Effects of reactive oxygen and nitrogen metabolites on MCP-1-induced monocyte chemotactic activity in vitro

ETSURO SATO, KEITH L. SIMPSON, MATTHEW B. GRISHAM, SEKIYA KOYAMA, AND RICHARD A. ROBBINS

Research Services, Tucson and Overton Brooks Veterans Affairs Medical Centers, and Department of Medicine, University of Arizona, Tucson, Arizona 85723; Department of Molecular and Cellular Physiology, Louisiana State University Medical Center, Shreveport, Louisiana 71131; and The First Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto 390-8621, Japan

Sato, Etsuro, Keith L. Simpson, Matthew B. Grisham, Sekiya Koyama, and Richard A. Robbins. Effects of reactive oxygen and nitrogen metabolites on MCP-1-induced monocyte chemotactic activity in vitro. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L543–L549, 1999.—Peroxynitrite, an oxidant generated by the interaction between superoxide and nitric oxide (NO), can nitrate tyrosine residues, resulting in compromised protein function. Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that attracts monocytes and has a tyrosine residue critical for function. We hypothesized that peroxynitrite would alter MCP-1 activity. Peroxynitrite attenuated MCP-1-induced monocyte chemotactic activity (MCA) in a dose-dependent manner (P < 0.05) but did not attenuate leukotriene B4 or complement-activated serum MCA. The reducing agents dithionite, deferoxamine, and dithiothreitol reversed the MCA inhibition by peroxynitrite, and exogenous L-tyrosine abrogated the inhibition by peroxynitrite. PAPA-NONOate, an NO donor, or superoxide generated by xanthine and xanthine oxidase did not show an inhibitory effect on MCA induced by MCP-1. The peroxynitrite generator 3-morpholinosydnonimine (SIN-1) was evaluated incubating with peroxynitrite or the peroxynitrite generator 3-morpholinosydnonimine (SIN-1) were evaluated in vitro. The results suggest that peroxynitrite may play a regulatory role in inflammation by regulating MCP-1-induced monocyte chemotaxis.

Methods

Measurement of MCA. Mononuclear cells for the chemotaxis assay were obtained from nonsmoking healthy volunteers by Ficoll-Hypaque density centrifugation (Histopaque 1077; Sigma Chemical, St. Louis, MO) to separate the red blood cells and neutrophils from the mononuclear cells (8). The mononuclear cells were harvested at the interface, centrifuged at 400 g for 5 min, and washed three times with Hanks’ balanced salt solution (HBSS; Biofluids, Rockville, MD). The resulting cell pellet routinely consisted of ~30% monocytes and 70% lymphocytes by morphology and esterase staining (Sigma) with >98% viable cells. The cells were suspended in Gey’s balanced salt solution (Life Technologies, Grand Island, NY) containing 2% BSA (Sigma) at a final concentration of 5 × 10^6 cells/ml.

To ensure that lymphocytes were not playing a major role in regulating human MCA, some experiments were performed using highly purified monocytes. Mononuclear cells obtained by Ficoll-Hypaque density centrifugation were suspended at a concentration of 2 × 10^6 cells/ml. Two milliliters of the cell suspension were placed in tissue culture flasks (Corning) for 90 min at 37°C. The supernatant fluids containing the nonadherent lymphocytes were removed, and the...
monocytes were detached by adding 0.2 ml of 0.05% trypsin and 0.53 mM EDTA.

MCA was assayed in 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD) as previously described (20). The bottom wells of the chamber were filled with 25 µl of the chemotactic stimulus or medium in duplicate. A 10-µm-thick ployvinylpyrrolidone-free polycarbonate filter with a pore size of 5 µm was placed over the samples. The silicon gasket and the upper pieces of the chamber were applied, and 50 µl of the cell suspension were placed in the upper wells. The chamber was incubated in humidified air in 5% CO2 at 37°C for 90 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. MCA is expressed as the mean number of migrated cells per high-power field from duplicate wells.

