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Lipopolysaccharide Down-Regulates the Leukotriene C4 Synthase Gene in the Monocyte-Like Cell Line, THP-1

Kenneth J. Serio, Scott C. Johns, Linda Luo, Craig R. Hodulik, and Timothy D. Bigby

We studied the effects of LPS on cysteinyl leukotriene (LT) synthesis and LTC4 synthase expression in mononuclear phagocytes. Conditioning of the monocyte-like cell line, THP-1, with LPS for 7 days resulted in significantly decreased ionophore-stimulated LTC4 release. The putative LPS receptor, Toll-like receptor 4, was expressed in THP-1 cells. LPS down-regulated LTC4 synthase mRNA in THP-1 cells in a dose- and time-dependent manner, with down-regulation observed as early as 4 h. Conditioning of actinomycin D-treated cells with LPS resulted in no change in the rate of LTC4 synthase mRNA decay. LPS treatment of THP-1 cells, transiently transfected with a LTC4 synthase promoter (1.35 kb)-reporter construct, decreased promoter activity. Neutralization of TNF-α and inhibition of mitogen-activated protein kinase kinase/extracellular signal-regulated kinase did not inhibit the effect of LPS. Treatment of cells with a Toll-like receptor 4-blocking Ab and an inhibitor of NF-κB activation resulted in inhibition of the LPS effect, while activation of NF-κB and p50/p65 overexpression down-regulated the LTC4 synthase gene. LPS down-regulates cysteinyl LT release and LTC4 synthase gene expression in mononuclear phagocytes by an NF-κB-mediated mechanism. The Journal of Immunology, 2003, 170: 2121–2128.

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Leukotrienes (LTs) are arachidonic acid-derived metabolites that are synthesized via the 5-lipoxygenase (5-LO) pathway. The formation of LTC4 by the LTC4 synthase enzyme represents the first committed step in the synthesis of the cysteinyl LTs, LTC4, LTD4, and LTE4. These potent mediators bind to the cysteinyl LT-1 receptor (expressed in lung smooth muscle cells, eosinophils, and macrophages) (1) and the cysteinyl LT-2 receptor (expressed in lymph node, spleen, heart, CNS cells, macrophages, and PBLs) (2). The cysteinyl LTs mediate a wide variety of biologic responses including enhanced vascular permeability, smooth muscle contraction, mucus hypersecretion, bronchial hyperreactivity (3), and eosinophil chemotaxis (4). The previously described role of the LTs strongly implicates them in the pathogenesis of allergic and inflammatory diseases, such as asthma and sepsis (3, 5–7).

LTC4 synthase (Enzyme Commission no. 2.5.1.37) is a selective, membrane-bound glutathione-S-transferase that catalyzes the conversion of LTA4 to LTC4. Tissue distribution of the LTC4 synthase enzyme is restricted to eosinophils, basophils, mast cells, platelets, and cells of monocyte/macrophage lineage. LTC4 synthase is a member of a superfamily of structurally related enzymes, which has been termed the membrane-associated proteins in eicosanoid and glutathione metabolism (8). Although the membrane-associated proteins in eicosanoid and glutathione metabolism famil

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3 Abbreviations used in this paper: LT, leukotriene; 5-LO, 5-lipoxygenase; TLR, Toll-like receptor; MAP, mitogen-activated protein; MEK, MAP kinase kinase; ERK, extracellular signal-regulated kinase.
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Materials and Methods

Cell culture

THP-1 cells were obtained from American Type Culture Collection (Manassas, VA). The monocyte-like cell line, THP-1, has been used as an effective model for the study of regulation of the 5-LO pathway in previous studies (23, 24, 12, 11). THP-1 cells were grown at 37°C with 5% CO₂ in RPMI 1640 medium supplemented with 10% heat-treated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml gentamicin. The media were changed every 2 days for all experiments.

