Differential Modulation of Leukotriene B₄ Synthesis and Degradation in Human Bronchoalveolar Lavage Cells by Lipopolysaccharide and Tobacco Smoke

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Abstract

Leukotrienes have been implicated to play a prominent inductive role in carcinogenesis. We previously reported that bronchoalveolar lavage (BAL) cells from smokers manifested higher levels of leukotriene B₄ (LTB₄) production than ex-smokers. This study aims to elucidate the underlying mechanism(s). BAL cells from current and former smokers were exposed to lipopolysaccharide (LPS) for up to 7 days. LPS induced the release of LTB₄ from BAL cells and down-regulated 5-lipoxygenase (5-LOX) mRNA expression in a dose-dependent manner, followed by a decrease in 5-LOX protein production and normalization of LTB₄ levels. Exogenous LTB₄ inhibited LPS-induced 5-LOX activity and accentuated the down-regulation of 5-LOX mRNA, whereas suppression of 5-LOX abrogated the LPS-induced changes, suggesting a negative feedback mechanism. LPS concomitantly induced expression and activity of the LTB₄ metabolizing enzyme LTB₄ ω-hydroxylase (LTB₄OH) in ex-smokers’ BAL cells, but not in smokers’ BAL cells. In vitro smoke exposure of ex-smokers’ BAL cells also abrogated the LPS-induced up-regulation of LTB₄OH mRNA expression. Furthermore, ex-smokers’ BAL cells expressed significantly higher LTB₄OH mRNA levels than smokers’ BAL cells. Such differential modulation of LTB₄ synthesis and degradation by LPS in the setting of tobacco smoke exposure suggests that mechanisms responsible for sustained elevation of LTB₄ levels in the lung microenvironment may contribute to the pathogenesis of tobacco-related respiratory diseases such as lung cancer. By regulating the balance of LTB₄ in the lung, LTB₄OH may function as a suppressor of lung carcinogenesis.

Arachidonic acid is converted within inflammatory cells to leukotrienes by lipoxygenases (LOX) and to prostaglandins by cyclooxygenases (COX; ref.1). Leukotrienes are generated via the action of the 5-LOX enzyme in concert with the 5-LOX activating protein (FLAP; ref. 2). The biologically active 5-LOX pathway metabolites are leukotriene B₄ (LTB₄) and the cysteinyl leukotrienes (LTC₄, LTD₄, and LTE₄). LTB₄ is rapidly and specifically catabolized via ω-oxidation by LTB₄ ω-hydroxylase (LTB₄OH; ref. 3). LTB₄OH belongs to a family of cytochrome P450 enzymes. This inactivation of endogenous LTB₄ has been studied in various cellular systems including the human neutrophils, which efficiently metabolize LTB₄ via a cytochrome P450 4F3-dependent pathway, to produce 20-hydroxy-LTB₄ (refs. 4, 5). The 5-LOX pathway–derived leukotrienes have been extensively implicated in the pathogenesis of a variety of respiratory diseases, including allergic rhinitis, asthma, chronic obstructive pulmonary disease, pneumonia, scleroderma lung disease, acute lung injury, and lung malignancies (6–12). Alveolar macrophages are the predominant effector cells in the lung microenvironment and are a major source of LTB₄, a potent leukocyte activator. LTB₄ is a powerful chemoattractant, promoting recruitment and adhesion of leukocytes to vascular endothelium. It also elicits the production and release of proinflammatory cytokines from macrophages and lymphocytes. Leukotrienes may be involved in regulation of angiogenesis (13) and have been reported to play a significant role in the pathogenesis and progression of a variety of cancers, with 5-LOX expression being reported in lung, colon, breast, prostate, and other cancers (1, 7, 14–18).

Lipopolysaccharide (LPS) is a glycolipid component of the Gram-negative bacterial cell wall. Among its myriad proinflammatory effects, it is known to modulate the production of arachidonic acid metabolites by monocytes and macrophages. LPS has been known to induce the synthesis of COX-derived prostaglandins (19, 20), whereas the role of LPS in modulating the 5-LOX pathway is less clear; prior work suggests that acute, high-dose LPS exposure primes for leukotriene release (21). Recent studies, however, indicate...
that this modulation is more complex than previously believed, as prolonged LPS exposure inhibits stimulated LTB₄ release from rat alveolar macrophages (22) and stimulated cysteiny1 leukotriene release from the monocyte-like cell line THP-1 (23).

