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Peroxy nitrite Mediates IL-8 Gene Expression and Production in Lipopolysaccharide-Stimulated Human Whole Blood

János G. Filep, Micheline Beauchamp, Chantal Baron, and Yves Paquette

Recent evidence indicates that free oxygen radicals, in particular hydroxyl radicals, may act as intracellular second messengers for the induction of IL-8, a potent chemoattractant and activator of neutrophil granulocytes. Here we report that peroxynitrite (ONOO⁻), formed by a reaction of nitric oxide (NO) with superoxide, mediates IL-8 gene expression and IL-8 production in LPS-stimulated human whole blood. The NO synthase inhibitors aminoguanidine and N⁵-nitro-L-arginine methyl ester (L-NAME) blocked IL-8 release by ~90% in response to LPS (1 μg/ml), but did not affect the production of IL-1β or TNF-α. Both aminoguanidine and L-NAME blocked the induction of IL-8 mRNA by LPS. Authentic ONOO⁻ (2.5–80 μM) augmented IL-8 mRNA expression and stimulated IL-8 release in a concentration-dependent manner, whereas the NO-releasing compounds, S-nitroso-N-acetyl-L.-penicillamine and sodium nitroprusside failed to induce cytokine production. Combination of the NO-generating chemicals with a superoxide-generating system (xanthine/xanthine oxidase) markedly increased IL-8 release. Enhanced ONOO⁻ formation was detected in granulocytes, monocytes, lymphocytes, and plasma after challenge with LPS. Furthermore, pyrrolidine dithiocarbamate, an inhibitor of activation of nuclear factor-κB, markedly attenuated the induction of IL-8 mRNA expression and IL-8 release by either LPS or ONOO⁻. Our study identifies ONOO⁻ as a novel signaling mechanism for IL-8 gene expression and suggests that inhibition of ONOO⁻ formation or scavenging ONOO⁻ may represent a novel therapeutic approach to inhibit IL-8 production that could lead to reduction of neutrophil accumulation and activation. The Journal of Immunology, 1998, 161: 5656–5662.

Interleukin-8 is a proinflammatory cytokine that promotes activation and accumulation of neutrophil granulocytes (1–4). IL-8, a member of the chemokine family, is produced by a wide variety of cell types, including neutrophils, monocytes-macrophages, T lymphocytes, and endothelial cells in response to inflammatory stimuli such as LPS, IL-1β, or TNF-α (1, 4). IL-8 induces neutrophil chemotaxis (1, 5), release of lysosomal enzymes (5, 6), shedding of L-selectin, and up-regulation of expression of Mac-1 (7) and promotes neutrophil adherence to endothelial cells (8) and transendothelial migration (7, 9). The presence of IL-8 has been detected in several disease states, including endotoxin shock (10, 11), inflammatory arthritis (12), and skin injury (13). Neutralizing Abs against IL-8 attenuate neutrophil accumulation and neutrophil-dependent tissue injury in endotoxemia and other experimental models (14, 15), indicating a pivotal role of IL-8 in recruitment and activation of neutrophils during acute inflammation.

Recent studies have identified oxidative stress as a ubiquitous mechanism for the induction of IL-8. Hydroxyl radical scavengers markedly suppress (16, 17) whereas H₂O₂ or deprivation of cellular glutathione enhances LPS-induced expression of the IL-8 gene (18). Recently, another radical, nitric oxide (NO)³ has also been implicated in the regulation of IL-8 production (19–22). Numerous pathophysiologic conditions, including endotoxin shock, are associated with simultaneously enhanced formation of oxygen radicals and NO. Superoxide and NO react together in a diffusion-limited manner to form peroxynitrite (ONOO⁻) (23), which might be responsible for some of the biologic actions attributed to NO. As a highly reactive oxidant, ONOO⁻ has the potential to produce oxidant stress. Therefore, we have studied whether ONOO⁻ mediates IL-8 gene expression and production in response to LPS in human whole blood.

