Peroxynitrite, formed by the reaction between nitric oxide and superoxide, has been shown to induce protein nitration, which compromises protein function. We hypothesized that peroxynitrite may regulate cytokine function during inflammation. To test this hypothesis, the neutrophil chemotactic activity (NCA) of interleukin-8 (IL-8) incubated with peroxynitrite was evaluated. Peroxynitrite attenuated IL-8 NCA in a dose-dependent manner (p < 0.01) but did not significantly reduce NCA induced by leukotriene B4 or complement-activated serum. The reducing agents, dithionite, deferoxamine, and dithiothreitol, reversed and exogenous L-tyrosine abrogated the peroxynitrite-induced NCA inhibition. Papain NONOate [N-(3-ammoniopropyl)-N-(n-propyl)aminol-diazene-1-ium-1,2-dialase] or sodium nitroprusside, NO donors, or a combination of xanthine and xanthine oxidase to generate superoxide did not show an inhibitory effect on NCA induced by IL-8. In contrast, small amounts of SIN-1, a peroxynitrite generator, caused a concentration-dependent inhibition of NCA by IL-8. Consistent with its ability to reduce NCA, peroxynitrite treatment reduced IL-8 binding to neutrophils. Nitrotyrosine was detected in the IL-8 incubated with peroxynitrite by enzyme-linked immunosorbent assay. These findings are consistent with nitration of tyrosine by peroxynitrite with subsequent inhibition of IL-8 binding to neutrophils and a reduction in NCA and suggest that oxidants may play an important role in regulation of IL-8-induced neutrophil chemotaxis.

Interleukin-8 (IL-8), an 8400-dalton protein, is synthesized and secreted by monocytes, macrophages, and other cells, including epithelial and endothelial cells, lymphocytes, fibroblasts, and keratinocytes (1–3). Current concepts suggest an important role for IL-8 in the pathogenesis of neutrophil-mediated inflammation because of the potent neutrophil chemotactic activity (NCA) of IL-8 and its detection in affected tissues or body fluids taken from patients suffering from neutrophilic inflammatory lesions (3). Peroxynitrite is produced by the rapid reaction of nitric oxide (NO) and superoxide (4, 5). In addition to its role in oxidative reactions, peroxynitrite also nitrates free or protein-associated tyrosine to form the stable product nitrotyrosine by addition of a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine (6). Several studies have shown that peroxynitrite-induced protein nitration may compromise protein function. For example, nitration of tyrosine residues on human IgG abrogates C1q binding (7) and inhibits protein phosphorylation by tyrosine kinase (8).

Recently, the tyrosine residue at position 13 of IL-8 has been shown to be important in neutrophil chemotaxis (9). We hypothesized peroxynitrite might regulate NCA by nitrating tyrosine residues. To test this hypothesis, the chemotactic responses of human neutrophils to IL-8 incubated with peroxynitrite and other compounds were evaluated in vitro. We found that peroxynitrite and 3-morpholinosydnonimine (SIN-1), a donor of peroxynitrite, significantly attenuated IL-8-induced NCA. In contrast activated serum and LTB4-induced NCA was not inhibited by peroxynitrite significantly. These data suggest that peroxynitrite plays an important role in regulating NCA during inflammation.

**EXPERIMENTAL PROCEDURES**

*Measurement of NCA—Human polymorphonuclear leukocytes were isolated from venous blood as described previously (10, 11). Briefly, 15 ml of venous blood was obtained from healthy volunteers and then sedimented with 3% dextran in isotonic saline for 45 min to separate white blood cells from red blood cells. The leukocyte-rich upper layer was collected, and neutrophils were separated from mononuclear cells by Ficoll-Paque density centrifugation (Histopaque 1077, Sigma). Contaminating red blood cells were removed by hypotonic lysis (0.1% KHCO3 and 0.8% NH4Cl). The suspension was then centrifuged at 400 × g for 5 min and washed three times in HBSS (Biofluids, Rockville, MD). The resulting cell pellet, as determined by trypan blue and erythrocyte exclusion, consisted of >96% neutrophils and >98% viable cells. The cells were suspended in Gey’s balanced salt solution (Life Technologies, Inc.) containing 2% bovine serum albumin (Sigma) at a final concentration of 3 × 106 cells/ml. NCA was assayed in 48-well microchemotaxis chambers (Neuroprobe, Inc., Cabin John, MD) as described previously (11). The bottom wells of the chamber were filled with 25 μl of the chemotactic stimulus or media in duplicate. A 10-μm thick polyvinylpyrrolidone-free polycarbonate filter with 3-μm pores was placed over the samples. The silicon gasket and the upper pieces of the chamber were applied, and 50 μl of the chemotactic stimulus were added to the bottom well. The chambers were incubated in humidified air in 5% CO2 at 37 °C for 30 min. Nonmigrated cells were wiped away from the filter. The filter was immersed in methanol for 5 min, stained with a modified Wright’s stain, and mounted on a glass slide. Cells that had completely migrated through the filter were counted using light microscopy. NCA was expressed as 1001; E-mail: Richard.Robbins2@med.va.gov.