We have previously shown that LPS exposure (for 24 hours) significantly increases LTB₄ production in ex vivo human bronchoalveolar lavage (BAL) cells obtained from both current and ex-smokers, with an enhanced responsiveness in BAL cells from smokers (24). Our study now explores the mechanisms underlying this important interaction between bacterial products, tobacco smoke, and leukotriene synthesis and degradation. We show that LPS induces the production of LTB₄ by human BAL cells while simultaneously down-regulating the expression of 5-LOX mRNA in a time- and dose-dependent manner. We further show that inhibition of guanylyl cyclase abrogates the LPS-induced down-regulation of 5-LOX mRNA expression in BAL cells whereas activation of guanylyl cyclase accentuates the effect of LPS, suggesting the involvement of the cyclic GMP (cGMP) signaling pathway. In addition, LPS induced a concomitant increase in LTB₄OH mRNA expression and enzymatic activity. However, the LPS-induced up-regulation of LTB₄OH mRNA expression was not observed in BAL cells obtained from current smokers. BAL cells from ex-smokers also had significantly higher level of constitutive LTB₄OH mRNA expression than BAL cells from smokers. These findings suggest that tobacco smoke impairs the regulatory mechanisms responsible for maintaining the balance of LTB₄ levels in the lung microenvironment both constitutively and in response to inflammatory stimuli such as LPS. Such differential patterns of expression and responsiveness may contribute to sustained elevation of LTB₄ in the lungs of smokers and the pathogenesis of tobacco-related respiratory diseases such as chronic obstructive pulmonary disease and lung cancer.

Materials and Methods

Human subjects

BAL cells were obtained from (a) 28 former heavy smokers, 45 y of age or older, with a smoking history of at least 30 pack-years, and who have abstained from smoking for at least 1 y, and (β) 14 active smokers with an average smoking history of 36 pack-years. All subjects underwent bronchoscopy with BAL. BAL cells were obtained from baseline bronchoscopy from smokers and ex-smokers participating in various chemoprevention trials. All bronchoscopies and sample processing were done using the same standard operating procedures. Written informed consent was obtained in accordance with the University of California at Los Angeles Institutional Review Board.

Bronchoscopy with BAL

Subjects were topically anesthetized with 20% benzocaine spray applied to the pharynx and 2% topical lidocaine applied as required. Conscious sedation was achieved using midazolam and meperidine applied to the pharynx and 2% topical lidocaine applied as required. A fiberoptic bronchoscope (Olympus) was advanced into the airway and wedged into a subsegment of the right middle lobe. Four 60-mL aliquots of room temperature saline were serially lavaged and recovered by manual syringe suction. Recovered fluid was passed through a 100-μm sterile nylon filter (Becton Dickinson) to remove mucus and particulates, pooled, and centrifuged at 300 × g for 8 min at 4°C. BAL cells were then harvested for ex vivo studies. Unfractionated BAL cells were used to expedite processing and minimize cell damage and loss from mechanical manipulations. It also allowed the cell population milieu to remain intact, providing a more physiologic assessment of cell signaling and interactions within the lung microenvironment.

Ex vivo stimulation of BAL cells

BAL cells were washed once in HBSS (Irvine Scientific) and resuspended in X-Vivo 15 serum-free medium (Biowhittaker) at a concentration of 0.5 × 10⁶/mL. BAL cells were conditioned with 0.01, 0.1, 1, and 5 μg/mL LPS (E. coli 026:B6, Sigma-Aldrich). Because the maximum effect of LPS was observed at 5 μg/mL without significantly affecting cell viability, subsequent characterizations were all done with
5 μg/mL LPS, including co-conditioning experiments with 5-LOX enzyme inhibitor zileuton (10 μg/mL; Abbott Laboratory), 5-LOX activation inhibitor MK-886 (5 μM/L; Calbiochem), guanylyl cyclase inhibitor LY-83583 (10 μM/L; Calbiochem), guanylyl cyclase activator YC-1 (20 μM/L; Calbiochem), LTB4OH inhibitor 17-oxoacyclic acid (Cayman Chemical), and/or exogenous LTB4 (200 pg/mL; Cayman Chemical). The conditioned supernatants were collected and total RNA or protein was extracted following various periods of incubation (6, 24, 44, 138, and 168 h) and stored at −80°C until analyzed. BAL cells obtained from ex-smokers were also exposed to ex vivo smoke from 1 cigarette (regular Marlboro; Philip Morris) for 3 min using the smoking chamber (Billups-Rothenberg) as previously described (25), followed by 24-h stimulation with LPS. Duplicate sets of BAL cell cultures were set up simultaneously in a separate chamber containing room air as controls. For prolonged culture conditions, equal volumes of fresh medium containing the same concentrations of LPS were added every 3 d. Each set of time course or dose-response experiments was done in BAL cells from the same subject to minimize intersubject variability.

Cell viability was determined by trypan blue staining. Counts indicated that >90% and 80% of cells were viable after 24 and 44 h of incubation in all conditions, respectively. Calcium ionophore (A23187) stimulation experiments were done to assess maximal synthetic capacity; alveolar macrophages were harvested and resuspended in fresh X-Vivo 15 serum-free medium (at 3 × 10⁶/mL). Cells were stimulated with 2 μM/L A23187 (Calbiochem) for 30 min and the supernatants were harvested and stored at −80°C until analyzed.

