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Covalent Cross-Linking of Immune Complexes by Oxygen Radicals and Nitrite¹

Masaaki Uesugi,* Takeshi Hayashi,† and Hugo E. Jasin²*

We have shown that polymorphonuclear neutrophils mediate the covalent cross-linking of immune complexes (ICs) using H₂O₂ and myeloperoxidase (MPO). Moreover, activated superficial chondrocytes produce large amounts of nitric oxide (NO), suggesting that high concentrations of these radicals may interact at the cartilage surface in rheumatoid arthritis. We describe the effects of the interaction of NO and its decay product, NO₂, with H₂O₂ and MPO on IC cross-linking. Cross-linking was measured by resistance to the guanidine extraction of plastic-bound ICs. The combination of H₂O₂, MPO, and NO in the absence of O₂ did not alter the magnitude of cross-linking. The addition of O₂ resulted in a significant enhancement of cross-linking (p < 0.004), suggesting that nitrite was responsible for the increase observed. Indeed, NaNO₂ greatly increased H₂O₂-dependent cross-linking (control: 29.2 ± 3.8; 1 mM NaNO₂: 58.4 ± 9.9; 10 mM: 60.4 ± 4.2% cross-linking, p < 0.0002). Sodium azide, which is an inhibitor of MPO, completely inhibited cross-linking. These results indicated that the product of interaction of H₂O₂ and NO₂ mediated by MPO may be responsible for the increase in cross-linking. The generation of nitrotyrosine was demonstrated when NO₂ was added to the cross-linking system. Cross-linking was also shown with an O₂⁻-generating system and NO. Peroxynitrite alone mediated cross-linking (100 μM ONOO⁻ : 40.3 ± 1.9% cross-linking; p < 0.0002), and the addition of MPO significantly enhanced this effect (100 μM: 57.7 ± 6.0%; p < 0.0002 with respect to no nitrite control). Oxygen radicals and NO are likely to interact at the cartilage surface in inflammatory arthritis, resulting in an increase in oxidative damage within the joint cavity. The Journal of Immunology, 1998, 161: 1422–1427.

Highly reactive oxygen-derived species (ROS) play important roles in defense mechanisms against infections (1) and in tissue injury in inflammatory reactions (2, 3). One of the main reactive products generated by activated phagocytic cells is hypochlorous acid (HOCl), which is formed by the enzyme myeloperoxidase (MPO) acting on H₂O₂ in the presence of chloride ion. This oxidant is mainly responsible for the intracellular killing of phagocytosed bacteria (1); it also plays a role in tissue injury because it mediates the oxidative modification of tissue macromolecules, the deamination and decarboxylation of proteins with the formation of aldehydes, sulfhydryl oxidation, and covalent cross-linking (4–10). We have shown previously that HOCl is responsible for the covalent cross-linking of proteins, namely IgG, resulting in the generation of immune complex (IC)-like aggregates with phlogogenic capacity (10, 11). Moreover, IgG aggregates with evidence of oxidative modification have been found in the synovial fluids of patients with rheumatoid arthritis (12). Previous work in our laboratory also indicated that the majority of articular cartilage specimens obtained from patients with rheumatoid arthritis contained ICs and complement that were tightly bound and sequestered on the pannus-free articular surface (13). It was suggested that these complexes gave rise to the phenomenon of frustrated phagocytosis (14, 15) on the cartilage surface, resulting in the egression of noxious neutrophil granular component such as ROS and proteases. Indeed, there is abundant evidence that a variety of neutrophil products are present at the cartilage surface and at the cartilage-pannus junction (16–19).

