Leukotrienes enhance the bactericidal activity of alveolar macrophages against *Klebsiella pneumoniae* through the activation of NADPH oxidase

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Leukotrienes (LTs) are lipid mediators that participate in inflammatory diseases and innate immune function. We sought to investigate the importance of LTs in regulating the microbicidal activity of alveolar macrophages (AMs) and the molecular mechanisms by which this occurs. The role of LTs in enhancing AM microbicidal activity was evaluated pharmacologically and genetically using in vitro challenge with *Klebsiella pneumoniae*. Exogenous LTs increased AM microbicidal activity in a dose- and receptor-dependent manner, and endogenous production of LTs was necessary for optimal killing. Leukotriene B_4 (LTB_4) was more potent than cysteinyl LTs. An important role for nicotinamide adenine dinucleotide (NADPH) oxidase in LT-induced microbicidal activity was indicated by the fact that bacterial killing was abrogated by the NADPH oxidase inhibitor diphenyleneiodonium (DPI; 10 μM) and in AMs derived from gp91phox-deficient mice. By contrast, LT-induced microbicidal activity was independent of the generation of nitric oxide. LTs increased H_2O_2 production, and LTB_4 was again the more potent agonist. Both classes of LTs elicited translocation of p47phox to the cell membrane, and LTB_4 induced phosphorylation of p47phox in a manner dependent on protein kinase C-δ (PKC-δ) activity. In addition, the enhancement of microbicidal activity by LTs was also dependent on PKC-δ activity. Our results demonstrate that LTs, especially LTB_4, enhance AM microbicidal activity through the PKC-δ–dependent activation of NADPH oxidase. (Blood. 2005;106:1067-1075)

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Introduction

Pneumonia is the leading cause of death from infection in the United States,[1] and its global mortality is 4.3 million people per year.[2] This problem is compounded by growing numbers of immunosuppressed patients and multidrug-resistant microorganisms. Developing more effective agents for the prevention and treatment of pneumonia requires a better understanding of how innate pulmonary defense mechanisms are regulated.

The alveolar macrophage (AM) patrols the epithelial surface of the distal lung and maintains sterility by phagocytosing and killing microorganisms.[3,4] AMs are the first line of defense against invading microorganisms and, as such, are capable of secreting cytokines, lipid mediators, and microbicidal molecules. Among the lipid mediators generated by AMs, the leukotrienes (LTs) play an important role in lung innate immunity, inducing neutrophil recruitment and enhancing macrophage antimicrobial functions.[5] LTs are derived from the metabolism of the cell-membrane fatty acid arachidonic acid (AA) through the enzyme 5-lipoxygenase (5-LO), in concert with its helper protein, 5-LO–activating protein (FLAP).[6] 5-LO oxygenates AA to the intermediate 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is either enzymatically reduced by 5-LO to the unstable epoxide leukotriene A_4 (LTA_4) or alternatively is reduced to 5-hydroxyeicosatetraenoic acid (5-HETE). LTA_4 can be hydrolyzed to form LTB_4 or can be conjugated with glutathione to form the cysteinyl LTs (cysLTs), LTC_4, LTD_4, and LTE_4.[6]

A role for LTs in host defense was first demonstrated by Wirth and Kierszenbaum[7,8] in experiments showing that both LTB_4 and LTC_4 increased the phagocytosis and killing of *Trypanosoma cruzi* by peritoneal macrophages. Subsequently, numerous in vitro and in vivo models have revealed key roles for LTs in augmenting monocyte/macrophage phagocytosis[9-12] and microbicidal activity.[7,8,11-20] With respect to pulmonary innate immunity, 5-LO–deficient mice demonstrated impaired bacterial clearance and enhanced mortality during pneumonia caused by *Klebsiella pneumoniae*.[11] In vitro, endogenous and exogenous LTs enhanced Fcγ-receptor–mediated phagocytosis by the AMs.[10,21] However, effects of LTs on the microbicidal activity of AMs and the relevant molecular mechanisms have not been carefully addressed.

Studies in non-AM cell types demonstrating LT-enhanced microbicidal activity reveal potent effects on myriad antimicrobial molecules, including nitric oxide (NO),[14,22,23] lysosomal enzymes,[24] defensins,[25] and the activation of NADPH oxidase (NADPHox).[23,26,27] NADPHox activation leads to the generation of reactive oxygen intermediates (ROIs) such as the superoxide anion (O_2^-) and H_2O_2, which are important weapons used by phagocytes (including AMs) to kill ingested bacteria.[28-31]

NADPHox functions as a large multisubunit complex assembled and activated through the phosphorylation and translocation of cytosolic subunits to the cytochrome b558 present in plasma membrane.[32,33] Although free AA is implicated in activation,[34] the

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mechanisms whereby LTs enhance NADPHox activity remain obscure. In eosinophils, LTB_4 was shown to enhance NADPHox activity, but the exact mechanisms responsible for this activation were not investigated.[35,36] In neutrophils, LTB_4 induced NADPHox activation through a Rac-dependent pathway; this effect was dependent on ERK and cytosolic phospholipase A_2 activities but not on p38 kinase[37]

Despite the critical role of AMs in innate immunity, little is known about the activation of NADPHox in these cells. Moreover, although AMs are known to have a far greater capacity for LT synthesis than macrophages from other sites,[38] virtually nothing is known about the influence of these lipid mediators on this process.