Materials and Methods

Reagents

LPS (Escherichia coli O111:B4), sodium nitroprusside, aminoguanidine hemisulfate, cycloheximide, pyrrolidine dithiocarbamate (PDTC), xanthine, xanthine oxidase (from buttermilk) and N⁵-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma (St. Louis, MO). N⁵-nitro-o-arginine methyl ester (D-NAME), S-nitroso-N-acetyl-L- penicillamine (SNAP), and 3-morpholinosydnonimine hydrochloride (SIN-1) were purchased from Research Biochemicals International (Natick, MA). Dihydro rhodamine 123 (DHR 123) and rhodamine were obtained from Molecular Probes (Eugene, OR). Human recombinant IL-1β and TNF-α were purchased from R&D Systems (Minneapolis, MN).

Experimental design

Venous blood (10 ml; anticoagulated with sodium heparin, 50 U/ml) was obtained from nonsmoking healthy volunteers (male and female, 23–46 yr old) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the clinical research committee. White blood cell counts were between 5000 and 9000 cells/μl. Whole blood aliquots (1 ml) were transferred to sterile 1.5-ml centrifuge tubes, and aminoguanidine (10 mM), L-NAME (10 mM), D-NAME (10 mM), cycloheximide (35.5 μg/ml), PDTC (100 μM), and LPS (1 μg/ml) were added as required. The blood

³ Abbreviations used in this paper: NO, nitric oxide; ONOO⁻, peroxynitrite; PDTC, pyrrolidine dithiocarbamate; L-NAME, N⁵-nitro-o-arginine methyl ester; D-NAME, N⁵-nitro-L-arginine methyl ester; SNAP, S-nitroso-N-acetyl-L.-penicillamine; SIN-1, 3-morpholinosydnonimine hydrochloride; DHR, 123, dihydrodichloride 123, GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase.
was then placed on a rotator and incubated at 37°C in 95% air/5% CO₂. At the designated time point, the plasma was harvested, diluted 1/5 with RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 1% FBS, and stored at −20°C for later cytokine analysis. For samples used in RNase protection assays, 100 μl of blood were mixed with an equal volume of lysis/denaturation solution (Ambion, Austin, TX) and stored at −20°C for later processing.

ONOÖ⁻ and control experiments

ONOÖ⁻ synthesized by using nitrite and hydrogen peroxide in a quenched flow reactor (24) was obtained from Alexis (San Diego, CA). Stock solutions of ONOO⁻ (160 mM/L) were stored at −70°C at pH 12.7. Since the ONOO⁻ stock solution contains nitrite, hydrogen peroxide (3.4 mM), and NaCl, we performed control experiments using the following solutions: 1) a pH-neutralized negative control solution (Alexis) that contains the same concentrations of nitrite, H₂O₂, and NaCl as the ONOO⁻ stock solution; 2) a solution of 3.4 mM H₂O₂ at pH 12.7, prepared and diluted 1/2000 (a dilution factor that achieved an 80 μM final ONOO⁻ concentration); and 3) NaOH at 0.15 mM (the final concentration of NaOH in 80 μM ONOO⁻ solution). A 10-μl aliquot of one of these solutions was added to some blood samples, and its effects were investigated.

Measurement of intracellular and plasma rhodamine levels

NO synthase blocker-inhibitable fluorescence of rhodamine, an oxidation product of DHR 123, was used as a marker of exposure of DHR 123 to ONOO⁻ (25). DHR 123 (20 μM) was mixed with whole blood samples; incubated with LPS (1 μg/ml), L-NAME (10 mM), or aminoguanidine (10 mM) for 3 h; and then incubated for an additional 60 min. At the end of the incubation period, erythrocytes were lysed, and leukocytes were prepared for flow cytometric analysis as described previously (26). Granulocytes, monocytes, and lymphocytes were gated by their forward and side scatter characteristics, and single-color fluorescence was analyzed by a cytofluorometer (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA) with LYSIS II software. The plasma rhodamine level was measured in a Perkin-Elmer fluorometer (excitation, 500 nm; emission, 526 nm; slit widths, 3 nm; Perkin-Elmer, Norwalk, CT) (25). The amount of plasma rhodamine was quantified using a rhodamine standard curve (0.8–400 nM) prepared in untreated plasma. Background fluorescence was subtracted from all samples.