Articular chondrocytes generate large amounts of nitric oxide (NO) when appropriately stimulated with cytokines or bacterial products (20, 21). We and others have recently shown that most of the oxidant gas is secreted by the chondrocytes located close to the articular surface (22, 23). Moreover, recent data indicate that the human neutrophil is also able to secrete NO when appropriately stimulated (24). Chemical data suggest that NO reacts with O₂⁻ and other reactive molecules to form stronger oxidant molecules such as peroxynitrite (25, 26) and nitryl chloride (27). Similarly, the dismutation of O₂⁻ by superoxide dismutase and the subsequent generation of H₂O₂ reacting with nitrite in the presence of MPO may give rise to another strong oxidant, nitrogen dioxide (NO₂) (28). Since the considerations discussed above indicate that high concentrations of ROS, neutrophil granular material such as MPO, and NO may interact at the articular surface in inflammatory arthritis, the present studies were conducted to study the effects of NO and its decay products on the protein cross-linking activity of neutrophil-derived ROS.

Materials and Methods

Reagents

Sodium hypochlorite and 5,5’dithiobis(2-nitrobenzoic acid) were purchased from Sigma (St. Louis, MO). Rabbit anti-BSA Ab was purified as described previously (10, 29). Purified human MPO was a gift from Dr. Isaac Ginsburg (Hebrew University, Hadassah School of Dental Medicine, Jerusalem, Israel) (10). NO gas (10% concentration) was purchased from Matheson (Montgomeryville, PA). HOCl was obtained by vacuum distillation of a 5% solution of sodium hypochlorite (pH 7.5) (30). The resulting

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¹ Abbreviations used in this paper: ROS, reactive oxygen-derived species; IC, immune complex; GO, glucose oxidase; MPO: myeloperoxidase; XO: xanthine oxidase; PMN, polymorphonuclear neutrophil; NO, nitric oxide; TNB, 5-thio-2-nitrobenzoic acid; PBS-RS, PBS containing 10% heat-inactivated rabbit serum; SNP, sodium nitroprusside.

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Preparation of NO-saturated HBSS

HBSS was saturated with NO gas as described by Harry et al. (32) with modifications. A total of 10 ml of HBSS without phenol red was bubbled for 15 min with nitrogen gas and subsequently with 10% NO gas followed by air for 15 min in studies in which the presence of O2 was required. NO-HBSS was prepared freshly before use. NO2 concentrations were determined by the Griess reaction (33). Briefly, 100 µl of diluted NO-saturated HBSS or standard solution was mixed with 50 µl of 0.1% naphthylenediamine dihydrochloride and 50 µl of 1% sulfanilamide, 5% phosphoric acid. Standards were prepared with 10 to 250 µM sodium nitrite. After a 5-min incubation, the OD at 540 nm was measured with a microplate reader (400ATC, SLT Labs, Triangle Park, NC).

Synthesis of 5-thio-2-nitrobenzoic acid (TNB)

The synthesis of TNB was conducted according to Ching et al. (34). A total of 20 mM of sodium borohydride was added to a 1 mM solution of 5,5’-dithiobis(2-nitrobenzoic acid) in 50 mM potassium phosphate buffer (pH 6.6) plus 5 mM EDTA. The solution was incubated at 37°C for 30 min. The concentrations were determined by spectrophotometric analysis (ε412 = 13,600 M⁻¹ cm⁻¹) (35).

Synthesis of peroxynitrite

Peroxynitrite was synthesized from sodium nitrite and hydrogen peroxide according to Rakesh and Victor (36). Two syringes containing 6 ml of 2 M cold NaNO2 and 6.6 ml of 30% cold H2O2 plus 5% HNO3 were used to mix the two solutions rapidly before neutralization with 6 ml of 4.2 M NaOH solution that had been stirred and kept in an ice bath. Residual H2O2 was removed by passing the solution through a column of granular MnO2 that had been rinsed with 0.5 M NaOH and kept at 4°C. The concentration of peroxynitrite was determined spectrophotometrically (ε390 = 1670 M⁻¹ cm⁻¹).