Here, we demonstrate that endogenous and exogenous LTs enhance bacterial killing in a manner dependent on NADPHox activation. We further demonstrate that LT-dependent activation of NADPHox is mediated by protein kinase C-δ (PKC-δ).

Materials and methods

Reagents

Dulbecco modified Eagle medium (DMEM) without phenol red and RPMI 1640 were purchased from Gibco-Invitrogen (Carlsbad, CA). Tryptic soy broth (TSB) was supplied by Difco (Detroit, MI). Saponin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and type IV horseradish peroxidase (HRP) were purchased from Sigma (St Louis, MO). LTB_4, LTC_4, LTD_4, 5(S)-HETE, phorbol myristate acetate (PMA), MK886 (FLAP inhibitor); AA861 (5-LO inhibitor); MK571 (CysLT1 receptor antagonist); the NADPHox-like flavoprotein inhibitor diphenyl-eneiodonium (DPI), and N^G-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor) were purchased from Biomol (Palo Alto, CA). CP105696 (B LT receptor 1 [BLT1] antagonist) was a generous gift of Dr Henry Showell (Pfizer, Groton, CT). Amplex Red (10-acetyl-3,7-dihydrophenoxazine) was purchased from Molecular Probes (Eugene, OR). PKC inhibitors rottlerin and Ro-32-0432 were supplied by Calbiochem (San Diego, CA). Compounds requiring reconstitution were dissolved in either ethanol or dimethyl sulfoxide (DMSO). Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls.

Animals

5-LO KO (129-Alox5^{tm1Fun})[39] and strain-matched wild-type (WT) sv129 mice were bred in the University of Michigan Unit for Laboratory Animal Medicine from breeders obtained from Jackson Laboratories (Bar Harbor, ME). All gp91phox (B6.129S6-$Cybb^{tm1Din}$/J)[40] and strain-matched C57BL/6 mice also were purchased from Jackson Laboratories, and female Wistar rats were obtained from Charles Rivers Laboratories (Portage, MI). The University Committee on Use and Care of Animals approved all animal protocols.

Cell isolation and culture

Resident AMs from mice and rats were obtained by ex vivo lung lavage, as previously described,[11,38] and were resuspended in RPMI to a final concentration of 2×10^6 cells/mL. Cells were allowed to adhere to tissue culture–treated plates for 1 hour (37°C, 5% CO_2); this was followed by one wash with warm RPMI, resulting in more than 99% of adherent cells identified as AMs by use of a modified Wright-Giemsa stain (Diff-Quik; American Scientific Products, McGraw Park, IL). Cells were cultured overnight in RPMI containing 10% fetal bovine serum and were washed twice the next day with warm medium so that nonadherent cells could be removed.

Tetrazolium dye reduction assay of bacterial killing

The ability of bacteria to survive within the AM was quantified using a tetrazolium dye reduction assay, as described elsewhere.[41,42] Briefly,

2×10^6/mL rat AMs, prepared as described previously, were seeded in duplicate 96-well tissue culture dishes. The next day, *K pneumoniae* were opsonized with 3% anti–*K pneumoniae* rat-derived immune serum, as previously described.[9] Cells were then treated with compounds of interest for 20 minutes, or as indicated in the figure legends, and were infected with a 0.1-mL suspension of opsonized *K pneumoniae* (1×10^7 colony-forming units [CFU]/mL; multiplicity of infection [MOI], 50:1) for 30 minutes to allow phagocytosis to occur. The bacterial killing protocol was assessed as described elsewhere.[42] The intensity of the absorbance at 595 nm was directly proportional to the number of intracellular bacteria associated with the macrophages.[41,42] Results were expressed as percentage of survival of ingested bacteria, where the survival of ingested bacteria = $100\% \times A_{595}$ control plate/A_{595} experimental plate. To determine the role of exogenous 5-LO metabolites in microbicidal activity, they were added 30 minutes after infection. The role of endogenous LT or PKC-α and -δ was assessed by including biosynthesis inhibitors, receptor antagonists, or specific PKC inhibitors 20 minutes before infection. Preliminary experiments compared this colorimetric assay with a conventional CFU-based (serial dilution) assay, and similar results were obtained (data not shown).

