in PMN nitrosation of IQ. The authenticity of neutrophil-derived N-NO-IQ was confirmed by its conversion to 2-chloro and 2-azido derivatives. Thus, MPO provides an alternative pathway for nitrosation that may help explain part of the significant amount of nitrosation occurring in vivo (6, 7).

**TABLE II**

| Conditions | NO2-IQ | NO2-IQ2 | IQ dimer |
|------------|-------|--------|---------|
| Complete   | 413 ± 23 | 237 ± 15 | 80 ± 6 |
| + 0.3 mM NaN3 | ND * | ND | ND |
| + 0.1 mM NADH | 337 ± 35 | 200 ± 6 | 80 ± 6 |
| + 100 mM NaCl | 143 ± 8 b | 85 ± 5 b | 63 ± 13 |
| + 0.3 mM ascorbic acid | ND | ND | ND |
| + 30 mM DMPO | 52 ± 6 b | 43 ± 5 b | ND |

* ND, not detected.

**TABLE III**

| Conditions | N-NO-IQ |
|------------|--------|
| Neutrophils | 18 ± 3 |
| + 0.05 mM PMA | 48 ± 5 a |
| + 0.05 mM PMA + 66 µg/ml catalase | 22 ± 5 b |
| + 0.05 mM PMA + 66 µg/ml SOD | 99 ± 14 b |
| + 0.05 mM PMA + 66 µg/ml SOD + 66 µg/ml catalase | 54 ± 5 |
| + 0.05 mM PMA + 66 µg/ml SOD + 1.0 mM NaN3 | 97 ± 9 |
| + 0.05 mM PMA + 66 µg/ml SOD + 0.1 mM NADH | 20 ± 2 |

* p < 0.001 versus neutrophils.
| p < 0.003 versus PMA. |
| p < 0.003 versus PMA + SOD. |
then form NO\(^+\). This proposed pathway is consistent with inhibition by both azide and NADH, as reported in Table I. The small decrease observed with NaCl is consistent with the reported reaction of biologically relevant nucleophiles with NO\(^+\) (26). Whereas inhibition by ascorbic acid might be attributed to its antioxidant properties on reactive intermediates, it also readily reduces MPO compounds I and II. DMPO inhibition suggests radical involvement (27). The lack of significant inhibition by catalase or SOD is consistent with neither H\(_2\)O\(_2\) nor superoxide playing a significant role. Thus, N-NO-IQ formation during autoxidation of NO is consistent with involvement of NO\(^+\) and NO\(_2\) or a NO\(_2\)-like species. The generation of these RNOS during autoxidation has been debated (4, 12, 23). Although individual test agents may have more than one action and may not provide unequivocal proof of the RNOS involved, they do characterize nitrosation attributed to autoxidation and allow comparisons with that process.

MPO potentiation of N-NO-IQ formation was distinct from that observed by autoxidation. Azide did not inhibit N-NO-IQ formation, suggesting that NO\(^+\) was not involved. Consistent with MPO-catalyzed metabolism, catalase completely inhibited N-NO-IQ formation. NADH caused complete inhibition, suggesting formation of a NO\(_2\)-like RNOS. Effects of ascorbic acid and DMPO were consistent with their antioxidant/reducing substrate and radical trapping effects, respectively. The lack of effect of SOD demonstrated that superoxide is not involved. Decreased N-NO-IQ formation at high levels of NO (Fig. 5) could be due to ligation of NO to the distal heme, which prevents access of H\(_2\)O\(_2\) to the catalytic site (9). Since NO\(^+\) has been proposed as the product of MPO metabolism of NO (8), results with azide and NADH were surprising. The lack of azide inhibition of nitrosation has been previously reported and attributed to oxidative nitrosylation (24). This pathway could involve a RNOS, such as NO\(_3\), oxidizing IQ to IQ\(^+\), with the latter combining with NO to form N-NO-IQ. The reaction elicited by the RNOS derived from MPO oxidation of NO is consistent with oxidative nitrosylation rather than nitrosation.