Leukocyte viability

At the indicated times, leukocytes were prepared as described above, and staining with propidium iodide (0.5 μg/ml) was analyzed with a FACScan cytofluorometer.

Measurement of IL-8, IL-1β, and TNF-α

The plasma concentrations of IL-8, IL-1β, and TNF-α were determined by highly selective ELISAs (R & D Systems). The detection limits of the IL-8, IL-1β, and TNF-α assays were 31, 4, and 15 pg/ml, respectively. Intra- and interassay coefficients of variation were typically <5% and <8%, respectively, in these assays. There was no cross-reactivity with the NO synthase inhibitors or PDTC in the assays.

IL-8 RNase protection assay

Human IL-8 and glyceroldehyde-3-phosphate dehydrogenase (GAPDH) primers (Clontech Laboratories, Palo Alto, CA) were used to amplify by PCR the positive control cDNA fragments provided by the primers’ manufacturer. The amplified products were analyzed on a 1% agarose gel, and single bands of the expected m.w. of 289 and 982 bp were obtained for IL-8 and GAPDH, respectively. The fragments were cloned in the Smal site of the pGEM3Zf vector (Promega, Madison, WI). The IL-8 cDNA clone was linearized with SmaI, which cuts once in the GAPDH cDNA sequence. The probe synthesized by using nitrite and hydrogen peroxide in a quenched flow reactor (24) was obtained from Alexis (San Diego, CA). Blood samples were stimulated with 1 μg/ml LPS in the absence and presence of aminoguanidine (AG; 10 mM) or L-NAME (10 mM) at 37°C for the indicated time intervals. D. Concentration-dependent inhibition of IL-8 production by L-NAME and aminoguanidine was assayed at 24 h post-LPS. The plasma cytokine concentrations were measured by ELISA.

Results

Aminoguanidine and L-NAME selectively inhibit IL-8 production

Consistent with previous observations (16, 27), our study also documents the biphasic pattern of IL-8 production (Fig. 1A). Previous studies have shown that the secondary phase of IL-8 production occurring between 12 and 24 h can be completely inhibited by neutralizing anti-IL-1 and anti-TNF Abs, whereas the early phase of IL-8 production (4–12 h) is unaffected by these Abs (16). Both L-NAME and aminoguanidine markedly reduced IL-8 production over the entire time course, indicating that their mechanism of inhibition is different from that of the anti-TNF and anti-IL-1 Abs. For instance, L-NAME and aminoguanidine at 10 mM suppressed IL-8 production, on the average, by 75 and 81% and by 90 and 87% (n = 3) at 4 and 24 h, respectively (Fig. 1A). Both NO synthase blockers exerted a concentration-dependent inhibitory action with an apparent maximum inhibition achieved at 10 mM, as assayed 24 h post-LPS (Fig. 1D). The inhibitory action of 10 mM L-NAME was completely reversed by 100 mM L-arginine, and
monocytes simultaneously produce both NO and superoxide anion, blood. None of these NO donors produced significant increases in and sodium nitroprusside (0.5–2 mM), were directly added to therefore performed in which the NO donors, SNAP (0.4 –2.4 mM) would induce IL-8 release. Concentration-response studies were capable of inhibiting IL-8 production. We next wanted to determine Our studies have established that NO synthase inhibitors are ca-

In unstimulated blood, the plasma IL-8 level was 0.6

vehicle (medium) for 24 h at 37°C. The plasma was then analyzed for IL-8. The results represent the mean ± SEM for six blood donors. *, p < 0.05 (compared with samples challenged with LPS, IL-1β, or TNF-α, as appropriate).