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1. The abbreviations used are: IL-8, interleukin-8; NCA, neutrophil chemotactic activity; NO, nitric oxide; SIN-1, 3-morpholinosydnonimine; LTB4, leukotriene B4; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; Papain NONOate, (2)-(3-ammoniopropyl)-N-(n-propyl)aminol-diazene-1-ium-1,2-dialase.

96% neutrophils and 98% viable cells.
the mean number of migrated cells per high power field from duplicate wells.

Effects of Peroxynitrite on IL-8-induced NCA—Peroxynitrite was evaluated for its capacity to modulate IL-8-induced NCA in vitro. Human recombinant IL-8 (R & D Systems, Inc., Minneapolis, MN) was incubated for 2 h at 37°C with each concentration of peroxynitrite (Calbiochem) before the NCA assay. In control experiments, IL-8 was incubated with medium alone.

Effects of Peroxynitrite on LTB4 and Activated Serum-induced NCA—The capacity of peroxynitrite to modulate LTB4 and activated serum-induced NCA was evaluated for comparison with IL-8. LTB4 (10 -6 M, Sigma) or complement-activated serum (1:10 dilution) was incubated with peroxynitrite (10 -2 M) for 2 h at 37°C before performing the NCA assay. The 2-h time point was chosen to ensure that all residual peroxynitrite had spontaneously decomposed prior to the chemotaxis assay.

Effects of NO Donors on IL-8-induced NCA—To evaluate the effects of NO to modulate IL-8 induced NCA, we utilized sodium nitroprusside or papa-NONOate (Alexis Corp., San Diego, CA) as a NO donor (13). IL-8 (10 -7 g/ml) was incubated with sodium nitroprusside (10 -3, 10 -4, 10 -5, 10 -6 M) or papa-NONOate (10 -3, 10 -4, 10 -5, and 10 -6 M) for 2 h at 37°C before performing the NCA assay. Effects of Xanthine/Xanthine Oxidase on IL-8-induced NCA—To evaluate the effect of superoxide on IL-8-induced NCA, xanthine (10 -2, 10 -3, 10 -4, 10 -5 M, Sigma) and xanthine oxidase (0.4 x 10 -3, 3.4 x 10 -3, and 3.4 x 10 -3 units/ml, Sigma) was combined to produce superoxide (14). IL-8 (10 -7 g/ml) was incubated with xanthine, xanthine oxidase, and xanthine oxidase for 2 h at 37°C before performing the NCA assay.

Effects of SIN-1 on IL-8-induced NCA—The capacity of SIN-1 (Alexis Corp.), a peroxynitrite generator (15), to modulate IL-8-induced NCA was evaluated. IL-8 (10 -7 g/ml) was incubated with SIN-1 (10 -4, 10 -5, 10 -6, 10 -7, and 10 -8 M Sigma) and xanthine oxidase (0.4 x 10 -3, 3.4 x 10 -3, and 3.4 x 10 -3 units/ml, Sigma) was combined to produce superoxide (14). IL-8 (10 -7 g/ml) was incubated with xanthine, xanthine oxidase, and xanthine oxidase for 2 h at 37°C before performing the NCA assay.

Effects of Reducing Agents on Peroxynitrite-induced Attenuation of NCA by IL-8—The capacity of the reducing agents dithiothreitol, deferoxamine, or dithionite to attenuate the effect of peroxynitrite on IL-8-induced NCA by peroxynitrite was assessed. Dithiothreitol (10 -3 M, Sigma), deferoxamine (50 μM, Sigma), dithionite (10 -3 M, Sigma), and peroxynitrite (10 -4 M) were added to IL-8 (10 -7 g/ml) and incubated for 2 h at 37°C before evaluating for NCA.

Effects of IL-8 Concentration on Peroxynitrite-induced Attenuation of NCA by IL-8—The capacity of IL-8 to preserve the attenuation of NCA by peroxynitrite was assessed. IL-8 (10 -7 g/ml) was incubated with peroxynitrite (10 -2 M) and tested for 2 h at 37°C before performing the NCA assay. IL-8 was also incubated with SIN-1 (10 -4 M) and sodium nitroprusside (10 -4, 10 -5, 10 -6, and 10 -7 M) and incubated for 2 h at 37°C before evaluating for NCA.

Detection of Nitrotyrosine on IL-8 Incubated with Peroxynitrite—Nitrotyrosine on IL-8 incubated with peroxynitrite was evaluated by addition of t-tyrosine (10 -3 M, Sigma) to IL-8 (10 -7 g/ml) before exposure to peroxynitrite (10 -2 M).