ELISA for LTC₄ release from LPS-conditioned THP-1 cells

THP-1 cells were conditioned for 7 days with Salmonella minnesota Re595 LPS (Re LPS) at 100 ng/ml. The cells were harvested and subsequently resuspended in HBS at a concentration of 10 × 10⁶ cells per ml. The cells were incubated at 37°C with catalase (at 60 μg/ml) (Sigma-Aldrich, St. Louis, MO) for 15 min to inhibit conversion of LTC₄. The calcium ionophore, A23187 (at 1 μM), was added and the cells were incubated for 15 min at 37°C. Supernatants were assayed for LTC₄ release by ELISA (Oxford Biomedical Research, Oxford, MI), per the manufacturer’s instructions.

RT-PCR determination of TLR expression in THP-1 cells

Total RNA was extracted from THP-1 cells by a single-step guanidinium thiocyanate method (29). A reverse-transcriptase reaction was performed on 5 μg of extracted total RNA using the Superscript II Kit (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions. Following cDNA synthesis, a PCR was then performed with primers specific for TLRs 1–5 (TLR1: forward 5′-GGGTCAGCTGGACTTCA with expected product size of 214 bp; TLR2: forward 5′-GGCCAGCAAATTACCT with expected product size of 167 bp; and TLR5: forward 5′-AACATCTGCTCAGGAGAC with expected product size of 212 bp). Following cDNA synthesis, a PCR was then performed with primers specific for TLRs 1–5 (TLR1: forward 5′-GGGTCAGCTGGACTTCA with expected product size of 214 bp; TLR2: forward 5′-GGCCAGCAAATTACCT with expected product size of 167 bp; and TLR5: forward 5′-AACATCTGCTCAGGAGAC with expected product size of 212 bp).

Northern blot analysis for LTC₄ synthase mRNA

THP-1 cells were conditioned with Re LPS at doses ranging from 0–100 ng/ml and for periods up to 24 h. Additional experiments were performed using the following: the NF-κB activator, paraoxon; the NF-κB inhibitor, bisperoxyvanadium (phen); the MAP kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) inhibitor, PD98059; the JID9 anti-TNF-α neutralizing Ab (30); the monoclonal HTA414 anti-TLR4 Ab (31); and/or actinomycin D. Re LPS was used in the studies because it is composed primarily of lipid A and lacks the O and the outer core polysaccharides (32).

Data analysis

Data are expressed as the mean ± SEM in all circumstances where mean values are compared. Data were analyzed by unpaired Student’s t test (InStat, version 2.03; GraphPad Software, San Diego, CA). Differences were considered significant when p < 0.05.

Results

LPS conditioning decreases calcium ionophore-stimulated LTC₄ release from THP-1 cells

To determine the effect of LPS conditioning on cytokin LT release, THP-1 cells (at 0.5 × 10⁶ cells per ml) were conditioned with Re LPS (at 100 ng/ml) for 7 days. The cells were harvested and stimulated with the calcium ionophore, A23187 (at 1 μM), with LTC₄ release quantitated by ELISA. Calcium ionophore stimulation has been shown to elicit LT release in previous studies (38, 39). LPS conditioning for 7 days significantly decreased ionophore-stimulated LTC₄ release from THP-1 cells, as compared with control (0.33 ± 0.03 vs 1.13 ± 0.03 ng LTC₄ released per million cells; mean ± SEM; n = 4, p < 0.0001, respectively) (Fig. 1). LPS conditioning of THP-1 cells for 7 days at the lower Re LPS dose of 10 ng/ml less potently, but significantly, decreased ionophore-stimulated LTC₄ release to 71% of control (mean ± SEM; n = 4, p < 0.05).

TLR expression in THP-1 cells

To determine the pattern of expression of the putative LPS receptor, TLR4, and the other TLRs in the THP-1 cell line, RT-PCR was
performed on 5 μg of extracted total RNA, followed by a PCR using primers specific for each of the TLRs 1–5. The mRNAs for TLR 1, 2, and 4 were detected in THP-1 cells (Fig. 2).

**FIGURE 2.** TLR4 is expressed in THP-1 cells. Total RNA was extracted from the cells and 5 μg were subjected to a reverse-transcription reaction, followed by a PCR using primers specific for each of TLRs 1–5. The mRNAs for TLR 1, 2, and 4 were detected in THP-1 cells. The expected 167-bp band for the putative LPS receptor, TLR4, is denoted.