### Isolation of mRNA from BAL cells and real-time PCR

Total RNA was isolated from conditioned BAL cells using RNAeasy miniprep kits according to the manufacturer’s instructions (Qiagen, Inc.). First-strand cDNA was synthesized using 2.5 μg of total RNA (DNase-treated) in a 50-μL reverse transcriptase reaction mixture from the iScript cDNA Synthesis Kit (Bio-Rad). The primer sets for β-actin, GAPDH, GAPDH, 5-LOX, LTB4, and LTB4 were amplified using specific primer pairs: β-actin, sense 5′-GTCACTCGGCATCTTGTAT-3′, antisense 5′-ATACATTGAGATGTCACAGCC-3′; GAPDH, sense 5′-CATGAGAAGGTATGACGCTCT-3′, antisense 5′-AGTCCTCACGATACAAAGT-3′; 5-LOX, sense 5′-AAGTGGCAGCTGAC-3′, antisense 5′-GTTCACCTCATCACCAG-3′; LTB4, sense 5′-GCAACCCTGTGCTACATT-3′, antisense 5′-TTTGAGTGTTTTCAAGTCCATCC-3′; and LTB4OH (cytochrome P450 4F3, input sequence ID: AB002416.2, http://www unpublishedlibrary.com). Roche Applied Science), sense 5′-TCCGAGAACCTCTTCCCATC-3′, antisense 5′-GCCCTCACGATCTGTCAT-3′ (IDT). The lengths of the amplified cDNA fragments were, for β-actin, 136 bp; GAPDH, 113 bp; 5-LOX, 96 bp; LTB4, 119 bp; and LTB4OH, 88 bp. All quantitative real-time PCR reactions were done in a 25-μL mixture containing 1/25 volume of cDNA preparation (2 μL) and 1× iQ SYBR Green Supermix (Bio-Rad). The PCR amplification protocol consisted of 1-min denaturation (95°C), 1-min annealing (60°C), and 1-min extension (72°C) for a total of 35 cycles. Real-time quantification was done with the Bio-Rad iCycler iQ system. The fluorescence threshold value was calculated using the iCycler iQ system software (PCR efficiency ranged from 90% to 97%). Values were first normalized to β-actin or GAPDH and then to the control values for each experiment. Because initial experiments using GAPDH as the reference housekeeping gene yielded similar results as those with β-actin, subsequent experiments were all done with β-actin. Data are depicted in fold changes normalized to control, with −2-fold changes representing 50% of control, −4-fold changes representing 25% of control, etc.

### Immunoblotting for 5-LOX

Conditioned BAL cells were harvested and disrupted in Laemmli buffer by repeatedly passing through a 25-gauge syringe. Bis-Tris HCl buffered PAGE was done (with 2 μg of protein per lane) by the technique of Laemmli under reducing and denaturing conditions, using 4% stacking and 4% to 12% gradient resolving gels (NuPAGE Novex 4-12% Bis-Tris Gel, Invitrogen). Following electrophoresis, the resolved proteins were electroblotted onto polyvinylidene difluoride membranes (Invitrogen, Invitrogen). Nonspecific binding was blocked by incubating polyvinylidene difluoride membranes with TBS containing 0.05% Tween 20 (TBST) and 5% milk at 25°C. Membranes were then probed with a mouse anti-human 5-LOX monoclonal antibody (1:1,000 dilution; BD Biosciences) in TBST containing 5% milk powder overnight at 4°C, then washed thrice with TBST + 5% milk. After washing, blots were incubated for 2 h at room temperature with horseradish peroxidase–conjugated horse anti-mouse IgG (1:2,000; Cell Signaling) in TBST with 5% milk. Immunoactive proteins were identified with a commercially available chemiluminescence kit (Amersham Life Sciences) developed according to the manufacturer’s instructions. The same membrane was then incubated in stripping buffer (Restore Western Blot Stripping Buffer, Pierce) thrice at 55°C for 30 min with agitation, followed by probing with a rabbit anti-human β-actin antibody (1:1,000; Sigma) as described above. The 78-kDa bands (5-LOX) and the 43-kDa bands (β-actin) on the developed X-ray films were quantitated by scanning densitometry (Image) software, NIH. The 5-LOX values were normalized to β-actin and then to control and expressed as arbitrary units.

### Measurement of LTB4

LTB4 concentrations in conditioned alveolar macrophages were measured by enzyme immunoassay with a LTB4 enzyme immunoassay kit (Cayman Chemical). Absorbance was determined at 405 nm with a microplate reader (Molecular Devices).

### Statistical analysis

Data were analyzed with paired t tests or ANOVA. Batch analyses were done for each subject/comparison group to minimize interassay variability.