Cell viability

Neither experimental compounds nor vehicles showed adverse effects on AMs or on bacterial viability, as determined by a cell-based MTT assay (data not shown).

H_2O_2 detection

AMs were plated in 96-well dishes, as described in "Tetrazolium dye reduction assay of bacterial killing." H_2O_2 secretion from AMs was determined colorimetrically using Amplex Red (Molecular Probes, Eugene, OR) reagent, according to the instructions of the manufacturer. A solution containing 50 μM Amplex Red reagent and 10 U/mL HRP was prepared in phosphate-buffered saline (PBS), and 0.1 mL of this was added to AMs (5×10^5 per well) at 37°C for 60 minutes to determine H_2O_2 generation. After this time, A_{560nm} was assessed. The H_2O_2 concentration was determined using a standard curve with known H_2O_2 concentrations. The detection limit of this method was 0.625 nM. To assess the effect of exogenous 5-LO metabolites on H_2O_2 production by AMs, compounds of interest were added to this solution before the addition to the AMs, as indicated in the legend of Figure 4. In separate experiments, AMs were pretreated with AA-861, MK886, or CP105696 for 20 minutes before the addition of the Amplex Red solution containing 50:1 opsonized *K pneumoniae*.

Cell fractionation, immunoprecipitation, and Western blotting

AMs (4×10^6) were plated in 6-well tissue culture dishes and were stimulated for 5 minutes with 1 nM LTB_4, 100 nM LTD_4, 10 nM PMA, or vehicle control. After this time, AMs were lysed by sonication in ice-cold lysis buffer containing 150 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 1 μg/mL leupeptin, followed by ultracentrifugation at 100 000*g* for 20 minutes at 4°C. The cytosolic (soluble) fraction was harvested, and the membrane (insoluble) fraction was washed and subjected to another ultracentrifugation step as described above. The resultant pellet was resuspended in lysis buffer and sonicated. Protein concentrations were determined by a modified Coomassie dye-binding assay (Pierce Chemical, Rockford, IL). The cytosolic fraction was used for immunoprecipitation, as described previously,[21] with some modifications. The fraction was incubated overnight at 4°C with anti–p47phox antibody (1:80; Upstate Biotechnology, Lake Placid, NY). Protein A-Sepharose was added to each sample and was incubated for 3 hours with rotation at 4°C. Beads were washed briefly 3 times with lysis buffer without Triton X-100, and samples containing 20 μg protein were separated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels transferred to nitrocellulose membranes. After blocking with 5% nonfat milk, membranes were probed with anti-p47phox antibody (1:500 dilution; Upstate Biotechnology); anti-FLAP (1:5000; provided by Dr J. Evans, Merck Frosst) or antiphosphoserine (1:900; KI-191, clone 1C8; Biomol) for 90 minutes,

followed by peroxidase-conjugated goat antirabbit (Amersham, Piscataway, NJ) or antimouse secondary (1:5000; Zymed, South San Francisco, CA), and were developed using enhanced chemiluminescence (ECL) detection (Amersham). Relative band densities were determined by densitometric analysis using National Institutes of Health Image software. In all instances, density values of bands were corrected by subtraction of the background values.

Statistical analysis

Data are represented as mean plus or minus SE and were analyzed with the Prism 3.0 statistical program (GraphPad Software, San Diego, CA). Comparisons between 2 experimental groups were performed with the Student t test. Comparisons among 3 experimental groups were performed with analysis of variance (ANOVA) followed by Bonferroni analysis. Differences were considered significant if P equaled .05. All experiments were performed on 3 separate occasions unless otherwise specified.

Results

5-LO products enhance the microbicidal activity of AMs infected with opsonized *K pneumoniae*

To determine the capacity of 5-LO products to modulate the microbicidal activity of AMs, we examined the effect of exogenously added LTB$_4$, LTC$_4$, LTD$_4$, and 5-HETE on the killing of immune serum–opsonized *K pneumoniae*. As demonstrated in Figure 1, all products caused a dose-dependent increase in microbicidal activity, reflected by a decrease in survival of bacteria that were ingested. The maximal effect (approximately 55% increase in killing) was similar for all metabolites, but potency differed— maximal effect was reached at 1 nM for LTB$_4$, 100 nM for cysLTs, and 1000 nM for 5-HETE.

