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Prolonged Exposure to Lipopolysaccharide Inhibits Macrophage 5-Lipoxygenase Metabolism Via Induction of Nitric Oxide Synthesis

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LPS from bacteria can result in the development of sepsis syndrome and acute lung injury. Although acute exposure to endotoxin primes leukocytes for enhanced synthesis of leukotrienes (LT), little is known about the effect of chronic exposure. Therefore, we determined the effect of prolonged LPS treatment on 5-lipoxygenase (5-LO) metabolism of arachidonic acid in alveolar macrophages (AM) and in peripheral blood monocytes. Pretreatment of AM with LPS caused time- and dose-dependent suppression of LT synthetic capacity. LPS pretreatment failed to inhibit arachidonic acid (AA) release. The fact that LPS inhibited LT synthesis from endogenous AA more than from exogenous AA suggested an effect on 5-LO-activating protein (FLAP). In addition, an inhibitory effect of LPS treatment on AM 5-LO activity was suggested by cell-free 5-LO enzyme assay. No effect on the expression of either 5-LO or FLAP proteins was observed. New protein synthesis was necessary for LPS-induced reduction of 5-LO metabolism in AM, and immunoblots demonstrated marked induction of NO synthase (NOS). Inhibition by LPS was reproduced by an NO donor and was abrogated by inhibitors of constitutive and inducible NOS. Compared with AM, peripheral blood monocytes exhibited no suppression by LPS of 5-LO metabolism and no induction of inducible NOS. We conclude that prolonged exposure to LPS impairs AM 5-LO metabolism by NO-mediated suppression of both 5-LO and FLAP function. Because LT contribute to antimicrobial defense, this down-regulation of 5-LO metabolism may contribute to the increased susceptibility to pneumonia in patients following sepsis.


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3 Abbreviations used in this paper: AA, arachidonic acid; AM, alveolar macrophage; COX, cyclooxygenase; EIA, enzyme-linked immunoasay; 5-HETE, 5-hydroxyeicosatetraenoic acid; iNOS, inducible NO synthase; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; LT, leukotriene; L-MMMA, N-monomethyl-L-arginine; L-NIL, L-Nω-(1-iminoethyl)lysine; PBM, peripheral blood monocyte; SNAP, S-nitroso-N-acetylimidodopropionyl.
suppression of LT synthesis than did AM. These effects of prolonged LPS exposure and the effect of NO on LT synthesis provide novel and clinically relevant insights into the regulation of LT synthesis.

Materials and Methods

Cell isolation and culture of rat AM and human PBM

The rat AM was studied because it is a good model of human AM 5-LO metabolism and has been well characterized in our laboratory (27). AM were obtained from 150-g specific pathogen-free female Wistar rats as previously described (26). Lavaged cells were >90% AM; they were >98% AM following adherence, as determined by differential staining. Viability was >95%, as assessed by trypan blue exclusion. PBMC were isolated from both AM and PBL by Ficol-Hypaque centrifugation and adherence, as previously described (28). Cells were >90% PBM by differential staining, and viability was >95% by trypan blue exclusion. Isolated AM and PBM were resuspended in LPS-free DMEM at 0.5 × 10^6/ml and were plated as follows: 0.2 ml/well in 96-well plates for enzyme immunoassay (EIA), 1 ml/well in 24-well plates for [3H]AA release studies, and 5 ml/50-mm culture plate for immunoblot analysis. Cells adhered for 1 h at 37°C in a humidified atmosphere of 5% CO2/95% O2. Nonadherent cells were removed by washing twice with DMEM, and adherent cells were cultured in DMEM containing 10% FCS, with or without various concentrations of LPS (Escherichia coli serotype 0111:4B, Sigma, St. Louis, MO)) and 10% human serum for various time periods.

