Peroxynitrite production by human neutrophils, monocytes and lymphocytes challenged with lipopolysaccharide

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Abstract To assess peroxynitrite formation in lipopolysaccharide (LPS)-stimulated human blood, we have measured nitric oxide (NO)-dependent intracellular oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine. LPS increased DHR 123 oxidation in neutrophil granulocytes, monocytes and lymphocytes in a time-dependent fashion. Greater extent of DHR 123 oxidation was detected in neutrophils and monocytes than in lymphocytes. These changes were accompanied by accumulation of rhodamine in the plasma. While intracellular DHR 123 oxidation and rhodamine accumulation in the plasma were not affected by inhibition of constitutive NO synthase at 30 and 60 min after addition of LPS, they were markedly attenuated by inhibition of inducible NO synthase at 4, 8, 16 and 24 h after addition of LPS. These results demonstrate that human leukocytes can produce high amounts of peroxynitrite in response to LPS, and may contribute to the elevated plasma peroxynitrite levels observed during endotoxic shock.

Key words: Peroxynitrite; Nitric oxide; Lipopolysaccharide; Endotoxin shock; Human leukocyte

1. Introduction

An increasing body of evidence indicates that several actions of nitric oxide (NO) are mediated in part by peroxynitrite ($ONOO^-$), a potent oxidant formed by the reaction of NO with superoxide at an almost diffusion-controlled rate [1,2]. In many pathologic conditions, simultaneous cellular production of superoxide and NO may occur, potentially leading to the formation of $ONOO^-$. Indeed, $ONOO^-$ formation has been demonstrated in activated macrophages [3] and endothelial cells [4], in acute respiratory distress syndrome [5], inflammatory bowel disease [6,7], ischemia reperfusion [1,6], rheumatoid arthritis [8] and septic shock [9,10].

Both the early and delayed phases of endotoxic shock are associated with enhanced $ONOO^-$ formation. Peroxynitrite-mediated oxidation of dihydrorhodamine 123 (DHR 123) was detected in the rat plasma in the early stages of endotoxic shock [10]. A substantial increase in nitrotyrosine immunoreactivity has been demonstrated in the rat lung and aorta in the delayed phase of endotoxic shock [9,11]. Nitrotyrosine formation is often considered as a specific 'footprint' of $ONOO^-$ [9,11,12], although recent studies suggest that tyrosine nitration may rather serve as an indicator of 'reactive nitrogen species' [13,14]. However, these studies did not investigate the cellular sources of peroxynitrite. Since both polymorphonuclear and mononuclear leukocytes are capable of producing high amounts of superoxide and NO in response to LPS, we have investigated the time course of peroxynitrite formation in human whole blood challenged with LPS. We have utilized the oxidation of DHR 123 to rhodamine coupled with the use of pharmacological inhibitors of NO synthase to assess the intracellular formation and plasma accumulation of biologically active peroxynitrite. This method has been employed for the measurement of peroxynitrite formation in vitro [9,15]. Our results indicate that peroxynitrite-mediated DHR 123 oxidation occurs in human granulocytes, monocytes and lymphocytes in whole blood at early phases in response to LPS, and these cells may contribute to the elevated plasma levels of peroxynitrite during endotoxic shock.

2. Materials and methods

2.1. Materials

Bacterial lipopolysaccharide (*E. coli*, serotype O111:B4), aminoguanidine hemisulfate and N^G-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). N^G-nitro-D-arginine methyl ester (D-NAME) was obtained from Research Biochemical International (Natick, MA, USA). DHR 123 and rhodamine were obtained from Molecular Probes (Eugene, OR, USA).

2.2. Whole blood incubation

Venous blood was collected into tubes containing heparin (25 U/ml) from healthy volunteers (male and female, 21 to 52 years 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 5500 and 9200 cells/μl. Aliquots of whole blood (1 ml) were placed on a rotator and challenged with LPS (1 μg/ml) in the presence or absence of L-NAME (10 mM), D-NAME (10 mM) or aminoguanidine (10 mM) at 37°C, 5% CO_2 for the indicated time periods. DHR 123 (20 μM) was added to the samples together with LPS or 60 min before termination of the incubation period as indicated.