Cross-linking of plastic-bound ICs

We have described previously the methodology for the generation of cross-linked ICs on plastic plates (10). Round-bottom, flexible, polivinyl chloride 96-well microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 50 µl BSA 20 µg/ml in saline solution. After a 2-h incubation, the wells were washed twice with 100 µl of PBS and blocked twice with 100 µl of PBS containing 1% heat-inactivated rabbit serum (PBS-RS) for 10 min. ICs were generated by 2 h of incubation with 50 µl of a 1:100 dilution of affinity-purified 125I-labeled rabbit-anti-BSA Ab in PBS-RS at room temperature. The cross-linking of ICs was achieved by incubation with MPO, glucose oxidase (GO), and glucose as a source of hydrogen peroxide. The wells contained 22 µl of MPO, 0.1 U/ml of GO with or without NaNO2, or 0.1 µU/ml of NO-saturated HBSS in a total volume of 200 µl. Additional experiments were conducted with 10 µM HOCl, MPO, and GO with or without 1.0 µM xanthine oxidase (XO) and 1.5 mM hypoxanthine with or without 2.0 mM sodium nitroprusside (SNP). After incubation with MPO, HOCl, or XO at 37°C for 1 h, the reaction was terminated by removing the HBSS and washing twice with 100 µl of PBS-RS. Sextuplicate wells were divided into two groups. The first group of triplicate wells was washed twice with 100 µl of 0.1 N HCl containing 10% rabbit serum and twice with 100 µl of 4 M guanidine solution (4 M guanidine, 0.1 M EDTA, 0.1 M NaCl, 0.05 M Tris, and 0.01 M sodium azide) for 10 min each to remove noncovalently bound IgG. The other group of wells was washed four times with PBS-RS for 10 min. Controls consisted of wells incubated without MPO. Plastic-bound radioactivity was measured in a Cobra-2 autogamma scintillation spectrometer (Packard Instrument Company, Meriden, CT). The quantification of the cross-linking of ICs was calculated as the percent radioactivity bound = cpm/well washed with HCl and 4 M guanidine/cpm/well washed with PBS × 100; net percent bound = percent bound with MPO – percent bound without MPO.

Colorimetric assay for oxidative activity

The oxidative activity of MPO or polymorphonuclear neutrophils (PMNs) was measured as described by Ching et al. (34). The wells containing 250 µl of MPO, 0.01 U/ml of GO, or 5 × 106 PMN/ml in and 70 µM of TNB in HBSS with or without 0.1 to 10 mM NaNO2 were incubated for 1 h at 37°C. The OD was measured at 412 nm.

![FIGURE 1. Effect of NO gas in air-saturated HBSS on the H2O2-MPO-mediated covalent cross-linking of ICs. Plastic-bound BSA/anti-BSA complexes were incubated for 1 h with 22 µM of MPO and 0.1 U/ml GO ± NO gas. *p < 0.0002; n = 4.](http://www.jimmunol.org/)

Colorimetric assay for H2O2

Hydrogen peroxide was measured as described by Pick and Keisari (37). The method is based on the colorimetric measurement of oxidized phenol red. Concentrations were calculated from a standard curve using serial dilutions of an H2O2 solution.

Detection of nitrotyrosine

The nitrosation of the tyrosine residues of BSA mediated by H2O2, MPO, and nitrite was detected by radioimmunooassay as described by Crow et al. with some modifications (38). We coated 96-well polystyrene plates with 50 µl BSA solution, 20 µg/ml for 2 h at room temperature. The coated wells were incubated with 44 µM of MPO, 0.1 U/ml of GO, and 0.1 to 10 mM NaNO2 in chloride-free buffer (0.02 M KH2PO4, 0.08 M NaH2PO4, 10 mg/ml MgSO4, and 100 mg/ml glucose (pH 7.4)) for 1 h at 37°C in the presence of increasing concentrations of sodium nitrite or sodium nitrate as control. Additional controls consisted of wells in which MPO was omitted. The wells were then washed twice with chloride-free phosphate buffer, twice with PBS, and twice with PBS and 0.05% Tween 20 for 10 min. Next, the wells were incubated with 50 µl of a 1:500 dilution of anti-nitrotyrosine Ab in PBS and 0.05% Tween 20 for 2 h at room temperature. After washing twice with PBS and blocking twice with PBS and 0.05% Tween 20, the wells were incubated with 50 µl of a 1:100 dilution of 125I-labeled goat anti-mouse Ig in PBS and 0.05% Tween 20 for 2 h. Subsequently, the wells were washed twice and counted with an autogamma scintillation spectrometer.