Quantitation of 5-LO metabolism in intact cells

The maximal capacity for 5-LO metabolism in intact cells was measured by EIA (Cayman Chemicals, Ann Arbor, MI) determination in cell-free supernatants of the predominant 5-LO product, LTB4. Following incubation with or without LPS, the cells were washed three times in DMEM and subsequently incubated with the Ca2+ ionophore A23187 (1 μM) to stimulate the release and metabolism of endogenous AA or exogenous AA (50 μM). This high concentration of AA has been shown to activate 5-LO enzyme activity as well as providing substrate for LT synthesis (28, 29). The EIA results were confirmed by reverse phase HPLC analysis. Briefly, cells were prelabeled overnight with [3H]AA in the presence or the absence of LPS. There was no effect of LPS on cellular uptake of radioactivity (data not shown). The eicosanoid profile was determined by HPLC analysis of 1H-mono- and dihydroxyeicosatetraenoic acid (thromboxane B2, PGE2, LTb4, and 5-hydroxyeicosatetraenoic acid (5-HETE)) released from A23187-stimulated cells as previously described (30). To assess total AA release, cells were stimulated in the presence of 0.1% BSA, which binds AA and prevents both metabolism and reacylation.

5-LO cell-free assay

The 5-LO activity of cell lysates (100 μg of total protein) or of purified recombinant 5-LO (gift from Denis Rendue, Merck Frosst, Montreal, Canada) was determined in reaction mixtures containing 20 μM AA (Cayman Chemicals; including −100,000 dpm of [3H]AA (DuPont-New England Nuclear, Boston, MA)) and 10 μM 13(S)-hydroperoxy-9-cis-11- trans-oxidatedicenoic acid (Cayman Chemicals) as activator in 1 ml of 50 mM Tris, 0.3 mM CaCl2, 0.6 mM EDTA, 0.1 mM ATP, and 12 μg/ml phosphotidylcholine (Avanti Polarlipids, Alabaster, AL) as previously described (31, 32). After a 30-min reaction at room temperature the reaction was stopped by adding 1 ml of ether/MeOH/H2O (3:1:1, v/v/v) and centrifuging at 3500 rpm for 5 min. The upper phase was removed, evaporated under nitrogen, and stored at −70°C. Lipid residues were dissolved in 250 μl of acetonitrile and analyzed by HPLC on a 5-μm Bondapak C18 column (30 × 0.4 cm; Waters Associates, Milford, MA) using a mobile phase of acetonitrile/water/trifluoroacetic acid at a flow rate of 2 ml/min as previously described (27). Radioactivity in 1 ml of eluate fractions was quantitated by on-line radioactivity detection. 5-LO-specific activity was calculated based on conversion of AA to 5-HPETE/5-HETE plus LTB4/LTB4 isomers, and was expressed as nanomoles per milligrams of protein per 10 min.

Immunoblot analysis of 5-LO, FLAP, and iNOS

The relative quantities of cellular 5-LO, FLAP, and iNOS proteins were determined by Western blot analysis. Crude lysates were prepared as previously described (27) and subjected to SDS-PAGE by the method of Laemmli (26). Proteins were transferred overnight to nitrocellulose membranes, and probed with rabbit polyclonal Abs against human leukocyte 5-LO (1/3000 dilution), amino acid residues 41–52 of the human FLAP sequence (1/5000 dilution; both provided by Dr. J. Evans, Merck Frosst) (28), or iNOS (1/2000 dilution; Biomol, Plymouth Meeting, PA). After washing, blots were incubated for 1 h with HRP-conjugated anti-rabbit IgG (Amersham) at a dilution of 1/5000. Membranes were then washed and incubated for 1 min with ECL chemiluminescence detection reagents (Amersham) and exposed to film for varying time periods to ensure that densitometric quantitation was performed under conditions in which band density and exposure time were linearly related. Video densitometry was performed using NIH Image software (Scion, Frederick, MD).

Modulation of NO

The levels of NO in cell cultures were modulated by a number of reagents. Exogenous NO was provided by addition of the NO donor S-nitroso-N-acetylpenicillamine (SNAP) (33). Inhibition of NO generation was accomplished by the addition of an analogue of l-arginine, N-nomonoethyl-l-arginine (l-NAME) (34). Finally, specific inhibition of iNOS-dependent NO generation was accomplished using the specific iNOS inhibitor l-N3-(1-iminoethyl)-lysine (l-NIL) (35).

Data analysis

Where indicated, data were expressed as the mean ± SEM. Intergroup differences were analyzed by ANOVA, with statistical significance assessed by Scheffe’s test; p < 0.05 was considered significant.