MPO nonnitrosative oxidation of IQ (Table II) was different from that observed in the presence of NO (Table I). With NO\(_2\) present, 0.3 mM NaN\(_3\) appears to be functioning as a heme poison and completely inhibits MPO metabolism of IQ. NO\(_2\) is a poor substrate for MPO (28). In contrast, NO is a good substrate for MPO and can bind the distal heme at the active site (8, 9). This may help explain why azide was a potent inhibitor of NO\(_2\), but not NO oxidation of IQ. MPO converts NO\(_2\) to NO\(_3\) (21). Whereas trapping of NO\(_3\) by NADH could explain reduced IQ nitration, this reduction was modest compared with that observed with N-NO-IQ formation. NADH is also a substrate for peroxidases and could function as a competitive inhibitor. However, since NO is a better substrate than NO\(_2\), one would expect more inhibition by NADH of the NO\(_2\)-like species. The generation of these NO\(_2\)-like species was significantly reduced the formation of the nitrated products more than 60%. Consistent with a previous study (8), this concentration of Cl\(^-\) did not alter MPO metabolism of NO (Table I). The lack of Cl\(^-\) inhibition of NO metabolism was previously attributed to higher substrate affinity of NO for MPO than Cl\(^-\) (8, 9). Effects of ascorbic acid and DMPO were consistent with their antioxidant/reducing substrate and radical trapping effects, respectively. Thus, the relative substrate and ligand affinities of NO, NO\(_2\), NaN\(_3\), and NaCl for MPO help to explain the diverse metabolite profiles observed.

![Figure 7](http://www.jbc.org/)

**Fig. 7.** NO-mediated nitrosation of IQ by human PMNs. PMNs were incubated with 0.01 mM \(^{[14]C}\)IQ and 1.2 \(\mu\)M NO/min for 36 min at 37 °C. Illustrated by HPLC mobile phase 1 are supernatants from PMNs (top), supernatants from PMNs stimulated by PMA plus SOD (middle), and the latter supernatants treated with 10 mM NaN\(_3\), pH 2.0, to convert N-NO-IQ to 2-N\(_3\)-IQ (bottom).
The diffusion-limited reaction of NO with superoxide to form peroxynitrite is thought to play an important role in nitrosation and oxidative nitrosylation (24, 32). Nearly equimolar fluxes of each reactant were shown to yield maximum nitrosation of the amines diaminonaphthalene and diaminofluorescein. An excess of one reactant over the other precipitated a decrease in nitrosation. This was attributed to secondary reactions occurring directly between peroxynitrite and excess NO or superoxide. Product formation was influenced by SOD, but not catalase. NO2, a metabolite of peroxynitrite, was proposed to facilitate nitrosation by combining with NO to form N2O3, which yields NO−, an azide-sensitive product. In addition, NO2 oxidized these amines to radicals, which then combine with NO forming the same product by oxidative nitrosylation. The latter reaction was not azide-sensitive but would be expected to be inhibited by NADH. Whereas nitrosation of NO-mediated nitrosylation by in situ generated peroxynitrite exhibits some differences in sensitivities to inhibitors from that observed with MPO, similar RNOS, such as NO2 or a NO2−-like species, may be involved in both reactions. MPO can potentiate NO consumption in the presence of a superoxide-generating system (8). Future studies will assess potentiation of N-NO-IQ formation by fluxes of NO and superoxide.

Human PMNs were used to simulate the in vivo condition. In the presence of SpN, PMNs produced increased amounts of N-NO-IQ following stimulation with PMA. The latter results in the release of MPO and causes an oxidant burst that produces superoxide, which dismutates to H2O2 (33). This oxidant burst is mediated by NADPH-oxidase reduction of molecular oxygen. Maximum N-NO-IQ formation was observed with SOD, suggesting that peroxynitrite was not involved. The increase observed with SOD could be due to decreased inhibition of MPO by superoxide and/or increased presence of H2O2 (33). Catalase, by hydrlyzing H2O2, reduced MPO activity and reduced N-NO-IQ formation. The lack of azide inhibition and inhibition by NADH is similar to that observed with MPO in Table I and consistent with oxidative nitrosylation. Studies assessing nitration and chlorination by PMNs have also demonstrated increased activity with SOD. However, nitration and chlorination were inhibited by catalase and azide (28, 34). N-NO-IQ is the only major product formed by stimulated PMNs, accounting for greater than 90% of the increased metabolism of IQ observed with PMA-stimulated PMNs treated with SOD. This increase in N-NO-IQ formation observed with MPO + SOD was not detected with PMNs from two unrelated MPO-deficient patients that exhibited a normal oxidant burst. Thus, MPO potentiates IQ nitrosylation by stimulated human PMNs exposed to NO.