Unlike L-NAME, D-NAME did not affect LPS-induced IL-8 release (data not shown). The inhibitory action of NO synthase inhibitors was highly specific for IL-8, as neither L-NAME nor amionoguanidine had a significant effect on the release of TNF-α and IL-1β (Fig. 1, B and C). Leukocyte viability was unaffected by L-NAME or aminoguanidine (data not shown).

Since the secondary phase of LPS-induced IL-8 production is mediated via TNF-α and IL-1β (16), we tested whether NO synthase inhibitors could also suppress IL-8 release evoked by these cytokines. Both aminoguanidine and L-NAME inhibited by >85% the amount of IL-8 released by TNF-α (370 ng/ml) or IL-1β (100 ng/ml; Fig. 2).

**Inhibition of IL-8 mRNA expression**

To determine whether the reduction of LPS-stimulated IL-8 release by NO synthase blockers is occurring at the level of transcription or translation, RNase protection assays were performed on RNA extracted from whole blood samples incubated for 4 h with LPS (1 μg/ml) in the presence and the absence of aminoguanidine or L-NAME. A significant reduction in LPS-stimulated IL-8 mRNA levels was detected upon incubation with either L-NAME or aminoguanidine (Fig. 3). IL-8 mRNA was similarly suppressed by both aminoguanidine and L-NAME in the absence and the presence of cycloheximide (Fig. 3), indicating that these compounds do not induce the synthesis of an intermediary protein; rather, they have a direct inhibitory effect on transcription.

**NO- and ONOO−-induced IL-8 release**

Our studies have established that NO synthase inhibitors are capable of inhibiting IL-8 production. We next wanted to determine whether exposure of whole blood to chemically generated NO would induce IL-8 release. Concentration-response studies were therefore performed in which the NO donors, SNAP (0.4–2.4 mM) and sodium nitroprusside (0.5–2 mM), were directly added to blood. None of these NO donors produced significant increases in IL-8 release (Table I). Since LPS-stimulated neutrophils and monocytes simultaneously produce both NO and superoxide anion, which react to form the potent oxidant ONOO− (24), we tested whether addition of either NO donor in combination with a superoxide-generating system (xanthine/xanthine oxidase) or authentic ONOO− to blood samples could enhance IL-8 production. Incubation of blood samples with SNAP or sodium nitroprusside together with xanthine/xanthine oxidase resulted in marked increases in IL-8 production, whereas IL-8 production was only slightly stimulated by xanthine/xanthine oxidase (Table I). The compound SIN-1, which simultaneously releases both NO and superoxide (28), caused a concentration-dependent increase in IL-8 accumulation (Table I). ONOO−-stimulated IL-8 release in a concentration-dependent manner, with the IL-8 concentrations measured after challenge with 80 μM ONOO− being comparable to those in samples incubated with 1 μg/ml LPS (Table I). On the other hand, 80 μM ONOO− produced only a slight increase in the TNF-α level (0.3 ± 0.1 ng/ml; n = 5), amounting to only 3% of the samples challenged with LPS (11.1 ± 2.3 ng/ml; n = 5). ONOO− up to a concentration of 80 μM did not affect leukocyte viability (data not shown). IL-8 concentrations in samples incubated with pH-neutralized (decomposed) ONOO− solution or NaOH were similar to those in control (unstimulated) samples (Table I). Furthermore, H2O2 at a 1.7-μM concentration, which is equivalent to its concentration in 80 μM ONOO− solution, did not induce IL-8 release (data not shown).

**ONO−-induced IL-8 mRNA expression**

RNase protection assays performed on RNA extracted from whole blood samples challenged for 4 h with an initial concentration of

**FIGURE 2.** NO synthase blockers inhibit IL-8 production in human blood challenged with LPS, IL-1β, or TNF-α. Blood samples were stimulated with 1 μg/ml LPS, 370 ng/ml TNF-α, or 100 ng/ml IL-1β in the presence of aminoguanidine (AG; 10 mM), L-NAME (10 mM), or their vehicle (medium) for 24 h at 37°C. The plasma was then analyzed for IL-8. In unstimulated blood, the plasma IL-8 level was 0.6 ± 0.2 ng/ml (n = 6). The results represent the mean ± SEM for six blood donors. *, p < 0.05 (compared with samples challenged with LPS, IL-1β, or TNF-α, as appropriate).