Endogenous LTs augment the killing of ingested bacteria by AMs

Given that exogenously added LTs enhance bacterial killing by AMs and given that AMs have a high capacity to generate endogenous LTs when challenged with *K pneumoniae*,[9] we next considered the role of endogenous LTs in AM killing by evaluating the microbicidal activity of AMs harvested from mice genetically deficient in 5-LO.[39] In these animals, we observed an increase of approximately 55% in the survival of intracellular bacteria when compared with the AMs from WT mice. (Figure 2A).
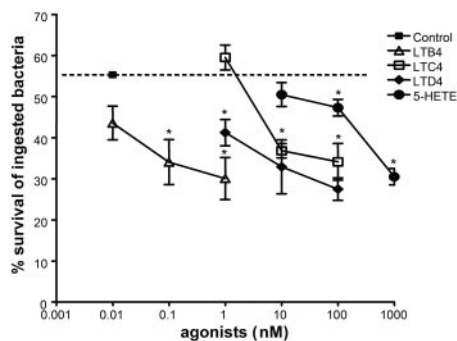

Figure 1. 5-lipoxygenase metabolites dose dependently increase microbicidal activity in AMs infected with opsonized *K pneumoniae*. Rat AMs were infected with 50:1 opsonized *K pneumoniae*. Thirty minutes after infection, the cells were or were not (horizontal dotted line) incubated with LTB$_4$, LTC$_4$, LTD$_4$, or 5-(S) HETE at the indicated concentrations. Microbicidal activity was assessed colorimetrically as described in "Materials and methods." Data are expressed as the mean ± SE percentage survival of ingested bacteria from 3 independent experiments, each performed in triplicate. *P < .05 versus control by ANOVA.
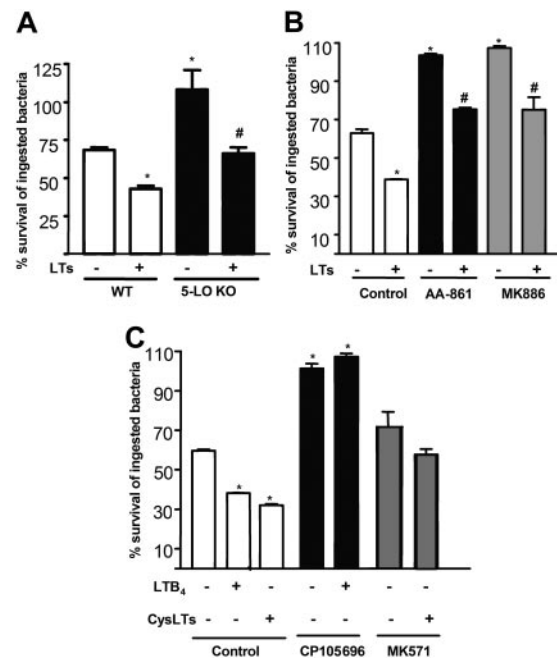

Figure 2. Role of endogenous LTs and their receptors in AM killing of opsonized *K pneumoniae*. (A) AMs from WT or 5-LO KO mice were infected with 50:1 *K pneumoniae* for 30 minutes, incubated without or with LT mixture (LTB$_4$, 1 nM; LTC$_4$ and LTD$_4$, 100 nM each), and bacterial killing was then assessed. (B) Rat AMs were pretreated with vehicle control, the 5-LO inhibitor AA-861 (10 μM), or the FLAP inhibitor MK-886 (1 μM) for 15 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes after infection, drugs were added back with or without LT mixture, and bacterial killing was assessed. (C) Rat AMs were treated with the LTB$_4$ receptor antagonist CP105696 (10 μM) or the cysLT receptor antagonist MK571 (10 μM) or vehicle control for 15 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes after infection, the antagonists were added back with or without 1 nM LTB$_4$ or LTC$_4$ plus LTD$_4$ (100 nM each). Microbicidal activity was assessed. Data are expressed as mean ± SE percentage of survival of ingested bacteria of quadruplicate values from 1 of 4 representative experiments. *P < .05 compared with untreated control by ANOVA. #P < .05 versus AA-861, MK 886, or 5-LO KO group by ANOVA.

As a complementary approach, we used pharmacologic means to inhibit the production of endogenous LTs by rat AMs or their ability to bind their respective receptors on the AM cell surface. As illustrated in Figure 2B, the inhibition of 5-LO by AA861 or FLAP by MK886 increased the survival of ingested bacteria by approximately 65% compared with untreated cells. To determine the specificity of these compounds, we performed "add-back" experiments, applying a mixture of optimal concentrations of exogenous LTs (1 nM LTB$_4$, 100 nM LTC$_4$, and 100 nM LTD$_4$) concomitantly with either AA861 or MK886 in rat AMs (Figure 2B) or in cells from 5-LO–deficient mice (Figure 2A). In all instances, exogenous LTs were able to overcome the effects of pharmacologic or genetic LT deficiency, restoring bacterial survival to the same degree observed in AMs from WT mice or untreated AMs from rat.