### Results

LPS dose-dependently induces LTB4 release and down-regulates 5-LOX mRNA expression in human BAL cells

To determine the effects of LPS on human BAL cell 5-LOX pathway activity, BAL cells were obtained from ex-smokers and smokers and treated with LPS (at doses ranging from 0.01 to 5 μg/mL) for 24 hours. Consistent with our previous findings, BAL cells from smokers were nearly twice as responsive in releasing LTB4 in response to LPS stimulation than those from ex-smokers (Fig. 1A). In ex-smokers, LPS significantly increased BAL cell LTB4 release into the culture media in a dose-responsive manner (Fig. 1B). LPS simultaneously induced a dose-dependent decrease in 5-LOX mRNA expression.
expression (Fig. 1C). Similarly, LPS-induced reduction of 5-LOX mRNA was observed in BAL cells obtained from current smokers (data not shown). In addition, LPS stimulation resulted in a significant reduction of FLAP mRNA expression (8.7 ± 3.2-fold decrease at 24 hours; data not shown). These data suggest that while LPS induces the acute and ongoing release of LTB₄ from BAL cells, it also inhibits the expression of mRNA coding for 5-LOX. Characteristics of BAL are shown in Table 1.

**LPS time-dependently modulates LTB₄ release, 5-LOX mRNA expression, and 5-LOX protein synthesis in BAL cells**

To determine the time course of the effects of LPS treatment on LTB₄ release, BAL cells from ex-smokers were conditioned for up to 138 hours with LPS (at 5 μg/mL). LPS induced LTB₄ release into culture media as early as 6 hours, with peak induction noted at 24 hours. By 138 hours, the LPS induction of LTB₄ release into media was no longer evident (Fig. 2A). BAL cells conditioned as above were also stimulated with the calcium ionophore A23187 to determine the functional synthetic capacity for maximal LTB₄ release. LPS induced ionophore-stimulated LTB₄ release at 44 hours and diminished by 138 hours (data not shown). The fact that A23187 continued to stimulate significant amounts of LTB₄ production at 138 hours indicates intact 5-LOX pathway functional activity even after prolonged culture and LPS exposure.

To determine the effects of LPS on 5-LOX mRNA abundance, BAL cells were conditioned for up to 6 days with LPS. LPS suppression of mRNA expression for 5-LOX gene was notably absent at 6 hours, with peak suppression observed at 24 hours, and suppression of 5-LOX mRNA in response to LPS diminished by 138 hours (Fig. 2B). Western blot analysis of 5-LOX protein levels showed that whereas no significant change in protein was observed at 24 hours, an ~70% reduction in 5-LOX protein was noted at day 7 (Fig. 2C and D). Collectively, these findings suggest that LPS exerts a delayed negative feedback mechanism involving an early induction of LTB₄ release, followed by a decrease in 5-LOX mRNA expression. As expected, 5-LOX protein levels subsequently decrease, resulting in resolution of LPS-associated induction of LTB₄.

**Exogenous LTB₄ decreases LTB₄ release and accentuates suppression of 5-LOX mRNA by LPS**

To determine if LTB₄ release exerts a negative feedback effect on the LPS suppression of 5-LOX pathway gene expression, BAL cells from ex-smokers were preconditioned with exogenous LTB₄ (at 200 pg/mL) for 1 hour before LPS conditioning for 24 hours. Exogenous LTB₄ significantly reduced LPS-induced LTB₄ release (Fig. 3A) and enhanced the suppression of 5-LOX mRNA expression even further than with LPS alone (Fig. 3B). The data in Fig. 3A represent the assayed LTB₄ concentrations from conditioned supernatants, without subtracting the amount of exogenous LTB₄ added to the cultures. These data indicate that LTB₄ directly inhibits LPS induction of 5-LOX pathway activity and exhibits a negative feedback effect on 5-LOX mRNA expression. To further examine the role of LTB₄ as an autocrine substance involved in a negative feedback loop, BAL cells were treated with the 5-LOX enzymatic inhibitor zileuton (at 10 μg/mL) and the leukotriene biosynthesis inhibitor MK-886 (at 5 μg/mL). Inhibition of 5-LOX pathway activity partially abrogated the LPS-induced decrease in 5-LOX mRNA expression (Fig. 3C and D), providing additional evidence that the LPS-induced modulation of the 5-LOX pathway is, in part, mediated via a negative feedback mechanism associated with LTB₄ generation.
Inhibitors and activators of guanylyl cyclase modulate the LPS-induced down-regulation of 5-LOX mRNA expression in BAL cells

To explore the role of cGMP/guanylyl cyclase in mediating the effects of LPS on 5-LOX mRNA expression, BAL cells from ex-smokers were preconditioned with the guanylyl cyclase inhibitor LY-83583 or the guanylyl cyclase activator YC-1 for 1 hour and then stimulated with LPS for 24 hours. Inhibition of guanylyl cyclase abrogated the LPS-induced decrease of 5-LOX mRNA expression whereas activation of guanylyl cyclase accentuated the effects of LPS (Fig. 4).