Inflammatory bowel disease is a chronic inflammatory disease associated with marked mucosal infiltration of macrophages, lymphocytes, and neutrophils in which high levels of iNOS and 3-nitrotyrosine, a marker of RNOS, are detected (35–37). The temporal and spatial association necessary for the various components (MPO, H2O2, and NO) to potentiate NO-mediated nitrosation probably exists in colons of inflammatory conditions such as inflammatory bowel disease, atherosclerosis, and asthma. Preliminary results in our laboratory have demonstrated ovine prostaglandin H synthase potentiation of N-NO-IQ formation. Indirect effects of NO attributed to autoxidation (2) might be due to peroxynitrite. Whereas nitrosation is thought to occur at sites of NO generation (2, 40), our results suggest that this highly diffusible stable gas initiates nitrosation at sites of neutrophil infiltration. This information provides insight for therapeutic intervention and prevention of certain inflammatory diseases.

Acknowledgment—We thank Priscilla De Haven for excellent technical assistance.

REFERENCES

1. Ignarro, L. J. (2000) *Nitric Oxide: Biology and Pathobiology*, Academic Press, New York.

2. Grisham, M. B., Jourd’Heuil, D., and Wink, D. A. (1999) *Am. J. Physiol.* 39, G315–G321.

3. Stamler, J. S., Singel, D. J., and Loscalzo, J. (1992) *Science* 258, 1198–1202.

4. Lewis, R. S., and Deen, W. M. (1994) *Chem. Res. Toxicol.* 7, 568–574.

5. Eich, R. F., Li, T., Lemon, D. D., Doherty, D. H., Curry, S. H., Atkine, J. F., Mathews, A. J., Johnson, J., Smith, R. D., Phillips, G. N., and Olson, J. S. (1996) *Biochemistry* 35, 6976–6983.

6. Feilisch, M., Rassaf, T., Moinmeh, S., Singh, N., Bryan, N. S., Jourd’Heuil, D., and Kelm, M. (2002) *FASEB J.* 16, 1775–1785.

7. Rassaf, T., Bryan, N. S., Kelm, M., and Feilisch, M. (2002) *Free Radic. Biol. Med.* 33, 1590–1596.

8. Abu-Ouda, H. M., and Hazen, S. L. (2000) *J. Biol. Chem.* 275, 37524–37532.

9. Nauseef, W. M., and Hazen, S. L. (2000) *J. Biol. Chem.* 275, 5425–5430.

10. Abu-Ouda, H. M., Khashawneh, M. Y., Sohn, J. T., Murray, P., Haxhiu, M. A., and Hazen, S. L. (2001) *Biochemistry* 40, 11866–11875.

11. Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W., Zhang, C., Toossan, A., Castro, L., Lu, A. J., Nauseef, W. M., White, C. R., and Freeman, B. A. (2002) *Science* 296, 2391–2394.

12. Espey, M. G., Miranda, K. M., Thomas, D. D., and Wink, D. A. (2001) *J. Biol. Chem.* 276, 30085–30091.

13. Laskymi, V. M., Hsu, F. F., and Zenser, T. V. (2002) *Chem. Res. Toxicol.* 15, 1059–1068.

14. Marugoe, C. M., Morley, D., Wink, D. A., Dunams, T. M., Saavedra, J. E., Hoffman, A., Aove, A. A., Isaac, L., Hrabie, J. A., and Keefler, L. K. (1991) *J. Med. Chem.* 34, 3242–3247.

15. Jones, M. C., and Henzel, W. J. (1996) *Biochemistry* 35, 3699–3675.

16. Tsuda, M., Negishi, C., Makino, R., Sato, S., Yamaizumi, Z., Hirayama, T., and Poore, C. M., Cook, J. A., and Fords, P. C. (1996) *Free Radic. Biol. Med.* 23, 709–716.