Statistics

The number of experiments (n) refers to the number of separate experiments, where the results for each experiment were the mean value of triplicate wells. The results were analyzed by ANOVA.

Results

Incubating ICs with GO-glucose as a source of H2O2 and also with MPO resulted in covalent cross-linking of high magnitude as shown previously (Fig. 1) (10). Incubating ICs with GO, MPO, and NO-saturated HBSS in the presence of O2, however, almost doubled cross-linking at an initial concentration of 1 mM (no NO: 31.7 ± 5.2%; 0.01 mM NO: 25.1 ± 5.2%; 0.1 mM NO: 43.6 ± 5.2%; 1 mM NO: 62.1 ± 5.7%).
4.0; 1 mM NO: 67.3 ± 5.9%; \(p < 0.0002\) (Fig. 1). In the absence of \(O_2\), no increase in covalent cross-linking was detected (data not shown).

The above results suggested that the oxidative product of NO, \(NO_2\), was responsible for the increase in IC cross-linking. Indeed, the \(H_2O_2\)-dependent cross-linking of plastic-bound ICs in the presence of MPO was significantly increased by 1 and 10 mM \(NaNO_2\) (no \(NO_2\): 29.2 ± 3.8%; 1 mM \(NaNO_2\): 58.4 ± 9.9%; \(p < 0.008\); 10 mM \(NaNO_2\): 60.4 ± 4.2%) (Fig. 2). Sodium nitrate in concentrations as high as 10 mM did not affect cross-linking (data not shown). Moreover, when \(NaNO_2\) was added to an optimal dilution of purified \(HOCl\), the salt inhibited protein cross-linking in a dose-dependent fashion (Fig. 3). As previously shown (10), \(H_2O_2\) and MPO in the absence of chloride resulted in insignificant cross-linking. However, the absence of chloride did not affect nitrite-dependent cross-linking (Fig. 4). These findings reinforced the suggestion that nitrite was able to mediate this phenomenon in the presence of \(H_2O_2\) and MPO. This conclusion was supported by the observation that sodium azide, which is an inhibitor of MPO, also inhibited nitrite-dependent cross-linking (Fig. 5). The aggregate of these results indicated that a nitrite-mediated enhancement of protein cross-linking required the presence of MPO and \(H_2O_2\); \(HOCl\) was not required, and its cross-linking capacity was inhibited by nitrite.

The next series of experiments was designed to investigate the effects of nitrite on MPO activity. The activity of MPO, as measured by the oxidation of TNB, was significantly enhanced by nitrite in a dose-dependent manner (Fig. 6). Similarly, the addition of increasing concentrations of nitrite to stimulated PMNs resulted in a significant increase in oxidative activity as measured with TNB as a substrate (Fig. 7). To rule out the possibility that the nitrite effects observed may have been due to increased \(H_2O_2\) production by \(GO\), the enzyme activity was measured in the presence or absence of sodium nitrite. As shown in Figure 8, 1 mM of sodium nitrite did not affect the production of \(H_2O_2\); 10 mM of sodium nitrite partially reduced its production in the face of a concomitant increase in protein cross-linking, as shown previously (Fig. 2).

Since the nitrosation of tyrosine can be generated by the product of \(H_2O_2\) and nitrite (28), experiments were conducted to examine this possibility. Plastic-bound BSA was incubated with the \(H_2O_2\)-generating system in the presence or absence of nitrite. As shown.