LPS up-regulates LTB4OH mRNA expression and enzymatic activity in a dose-dependent manner in BAL cells from ex-smokers

To evaluate the effects of LPS on the expression of LTB4OH, the enzyme responsible for the metabolism of LTB4 in the local microenvironment, BAL cells from ex-smokers were stimulated with LPS (at doses ranging from 0.01 to 5 μg/mL) for 24 hours. LPS induced a dose-dependent up-regulation of LTB4OH mRNA expression at doses as low as 10 ng/mL (Fig. 5A). Inhibition of LTB4OH activity with 17-octadecynoic acid induced a dose-dependent increase in LTB4 levels in the presence of LPS (Fig. 5B).

Effects of tobacco smoke on LTB4OH mRNA expression in BAL cells

When BAL cells from current smokers were treated with LPS (at 5 μg/mL) for 24 hours, LPS did not induce an up-regulation of LTB4OH mRNA expression (Fig. 6A), in contrast to that observed in BAL cells from ex-smokers (Fig. 5A). To further explore whether cigarette smoke may reduce LPS-induced LTB4OH mRNA expression, BAL cells from ex-smokers were first exposed ex vivo to cigarette smoke for 3 minutes, followed by 24 hours of LPS (5 μg/mL) conditioning. In vitro smoke exposure of BAL cells from ex-smokers also abrogated the LPS-induced up-regulation of LTB4OH mRNA expression (Fig. 6B). These findings suggest that tobacco smoke alters BAL cell LTB4OH mRNA expression in response to LPS exposure.

BAL cells from smokers express significantly less LTB4OH mRNA than BAL cells from ex-smokers

To ascertain whether or not tobacco smoking alters constitutive LTB4OH mRNA expression in BAL cells in vivo, total RNA isolated from freshly harvested BAL cells from both current smokers and ex-smokers (matched by gender and age) was measured using quantitative PCR. Expression of LTB4OH mRNA in ex-smokers' BAL cells is, on average, 3.8-fold higher than that in smokers' BAL cells (Fig. 7).

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**Fig. 3.** Exogenous LTB4 inhibits LPS-induced LTB4 release and accentuates the down-regulation of 5-LOX mRNA expression in human BAL cells, and inhibition of 5-LOX pathway activity abrogates LPS-induced down-regulation of 5-LOX mRNA expression in ex vivo human BAL cells. BAL cells from ex-smokers were preconditioned with exogenous LTB4 (at 200 pg/mL) for 1 h, then stimulated with LPS (at 5 μg/mL) for 24 h. A, exogenous LTB4 results in inhibition of LPS-induced LTB4 release. Columns, mean (n = 3); bars, SE. *, P < 0.02. B, exogenous LTB4 results in decreased LTB4 mRNA expression, accentuating the down-regulatory effect of LPS. Columns, mean (n = 3); bars, SE. *, P < 0.05. BAL cells were also conditioned with 5 μg/mL LPS with or without the 5-LOX inhibitor zileuton (at 10 μg/mL) for 24 h; zileuton abrogated the LPS-induced LTB4 production without affecting cell viability; data not shown. C, zileuton partially reversed the down-regulatory effect of LPS on 5-LOX mRNA expression. Columns, mean (n = 3); bars, SE. *, P < 0.05. In addition, BAL cells were conditioned with 5 μg/mL LPS with or without the leukotriene biosynthesis inhibitor MK-886 (at 5 μmol/L) for 24 h. D, MK-886 also partially reversed the LPS-induced down-regulation of 5-LOX mRNA expression without affecting cell viability. Columns, mean (n = 3); bars, SE. *, P < 0.05.