Effect of peroxynitrite on MCP-1-induced MCA. Peroxynitrite was evaluated for its capacity to modulate MCP-1-induced MCA in vitro. Recombinant human MCP-1 (R&D Systems, Minneapolis, MN) was incubated for 2 h at 37°C with each concentration of peroxynitrite (Calbiochem, La Jolla, CA) before the MCA assay was performed. In control experiments, MCP-1 was incubated with medium alone.

Effect of peroxynitrite on leukotriene B4- and activated serum-induced MCA. The capacity of peroxynitrite to modulate leukotriene B4 (LTB4)- and activated serum-induced MCA was similarly evaluated and compared with MCP-1. LTB4 (10^{-6} M; Sigma) or complement-activated serum (1:10 dilution; see Ref. 40) was incubated with peroxynitrite (10^{-4} M) for 2 h at 37°C before the MCA assay was performed.

Effect of SIN-1 (0.5 to 10 M) on MCP-1-induced MCA. To evaluate the effect of NO alone on MCP-1-induced MCA, PAPANONOate (Alexis, San Diego, CA) was used as an NO donor (47). MCP-1 (10^{-4} M) was incubated with PAPANONOate (10^{-3} to 10^{-6} M) for 2 h at 37°C before the MCA assay was performed. The samples were dialyzed overnight at 4°C against HBSS using tubing with a molecular-mass cutoff of 3 kDa to remove NO because NO has been reported to effect cell migration. The half-life of PAPANONOate is 15 min in physiological buffer at 37°C, and two moles of NO are released per mole of PAPANONOate (24).

Effect of xanthine/xanthine oxidase on MCP-1-induced MCA. To evaluate the effect of superoxide on MCP-1-induced MCA, xanthine (10^{-6} to 10^{-3} M; Sigma) and xanthine oxidase (3.5 x 10^{-4} M; Sigma) or complement-activated serum (1:10 dilution; see Ref. 40) was incubated with peroxynitrite (10^{-4} M) for 2 h at 37°C before the MCA assay was performed. The samples were dialyzed overnight at 4°C against HBSS using tubing with a molecular-mass cutoff of 3 kDa to remove NO because NO has been reported to effect cell migration. The half-life of PAPANONOate is 15 min in physiological buffer at 37°C, and two moles of NO are released per mole of PAPANONOate (24).

Effect of xanthine/ xanthine oxidase on MCP-1 MCA. To evaluate the effect of xanthine/xanthine oxidase on MCP-1-induced MCA, xanthine was also added to the reaction mixture.

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without peroxynitrite was incubated with monocytes (10^6 cells) at 4°C for 30 min. Next, supernatants were removed, and monocytes were washed three times by HBSS. Monocytes were suspended in 1 ml PBS-Tween, sonicated for 20 s (MSE Soniprep, Crawley, UK), and then centrifuged at 20,000 rpm for 30 min in a refrigerated microcentrifuge to obtain a supernatant (soluble) and particulate fraction. MCP-1 was measured using a commercially available MCP-1 ELISA (R&D Systems).

Statistics. Data were analyzed by one-way ANOVA. In all cases, a P value of <0.05 was considered significant. The data are expressed as means ± SE.

RESULTS

Effect of peroxynitrite on MCP-1. Differing concentrations of MCP-1 were incubated with peroxynitrite (100 µM). At each concentration, exposure to peroxynitrite caused a reduction in MCA (Fig. 1; n = 4 experiments, P < 0.05). Incubation of MCP-1 (100 ng/ml) with various amounts of peroxynitrite induced a significant, concentration-dependent attenuation of MCA (Fig. 2; n = 4, P < 0.05). The lowest dose of peroxynitrite tested, 10^-5 M, significantly inhibited MCA (P < 0.05). Peroxynitrite itself was not chemotactic for monocytes (data not shown). Similarly, incubation of peroxynitrite (100 µM) with the monocytes before the chemotaxis assay did not inhibit MCA to MCP-1 (data not shown).

Effect of peroxynitrite on LTB4- and activated serum-induced MCA. To ensure that the effect of peroxynitrite was not a nonspecific effect on monocyte chemotaxis, the effect of peroxynitrite on MCA induced by MCP-1 (Fig. 3A), complement-activated serum (Fig. 3B), and LTB4 (Fig. 3C) was assessed. Peroxynitrite did not significantly inhibit the MCA of LTB4 or complement-activated serum.