Effects of Peroxynitrite on IL-8 Binding to Neutrophils—To investigate the peroxynitrite effect on IL-8 binding to neutrophils, IL-8 was incubated with 10 -4 M of peroxynitrite for 2 h at 37°C. In control experiments, IL-8 was incubated with medium alone. Subsequently, IL-8 with or without peroxynitrite was incubated with neutrophils (10 6 or 10 5 cells) at 4°C for 30 min. Then supernatants were removed, and neutrophils were washed three times by HBSS. Neutrophils were suspended in 1 ml of PBS/Tween, sonicated for 20 s, and then centrifuged at 20,000 x g for 30 min in a refrigerated microcentrifuge to obtain a supernatant (soluble) and cellular fraction. Then IL-8 was measured in the cellular fraction using a commercially available IL-8 ELISA (R & D Systems).

**RESULTS**

Effects of Peroxynitrite on NCA by IL-8—IL-8 was incubated with peroxynitrite (10 -4 M). At each IL-8 concentration tested, peroxynitrite reduced NCA by IL-8 (Fig. 1; n = 6, p < 0.01). Incubation of IL-8 (100 ng/ml) with various amounts of peroxynitrite induced a significant, concentration-dependent attenuation of NCA (data not shown; n = 6, p < 0.01). The lowest dose of peroxynitrite tested, 10 -5 M, significantly inhibited NCA (p < 0.01). Peroxynitrite itself was not chemotactic for neutrophils (data not shown).

Effects of Peroxynitrite on LTB4 and Activated Serum-induced NCA—To ensure that the effect of peroxynitrite was not a nonspecific effect on neutrophil chemotaxis, the effect of peroxynitrite on NCA induced by LTB4 or complement-activated serum was assessed. Peroxynitrite did not significantly inhibit the NCA of LTB4 or complement-activated serum (Fig. 2; n = 4, p < 0.01).

Effects of NO Donors on IL-8-induced NCA—Neither sodium nitroprusside nor papa-NONOate significantly changed NCA induced by IL-8 at any of the concentrations tested (data not shown; n = 8–10, p > 0.05 all comparisons).

Effects of Xanthine/Xanthine Oxidase on IL-8-induced NCA—Incubation of IL-8 with xanthine, xanthine oxidase, or xanthine and xanthine oxidase did not significantly alter NCA to IL-8 (data not shown; n = 4, p > 0.05 all comparisons).

Effects of SIN-1 on IL-8-induced NCA—SIN-1 induced a significant, concentration-dependent attenuation of NCA by IL-8 (Fig. 3; n = 6, p < 0.01). The lowest dose of SIN-1 to inhibit NCA was 10 -6 M (p < 0.05). 10 -4 M of SIN-1 induced 80% attenuation of NCA by IL-8. SIN-1 itself was not chemotactic for neutrophils (data not shown). Addition of sodium nitroprusside (10 -4, 10 -5, 10 -6, 10 -7, and 10 -8 M) to SIN-1 (10 -4 M) did not differ from SIN-1 (10 -4 M) alone in reducing IL-8 NCA.
alone at any of the concentrations tested (data not shown, $p > 0.05$).

**Effects of Dithionite, Dithiothreitol, and Deferoxamine on Peroxynitrite-induced Attenuation of NCA by IL-8**—Addition of the reducing agents, dithiothreitol, deferoxamine, and dithionite to IL-8 prior to incubation with peroxynitrite attenuated the inhibition of NCA induced by peroxynitrite (Fig. 3B; $n = 4, p < 0.01$).

**Effects of l-Tyrosine on Peroxynitrite-induced Attenuation of NCA by IL-8**—Addition of l-tyrosine to IL-8 before incubating with peroxynitrite abrogated the attenuation of NCA induced by peroxynitrite (Fig. 3C; $n = 4, p < 0.01$). The addition of $10^{-4}$ M of l-tyrosine prevented the inhibition of NCA induced by $10^{-5}$ M peroxynitrite.

**Detection of Nitrotyrosine on IL-8 Incubated with Peroxynitrite**—Optical density of IL-8 with peroxynitrite incubation was significantly higher than IL-8 without peroxynitrite incubation. Peroxynitrite resulted in nitrotyrosine formation on IL-8 (Fig. 4; $n = 6, p < 0.01$). There was also an increase in the absorbance of IL-8 exposed to peroxynitrite at 428 nm consistent with the formation of 3-nitrotyrosine (16).

**Effects of Peroxynitrite on IL-8 Binding to Neutrophils**—Addition of peroxynitrite to IL-8 resulted in a reduction of IL-8 bound to the neutrophils (Fig. 5; $n = 4, p < 0.01$).