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Discussion

In a previous report, to determine the effects of inflammatory stimuli known to modulate activity of the 5-LOX pathway in monocytes/macrophages (20, 22), we examined the LPS responsiveness of BAL cells (>90% of which are alveolar macrophages) obtained from current and ex-smokers. We showed that LPS exposure (for 24 hours) increased LTB₄ release in human BAL cells from both smokers and ex-smokers via an increase in 5-LOX enzymatic activity (24). BAL cells from ex-smokers, however, were less responsive to LPS stimulation than BAL cells from smokers. To further explore the underlying molecular regulatory mechanisms, we have now evaluated the effects of LPS on mechanisms responsible for LTB₄ synthesis and degradation. We first assessed 5-LOX mRNA expression in BAL cells. At 24 hours, LPS significantly induced LTB₄ release while simultaneously down-regulating the expression of 5-LOX mRNA in both smokers and ex-smokers. This pattern of LPS-induced changes was dose dependent (at doses as low as 10 ng/mL) and time dependent, with peak induction of LTB₄ release and suppression of 5-LOX pathway gene expression occurring at 24 hours. Our data suggest that the dichotomous LPS induction of LTB₄ production and suppression of 5-LOX mRNA expression is due, in part, to a negative feedback mechanism, in which the early increase in LTB₄ release from an enhanced 5-LOX enzymatic activity detected within 6 hours leads to a delayed suppression of 5-LOX mRNA expression, occurring by 24 hours, followed by a reduction of 5-LOX protein production and functional synthetic capacity by 7 days. Consistent with this proposed negative feedback mechanism, exogenous LTB₄ inhibited LPS-induced LTB₄ synthesis, and 5-LOX enzymatic inhibition partially abrogated the down-regulation of 5-LOX mRNA in response to LPS. Inhibition of guanylate cyclase reversed the LPS-induced reduction of 5-LOX mRNA expression, whereas activation of the enzyme further enhanced the down-regulation, suggesting the role of cGMP in mediating this effect of LPS. We then show that LPS induces up-regulation of LTB₄OH mRNA expression and enzymatic activity in ex-smokers’ BAL cells. However, the up-regulation of LTB₄OH mRNA expression was not seen in BAL cells obtained from current smokers. In vitro smoke exposure of BAL cells from ex-smokers also abrogated the LPS-induced up-regulation of LTB₄OH mRNA expression (Fig. 6). Interestingly, the constitutive expression of LTB₄OH mRNA in ex-smokers is significantly higher than that observed in smokers. Such findings likely contribute to the higher level of LTB₄ observed from smokers’ BAL cells. To our knowledge, this is the first time tobacco smoke has been shown to impair the downstream counterregulatory mechanisms that may be responsible for maintaining the balance of LTB₄ levels in the lung microenvironment, both constitutively and under stimulation. The differential responsiveness of BAL cells to LPS in the setting of tobacco exposure supports the notion that sustained elevation of 5-LOX pathway metabolites may contribute to tobacco-related diseases such as lung cancer and chronic obstructive pulmonary disease (1, 8).

Inflammation represents a natural biological defense against insults such as infection or injury. Unregulated inflammation, however, may lead to undesirable host cellular or tissue damage. There has been increased recognition in recent years that aberrant levels of inflammatory mediators such as leukotrienes also play pivotal roles in the pathogenesis of lung cancer. Therefore, it is reasonable to expect that timely intrinsic negative regulatory mechanisms must be available to extinguish inflammatory responses to prevent untoward effects.

Fig. 4. The LPS-induced down-regulation of 5-LOX mRNA expression in BAL cells may be mediated via the cGMP signaling pathway. BAL cells from ex-smokers were preconditioned with the guanylyl cyclase inhibitor LY-83583 (at 10 μmol/L) or the guanylyl cyclase activator YC-1 (at 20 μmol/L) for 1 h, then stimulated with LPS (at 5 μg/mL) for 24 h. Inhibition of cGMP formation abrogated the LPS-induced decrease of 5-LOX mRNA expression whereas activation of guanylyl cyclase further accentuated the down-regulation. n = 3. * P < 0.05.

Fig. 5. LPS up-regulates LTB₄OH mRNA expression and enzymatic activity in a dose-dependent manner in BAL cells from ex-smokers. BAL cells from ex-smokers were stimulated with varying doses of LPS for 24 h. A, LPS induced a dose-dependent up-regulation of LTB₄OH mRNA expression at doses as low as 10 ng/mL. Columns, mean (n = 3); bars, SE. * P < 0.05. B, inhibition of LTB₄OH activity with 10 and 20 μmol/L of 17-octadecynoic acid (17-ODA) induced an average of 44% and 72% increase in LTB₄ levels in the presence of LPS (at 5 μg/mL), respectively. Following LPS treatment, LTB₄ levels ranged from 598 to 882 pg/mL (without 17-octadecynoic acid) to 1,093 to 1,565 pg/mL (with 20 μmol/L 17-octadecynoic acid). Columns, mean (n = 3); bars, SE. * P < 0.05.
A BAL cell's mRNA expression in smokers and former smokers did not increase LTB4OH. In vitro, LPS stimulation of BAL cells from smokers and former smokers caused upregulation of LTB4OH mRNA expression. Columns, mean (n = 3); bars, SE. B, in vitro smoke exposure abrogated the LPS-induced up-regulation of LTB4OH mRNA expression in BAL cells from ex-smokers. Further support for the role of tobacco smoke in impairing LTB4 metabolism, BAL cells from ex-smokers were first exposed to cigarette smoke for 3 min, followed by 24-h LPS stimulation. In vitro smoke exposure of BAL cells from former smokers also abrogated the LPS-induced LTB4OH mRNA expression. Columns, mean (n = 3); bars, SE.

consequences. Consistent with this concept, our data show, for the first time, that LTB4 (induced in the presence of LPS) directly and negatively feeds back on 5-LOX metabolism in human BAL cells via an autocrine loop. We further show that LPS induces the activity and expression of LTB4OH, the enzyme responsible for LTB4 degradation. These mechanisms act in concert to normalize excess LTB4 levels and provide evidence of novel mechanisms governing the regulation of LTB4 levels in the lung microenvironment. Tobacco smoke exposure, however, perturbs this balance, in part through inhibition of LTB4OH expression.