17. Naus239eef, W. M., Cogley, M., and McCormick, S. (1996) *J. Biol. Chem.* 271, 9546–9549.

18. Nausneef, W. M., Cogley, M., Bock, S., and Petrides, P. E. (1998) *J. Leukocyte Biol.* 63, 364–369.

19. Bahir, B., Kipnes, R. S., and Carmute, J. T. (1973) *J. Clin. Invest.* 52, 741–744.

20. Laskymi, V. M., Hsu, F. F., and Zenser, T. V. (2004) *Chem. Res. Toxicol.* 17, 709–716.

21. Brennan, N. A., Fu, X., Shen, Z., Song, W., Frost, H., Vade, C., Narine, L., Lenkwiescev, E., Borchers, M. T., Lu, A. J., Lee, J. J., Lee, N. A., Aube-Soud, H. M., Ischiropoulos, H., and Hazen, S. L. (2002) *J. Biol. Chem.* 277, 17415–17427.

22. Rettori, D., Yang, J., Dias Jr., L. C., and Cadenas, E. (2002) *Free Radic. Biol. Med.* 33, 685–690.

23. Wink, D. A., Darkshire, J. F., Nima, R. W., Saavedra, J. E., and Ford, P. C. (1993) *Chem. Res. Toxicol.* 6, 23–27.

24. Espey, M. G., Thomas, D. D., Miranda, K. M., and Wink, D. A. (2002) *Proc. Natl. Acad. Sci.* 99, 11127–11132.

25. Goldstein, S., and Czapski, G. (2000) *Chem. Res. Toxicol.* 13, 736–741.

26. Mottley, C., and Mason, R. P. (1989) *Chem. Res. Toxicol.* 2, 159–166.

27. Mottley, C., and Mason, R. P. (1989) *Chem. Res. Toxicol.* 2, 159–166.

28. Van Dalen, C. J., Winterbourn, C. C., Senthilmohan, R., and Kettle, A. J. (1996) *Free Radic. Biol. Med.* 275, 11866–11875.

29. van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. C. (1997) *J. Biol. Chem.* 272, 7617–7625.

30. Laskymi, V. M., Hsu, F. F., Davis, B. B., and Zenser, T. V. (2000) *Chem. Res. Toxicol.* 13, 891–899.

31. Jiang, Q., and Hurst, J. K. (1997) *J. Biol. Chem.* 272, 32767–32772.

32. Jourd’Heuil, D., Jourd’Heuil, F. L., Kutchuckian, P. S., Musah, R. A., Wink, D. A., and Grisham, M. B. (2001) *J. Biol. Chem.* 276, 28799–28805.
33. Kettle, A. J., and Winterbourn, C. C. (1997) *Redox Rep.* 3, 3–15
34. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) *Nature* 391, 393–397
35. Hussain, S. P., Amstad, P., Raja, K., Ambs, S., Nagashima, M., Bennett, W. P., Shields, P. G., Ham, A.-J., Swenberg, J. A., Marrogi, A. J., and Harris, C. C. (2000) *Cancer Res.* 60, 3333–3337
36. Singer, I. I., Kawka, D. W., Scott, S., Weidner, J. R., Mumford, R. A., Riehl, T. E., and Stenson, W. F. (1996) *Gastroenterology* 111, 871–885
37. Dijkstra, G., Moshage, H., Van Dullemen, H. M., De Jager-Krikken, A., Tiebosch, A. T. M. G., Kleibeuker, J. H., Jansen, P. L. M., and Van Goor, H. (1998) *J. Pathol.* 186, 416–421
38. Sugimura, T. (2000) *Carcinogenesis* 21, 387–395
39. O'Donnell, V. B., Coles, B., Lewis, M. J., Crews, B. C., Marnett, L. J., and Freeman, B. A. (2000) *J. Biol. Chem.* 275, 38239–38244
40. Espey, M. G., Miranda, K. M., Thomas, D. D., Xavier, S., Citrin, D., Vitek, M. P., and Wink, D. A. (2002) *Ann. N. Y. Acad. Sci.* 962, 195–206
Myeloperoxidase Potentiates Nitric Oxide-mediated Nitrosation
Vijaya M. Lakshmi, William M. Nauseef and Terry V. Zenser

J. Biol. Chem. 2005, 280:1746-1753.
doi: 10.1074/jbc.M411263200 originally published online November 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411263200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 38 references, 15 of which can be accessed free at
http://www.jbc.org/content/280/3/1746.full.html#ref-list-1