**FIGURE 3.** Aminoguanidine and L-NAME reduce LPS-stimulated IL-8 mRNA expression. Blood samples were incubated with LPS (1 μg/ml), aminoguanidine (AG; 10 mM), L-NAME (10 mM), the NF-κB inhibitor PDTC (100 μM), or the protein synthesis inhibitor cycloheximide (35.5 μM) as indicated for 4 h at 37°C A. Representative RNase protection assay using probes for IL-8 and GAPDH. Lane 1, Negative control (probes only); lane 2, unstimulated; lane 3, LPS; lane 4, aminoguanidine plus LPS; lane 5, L-NAME plus LPS; lane 6, cycloheximide plus LPS; lane 7, PDTC plus LPS; lane 8, aminoguanidine plus cycloheximide plus LPS; lane 9, L-

NAME plus cycloheximide plus LPS. B. Densitometric analysis of autoradiograms of the samples probed for IL-8 and GAPDH. The IL-8 results represent the mean ± SEM of blots from five blood donors. *, p < 0.05 (compared with samples incubated with LPS alone).
Table I. Effect of NO donors and peroxynitrite on IL-8 production in human whole blood

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Concentration</th>
<th>n</th>
<th>IL-8 (ng/ml Plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated</td>
<td>—</td>
<td>5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>LPS</td>
<td>1 μg/ml</td>
<td>5</td>
<td>12.9 ± 0.4**</td>
</tr>
<tr>
<td>SNAP</td>
<td>0.4 mM</td>
<td>4</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Xanthine/xanthine oxidase</td>
<td>—</td>
<td>3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>0.5 mM</td>
<td>4</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Xanthine/xanthine oxidase + SNAP</td>
<td>1.2 mM</td>
<td>3</td>
<td>6.3 ± 2.0*</td>
</tr>
<tr>
<td>Xanthine/xanthine oxidase + NaOH</td>
<td>1.0 mM</td>
<td>3</td>
<td>6.2 ± 1.3*</td>
</tr>
<tr>
<td>SIN-1</td>
<td>0.001 mM</td>
<td>3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>OONO⁻</td>
<td>2.5 μM</td>
<td>5</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>5.0 μM</td>
<td>5</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>10 μM</td>
<td>5</td>
<td>3.1 ± 0.4*</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>20 μM</td>
<td>5</td>
<td>3.9 ± 0.8*</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>40 μM</td>
<td>5</td>
<td>7.0 ± 1.2*</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>80 μM</td>
<td>5</td>
<td>10.3 ± 1.8*</td>
</tr>
<tr>
<td>Decomposed ONOO⁻</td>
<td>—</td>
<td>5</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.15 mM</td>
<td>5</td>
<td>0.2 ± 0.03</td>
</tr>
</tbody>
</table>

* Blood samples were left unstimulated or challenged with LPS, SNAP, sodium nitroprusside, superoxide generated by oxidation of xanthine (20 μM) by xanthine oxidase (0.01 U/ml), SIN-1, authentic ONOO⁻, or NaOH (0.15 mM), the concentration of NaOH in 80 μM OONO⁻ solution) for 4 h at 37°C. Values are means ± SEM for n independent experiments. ** p < 0.01 (compared to unstimulated).

80 μM ONOO⁻ revealed a 2.4-fold increase in IL-8 mRNA expression, whereas IL-8 mRNA in samples incubated with decomposed ONOO⁻ or NaOH (final concentration, 0.15 mM) did not differ from that detected in control samples (Fig. 4).

**PDTC inhibits the production of IL-8 in response to LPS and ONOO⁻**

Since the IL-8 gene contains functional NF-κB binding sites in its promoter that are necessary for the transcriptional activation of this gene (29, 30), we studied whether PDTC, a specific inhibitor of the NF-κB activation (31), would affect induction of IL-8 by LPS and ONOO⁻. In the presence of PDTC, both IL-8 release (Fig. 5) and expression of IL-8 mRNA were markedly suppressed in blood samples challenged with either LPS (Fig. 3) or ONOO⁻ (Fig. 4).