These data show a role for endogenous LTs but do not distinguish between LTB$_4$ and cysLTs. To determine the receptors through which the effects on bacterial killing of endogenously produced LTs were mediated, we used CP105696 (BLT1 antagonist) and MK571 (cysLT1 antagonist). Interestingly, AMs treated with the BLT1 antagonist exhibited suppressed intracellular killing, as evidenced by an increase of approximately 65% in the survival of ingested bacteria, whereas treatment of the AMs with MK571 had no effect. To exclude a possible role for cysLT2-mediating AM microbicidal activity, we examined the effect of a dual cysLT1/ cysLT2 antagonist (BAY-u9773, 10 μM) and observed no effect on microbicidal activity compared with untreated controls (data not
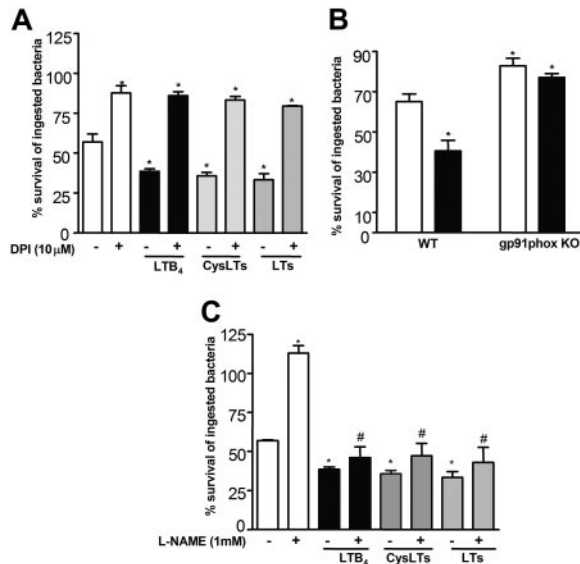
Figure 3. Role of ROIs and NO in basal and LT-enhanced AM microbicidal activity. (A) Rat AMs were pretreated with the NADPH oxidase inhibitor DPI (10 μM) for 20 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes later, DPI was added back with or without LTB$_4$ (1 nM), LTC$_4$ plus LTD$_4$ (100 nM each, cysLTs), or LT mixture (LTB$_4$, 1 nm; LTC$_4$ and LTD$_4$, 100 nM each). (B) AMs from WT or gp91phox KO mice were infected, and LT mixture was added 30 minutes after infection. □ indicates control; ■, LTs. (C) Rat AMs were pretreated with the NO synthase inhibitor L-NAME (1 mM) for 20 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes later L-NAME was added back with or without LTB$_4$ (1 nM), LTC$_4$ plus LTD$_4$ (100 nM each, cysLTs), or LT mixture (LTB$_4$, 1 nm; LTC$_4$ and LTD$_4$, 100 nM each). Microbicidal activity was assessed colorimetrically. Data are expressed as the mean ± SE percentage of survival of ingested bacteria from quadruplicate values from 1 of 2 representative experiments. *P < .05 versus control. #P < .05 versus L-NAME group by ANOVA.

shown). Furthermore, no enhancement in bactericidal activity was elicited by LTB$_4$ or cysLTs when AMs were pretreated with BLT1 or cysLT1 antagonists, respectively (Figure 2C).

These results indicate that though LTB$_4$ and cysLTs are capable of enhancing bactericidal activity in AMs, LTB$_4$ is the endogenously produced metabolite primarily responsible for this activity in the rat AM. This is consistent with the known ability of rat AMs to produce 10-fold more LTB$_4$ than cysLTs.[38]

LTs increase AM microbicidal activity through the activation of NADPHox

ROIs generated by NADPHox are an important bactericidal mechanism following Fcγ-receptor–mediated phagocytosis,[43] and LTs have been shown to activate this pathway in other cell types.[35,36,44] Therefore, we evaluated the ability of LTs to modulate NADPHox activation during bacterial infection of the AM. In these experiments, we used the potent and specific NADPHox inhibitor DPI.[45] DPI alone increased the percentage of viable ingested *K pneumoniae* by approximately 52% compared with untreated cells, indicating an important role for NADPHox. In the presence of DPI, exogenous LTB$_4$, cysLTs, or a mixture of LTs failed to overcome this defect (Figure 3A), suggesting a dependence of LT-induced killing on NADPHox. To confirm this result, we performed similar experiments using AMs from mice lacking NADPHox activity (gp91phox null mice[40]). AMs from these animals demonstrated an increase of approximately 30% in the number of viable ingested bacteria compared with the cells from WT mice. Importantly, the exogenous addition of a mixture of LTs did not enhance the microbicidal capacity of AMs from the gp91phox null mice (Figure

3B). Taken together, these data suggest that enhancement of AM microbicidal activity by LTs requires activation of NADPHox.