2.3. Isolation and stimulation of lymphocytes

Mononuclear cells isolated from heparinized blood by centrifugation through Ficoll-Hypaque gradients (Pharmacia Diagnostics AB, Uppsala, Sweden) were incubated on plastic culture dishes for 1 h at 37°C in 5% CO_2 in air. Non-adherent cells were then harvested and resuspended in autologous plasma at 5×10^5 cells/ml. The resulting cell preparation contained $>94\%$ lymphocytes. Lymphocytes were challenged with LPS (1 μg/ml) for 4 h. DHR 123 (20 μM) was added for the last 60 min of incubation.

2.4. Flow cytometry analysis

At the end of the incubation period, samples were prepared for flow cytometry analysis as described previously [16]. In brief, 100 μl of blood samples were mixed with 2 ml of a lysing medium (FACS Lysing Solution, Becton-Dickinson) to lyse erythrocytes and fix leukocytes, centrifuged and washed two times. Leukocytes were resuspended in PBS containing 0.5% formaldehyde. Single color fluorescence staining was analyzed by a cytofluorometer (FACScan, Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) with Lysis II software. Data from 10 000 events per sample were

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acquired. Mean fluorescence intensity was determined after gating for monocytes, lymphocytes and granulocytes by their forward and side scatter characteristics. Background fluorescence was subtracted from all samples. Leukocyte viability was assessed by using propidium iodide (0.5 μg/ml) as described previously [16].

2.5. Measurement of rhodamine accumulation in plasma

Plasma was harvested at the indicated times, and plasma rhodamine level was measured with a spectrofluorometer (LS-5, Perkin-Elmer Corporation, Norwalk, excitation 500 nm, emission 536 nm, slit widths 3 nm). The amount of plasma rhodamine was quantitated by using a rhodamine standard curve (0.8–400 nM) prepared in human untreated plasma. Autofluorescence and rhodamine concentrations detected in autologous plasma incubated with DHR 123 for the same time period have been subtracted from each sample.

2.6. Statistical analysis

Values are reported as mean ± S.E.M. of n observations. Statistical analysis was performed by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to evaluate differences between various treatments. A P value >0.05 was considered significant for all tests.

3. Results

Addition of LPS to human whole blood caused, on average, a 2.4-fold increase in DHR 123 oxidation in granulocytes within 30 min (Fig. 1, upper panel). The highest levels of rhodamine were detected at 4 h following LPS challenge (Fig. 1, upper panel), thereafter the amount of DHR 123 oxidized per 60 min decreased, but was still significantly higher than those detected at 1 h. The increases in DHR 123 oxidation at 30 and 60 min after addition of LPS were unaffected by L-NAME and aminoguanidine (Fig. 1, upper panel). By contrast, the increased rhodamine fluorescence observed after incubation of blood and LPS for longer periods of time (4–24 h) were markedly, though never completely, attenuated by inhibition of NO synthase with L-NAME or aminoguanidine (Fig. 1, upper panel). For instance, L-NAME and aminoguanidine reduced DHR 123 oxidation by 72 and 74%, respectively at 4 h following LPS. L-NAME and aminoguanidine appeared to be equally potent inhibitors of DHR 123 oxidation at all time points studied. The D-enantiomer of L-NAME, D-NAME had no significant effect on LPS-induced DHR 123 oxidation at any time points studied (data not shown). Similar trends were observed in monocytes and lymphocytes (Fig. 1, middle and lower panel). Rhodamine fluorescence was similar in granulocytes and monocytes, whereas it was one order of magnitude lower in lymphocytes (Fig. 1, lower panel). As with granulocytes, both L-NAME and aminoguanidine failed to prevent DHR 123 oxidation at 30 and 60 min after LPS, whereas both NO synthase inhibitors significantly attenuated DHR 123 oxidation at later phases (Fig. 1, middle and lower panel). Viability of leukocytes did not differ significantly in the absence or presence of NO synthase inhibitors at any time point studied (data not shown).