**Detection of ONOO⁻ formation in human blood challenged with LPS**

Incubation of blood samples with LPS for 4 h resulted in increases in intracellular rhodamine fluorescence in neutrophils, monocytes, and, to a lesser extent, lymphocytes (Fig. 6). DHR 123 oxidation was partially prevented by inhibition of NO synthase with either aminoguanidine or L-NAME (Fig. 6). Parallel with intracellular DHR 123 oxidation, an increase in the plasma rhodamine concentration was observed at 4 h post-LPS, which was prevented by both aminoguanidine and L-NAME (Fig. 6). By contrast, LPS-induced increases in intracellular DHR 123 oxidation were not affected significantly by L-NAME at 1 h post-LPS. Rhodamine fluorescence in neutrophils increased from 6.2 ± 0.4 to 14.8 ± 1.4 and 14.1 ± 1.4 relative fluorescence units in response to LPS in the absence and the presence of L-NAME, respectively (n = 4; both p < 0.05 compared with unstimulated); in monocytes from 6.0 ± 1.2 to 20.6 ± 2.1 and 19.6 ± 2.7 relative fluorescence units in response to LPS and L-NAME plus LPS, respectively (n = 4; both p < 0.05 compared with unstimulated); and in lymphocytes from 0.9 ± 0.1 to 1.6 ± 0.3 and 1.5 ± 0.2 relative fluorescence units in response to LPS and L-NAME plus LPS, respectively (n = 4; both p < 0.05 compared with unstimulated).

**FIGURE 4.** ONOO⁻ induces IL-8 mRNA expression. Whole blood samples were incubated with PDTC (100 μM) or cycloheximide (35.5 μM) as indicated for 30 min, and then challenged with ONOO⁻ (80 μM), decomposed ONOO⁻, or 0.15 mM NaOH for 4 h at 37°C. A, Representative RNase protection assay using probes for IL-8 and GAPDH. Lane 1, Negative control (probes only); lane 2, unstimulated; lane 3, LPS; lane 4, ONOO⁻; lane 5, decomposed ONOO⁻; lane 6, PDTC plus ONOO⁻; lane 7, NaOH; lane 8, cycloheximide plus ONOO⁻. B, Densitometric analysis of autoradiograms of the samples probed for IL-8 and GAPDH. The IL-8 results were adjusted based on the GAPDH values to represent equivalent RNA loading in each lane, and the results were then expressed as a percentage of the band obtained from unstimulated blood. The results represent the mean ± SEM of blots from five blood donors. * p < 0.05 (compared with control); #, p < 0.05 (compared with ONOO⁻) by Dunn’s multiple contrast hypothesis test.

**FIGURE 5.** PDTC suppresses LPS and ONOO⁻-stimulated IL-8 production. Blood samples were incubated with PDTC (100 μM) for 30 min at 37°C and challenged with LPS (1 μg/ml) or ONOO⁻ (80 μM) for 4 h, and the plasma was then harvested for IL-8 analysis. Values are the mean ± SEM (n = 4). Unstimulated blood plasma contained 0.2 ± 0.1 ng IL-8/ml (n = 5). *, p < 0.05 (compared with LPS or ONOO⁻ without PDTC, respectively).
Discussion

Reactive oxygen radicals are produced and act as modulators of cellular signaling for several stimuli, including TNF-α (32), platelet-derived growth factor (33), TGF-β (34), and LPS (35). Our study identifies ONOO⁻ as a novel signaling mechanism regulating IL-8 gene expression. Three lines of evidence support a role for ONOO⁻ in mediating LPS-induced IL-8 release in human whole blood: 1) enhanced ONOO⁻ formation can be detected intracellularly in leukocytes as well as in plasma; 2) inhibition of NO synthesis, and consequently ONOO⁻ formation, prevents IL-8 mRNA expression and IL-8 release; and 3) authentic ONOO⁻ enhances IL-8 gene expression and release.