Effect of PAPA-NONOate on MCP-1-induced MCA. To investigate the capacity of NO to modulate MCP-1-induced MCA, the effect of the NO donor PAPA-NONOate was evaluated. PAPA-NONOate (10^-3 to 10^-6 M) did not significantly change MCA induced by MCP-1 (P > 0.05, all comparisons).

Effect of xanthine/xanthine oxidase on MCP-1-induced MCA. To evaluate the effect of superoxide on MCA induced by MCP-1, MCP-1 was incubated with xanthine (10^-6 to 10^-3 M), xanthine oxidase (3.4 x 10^-6, 3.4 x 10^-5, 3.4 x 10^-4, and 3.4 x 10^-3 U/ml), or xanthine and xanthine oxidase. None significantly altered MCA to MCP-1 (P > 0.05, all comparisons).

Effect of SIN-1 on MCP-1-induced MCA. SIN-1 spontaneously decomposes under aqueous conditions, generating first O2 and then NO at comparable rates. SIN-1 induced a significant, concentration-dependent attenuation of MCA by MCP-1 (Fig. 4; n = 6, P < 0.05). The lowest dose of SIN-1 to inhibit MCA was 10^-6 M (P < 0.05). One hundred micromolar SIN-1 induced ~80% inhibition of MCA by MCP-1. SIN-1 itself was not chemotactic for monocytes (data not shown).

Effect of dithionite, dithiothreitol, and deferoxamine on peroxynitrite-induced attenuation of MCA by MCP-1. The reducing agents dithiothreitol, deferoxamine, and dithionite were added to MCP-1 before incubation with peroxynitrite. Each inhibited the peroxynitrite effect on MCA (Fig. 5; n = 4, P < 0.05). Dithionite, dithiothreitol, and deferoxamine alone were not chemotactic for monocytes (data not shown).

Effect of L-tyrosine on peroxynitrite-induced attenuation of MCA by MCP-1. One mechanism of peroxynitrite inhibition may be through nitrating tyrosine residues. Therefore, the effect of L-tyrosine addition to MCP-1 before incubation with peroxynitrite was investigated.
Addition of L-tyrosine to MCP-1 abrogated the attenuation of MCA induced by peroxynitrite (Fig. 6; \( n = 4 \), \( P < 0.05 \)). The addition of 100 µM L-tyrosine prevented the inhibition of MCA induced by 100 µM of peroxynitrite. L-Tyrosine itself was not chemotactic for monocytes (data not shown).

Detection of nitrotyrosine on MCP-1 incubated with peroxynitrite. Optical density of MCP-1 with peroxynitrite incubation was significantly higher than that of MCP-1 without peroxynitrite incubation. Peroxynitrite resulted in nitrotyrosine formation on MCP-1 (Fig. 7; \( n = 6 \), \( P < 0.05 \)).

Effect of peroxynitrite on MCP-1 binding to monocytes. MCP-1 induces chemotactic activity by binding to monocytes. Addition of peroxynitrite to MCP-1 resulted in an inhibition of MCP-1 binding to monocytes (Fig. 8; \( n = 4 \), \( P < 0.05 \)).

**DISCUSSION**

The results of this study show that peroxynitrite significantly attenuated MCP-1-induced MCA in vitro. Sodium dithionite, deferoxamine, dithiothreitol, or tyrosine attenuated the inhibition. NO or superoxide did not cause a reduction in MCP-1-induced MCA because PAPA-NONOate and xanthine/xanthine oxidase did not show an inhibitory effect. The peroxynitrite donor SIN-1 induced a significant, concentration-dependent inhibition of MCA by MCP-1. Nitrotyrosine was detected in the MCP-1 incubated with peroxynitrite by ELISA. These data suggest that peroxynitrite may play an important role in regulating human monocyte locomotion in response to MCP-1.