Results

Reduced 5-LO metabolism in cultured AM, and not PBM, following LPS treatment

AM treated with LPS overnight consistently demonstrated a marked decline in LTB4 synthesis from endogenous AA. This suppression of 5-LO metabolism was dose dependent (Fig. 1A). A dose of 1 μg/ml suppressed LT synthesis by 81.3 ± 6.3% (n = 6; p < 0.001) compared with that in untreated cells (Fig. 1B). A 1-h incubation with LPS at any dose failed to suppress LTb4 synthesis. In fact, consistent with literature reports, such short term LPS pre-treatment increased AA release (data not shown). A modest decrease in LTB4 synthesis was also seen in PBM treated overnight.
with LPS (69.4 ± 9.5% of untreated cells; n = 4; p = 0.02), but this difference reached statistical significance only when comparing relative (data expressed as a percentage of the value in untreated cells), as shown above, and not absolute levels of LTB4 (as shown in Fig. 1B).

LT synthesis from endogenous AA requires both phospholipase A2-mediated deacylation of AA and FLAP-dependent binding and presentation of AA to 5-LO. We next examined the effect of LPS pretreatment on LT synthesis from exogenous AA. Exogenous AA bypasses the necessity for AA release by cytosolic phospholipase A2 and also reduces the reliance on FLAP by presenting large amounts of the substrate AA directly to the 5-LO enzyme (28). Interestingly, this suppressive effect of overnight LPS treatment of AM was much less marked when LTB4 synthesis from exogenously supplied AA was analyzed (56.0 ± 4.9% of cells untreated with LPS; n = 3; p = 0.05). PBM demonstrated no inhibitory effect of overnight LPS on LT synthesis from exogenous AA (98.2 ± 8.5% of cells untreated with LPS; n = 4; not significant).

LPS has no effect on AA release in macrophages

We next wished to specifically examine the effects of LPS pretreatment on AA release. AM phospholipids were prelabeled overnight with [3H]AA and then stimulated with A23187. Although all the 5-LO products were diminished following LPS treatment (Fig. 2), the synthesis of [3H]COX products (predominantly thromboxane) and of 12-HETE was unchanged. This argues against a reduction in AA release being responsible for the decrease in LT synthesis. This was confirmed in experiments conducted to conclusively measure deacylation of AA by stimulating cells in the presence of 0.1% albumin. Release of AA from macrophages was not affected by LPS treatment (untreated cells, 1.64 ± 0.27% of incorporated radioactivity; LPS-treated cells, 1.93 ± 0.61%; n = 3), indicating that this was not a mechanism for limiting LT synthesis.

LPS treatment reduces 5-LO cell-free activity

The reduction in LT synthesis from exogenous AA, albeit less pronounced than that observed with endogenous AA, suggested an inhibitory effect of LPS on the activity of the 5-LO enzyme itself. We further tested this possibility by performing cell-free 5-LO assays on crude cellular lysates of AM following treatment with or without LPS. Treatment of AM with LPS indeed resulted in reduced 5-LO activity in crude cell lysates (33.5 ± 11.5% of cells untreated with LPS; n = 2). The extent of this suppression of 5-LO activity by LPS treatment was greater than that of LT synthesis by intact AM incubated with exogenous AA, and not as great as that seen with A23187 stimulation of intact AM. These observations indicate that in addition to possible effects on FLAP function, LPS probably has a direct inhibitory effect on the 5-LO enzyme itself.

No effect of LPS on 5-LO and FLAP expression

A reduction in 5-LO metabolism that is slow in onset could theoretically be explained by reduced 5-LO and/or FLAP protein expression. The data presented to date suggested impairment of both these components of 5-LO metabolism in LPS-treated cells. However, Western blot analysis did not demonstrate any reduction in 5-LO or FLAP expression in AM upon overnight treatment with LPS (Fig. 3). Therefore, the mechanism of the LPS effect is likely to involve impaired 5-LO and FLAP actions.