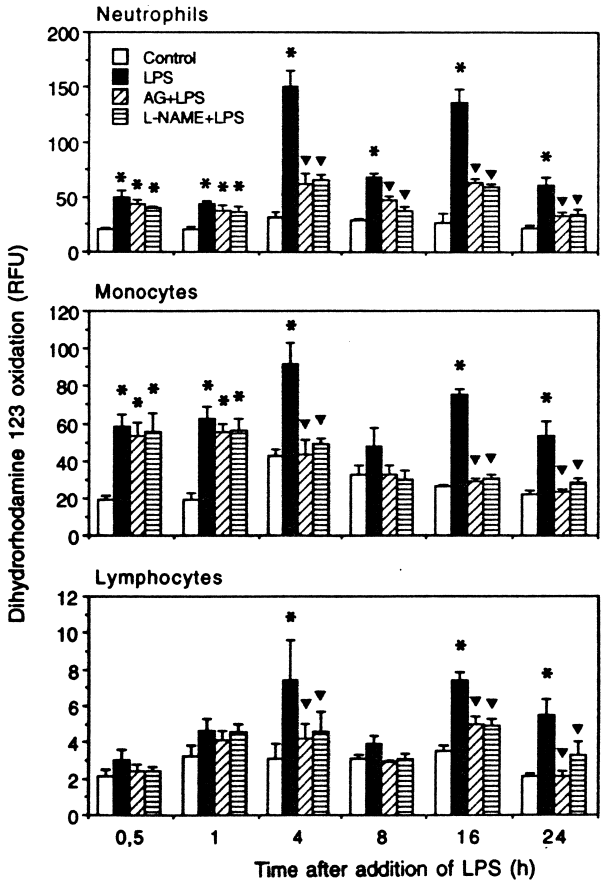

Fig. 1. Effects of L-NAME and aminoguanidine (AG) on LPS-induced dihydrorhodamine 123 (DHR 123) oxidation in human granulocytes (upper panel), monocytes (middle panel) and lymphocytes (lower panel). Whole blood aliquots were left unstimulated (control) or challenged with LPS (1 μg/ml) in the absence or presence of aminoguanidine (AG, 10 mM) or L-NAME (10 mM). DHR 123 was added during the last 60 min of incubation. Relative fluorescence intensity (RFU) was determined by cytofluorometry. Values are means ± S.E.M. for four to five separate experiments. $*P<0.05$ (compared with control); $\blacktriangledown P<0.05$ (compared with LPS).

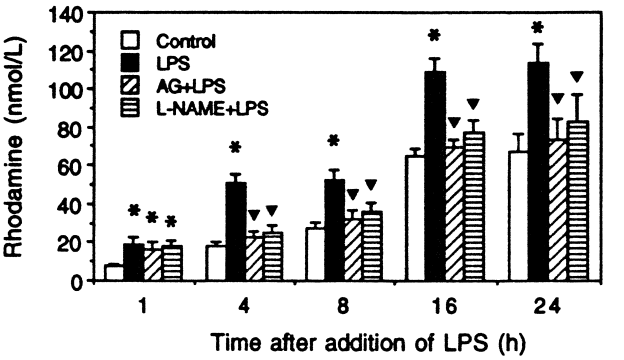

Fig. 2. Plasma levels of rhodamine after LPS. Aliquots of human whole blood were left unstimulated (control) or challenged with LPS (1 μg/ml) in the absence and presence of aminoguanidine (AG, 10 mM) or L-NAME (10 mM) for the indicated time periods. DHR 123 (20 μM) was added to the samples together with LPS. Values are means ± S.E.M. for four to five independent experiments. $*P<0.05$ (compared with control); $\blacktriangledown P<0.05$ (compared with LPS).

Increased rhodamine fluorescence was also observed in isolated lymphocytes challenged with LPS for 4 h (relative fluorescence intensity was 201 ± 25% of unstimulated cells, $n=5$) that was markedly attenuated by either aminoguanidine or L-NAME (RFU: 114 ± 4% and 131 ± 8% of unstimulated cells, respectively, $n=5$, both $P<0.05$).