NO has been reported to inhibit monocyte chemotaxis (4) and to inhibit MCP-1 expression (44). Other studies have reported that NO synthase inhibitors attenuate MCA to a variety of stimuli (6). However, in this study, we examined the effect of an end product of NO production, peroxynitrite, on a chemotactic factor itself.

The concentration of peroxynitrite in vivo is unknown. Peroxynitrite is a transient intermediate in free radical chemistry and is highly reactive at physiological pH. Thom et al. (43) reported that nitrotyrosine concentrations in lung homogenates were 30–60 ng/mg protein. If peroxynitrite concentrations were of the same order, the peroxynitrite concentration in this experiment should be sufficient to modulate MCP-1 function.

MCP-1 is a prototypical C-C chemokine that was isolated on the basis of its ability to attract monocytes in Boyden chambers in vitro (31, 45, 50). MCP-1 expression has been detected in a variety of pathological conditions including atherosclerosis and rhue-
toid arthritis (19, 26, 33, 42, 49). Various inflammatory mediators are reported to enhance production of NO and superoxide, and they may lead to the formation of peroxynitrite during inflammation. Evidence of peroxynitrite reaction products, specifically immunoreactive 3-nitrotyrosine residues, occurs in lung tissue sections from patients with bronchial asthma (38) and adult respiratory distress syndrome (27) and in atherosclerotic blood vessels (10). Chemotactic factors, including MCP-1, are likely to be exposed to high local concentrations of NO, superoxide, and peroxynitrite at inflammatory sites.

Coincubation of MCP-1 with several peroxynitrite scavengers ameliorated the peroxynitrite-induced MCA inhibition. The protective effect of dithiothreitol on MCP-1 has been suggested to be by attenuating the effects of nitrotyrosine on cysteine residues; however, dithiothreitol has also been reported to prevent peroxynitrite-mediated nitration of tyrosine (37). The iron chelator deferoxamine also inhibited peroxynitrite-induced inhibition of MCP-1 chemotactic activity but is also a scavenger of peroxynitrite reaction independent of iron chelation (13). Dithionite, which has been proposed to modify 3-nitrotyrosine by substitution of an amine group (2), ameliorated the peroxynitrite inhibition. In addition, L-tyrosine abrogated the peroxynitrite MCA inhibition. These results are consistent with tyrosine nitration by peroxynitrite as a mechanism for MCP-1 inhibition.

MCP-1 is a 76-amino acid chemokine, and it has two tyrosine residues. Several studies reported that tyrosine nitration is related to inactivation of protein and enzymatic activity (30, 48). Consistent with the concept that tyrosine is important in binding of MCP-1 to its receptor, Zhang et al. (51) reported that changing tyrosine-28 to aspartate or arginine-30 to leucine produced proteins with essentially no monocyte chemoattractant activity. Steitz et al. (41) reported that point mutations of tyrosine-13 greatly lowered MCP-1 receptor binding and activity. Our findings of nitrotyrosine formation on MCP-1 after peroxynitrite are consistent with these observations and suggest that tyrosine nitration by peroxynitrite on MCP-1 receptor is a likely mechanism altering MCP-1 binding and chemotactic function. However, deletion or mutational analysis of MCP-1 shows that other regions of the molecule contribute to its activity. Peroxynitrite may potentially affect protein function by other mechanisms, including methionine (46), tryptophan (1), or formation of S-nitrosothiol groups on cysteine residues (15).

Although NO and peroxynitrite are physiological regulators, they have been shown to alter respiration (7, 39) and induce cell death (9). To estimate the effect of peroxynitrite on monocytes, we incubated monocytes with peroxynitrite for 90 min at 37°C before chemotaxis experiments. It induced no significant cytotoxicity, as assessed by trypan blue exclusion, compared with medium alone, and it had no significant effect on MCA by MCP-1.

In summary, we found that peroxynitrite modulates MCP-1-induced MCA in vitro. The results are consistent with the mechanism of inhibition being nitration of a tyrosine residue. These data demonstrate that peroxynitrite attenuates MCP-1 chemotactic activity and suggest a role for peroxynitrite in regulating human monocyte locomotion during inflammation.
REFERENCES


