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. The Journal of Immunology, 2000, 165: 3592–3598.

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3 Abbreviations used in this paper: AA, arachidonic acid; AM, alveolar macrophage; COX, cyclo-oxygenase; ELA, enzyme-linked immunosassay; 5-HETE, 5-hydroxyeicosatetraenoic acid; iNOS, inducible NO synthase; 5-LO, 5-lipoxygenase; FLAP, 5-LO-activating protein; LT, leukotriene; L-NMMA, N-monomethyl-L-arginine; L-NIL, N-((1-iminoethyl)-lysine; PBM, peripheral blood monocyte; SNAP, S-nitroso-N-acetylpenicillamine.

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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. AM were isolated from rat BM (by Ficoll-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) 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 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 [3H]-radiolabeled eicosanoids (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 recycling.

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 Riendeau, 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-octadecadienoic 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 phosphatidylcholine (Avanti Polarlipids, Alabaster, AL) 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/S-HETE plus LTB4/LTB2 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 (28). 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; Biocon, 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 t-arginine, N-monomethyl-L-arginine (t-NMMA) (34). Finally, specific inhibition of iNOS-dependent NO generation was accomplished using the specific iNOS inhibitor 1-NN-(1-iminoethyl)-lysinine (t-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 pretreatment increased AA release (data not shown). A modest decrease in LTB4 synthesis was also seen in PBM treated overnight.
NO INDUCTION BY LPS SUPPRESSES 5-LO METABOLISM

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. Prelabelled 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.
iNOS plays a role in the suppression of 5-LO metabolism in AM, but not in PBM. A, AM and PBM were incubated for 16 h with or without LPS (1 μg/ml). Crude cell lysates were prepared, and equal amounts (20 μg) of crude cellular lysate were subjected to immunoblot analysis for iNOS as described in Materials and Methods. Shown is a representative autoradiograph from three separate experiments. B, AM were incubated for 16 h with or without LPS (1 μg/ml) in the presence or the absence of the selective iNOS inhibitor t-NIL. Cells were then stimulated with A23817 (1 μM) for 30 min at 37°C, and medium was analyzed for LTB₄ by EIA. Data shown are from a representative experiment of three performed.

Like t-NMMA, t-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.

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 provide crucial new insights into the control of this pathway.

LPS is present on the outer membrane of Gram-negative bacteria and plays an important role in inflammatory reactions that occur in response to these infections (1). It is well known that LPS activates macrophages to release proinflammatory mediators, including superoxide (38), cytokines (39), NO (17), and AA metabolites (40). Short term (2-h) treatment with LPS has been reported to prime PBM for increased AA release and enhanced LT synthesis following stimulation with FMLP (41). However, this priming effect waned after 2 h. Other investigators have demonstrated that i.v. LPS increased ex vivo synthesis of LTB₄ by rat AM on day 1, but this returned to control levels on day 3 (42). LPS for 1–2 h in vitro primed human AM for A23187-stimulated LTB₄ synthesis (8, 43).

Our data suggest that multiple mechanisms underlie the reduced 5-LO metabolism by LPS. As discussed above, the effect is not at the phospholipase A₂ level, because there was no reduction in AA release itself or in the synthesis of eicosanoids derived from AA via the COX and 12-LO pathways with LPS treatment. LPS globally suppressed the formation of all 5-LO metabolites, but did not affect the expression of 5-LO or FLAP. Separate effects on the function of these two proteins appear to be involved. First, cell-free 5-LO activity was reduced. NO has been proposed to inactivate lipoxygenases by reducing the ferric enzyme to the ferrous form, which is inactive (33, 44). This also explains why LT synthesis is reduced in intact cells incubated with exogenous AA. An additional inhibition by LPS of FLAP activity was suggested by the observation that a reduction in LT synthesis was less marked when the cells were incubated under conditions that are relatively FLAP independent (exogenous AA) than when they were stimulated under conditions that are highly FLAP dependent (endogenous AA mobilized by A23187).

Induction of COX-2-dependent synthesis of PGE₂ by LPS treatment represented one possible mechanism by which LT synthesis was suppressed in AM. However, a number of lines of evidence argue against such a mechanism. First, the degree of PGE₂ augmentation by LPS was quite modest, consistent with previous observations in AM from the rat (45). Second, PG production was greater in LPS-treated AM incubated with exogenous AA, whereas the suppressive effect of LPS was more pronounced in A23187-treated cells. Finally, inhibition of PG production with indomethacin did not restore LT synthetic capacity in LPS-treated AM. The nature of the interaction of NO with COX has proven controversial. NO has been reported to both augment (21, 22) and suppress (23) COX activity in various systems. In our studies inhibition of NO induction tended to augment COX product synthesis to a minor degree.

There is less information available about the interactions of NO 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, which was associated with reduced coronary perfusion pressure (34). Our work extends the observation that endogenous NO inhibits lipoxygenase metabolism (47). Although the above studies suggest that NO may inhibit lipoxygenase metabolism, other investigators have noted that NO up-regulates the activity of lipoxygenases (48, 49), including 5-LO (50), in different models.

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 micro-organisms (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.

References