New protein synthesis is necessary for inhibition of 5-LO metabolism by LPS

We next investigated whether new protein synthesis was necessary for the suppression of LT synthesis in AM treated with LPS overnight. Treatment with the protein synthesis inhibitor cycloheximide overnight blocked the inhibitory effect of LPS on 5-LO metabolism (169.5 ± 10.31% of LPS-treated AM; n = 3; p = 0.003). These observations suggested that LPS treatment induced de novo synthesis of a protein that, in turn, was responsible for reduced 5-LO metabolism in AM.

COX metabolism plays only a modest role in inhibition of 5-LO metabolism by LPS

One plausible candidate for such a newly synthesized protein is COX-2, because LPS is known to induce COX-2 in AM (5, 6), and PGE2 is known to inhibit LT synthesis (36, 37). Although A23187-stimulated PGE2 synthesis was indeed increased after LPS treatment (696.7 ± 196.6 vs 259.3 ± 59.5 pg/ml; n = 3; p = 0.01), relatively less of an increase was observed in cells incubated with exogenous AA (1629 ± 59.3 vs 1296.0 ± 138.2 pg/ml; n = 3; p = 0.09). Thus, there was dissociation between LPS-induced enhancement of PGE2 synthesis and inhibition of 5-LO metabolism under the two experimental conditions. Furthermore, the COX inhibitor indomethacin failed to abrogate the LPS-induced inhibition of LT synthesis (LPS-treated cells, 24% of untreated cells; LPS plus indomethacin-treated cells, 26% of untreated cells), conclusively demonstrating that enhanced PG synthesis did not play a significant role in the suppression of 5-LO metabolism.

FIGURE 2. Reduced [3H]-labeled 5-LO products in AM incubated with LPS. Prelabeled cells incubated for 16 h with (solid line) or without (dotted line) LPS were stimulated with A23187 (1 μM) for 30 min, and [3H]AA metabolites were identified by HPLC. Peaks were identified by coelution with authentic standards, and the products were expressed as a percentage of incorporated radioactivity. A representative profile of three separate experiments is shown.
Suppression of 5-LO metabolism in LPS-treated AM involves NO generation

Because LPS also enhances NO production, we examined the effect of this reactive nitrogen intermediate on 5-LO metabolism. When L-NMMA, an analogue of L-arginine that suppresses cellular NO synthesis, was coincubated overnight with LPS-treated AM, it dose-dependently increased LT synthesis (Fig. 4). At L-NMMA doses >10 μg/ml, LPS-induced suppression was completely overcome, and at doses >100 μg/ml, LTB₄ synthesis far exceeded that in the cells untreated with LPS. The effect of treatment of LPS-untreated AM with L-NMMA showed a trend toward an increase in 5-LO metabolism, but this did not reach statistical significance (Fig. 4).

The ability of L-NMMA treatment to overcome the reduction in 5-LO metabolism in LPS-treated AM suggested an important role for NO in regulating LT synthesis under these conditions. Therefore, we added exogenous NO, in the form of the donor compound SNAP, and examined its effect on L-NMMA-treated cells. SNAP reversed the effect of L-NMMA on 5-LO metabolism, further confirming a role for NO in suppressing LT synthesis in LPS-treated AM (Fig. 5A). Furthermore, overnight treatment of rat AM with SNAP alone in the absence of LPS was able to significantly suppress 5-LO metabolism (Fig. 5A). Finally, when purified 5-LO enzyme was incubated for 5 min with the NO donor SNAP, there was a decrease in cell-free enzymatic activity (Fig. 5B). These observations confirm a role for NO in the suppression of 5-LO metabolism by LPS, and indicate that this suppression involves at least in part a direct effect on 5-LO.

In view of the involvement of NO and the dependence on new protein synthesis of the LPS suppression of 5-LO metabolism, we hypothesized that LPS acted via induction of iNOS. Indeed, LPS treatment of AM resulted in markedly increased expression of iNOS (Fig. 6A). Consistent with the fact that suppression of 5-LO metabolism was much more modest in PBM than in AM, PBM failed to demonstrate iNOS induction by LPS (Fig. 6A). Furthermore, in view of the controversy on the elaboration of NO by human AM, we examined the effect of LPS overnight on 5-LO metabolism and NO generation. LPS treatment of human AM for 16 h did not suppress LT synthesis and was not associated with iNOS induction (data not shown). However, exogenous NO, in the form of SNAP, suppressed human AM 5-LO product formation.