**FIGURE 2.** Effect of sodium nitrite on the \(H_2O_2\)-MPO-mediated cross-linking of ICs. For experimental details, see Figure 1. *\(p < 0.008\); **\(p < 0.0006\); \(n = 4\).

**FIGURE 3.** Effect of sodium nitrite on the HOCl-mediated cross-linking of ICs. Plastic-bound ICs were incubated with 10 \(\mu M\) freshly distilled \(HOCl\) ± sodium nitrite. *\(p < 0.02\); **\(p < 0.004\); \(n = 3\).

**FIGURE 4.** Effect of sodium nitrite on the chloride-free \(H_2O_2\)-MPO-mediated cross-linking of ICs. *\(p < 0.01\); **\(p < 0.0001\); \(n = 3\).

**FIGURE 5.** Effect of sodium azide on the \(H_2O_2\)-MPO-mediated cross-linking of ICs. Control: no sodium nitrite or azide (100%); Open bars: with sodium azide; stippled bars: no sodium azide. *\(p < 0.04\) with respect to an absence of azide control; \(n = 3\).
in Figure 9, easily detectable nitrosation was seen after a 1-h incubation with 0.1 mM nitrite, reaching a maximal increase in nitrotyrosine at a nitrite concentration of 1 mM. No nitrosation was detected with nitrate or when MPO was omitted; this observation supports our previous findings, which indicated that H₂O₂ and nitrite were necessary and sufficient for the generation of nitrotyrosine and protein cross-linking.

The aggregate of these results suggested that the product of interaction between NO₂⁻ and H₂O₂ that was catalyzed by MPO may have been responsible for the increase in IC cross-linking observed. Since activated PMNs generate O₂⋅*, an alternative pathway may involve the product of the interaction of this radical with NO, namely peroxynitrite. The next series of experiments were designed to explore this possibility. Adding increasing concentrations of NaNO₂ to the O₂⋅*-generating system did not result in any detectable cross-linking or nitrotyrosine generation (data not shown). However, significant protein cross-linking was obtained (0.1 mU/ml XO: 4.1 ± 0.9%, p < 0.02; 1.0 mU/ml XO: 5.2 ± 1.4%, p < 0.05, crosslinking over control without SNP) when a NO donor (SNP, 1 mM) was added to the O₂⋅*-generating system, suggesting that the resulting peroxynitrite was responsible for these results. Indeed, increasing concentrations of freshly synthesized peroxynitrite alone mediated cross-linking (10 μM ONOO⁻: 2.2 ± 3.7%; 10 μM ONOO⁻: 0.7 ± 3.7%; 100 μM ONOO⁻: 40.3 ± 1.9% cross-linking; p < 0.002) and the addition of MPO significantly enhanced this effect (1.0 μM ONOO⁻: 6.0 ± 5.1%; 10 μM ONOO⁻: 7.1 ± 5.5%; 100 μM ONOO⁻: 55.7 ± 6.0%; p = 0.05 vs no MPO) (Fig. 10).

Discussion

There is considerable evidence that in inflammatory foci such as the involved joint in rheumatoid arthritis, ROS and NO-derived reactive products play important pathogenic roles in tissue damage (39). Thus, evidence of oxidative damage of rheumatoid synovial fluid proteins has been reported by us (12) and others (40); in addition, increased concentrations of nitrite (41) and the presence of nitrotyrosine (42) in synovial fluids indicate that NO-derived products also play a significant role. There is also abundant evidence that a variety of neutrophil components are present at the cartilage surface and at the cartilage-pannus junction (16-19). We have previously shown that one of the major oxidative products of PMNs, namely HOCl, is responsible for the covalent cross-linking of ICs (10). Moreover, we have also obtained evidence that superficial articular chondrocytes generate large amounts of NO when appropriately stimulated with cytokines or bacterial products.
Recent data by other investigators indicate that the human neutrophil is also able to secrete NO when appropriately stimulated (24). Chemical data suggest that NO reacts with $O_2^-$ and other reactive molecules to form strong oxidant molecules such as peroxynitrite (25, 26) and nitryl chloride (27). Alternatively, $O_2^-$ derived $H_2O_2$ reacting with nitrite also gives rise to another strong oxidant, probably NO$_2^-$ (28). However, NO has been shown to have both deleterious and protective effects in pathologic states. The considerations discussed above suggest that high concentrations of ROS, PMN granular products, and NO may interact at the articular surface in inflammatory arthritis; this finding prompted the present studies dealing with the effects of NO and its decay products on the oxidizing activity of PMN-derived ROS.