The increases in intracellular DHR 123 oxidation were accompanied by a time-dependent accumulation of rhodamine in the plasma (Fig. 2). A small, but statistically significant increase in plasma rhodamine concentration was detected as

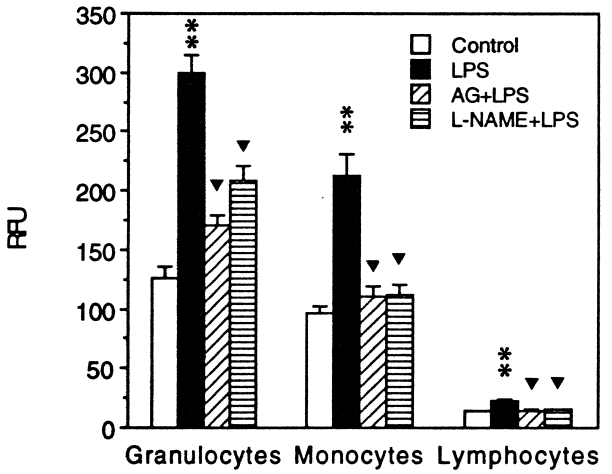

Fig. 3. Intracellular oxidation of dihydrorhodamine 123 (DHR 123) in human leukocytes challenged with LPS for 24 h. Human whole blood aliquots were left unstimulated (control) or stimulated with LPS (1 μg/ml) in the absence or presence of aminoguanidine (AG, 10 mM) or L-NAME (10 mM). DHR 123 was added to the samples together with LPS. Relative rhodamine fluorescence intensity (RFU) was determined by cytofluorometry. Values are means ±S.E.M. for five independent experiments. $^{**}P < 0.01$ (compared with control); $\blacktriangledown P < 0.05$ (compared with LPS).

early as 1 h after addition of LPS (Fig. 2). This was not inhibited by aminoguanidine or L-NAME. As with intracellular DHR 123 oxidation, both aminoguanidine and L-NAME markedly attenuated plasma rhodamine levels at 4, 8, 16 and 24 h incubation with LPS. No significant differences could be detected between the inhibitory effects of the two NO synthase inhibitors used (Fig. 2).

We have made an attempt to assess the amount of DHR 123 oxidized by leukocytes during 24 h incubation with LPS. In these experiments, DHR 123 was added together with LPS. Rhodamine fluorescence was on average 2.4-, 2.2- and 1.6-fold higher in granulocytes, monocytes and lymphocytes in LPS-stimulated blood than in unstimulated blood and was markedly attenuated by aminoguanidine or L-NAME (Fig. 3).

4. Discussion

In this study, we have utilized the NO-dependent oxidation of DHR 123 to rhodamine to assess intracellular formation of peroxynitrite. This method is sensitive and specific, because while authentic peroxynitrite oxidizes DHR 123, neither NO nor superoxide causes DHR 123 oxidation [15]. Therefore, it is conceivable that DHR 123 oxidation that is blocked by NO synthase inhibitors can be attributed to peroxynitrite formation. Rhodamine is retained intracellularly, since it is a selective vital stain for mitochondria in living cells [17].

Enhanced DHR 123 oxidation was detected in granulocytes and monocytes as early as at 30 and 60 min after addition of LPS to whole blood. However, this appears to be independent of peroxynitrite formation, since inhibition of constitutive NO synthesis with L-NAME failed to affect DHR 123 oxidation. Instead, the early DHR 123 oxidation could be attributed to superoxide plus hydrogen peroxide, which in the presence of a metal catalyst is known to oxidize DHR 123 to rhodamine [18]. Previous studies have reported that NO donor compounds inhibit peroxynitrite-mediated lipid peroxidation [19] and endothelial dysfunction [20]. These findings coupled with the observation of enhanced DHR 123 oxidation following administration of L-NAME in normal, control rats [10,21] raised the possibility of an NO-mediated inhibition of DHR 123 oxidation. Although both neutrophils and monocytes are known to express constitutive NO synthase [22,23], the amount of NO produced via this pathway might have been insufficient to inhibit effectively DHR 123 oxidation in human leukocytes. The lack of effect of L-NAME on DHR 123 oxidation by leukocytes at 30 and 60 min post-LPS would lend further support to the notion that peroxynitrite detected in the rat plasma within 1 h of the onset of endotoxic shock is formed by a reaction of superoxide (produced by activated blood-borne and/or endothelial cells) with NO derived from the constitutive, endothelial isoform of NO synthase [10]. L-NAME inhibited endothelial NO production, leading to a reduction of DHR 123 oxidation in the plasma of endotoxic rats [10].