LPS, or endotoxin, is a component of Gram-negative bacteria cell wall known to induce inflammation and potent immune responses that can contribute to host cell damage. Various counterregulatory mechanisms involving other eicosanoid metabolites or signaling pathways have been implicated in the modulation of 5-LOX pathway activity by LPS. For example, previous studies in peritoneal macrophages showed that prolonged LPS exposure (16 hours) inhibited leukotriene production via the combined action of COX and nitric oxide synthase products (26). In contrast to these previous findings, we did not observe a significant change in LTB4 production with COX-2 inhibition (data not shown). Likewise, inhibition of nitric oxide also did not significantly change LTB4 production (data not shown), in contrast to findings in rat alveolar macrophages, in which nitric oxide pathway inhibition blocked the effects of prolonged LPS exposure (16 hours) on 5-LOX metabolism (22). Interspecies variations in cellular responsiveness to timing and dosing of LPS most likely contribute to the disparity observed between human and rat alveolar macrophages. In support of this notion is the fact that human peripheral blood monocytes exhibit no suppression of 5-LOX metabolism or induction of nitric oxide synthase following 16 hours of LPS exposure (22). Another plausible explanation is that prior studies present only incomplete pictures by studying the effects of LPS at a single time point (2 or 16 hours) without systematic characterization over time (up to 7 days in our study). To our knowledge, no prior report has shown that LTB4 plays a direct role in regulating its own metabolism in human BAL cells. We speculate that this negative feedback mechanism might play some role in the development of “LPS tolerance,” in which prolonged or repetitive exposure to LPS leads to an immunosuppressive state characterized by hyporesponsiveness to LPS (27, 28).

cGMP is a second messenger, formed from GTP by guanylyl cyclase. cGMP plays pivotal roles in a variety of cell signaling mechanisms; it can regulate cation channels, cGMP-dependent kinases, and various isoforms of phosphodiesterase (29). The guanylyl cyclase pathway has previously been described to mediate neutrophil responses to the bacterial chemotactic peptide N-formylmethionyl-leucyl-phenylalanine in the presence of granulocyte macrophage colony-stimulating factor priming (30). As such, this signaling pathway may be a possible candidate in mediating the effects of other bacterial products, such as LPS, on inflammatory cells. In our study, we found that inhibition of guanylyl cyclase with LY-83583 abrogates the LPS-induced down-regulation of 5-LOX mRNA expression, whereas activation of the enzyme with YC-1 further accentuates the LPS-induced changes, suggesting the role of cGMP in mediating these events. Precisely how cGMP/guanylyl cyclase may mediate the down-regulation of 5-LOX mRNA expression by LPS is currently unclear and remains to be elucidated in future studies.

The maintenance of balanced LTB4 levels in the lung microenvironment involves a dynamic process that is dependent on the rate of synthesis and metabolism. Previously, we showed that the degree of LTB4 increase in response to LPS was less in BAL cells obtained from ex-smokers than in BAL cells from current smokers (24). Whereas tobacco exposure may prime

Fig. 6. LPS does not induce a dose-dependent up-regulation of LTB4OH mRNA expression in BAL cells from smokers. A, BAL cells from current smokers were harvested and conditioned with LPS (5 μg/mL) for 24 h. LPS exposure did not increase LTB4OH mRNA expression in smokers’ BAL cells. Columns, mean (n = 3); bars, SE. B, in vitro smoke exposure abrogated the LPS-induced up-regulation of LTB4OH mRNA expression in BAL cells from ex-smokers. For example, previous studies in peritoneal macrophages showed that prolonged LPS exposure (16 hours) inhibited leukotriene production via the combined action of COX and nitric oxide synthase products (26). In contrast to these previous findings, we did not observe a significant change in LTB4 production with COX-2 inhibition (data not shown). Likewise, inhibition of nitric oxide also did not significantly change LTB4 production (data not shown), in contrast to findings in rat alveolar macrophages, in which nitric oxide pathway inhibition blocked the effects of prolonged LPS exposure (16 hours) on 5-LOX metabolism (22). Interspecies variations in cellular responsiveness to timing and dosing of LPS most likely contribute to the disparity observed between human and rat alveolar macrophages. In support of this notion is the fact that human peripheral blood monocytes exhibit no suppression of 5-LOX metabolism or induction of nitric oxide synthase following 16 hours of LPS exposure (22). Another plausible explanation is that prior studies present only incomplete pictures by studying the effects of LPS at a single time point (2 or 16 hours) without systematic characterization over time (up to 7 days in our study). To our knowledge, no prior report has shown that LTB4 plays a direct role in regulating its own metabolism in human BAL cells. We speculate that this negative feedback mechanism might play some role in the development of “LPS tolerance,” in which prolonged or repetitive exposure to LPS leads to an immunosuppressive state characterized by hyporesponsiveness to LPS (27, 28).