Leukotrienes do not augment nitric oxide–dependent killing of *K pneumoniae*

We assessed the effect of LTs on another important bactericidal molecule, NO. This reactive species has been shown to be crucial for the control of *K pneumoniae* infection in murine and in human AMs.[46,47] For these experiments, AMs were pretreated with L-NAME (1 mM), a nonselective NO synthase inhibitor (or vehicle control), and then stimulated with LTB$_4$, cysLTs, or the LT mixture. As evidenced in Figure 3C, inhibition of NO completely abolished the ability of AMs to control *K pneumoniae* infection, increasing the number of ingested bacteria by approximately 98%, but this defect was reversed by treatment with LTs. These data suggest that though NO has important bactericidal activities in AMs, it does not account for the enhanced killing induced by LTs.

5-LO products induce H$_2$O$_2$ secretion by rat AMs in a dose-dependent manner

The demonstration of a role for NADPHox activation in LT-mediated microbicidal activity suggested that LTs and 5-HETE could directly stimulate ROI production by AMs. Although LTs can induce ROI production in many cell types, including human and guinea pig eosinophils,[48] murine and human neutrophils,[49,50] and astroglioma cell,[51] only LTC$_4$ has been shown to stimulate its production by peritoneal macrophages.[52] As illustrated in Figure 4A, the stimulation of AMs with LTs or 5-HETE induced the secretion of H$_2$O$_2$ in a dose-dependent manner. Similar to the effects on bacterial killing, LTB$_4$ was the most effective NADPHox activator in terms of magnitude (LTB$_4$ = 5-HETE > LTD$_4$ and LTC$_4$) and potency (LTB$_4$ > LTD$_4$ and LTC$_4$ > 5-HETE).

The impact of endogenously produced LTs on cellular H$_2$O$_2$ production in response to *K pneumoniae* infection was explored using the LT biosynthesis inhibitors AA861 and MK886 and the BLT1 antagonist CP105696. As shown in Figure 4B, *K pneumoniae* infection induced H$_2$O$_2$ production by rat AMs; this was inhibited by LT synthesis inhibitors and the BLT1 receptor antagonist, suggesting that endogenous LTB$_4$ is involved in NADPHox activation and release of H$_2$O$_2$ during *K pneumoniae*
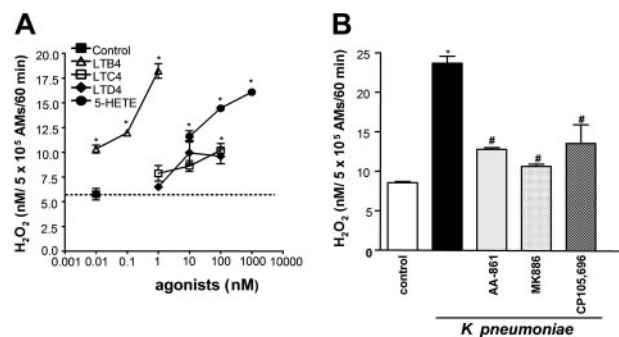

Figure 4. Effect of exogenous and endogenous 5-LO metabolites on H$_2$O$_2$ release in rat AMs. (A) 5×10^5 rat AMs were plated and incubated with indicated concentrations of LTB$_4$, LTC$_4$, LTD$_4$, 5-(S) HETE, or vehicle control (—) for 60 minutes. (B) 5×10^5 rat AMs were plated and preincubated with 10 μM AA-861, 1 μM MK886, 10 μM CP105696, or 10 μM MK571 for 20 minutes and then infected with 50:1 *K pneumoniae*. The H$_2$O$_2$ concentration in medium was determined using Amplex red as a probe. Results represent the mean ± SE from 3 independent experiments performed in triplicate. *P < .05 versus control. #P < .05 versus *K pneumoniae* group by ANOVA.
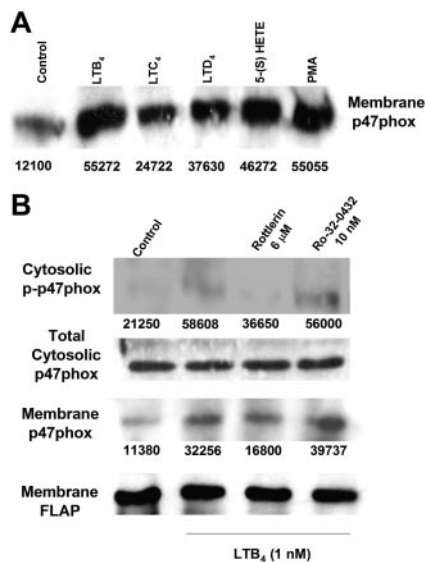
Figure 5. LTB₄ elicits translocation and phosphorylation of p47phox in a PKC-δ–dependent manner. (A) Rat AMs (4×10^6/well) were stimulated with 1 nM LTB₄, 100 nM LTC₄, 100 nM LTD₄, and 1000 nM 5(S)-HETE or 10 nM PMA for 5 minutes and then were harvested and fractionated as described in "Materials and methods." Immunoblotting of membrane fractions was performed using anti-p47phox (1:500). Results are representative of those from 3 experiments. (B) Rat AMs (4×10^6/well) were pretreated with 10 nM RO32-0432 (PKC-α inhibitor), 6 μM rottlerin (PKC-δ inhibitor), or vehicle control for 20 minutes, stimulated with 1 nM LTB₄ for 5 minutes, and then harvested and fractionated as described in "Materials and methods." The cytosolic fraction was immunoprecipitated, and immunoprecipitated protein was immunoblotted using antiphosphoserine (1:900). The total cytosolic fraction and the membrane fraction were probed for total p47phox (1:500). Results are representative of those from 2 experiments. Densitometry analysis is from phosphorylated p47phox or membrane-translocated p47phox. In all instances, density values of bands were corrected by subtraction of the background values. Numbers under lanes indicate relative density of phosphorylated or membrane-translocated p47phox. To ensure equal protein loading, blots were stripped and reprobed for FLAP.