We monitored the NO-dependent oxidation of DHR 123 to rhodamine as a marker of ONOO⁻ formation (25) and detected increases in rhodamine fluorescence in neutrophils, monocytes, lymphocytes, and plasma in response to LPS. The enhanced oxidation of DHR 123 at 4 h post-LPS can presumably be attributed to ONOO⁻, because it is dependent on an NO-related species, for it can be inhibited by aminoguanidine or L-NAME, inhibitors of NO synthase (36–38), whereas NO by itself does not increase oxidation of DHR 123 (25). It is conceivable that ONOO⁻ is formed by a reaction of superoxide produced by activated leukocytes with NO produced by the inducible form of NO synthase (iNOS), since LPS is a potent inducer of iNOS expression (39, 40), and it can be inhibited by aminoguanidine, a selective inhibitor of iNOS (37, 38). Since L-arginine binding to the catalytic site of constitutive NO synthase (cNOS) is preferential to aminoguanidine by a factor >1600 (41), a normal plasma concentration of L-arginine (~1 mM) would protect cNOS against aminoguanidine-induced inactivation. Furthermore, intracellular DHR 123 oxidation at 1 h post-LPS was not affected significantly by L-NAME, indicating that activation of cNOS by LPS (which occurs within minutes after addition of LPS) cannot reasonably account for the increases in ONOO⁻ formation. In addition, aminoguanidine may also scavenge an intermediate derived from ONOO⁻ (42). Our results imply that neutrophils, monocytes, and lymphocytes are potential sources of ONOO⁻. The low rhodamine fluorescence intensity detected in lymphocytes may indicate their low capacity to produce ONOO⁻ or, alternatively, that ONOO⁻ produced and released by neutrophils or monocytes diffused into lymphocytes and resulted in DHR 123 oxidation. ONOO⁻ is released into the plasma, as evidenced by increases in the plasma rhodamine concentration. However, the amounts of ONOO⁻ accumulated in plasma during 4-h incubation with LPS cannot be precisely calculated, because ONOO⁻ could react with molecules other than DHR 123 in plasma.

The present study shows that incubation of blood samples with either aminoguanidine or L-NAME selectively inhibited IL-8 release. These observations are consistent with those obtained with L-NAME (43). Although scavenging superoxide with superoxide dismutase could also be expected to reduce ONOO⁻ formation, superoxide dismutase does not affect IL-8 production (16). This may reflect the inability of this charged, high m.w. protein to cross cell membranes (44). The NO donors, SNAP, SIN-1, S-nitrosogluthathione, and 2,2′-(hydroxy nitrosohydrazino)bis-ethaneamine, increased IL-8 secretion from melanoma cells (20) and ECV304 endothelial cells (21), whereas NO inhalation reduced the bronchoalveolar IL-8 levels in patients with acute respiratory distress syndrome (22). Furthermore, NOS inhibitors had no effect on the respiratory syncytial virus-induced release of IL-8 from epithelial cells (45). These discordant observations might be attributed to differences in the balance between NO and superoxide generation that appears to be a critical determinant in the induction of IL-8 release. Furthermore, the observations with NO donors may not necessarily indicate that NO per se is responsible for IL-8 release. For instance, SIN-1 simultaneously releases both superoxide and NO (28), and even exogenously added NO might react with superoxide produced by mitochondria (46), leading to the formation of ONOO⁻. Recently, the hydroxyl radical has also been implicated in mediating 2,2′-(hydroxynitrosodiazirino)bis-ethaneamine-induced IL-8 release in blood (43), although the mechanism by which this NO donor might generate hydroxyl radical is unknown at present.