cGMP is a second messenger, formed from GTP by guanylyl cyclase. cGMP plays pivotal roles in a variety of cell signaling mechanisms; it can regulate cation channels, cGMP-dependent kinases, and various isoforms of phosphodiesterase (29). The guanylyl cyclase pathway has previously been described to mediate neutrophil responses to the bacterial chemotactic peptide N-formylmethionyl-leucyl-phenylalanine in the presence of granulocyte macrophage colony-stimulating factor priming (30). As such, this signaling pathway may be a possible candidate in mediating the effects of other bacterial products, such as LPS, on inflammatory cells. In our study, we found that inhibition of guanylyl cyclase with LY-83583 abrogates the LPS-induced down-regulation of 5-LOX mRNA expression, whereas activation of the enzyme with YC-1 further accentuates the LPS-induced changes, suggesting the role of cGMP in mediating these events. Precisely how cGMP/guanylyl cyclase may mediate the down-regulation of 5-LOX mRNA expression by LPS is currently unclear and remains to be elucidated in future studies.

The maintenance of balanced LTB4 levels in the lung microenvironment involves a dynamic process that is dependent on the rate of synthesis and metabolism. Previously, we showed that the degree of LTB4 increase in response to LPS was less in BAL cells obtained from ex-smokers than in BAL cells from current smokers (24). Whereas tobacco exposure may prime

Fig. 7. BAL cells from smokers express significantly less LTB4OH mRNA than BAL cells from ex-smokers. LTB4OH mRNA expression from freshly harvested BAL cells from both smokers and ex-smokers were measured by quantitative PCR. Expression in ex-smokers’ BAL cells is 3.8-fold higher than that in smokers’ BAL cells. Columns, mean (n = 10); bars, SE. *, P < 0.05.
cells for enhanced LTB₄ release, our current study provides an additional mechanism that may also account for the differential LTB₄ levels in the presence of LPS (Fig. 8). To our knowledge, this is the first report showing that tobacco smoke may impede the elimination of LTB₄ both constitutively and in the presence of LPS via the reduction of LTB₄OH expression. This notion is further supported by the fact that in vitro smoke exposure decreased LTB₄OH expression in alveolar macrophages obtained from ex-smokers in the presence of LPS. Our observations provide evidence that ongoing smoke exposure may modulate the leukotriene-related responsiveness of BAL cells to bacterial respiratory infection, which may play an important role in conditions such as chronic obstructive pulmonary disease exacerbation and lung cancer. The presence of chronic obstructive pulmonary disease is a known independent risk factor for lung cancer.

As shown by Western blot analysis, the normalization of LPS-induced LTB₄ release at 138 hours was due to a reduction of 5-LOX protein mass, as a consequence of the down-regulation of mRNA. The observation that control LTB₄ levels did not change substantially from 44 to 138 hours and that the control 5-LOX protein levels were sustained at day 7 argues against a significant loss of 5-LOX enzymatic activity or protein mass as a result of prolonged culture. Because we did not measure the total release of arachidonic acid, the possibility that LPS may increase substrate release at the level of phospholipase cannot be completely excluded. We further acknowledge that increased LTB₄ with LTB₄OH inhibition in the setting of LPS exposure provides indirect evidence for the effects of LPS on LTB₄OH activity.

In summary, we delineate for the first time the temporal sequence of alterations in the 5-LOX pathway in human BAL cells in response to LPS exposure. We show that the up-regulation of LTB₄ production in response to LPS is coupled to down-regulation of 5-LOX and FLAP mRNA, and that exogenous LTB₄ abrogates these changes, suggesting the presence of a negative feedback mechanism. In addition, LPS exposure increases the expression and activity of the downstream metabolizing enzyme LTB₄OH. This pattern of LTB₄ pathway responses to LPS indicates that the inflammatory response is tightly regulated by a series of checks and balances. Exposure to tobacco smoke, however, perturbs this crucial balance. These findings may have significant implications for understanding the pathogenesis of tobacco-related diseases such as chronic obstructive pulmonary disease and lung cancer. It is conceivable that any major deviation from these regulatory mechanisms caused by tobacco exposure, whether it is from genetic or epigenetic alterations, could influence susceptibility to such diseases and the eventual clinical outcome. Our findings also suggest an additional way to target LTB₄ balance and may have potential implications in lung cancer chemopreventive strategies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**

Differential Modulation of Leukotriene B4 Synthesis and Degradation in Human Bronchoalveolar Lavage Cells by Lipopolysaccharide and Tobacco Smoke

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