**FIGURE 1.** LPS conditioning decreases calcium ionophore-stimulated LTC4 release from THP-1 cells. THP-1 cells were conditioned with Re LPS at 100 ng/ml for 7 days. The cells were stimulated with the calcium ionophore, A23187, at 1 μM for 15 min. The supernatants were assayed for LTC4 release by ELISA. LPS conditioning decreased ionophore-stimulated LTC4 release from THP-1 cells. Data represent the mean ± SEM, n = 4 (*, p < 0.0001).

**FIGURE 3.** LPS dose-dependently down-regulates LTC4 synthase mRNA accumulation in THP-1 cells. THP-1 cells were conditioned for 24 h with Re LPS at doses ranging from 0.001 to 100 ng/ml. The dose of 100 ng/ml was chosen for the transfection experiments because it was deemed to be well above the IC50 (of 1 ng/ml) that was observed for the LPS effect on LTC4 synthase mRNA. Conditioning with Re LPS conditioning resulted in a significant decrease in LTC4 synthase mRNA accumulation with an IC50 of ~1 ng/ml (Fig. 3).

**LPS down-regulates LTC4 synthase mRNA accumulation in a dose-dependent manner in THP-1 cells**

To determine the effect of LPS on LTC4 synthase mRNA accumulation, THP-1 cells (at 0.5 × 10⁶ cells per ml) were conditioned for 24 h with Re LPS (10 ng/ml). Treatment with Re LPS resulted in a dose-dependent decrement in LTC4 synthase mRNA accumulation with an IC50 of ~1 ng/ml (Fig. 3).

**LPS down-regulates LTC4 synthase mRNA accumulation in a time-dependent manner in THP-1 cells**

To determine the time course of the effect of LPS on LTC4 synthase mRNA accumulation, THP-1 cells (at 0.5 × 10⁶ cells per ml) were conditioned for periods up to 24 h with Re LPS (10 ng/ml). Treatment with Re LPS resulted in a time-dependent decrement in LTC4 synthase mRNA, with a significant decrease observed as early as 4 h (0.83 ± 0.09 densitometric units normalized to control; mean ± SEM; n = 3, p < 0.0001) (Fig. 4, A and B).

**LPS does not modulate the rate of LTC4 synthase mRNA decay in THP-1 cells**

To determine whether the down-regulatory effect of LPS on LTC4 synthase mRNA was associated with enhanced mRNA degradation, THP-1 cells (at 0.5 × 10⁶ cells per ml) were conditioned with Re LPS (10 ng/ml) and actinomycin D (2 μg/ml). Conditioning with Re LPS did not result in shortened LTC4 synthase mRNA half-life, as determined by the slope of mRNA decay of the LPS-treated cells, as compared with the slope for control cells (Fig. 4, C and D). These data suggest that LPS does not accelerate the rate of LTC4 synthase mRNA decay, indicating a likely transcriptional mechanism. In addition, at early time points, actinomycin appears to block the effect of LPS, suggesting that transcription of an intermediary gene may be required for the effect of LPS (Fig. 4, C and D).

**LPS down-regulates LTC4 synthase promoter activity in THP-1 cells**

To determine whether LPS down-regulates LTC4 synthase gene transcription, THP-1 cells were transiently transfected with the 1.35-LTCS-pGL3 construct and were subsequently conditioned with Re LPS (at 100 ng/ml). The dose of 100 ng/ml was chosen for the transfection experiments because it was deemed to be well above the IC50 (of 1 ng/ml) that was observed for the LPS effect on LTC4 synthase mRNA. Conditioning with Re LPS conditioning resulted in a significant decrease in reporter activity, suggesting that the down-regulatory effect of LPS is mediated by an inhibition of LTC4 synthase gene transcription (Fig. 5).