Unlike the NO donors, authentic ONOO⁻ produced concentration-dependent IL-8 release, with maximal increases similar to those observed with 1 μg/ml LPS. Furthermore, ONOO⁻ (80 μM) increased IL-8 mRNA expression by 2.4-fold. This action of ONOO⁻ was due to ONOO⁻ per se and was not a result of residual contaminants that are present in the ONOO⁻ stock solution, because neither the decomposed and pH-neutralized ONOO⁻ solution that contains all residual contaminants nor NaOH at 0.15 mM (final concentration in ONOO⁻ dilutions) induced IL-8 gene expression and protein release. Since ONOO⁻ was used in the range of 2.5–80 μM, one should consider its rapid decomposition. Thus, much higher concentrations of exogenous ONOO⁻ may be required to achieve biologic responses similar to those produced by much lower concentrations of continuously produced endogenous ONOO⁻ (24).

Since the half-life of ONOO⁻ at pH 7.4 is on the order of seconds, we cannot conclude at present whether induction of the IL-8 gene expression was a direct effect of ONOO⁻ or one of its more...
stable decomposition products. These decomposition products are still a subject of debate and appear to depend on the cellular and chemical environment (23, 47–49). One theory assumes that peroxynitrous acid undergoes homolysis to form hydroxyl radicals (23, 47). Although this would explain the effectiveness of hydroxyl radical scavengers to inhibit LPS-stimulated IL-8 release (16), the selectivity of such scavengers can be questioned. For instance, thioreua and dimethylthiourea have been found to protect against ONOO−-mediated damage, indicating their capability of scavenging ONOO− (50). The second hypothesis proposes that a reactive form of peroxynitrous acid, HOONO−, is the proximate oxidant (48, 49). In the present experiments, ONOO− was added to whole blood. The presence of metal ions, amino acids, and proteins in the plasma might have either scavenged ONOO− or influenced its decomposition characteristics, thereby decreasing its apparent potency or concentration. This may explain why much higher concentrations of exogenous ONOO− were required to induce IL-8 release than those detected in plasma. Furthermore, the plasma levels of ONOO− may not correctly reflect ONOO− formation, because significant portions of ONOO− formed by leukocytes might be retained and decomposed intracellularly.

Consistent with previous studies on LPS-induced transcription of IL-8 gene in endothelial cells (51), the present study also documents the ability of PDTC to abrogate both LPS- and ONOO−-induced IL-8 production in human whole blood to a similar degree. NF-κB activation takes place following phosphorylation of the inhibitory subunit IκB-α (52–54). The mechanism of action of PDTC has not yet been fully defined, but probably involves the inhibition of formation of reactive oxygen radicals that would result in the activation of an IκB-α kinase or, alternatively, in the activation of a process involved in phosphorylation of the inhibitor of NF-κB (31, 52, 55). This would suggest that NF-κB activation can be attributed to decomposition products of ONOO−, rather than to the parent molecule itself. We cannot exclude the possibility that PDTC may also function as a scavenger of ONOO− or one of the intermediates derived from ONOO−. The 50–60% inhibition observed with PDTC suggests a role for NF-κB in the induction of IL-8 by ONOO−. In contrast to IL-8, ONOO− did not induce expression of the TNF-α gene, which also features κB binding motifs in its 5′-regulatory region (56). This suggests that NF-κB may be necessary, but not sufficient, for the induction of TNF-α. Indeed, deletion of κB binding sites from the human TNF-α promoter had little influence on the induction of the gene by LPS (57). It is not known at present whether NF-κB by itself may be sufficient for the induction of IL-8. The transcription factors activating protein-1 (51) and NF-IL-6 (58) have been reported to act synergistically with NF-κB to activate IL-8 transcription. Therefore, it is plausible to assume that ONOO− might have also activated these or other transcription factors. Clearly, further studies are required to assess the multiple promoter regions and factors that may act in an inducer-specific manner.

In summary, the present study provides evidence that endogenously produced ONOO− directly induces the transcription and translation of IL-8 in human whole blood in response to LPS. This occurs in part through activation of NF-κB. The results suggest that inhibition of the formation and/or actions of ONOO− may represent a novel therapeutic approach to attenuate IL-8 production that could lead to a reduction in neutrophil recruitment/activation and consequently to inhibition of inflammatory responses.

References