At later phases (4 to 24 h) of LPS stimulation, intracellular DHR 123 oxidation can be prevented by either L-NAME, which inhibits all forms of NO synthase, or aminoguanidine, a selective inhibitor of inducible NO synthase [24,25], indicating formation of peroxynitrite in granulocytes, monocytes and to a lesser extent in lymphocytes. While LPS-induced expression of inducible NO synthase in human neutrophil granulocytes [22,23,26] and monocytes [22] has been described, little is known about the expression of inducible NO synthase in lymphocytes. Interleukin-2-stimulated murine T lymphocytes [27] and activated natural killer cells [28] were reported to synthesize NO, most likely via inducible NO synthase. LPS induced increases in rhodamine fluorescence in isolated lymphocytes, that can be prevented by either aminoguanidine or L-NAME, indicating that DHR 123 oxidation detected in lymphocytes can be attributed, at least in part, to peroxynitrite formation by these cells. However, we cannot exclude the possibility that DHR 123 oxidation was due to peroxynitrite that might have diffused into lymphocytes after its production by granulocytes and monocytes. Indeed, a recent study suggests that peroxynitrite could diffuse across the plasma membrane and will be able to reach and diffuse into neighboring cells [29]. One should consider the possibility that rhodamine might have diffused out of some cells and into others. However, addition of rhodamine to unstimulated blood at concentrations (25 nM) similar to those detected in the plasma at 24 h post-LPS, produced only slight increases in intracellular fluorescence, amounting to only 1–2% of the fluorescence detected in leukocytes challenged with LPS. These observations coupled with the fact that plasma rhodamine concentrations in LPS-treated blood were three orders of magnitude lower than those required for optimal staining of mitochondria (i.e. 20–30 μM) [17] suggest that increases in rhodamine fluorescence can be attributed to intracellular DHR 123 oxidation in neutrophils, monocytes and lymphocytes. In addition to inhibition of inducible NO synthase, guanidines may also scavenge an intermediate derived from peroxynitrite [30]. Although this action of aminoguanidine might have contributed to prevention of DHR 123 oxidation, aminoguanidine appears to be a rather weak peroxynitrite scavenger [30]. Consistent with this observation, we could not detect significant differences between the inhibitory effects of L-NAME and aminoguanidine on LPS-induced DHR 123 oxidation. L-NAME is unlikely to be a scavenger of peroxynitrite [31].

Modest, though statistically significant increases in plasma rhodamine concentration were detected as early as after 1 h incubation with LPS. However, these were not due to peroxynitrite production, because they cannot be prevented by the NO synthase inhibitors. By contrast, at later phases of LPS incubation, both L-NAME and aminoguanidine markedly attenuated increases in plasma concentrations of rhodamine. For instance, after 24 h incubation with LPS, about 66 to 86% of rhodamine accumulation in the plasma can be attributed to peroxynitrite formation. Recent results suggest that peroxynitrite could react at a distance from its site of production (i.e. 1–2 cell diameters) even in the presence of excess target molecules [29]. Therefore, peroxynitrite could also oxidize plasma DHR 123 following its release from leukocytes. DHR 123 oxidation in the plasma has previously been used to monitor peroxynitrite production in the rat [10]. However, it is questionable whether rhodamine in plasma can be used as a quantitative assay of peroxynitrite released from leukocytes, because the plasma is a powerful peroxynitrite scavenger. Based on rhodamine accumulation, the amount of peroxynitrite accumulated in the plasma during 24 h incubation with LPS can be estimated as at least 60 to 80 nM, because oxidation of each molecule of DHR 123 requires two molecules of peroxynitrite [15]. The amounts of peroxynitrite produced in whole blood might be even higher, for a significant portion of peroxynitrite produced has been retained intracellularly, as evidenced by the high rhodamine fluorescence detected in granulocytes and monocytes at 24 h after LPS stimulation.

In conclusion, the present study demonstrates that human leukocytes, in particular neutrophil granulocytes and monocytes, can produce and release significant amounts of peroxynitrite in response to LPS, thereby contributing to the increased plasma levels of peroxynitrite in endotoxic shock. Thus, it is plausible to assume that leukocyte-derived peroxynitrite is one of the mediators of endothelial and vascular dysfunction/damage.

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