Neutralization of TNF-α does not inhibit the down-regulatory effect of LPS on LTC4 synthase mRNA accumulation in THP-1 cells

To determine whether TNF-α secretion by THP-1 cells mediates the down-regulatory effect of LPS on LTC4 synthase mRNA via an autocrine/paracrine effect, cells (at 1 × 10⁶ cells per ml) were pre-conditioned with anti-TNF-α neutralizing Ab (at 5 μg/ml), for 2 h, followed by the further addition of Re LPS (at 10 ng/ml) for an additional 12 h. In the presence of anti-TNF-α, the down-regulatory effect of LPS on LTC4 synthase mRNA was not inhibited (Fig. 6). These data suggest that the down-regulatory effect of LPS on the LTC4 synthase gene acts via a TNF-α-independent mechanism. Anti-TNF-α treatment alone had no effect on LTC4 synthase mRNA (0.96 densitometric units, normalized to control; mean, n = 1).
Blockade of TLR4 inhibits the down-regulatory effect of LPS on LTC₄ synthase mRNA accumulation in THP-1 cells

To determine whether LPS acts via TLR4 to down-regulate LTC₄ synthase mRNA, THP-1 cells (at 1 x 10⁶ cells per ml) were preconditioned with anti-TLR4 Ab (at 5 μg/ml), for 2 h, followed by the further addition of Re LPS (at 10 ng/ml) for an additional 12 h. In the presence of anti-TLR4, the down-regulatory effect of LPS on LTC₄ synthase mRNA was inhibited (Fig. 6). These data suggest that the down-regulatory effect of LPS on the LTC₄ synthase gene acts via the putative LPS receptor, TLR4. Anti-TLR4 treatment alone minimally increased LTC₄ synthase mRNA (1.17 densitometric units, normalized to control; mean; n = 2).

Inhibition of MEK/ERK activity does not inhibit the down-regulatory effect of LPS on LTC₄ synthase mRNA accumulation in THP-1 cells

To determine whether the ERK pathway mediates the down-regulatory effect of LPS on LTC₄ synthase mRNA, THP-1 cells (at 1 x 10⁶ cells per ml) were preconditioned with the MEK/ERK inhibitor, PD98059 (at 10 μM), for 4 h, followed by the further addition of Re LPS (at 10 ng/ml) for an additional 16 h. In the presence of PD98059, the down-regulatory effect of LPS on LTC₄ synthase mRNA was not inhibited (Fig. 7). These data suggest that the MEK/ERK pathway does not mediate the down-regulatory effect of LPS on the LTC₄ synthase gene. In addition, MEK/ERK...
inhibition independently increases LTC₄ synthase mRNA accumulation, suggesting that basal activation of this pathway may serve to constitutively inhibit LTC₄ synthase gene expression.

Inhibition of NF-κB activation blocks the down-regulatory effect of LPS on LTC₄ synthase mRNA in THP-1 cells

To determine whether the down-regulatory effect of LPS on LTC₄ synthase mRNA was mediated by the transcription factor, NF-κB, THP-1 cells (at 1 × 10⁶ cells per ml) were preconditioned for 2 h with the NF-κB activation inhibitor, parthenolide (at 10 μM), followed by the addition of Re LPS (at 10 ng/ml) for an additional 12-h incubation. Inhibition of the MEK/ERK pathway activity does not inhibit the down-regulatory effect of LPS on LTC₄ synthase mRNA (Re LPS + PD98059). Inhibition of the MEK/ERK pathway increases LTC₄ synthase mRNA accumulation (PD98059). Data represent mean ± SEM, n = 3 (*, p < 0.0001; **, p < 0.05).

Bisperoxynavadium (phen) down-regulates LTC₄ synthase mRNA in a time-dependent manner in THP-1 cells

To determine the functional role of the transcription factor, NF-κB, in the LPS-induced down-regulation of LTC₄ synthase promoter activity, THP-1 cells (at 1 × 10⁶ cells per ml) were conditioned with the NF-κB activator, bisperoxynavadium (phen) (at 10 μM), for periods up to 24 h. The addition of bisperoxynavadium (phen) resulted in a time-dependent decrease in LTC₄ synthase mRNA accumulation, occurring as early as 8 h (Fig. 9).