infection. As expected, DPI abolished H₂O₂ production in infected AMs, whereas L-NAME treatment did not alter H₂O₂ (data not shown).

LTB₄ activates p47phox phosphorylation and translocation by increasing PKC-δ activity

To characterize the mechanisms through which LTs activate NADPHox, we evaluated the translocation of the cytosolic p47phox subunit to the plasma membrane, a requisite step in NADPHox activation. Translocation of p47phox was determined by immunoblot analysis of cell-membrane proteins. Stimulation of AMs with either LTs or 5-HETE increased the translocation of p47phox to the membrane fraction compared with untreated cells (Figure 5A). Consistent with the magnitude of actions of 5-LO products on H₂O₂ production, LTB₄ and 5-HETE induced p47phox translocation to a higher degree than either LTD₄ or LTC₄.

The translocation of p47phox is associated with its phosphorylation, and its phosphorylation and translocation are stimulated by various PKC isoforms in neutrophils and monocytes.[53] In this study, we specifically evaluated the roles of PKC-α and -δ because these isoforms have been implicated in p47phox phosphorylation and are more abundant and more easily activated in AMs than in peritoneal macrophages and monocytes.[54,55] Thus, AMs were pretreated with selective inhibitors of PKC-α (10 nM Ro-32-0432)[56] or PKC-δ (6 μM rottlerin)[57] and were then stimulated with LTB₄ (1 nM) for 5 minutes. As observed in Figure 5B, LTB₄ increased the serine phosphorylation of p47phox compared with the untreated control. Interestingly, treatment with the PKC-α

inhibitor did not alter the effect of LTB₄, whereas the PKC-δ inhibitor suppressed the phosphorylation of p47phox when compared with LTB₄ alone. The total amount of cytosolic p47phox did not change in any condition tested (Figure 5B). In addition, we observed the same role of PKC isoforms in p47phox translocation that we observed for its phosphorylation (Figure 5B), supporting a contribution of PKC-δ but not -α. Inhibition of PKC activity in AMs by Ro-32-0432 or rottlerin was isoform specific, as determined by immunoblot analysis of the cytosolic protein fraction against phospho-PKC-α and -δ (data not shown). In addition, to ensure equal protein loading, blots were stripped and reprobed against FLAP. Thus, our data show that LTB₄-induced phosphorylation and subsequent translocation of p47phox are mediated by PKC-δ.

LTB₄ enhances AM microbicidal activity by increasing PKC-δ activity

Once it was determined that LTB₄ was able to activate NADPHox through p47phox phosphorylation and membrane translocation in a manner dependent on PKC-δ, we sought to evaluate whether this phenomenon was also important for LTB₄-induced microbicidal activity in AMs. To do this, AMs were pretreated with selective inhibitors of PKC-α (10 nM Ro-32-0432) or PKC-δ (6 μM rottlerin) and then by LTB₄ (1 nM) after 30 minutes of infection. As observed in Figure 6, LTB₄ increased AM microbicidal activity by approximately 34%; however, this effect was abolished when the cells were pretreated with PKC-δ inhibitor. Interestingly, treatment with the PKC-α inhibitor did not alter the effect of LTB₄. Thus, this result establishes that PKC-δ is an upstream target for the effects of LTB₄ on enhancing microbicidal activity, which results in NADPHox activation and bacterial killing.