We next used a selective iNOS inhibitor to examine the role of NO derived from this synthase isoform in LPS-treated rat AM.
**Discussion**

In this study we examined the effect of prolonged LPS treatment on 5-LO metabolism in macrophages, and now report a number of novel findings: 1) overnight LPS treatment resulted in marked suppression of LT synthesis in AM, but only a modest degree of suppression in PBM; 2) the suppressive effect of LPS treatment on 5-LO metabolism in macrophages entails reductions in both 5-LO and FLAP activity; 3) the LPS effect is independent of changes in AA release as well as 5-LO and FLAP expression; 4) the reduction in 5-LO metabolism is largely explained by LPS-induced synthesis of NO by increased iNOS. Considering their important pathophysiological roles in inflammatory states, synthesis of LTs must be tightly regulated. The observations that LPS and NO can both suppress 5-LO metabolism in intact cells incubated with exogenous AA, and 5-LO, and the information that is available is derived from complex experimental models. In platelet-neutrophil cocultures, exogenous nitroprusside had opposing effects on 5-LO-derived products, reducing LT synthesis and increasing lipoxin synthesis (46). Others have shown in a rabbit leukocyte-perfused heart model that treatment with l-arginine reduced cysteinyl-LT levels, (46). Others have shown in a rabbit leukocyte-perfused heart model that treatment with l-arginine reduced cysteinyl-LT levels, which was associated with reduced coronary perfusion pressure (34). Our work extends the observation that endogenous NO inhibits lipoxigenase metabolism (47). Although the above studies suggest that NO may inhibit lipoxigenase metabolism, other investigators have noted that NO up-regulates the activity of lipoxigenases (48, 49), including 5-LO (50), in different models.

Like L-NMMA, L-NIL treatment dose-dependently overcame the inhibitory effect of LPS on AM 5-LO metabolism (Fig. 6B). Selective iNOS inhibitors had no effect on LT synthesis in LPS-treated PBM (data not shown). These data suggest that iNOS was an important source of NO, which resulted in reduced 5-LO metabolism in LPS-treated AM.

**What are the clinical implications of our findings?** Although human AM may not elaborate NO under normal conditions, there is evidence that NO is produced in AM in inflammatory states, e.g., acute lung injury (51), emphysema (52), and idiopathic pulmonary fibrosis (53). In addition, we have demonstrated that exogenous NO suppresses 5-LO metabolism in human AM. Furthermore, in the human lung other cell types, e.g., epithelial and endothelial cells, elaborate large amounts of NO, which, in turn, may suppress AM LT synthesis. The reduction in the synthesis of proinflammatory, smooth muscle constrictive, and edemagenic LTs may help to explain the salutary effects of inhaled NO in various models. These effects include relaxation of airway tone in asthmatics (54, 55) and of pulmonary vascular tone in pulmonary
hypertension (56, 57), and inhibition of neutrophil migration (58) and endothelial permeability in models of acute lung injury (59). Therefore, exogenous treatment with NO may be a method of suppressing excessive 5-LO product formation that contributes to the pathogenesis of asthma (60), idiopathic pulmonary fibrosis (61), and acute lung injury (62). In contrast, prolonged exposure of macrophages to LPS is relevant in patients who survive the acute phase of sepsis secondary to Gram-negative bacteria (63). Our data predict that the macrophage’s capacity to elaborate LT would be compromised under these circumstances. This, in turn, might be expected to impair host defense mechanisms, because LT enhance the phagocytosis and killing of microorganisms (14, 64, 65). In summary, we have demonstrated for the first time that exposure of AM to LPS for a prolonged period suppresses LT synthesis in vitro via an NO-dependent mechanism. The induction of iNOS with associated NO generation results in reduced 5-LO and FLAP activity. This reduction in 5-LO metabolism in the setting of prolonged LPS exposure may represent an endogenous means to limit the inflammatory response, but may at the same time increase susceptibility to infection. iNOS inhibitors may have utility in restoring LT synthetic capacity and thereby enhancing host defense capabilities in subjects surviving sepsis.

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