Overexpression of p50 and p65 down-regulates LTC₄ synthase promoter activity in THP-1 cells

To determine the functional role of the NF-κB component proteins, p50 and p65, THP-1 cells were transiently cotransfected with the 1.35LTCS-pGL3 construct, a Renilla luciferase pHRL-TK construct, and pRSV-p50 and pRSV-p65 expression vectors. Following transfection, the cells were incubated for 24 h, lysed, and assayed for luciferase activities. Overexpression of the NF-κB components, p50 and p65, resulted in significant decreases in LTC₄ synthase promoter activity (Fig. 10).
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FIGURE 10. Overexpression of p50 and p65 down-regulates LTC₄ synthase promoter activity in THP-1 cells. THP-1 cells were transiently co-transfected with the 1.35 LTCS-pGL3 construct, a Renilla luciferase pRL-TK construct, and pRSV-p50 and pRSV-p65 expression vectors. The cells were incubated for 24 h, lysed, and assayed for luciferase activities. Overexpression of the NF-κB components, p50 and p65, resulted in a significant decrease in LTC₄ synthase promoter activity. Data represent the firefly luciferase values normalized to Renilla luciferase values, mean ± SEM, n = 6 (*, p < 0.05; **, p < 0.01).

Discussion

In this report, we demonstrate that prolonged exposure to the bacterial component, LPS, inhibits cysteinyl LT release by the monocyte-like cell line, THP-1. LPS down-regulates LTC₄ synthase mRNA at biologically relevant concentrations (40). We demonstrate that the effect of LPS on LTC₄ synthase mRNA is dose- and time-dependent, occurring as early as 4 h after exposure. Transfection assays and actinomycin D half-life studies indicate that LPS acts to suppress LTC₄ synthase gene transcription but does not affect the rate of mRNA degradation. The effect of LPS on LTC₄ synthase mRNA does not appear to be mediated via the NF-κB components, p50 and p65, but not in human monocytes, THP-1 cells have been demonstrated to release TNF-α by itself produces a similar effect (39). Although the majority of prior studies have addressed the effects of acute LPS exposure, our studies demonstrate that LPS exposure decreases LTC₄ synthase gene expression within hours (with an effect persisting through 24 h) and that prolonged LPS exposure (for up to 7 days) results in a sustained decrease in the capacity for LTC₄ synthase synthesis by THP-1 cells. The observed effects of LPS on the LTC₄ synthase gene stand in the context of prior work that implicates the LTs in the pathogenesis of the sepsis syndrome. Previous studies investigating the role of the cysteinyl LTs in animal sepsis models (24, 25, 42) suggest that these mediators may function in sepsis to mediate alterations in systemic vascular tone and permeability. Prior work suggests that LTs derived from the 5-LO pathway may serve a protective role in response to bacterial infection (43) and the decreased capacity to synthesize cysteinyl LTs has been associated with increased mortality in sepsis (7). However, the biologic consequences of altered cysteinyl LT synthesis by monocytes/macrophages in response to LPS exposure is unclear. Importantly, our data provide a possible mechanism for the observed findings in sepsis, in that LPS suppression of LTC₄ gene expression in mononuclear phagocytes may contribute to the observed in vivo decrease in synthetic capacity.

Specific autocrine/paracrine mechanisms of mediator release are believed to account for the biologic effects of LPS. LPS acts on inflammatory cells to enhance generation of arachidonic acid (44, 45) and its metabolites (16), as well as to increase the formation of NO (17). LPS exposure also induces the release of various cytokines/growth factors, such as IL-1, IL-6, IL-8, TNF-α (46–48).

The specific induction of TNF-α by LPS (47) may significantly contribute to the observed biologic effects ascribed to LPS in various cell systems (48) and in sepsis (49, 50). In response to LPS, THP-1 cells have been demonstrated to release TNF-α (51). Importantly, our studies indicate that the down-regulatory effect of LPS on expression of the LTC₄ synthase gene does not involve a TNF-α-dependent autocrine/paracrine mechanism. In addition, our findings suggest that LPS acts via the LPS receptor, TLR4, to elicit intracellular signaling events that result in the decreased transcription of LTC₄ synthase mRNA.