**DISCUSSION**

The results of this study show that the peroxynitrite significantly attenuated IL-8-induced NCA *in vitro*. This was not explained by an inhibition of chemotaxis by peroxynitrite. Incubation of peroxynitrite ($10^{-4}$ M) with the neutrophils prior to the chemotaxis assay did not inhibit NCA to IL-8 (data not shown), and the inhibitory effects of peroxynitrite were not significant on NCA induced by LTB$_4$ or complement-activated serum. Sodium dithionite, deferoxamine, dithiothreitol, or tyrosine attenuated the inhibition but did not affect chemotaxis alone (data not shown). NO or superoxide did not cause the reduction in IL-8 NCA because papain-NONOate and xanthine/xanthine oxidase did not show an inhibitory effect. The peroxynitrite donor, SIN-1, induced a significant concentration-dependent inhibition of NCA by IL-8. Nitrotyrosine was detected in the IL-8 incubated with peroxynitrite by ELISA. These data suggest that peroxynitrite plays an important role in regulating human neutrophil locomotion by IL-8.

**Neutrophils** migrate from the bloodstream across vascular endothelium and smooth muscle to vascular sites of inflammation, play an important role in host defenses during tissue
injury and inflammation, and also play an important role in tissue remodeling after injury (17, 18). Current concepts suggest that neutrophils are recruited from the bloodstream by chemotactic factors, such as IL-8, generated and released locally in injured tissues that may also generate NO and superoxide. Therefore, IL-8 is likely to encounter high local concentrations of peroxynitrite. Neutrophils have been shown to respond to NO (19, 20); however, NO donors or NO synthase inhibitors have produced variable effects on neutrophil chemotaxis in vitro (21–24).

Co-incubation of IL-8 with several peroxynitrite scavengers ameliorated peroxynitrite-induced NCA inhibition. The protective effect of dithiothreitol on IL-8 may suggest the involvement of cysteine residues; however, dithiothreitol has also been shown to prevent peroxynitrite-mediated nitration of tyrosine (25). The iron chelator, deferoxamine, also inhibited peroxynitrite-induced inhibition of IL-8 NCA but is also a scavenger of peroxynitrite reaction independent of iron chelation (26). Di-thionite, which has been proposed to modify 3-nitrotyrosine by substituting an amine group (27), ameliorated the peroxynitrite inhibition. In addition, L-tyrosine abrogated the peroxynitrite NCA inhibition. These results are consistent with tyrosine nitration by peroxynitrite as a mechanism for IL-8 inhibition.

IL-8 is a stimulatory peptide for neutrophils that appears to play an important role in the inflammatory process (1–3). Local injection or genetic overexpression of IL-8 in experimental animals causes specific neutrophil migration to the targeted site (28). Current concepts suggest that the mechanisms that lead to neutrophil locomotion in response to IL-8 are by binding to receptors on neutrophils (29–31). Consistent with this concept, our results demonstrate that peroxynitrite-treated IL-8 exhibited decreased binding to neutrophils.

Binding of IL-8 to neutrophil receptors has recently been studied by Schraufstatter et al. (9) who reported the importance of Tyr13 and Lys15. Mutation of amino acids 13–15 of the rabbit (His-Ser-Thr) to the human sequence (Tyr-Ser-Lys) confers the high affinity for the human IL-8 receptor to this mutated form of rabbit IL-8. Consistent with the concept that tyrosine is important in binding of IL-8 to its receptor, Clark-Lewis et al. (32) reported a 6-fold decrease in affinity for a human IL-8 molecule in which Tyr 13 was replaced with Thr. Our findings of nitrotyrosine formation on IL-8 after peroxynitrite treatment are consistent with these observations and suggest that tyrosine nitration by peroxynitrite on IL-8 is a likely mechanism altering IL-8 binding and chemotactic function. However, peroxynitrite may potentially affect protein function by other mechanisms including nitration of methionine (33) or tryptophan (34) or formation of S-nitroso-thiol groups on cysteines (35). IL-8 does not contain methionine, but cysteines are present and form disulfide bonds.

Although NO and peroxynitrite are physiological regulators, they have been shown to alter cell respiration (36, 37) and induce cell death (38). In the studies with SIN-1, neutrophils may have been exposed to peroxynitrite during their migration across the polycarbonate filter. However, neutrophils had no significant cytotoxicity as assessed by trypan blue exclusion after incubation with peroxynitrite. Furthermore, incubation of neutrophils with peroxynitrite had no significant effect on NCA induced by IL-8.

In summary, we found that peroxynitrite modulates IL-8
induced NCA in vitro. These data suggest that peroxynitrite generated at sites of inflammation may attenuate IL-8 chemo-
tactic activity and play an important role in regulating human
neutrophil locomotion during inflammation.

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