The results obtained indicated that NO gas under anaerobic conditions did not interfere with HOCl-mediated protein cross-linking. However, NO significantly enhanced cross-linking in the presence of $O_2$, suggesting that the oxidation end-product of NO, namely nitrite, may have been responsible for this enhancement. Additional experiments clearly indicated that NO$_2^-$ and not NO$_2$ was able to increase MPO-mediated cross-linking in the absence of chloride ion. Indeed, NO$_2^-$ was shown to partially quench HOCl-mediated protein cross-linking, suggesting that cross-linking was solely the result of the interaction of NO$_2$ with MPO and $H_2O_2$. The enhanced oxidative capacity of MPO in the presence of NO$_2$ was also evident when an alternative substrate (TNB) was used, and, more pertinent to biology, when PMNs were the source of ROS. These findings are also in accordance to previous work showing that MPO reacts directly both with NO$_2$ (28, 43) and ONOO$^-$ (44).

Finally, the covalent cross-linking of ICs was demonstrated with a source of $O_2^-$ and a NO donor, and in addition with a low concentration of freshly synthesized ONOO$^-$ that was further enhanced by the addition of MPO. The modest increase in cross-linking observed when MPO was added suggests that ONOO$^-$ was able to mediate this reaction by itself, since most of the cross-linking observed took place with the oxidant alone.

The chemical pathways resulting in the modification of macromolecules as found in inflammatory foci in vivo are complicated and have not been completely clarified. The $t_{1/2}$ of NO is probably very short in inflammatory fluids (45), reacting with various substrates and decaying to its oxidized byproduct, NO$_2$, which has been found to be increased in inflammatory synovial fluids (41). Since NO is generated intracellularly, it may react with $O_2^-$ to

form ONOO$^-$, which is a well-known nitrosating and cross-linking species (38, 39). Moreover, $O_3^-$ generated by inflammatory cells would readily dismutate to $H_2O_2$ in the presence of the superoxide dismutase found in inflammatory cells and rheumatoid synovial fluids (46, 47). This oxidant may mediate the same effects in conjunction with nitrite and MPO as shown in these studies. To complicate things even further, tyrosine chlorination and nitrosation may also occur as a result of the interaction of HOCl and NO, giving rise to nitryl chloride or similar chloride- and NO-derived free radicals (27, 48). Finally, ONOO$^-$ can decay spontaneously to form hydroxyl radical and nitrogen dioxide (25), another molecule which is thought to mediate tyrosine nitrosation (28). The presence of the inhibitors of these reactions (chloride, as shown here, or bicarbonate (49) in body fluids) introduces yet another complicating factor. At the present time, it is not possible to determine which or how many of these pathways may be operative in inflammation.

Regardless of the relative importance of each of the reactions outlined above, one of the possible corollaries of the results presented stems from our previous observations that both in the superficial layers of cartilage obtained from an experimental model of rheumatoid arthritis (50) and in superficial cartilage from patients with this disease (13), we found large amounts of ICs tightly bound to the tissue, so that even exposure to high molar guanidine solutions was not sufficient to extract them. Extraction could only be achieved with a dissolution of the tissue using bacterial collagenase (51), suggesting that the ICs were covalently bound to the underlying cartilage macromolecules. Ongoing studies in our laboratory may help clarify some of the unresolved issues discussed above.

References