We demonstrate the presence of mRNA for the LPS receptor, TLR4, in THP-1 cells, consistent with previous reports (52). Our studies indicate that LPS acts via TLR4, which is believed to be responsible for LPS signal transduction in monocytes/macrophages (53–56), to down-regulate LTC₄ synthase gene expression. Following LPS binding to TLR4, activation of various MAP kinase pathways in monocytes/macrophages may occur, such as ERK (57), p54 stress-activated protein kinase/c-Jun N-terminal kinase (58), and p38 MAP kinase (59). Our studies indicate that the MEK/ERK pathway does not mediate the observed LPS inhibition of LTC₄ synthase gene expression in THP-1 cells. However, our data indicate that pharmacologic inhibition of the MEK/ERK pathway increases LTC₄ synthase mRNA accumulation. These findings suggest that a basal level of ERK activation functions to inhibit constitutive LTC₄ synthase expression. Although previous studies have not specifically examined the role of MEK/ERK activation in the regulation of LTC₄ synthase gene expression, prior work suggests that ERK activation (by fMet-Leu-Phe in IL-5-primed human eosinophils) enhances immediate LTC₄ synthesis (60). The contrasting effects of ERK activation on LTC₄ synthase gene expression and/or activity may be related to differences in cell type, type of bacterial stimulus, or the time-course of ERK activation. Elucidation of the mechanisms involved in inhibition of constitutive LTC₄ synthase gene expression requires further study.

The molecular mechanisms that mediate the downstream effects of LPS may involve the activation of multiple classes of transcription factors, including NF-κB (61–63), Sp1 (59), c-Jun (58, 64), Ets (64), and Egr-1 (64). Results obtained from the actinomycin D and transient transfection studies indicate that LPS does not alter the rate of LTC₄ synthase mRNA degradation, but does down-regulate gene transcription through an element located within the first 1.35 kb of the 5′-UTR. The demonstration of LPS inhibition of LTC₄ synthase mRNA expression within hours is consistent with a transcriptional mechanism of action. Our studies implicate NF-κB in this mechanism, as suppression of NF-κB activation inhibits the effect of LPS on LTC₄ synthase gene expression and induction of NF-κB activation by itself produces a similar effect (with a similar time-course) to that of LPS. Pharmacologic activation (by bisperoxynitridum) of NF-κB by tyrosine phosphorylation may be accompanied by the phosphorylation of other proteins, as well. However, our data further support the role of NF-κB in mediating the effect of LPS by demonstrating that overexpression of the NF-κB components, p50 and p65, decreases LTC₄ synthase promoter activity. Although database analysis reveals that
the LTC4 synthesize promoter contains multiple partial NF-κB consensus sites, no functional NF-κB sites have been identified. Although NF-κB has been reported to up-regulate the transcription of over 150 genes (61), the demonstration of repression of gene tran-
scription by NF-κB family members has been reported to a more
limited extent (65, 66). Our data does not specifically address the
direct binding of NF-κB to the LTC4 synthesize promoter or the
potential synergistic or antagonistic interaction with other
transcription factors (67–69). A large body of evidence indicates that
LPS induces NF-κB activation in THP-1 and other monocyteic cells
(61) and a correlation has been demonstrated between NF-κB
binding activity and sepsis mortality (62). However, other studies
indicate that levels of the active NF-κB heterodimer, p50-p65, are
decreased in the monocytes of patients with sepsis (70). These
conflicting data suggest that further study of the effects of LPS and
other bacterial components on the regulation of inflammatory me-
diator genes in monocytes/macrophages is necessary. Future stud-
ies will examine whether NF-κB acts by direct inhibition of LTC4
synthase gene transcription or by induction of an intermediary
suppressor gene/protein.

Our findings demonstrate that cysteiny1 LT synthesis is down-
regulated by the bacterial component, LPS, in mononuclear phago-
cytes via an NF-κB-mediated mechanism. This mechanism in-
volves TLR4-mediated down-regulation of LTC4 synthase mRNA
expression that occurs in a TNF-α-independent manner. We be-
lieve that this observation may have important implications for the
modulation of cysteiny1 LT synthesis and the role of these potent
mediators in the systemic response to inflammatory conditions
such as the sepsis syndrome. The molecular mechanisms that me-
diate LPS modulation of the LTC4 synthase gene remain to be
determined.

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