Discussion

This study establishes the importance of LTs in AM bactericidal activity and characterizes some of the molecular mechanisms involved in this phenomenon. Our results show that (1) LTs greatly enhance the microbicidal activity of AMs infected by the relevant Gram-negative pathogen *K pneumoniae*; (2) LTB₄ is the major
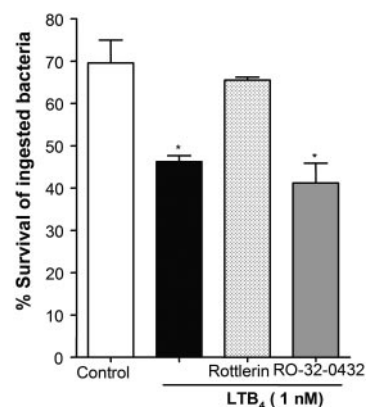

Figure 6. Role of PKC-δ and -α in LTB₄ enhancing AM killing of opsonized K pneumoniae. Rat AMs were pretreated with vehicle control, the PKC-δ inhibitor rottlerin (6 μM), or the PKC-α inhibitor RO32-0432 (1 nM) for 20 minutes before the addition of opsonized *K pneumoniae*. Thirty minutes after infection, drugs were added back with or without 1 nM LTB₄, as described above. Microbicidal activity was assessed; data are expressed as mean ± SE. Percentage of survival of ingested bacteria of quadruplicate values from 1 of 4 representative experiments. *$P < .05$ compared with untreated control; ANOVA.

contributing endogenous LT, and its effects are mediated by the BLT1 receptor; (3) the microbicidal activity induced by LTs occurs because of the activation of NADPHox, not because of NO generation; (4) LTs increase the phosphorylation and translocation of p47phox in a manner dependent on PKC-δ activation; (5) PKC-δ activation is also a determinant of the AM microbicidal activity enhanced by LTB$_4$.

A role for LTs in amplifying leukocyte microbicidal activity has been suggested by studies documenting enhanced leukocyte functions, such as phagocytosis, pathogen killing, release of neutrophil granules, defensin production, and generation of antimicrobial oxidants.[7-9,11-14,16,19,25,58,59] However, information is lacking regarding the relative importance of the various LTs in immunoregulation and which molecular mechanisms evoked by LTs are involved in these processes. In particular, there is a paucity of information in the AM, a cell type critical to the antimicrobial defense of the lung.

To examine these issues, our first approach was to stimulate the AM with exogenous LTB$_4$, cysLTs, and 5-HETE and to assess their individual effects on the killing of ingested *K pneumoniae*. Because LTs are known to enhance Fc-γ receptor–mediated phagocytosis by AMs,[10] it was important to use a bactericidal assay that could quantify the effects on bacterial killing independently of effects on phagocytosis. To achieve this, we used a well-characterized bactericidal assay[41] that allowed the addition of LTs after phagocytosis had occurred and that included a (4°C) control to account for differences in bacterial ingestion.

Our results indicate that all the 5-LO products tested enhanced bacterial killing but that LTB$_4$ was the most potent. This finding is of interest because cysLTs were slightly more potent than LTB$_4$ in promoting bacterial phagocytosis by AMs.[9] Wirth and Kierszenbaum[7,8] showed that LTB$_4$ and LTC$_4$ increased phagocytosis and *T cruzi* killing; however, they did not evaluate the relative potency of both LTs. We confirmed that the effects of LTB$_4$ and the cysLTs were specifically mediated by the BLT1 and cysLT1 receptors, respectively. Comparison between these data also indicates that a lower concentration of exogenous cysLTs is required to enhance phagocytosis (1 nM)[9] than bacterial killing (100 nM). It is notable that blocking the BLT1 receptor itself suppressed the ability of AMs to kill ingested bacteria, illustrating the importance of endogenously produced LTB$_4$ in AM function. However, pharmacologic blockade of the cysLT receptors did not affect basal rat AM microbicidal activity. These results may best be explained by the fact that the rat (and human) AM capacity to produce cysLTs is approximately 10 times lower than that of LTB$_4$.[9] These results are in contrast to studies of phagocytosis of *K pneumoniae*, in which blocking cysLT1 suppressed rat AM phagocytosis.[9]

We can contrast these findings with our examination of the link between the NO cascade and LT signaling (Figure 3C). Although NO production is required for the AM to kill *K pneumoniae*, blocking NO production did not abrogate the microbicidal enhancement induced by LTs. Other investigators have found a relationship between LT-induced antimicrobial activity and the NO cascade.[14,38] For example, Talvani et al[14] showed that LTs enhanced the ability of peritoneal macrophages to kill *T cruzi* organisms in a manner dependent on NO production. The reasons for this discrepancy are unclear but may reflect differences in the pathogen studied or the experimental design. Unlike our model, in which infection with *K pneumoniae* was of a short duration (90 minutes), the *T cruzi* infection model was more prolonged (7 days).

Because LTs enhance Fc-γ receptor–mediated phagocytosis by the AM[10] and immunoglobulin G (IgG)–opsonized targets induce the activation of NADPHox,[60] we wanted to examine the ability of LTs to stimulate NADPHox. To accomplish this, we used genetic and pharmacologic approaches to characterize relationships between LT signaling and NADPHox activity. Using the specific NADPHox inhibitor DPI and AMs derived from gp91phox-deficient mice, we confirmed the important role of this enzyme complex in generating ROIs lethal to invading bacteria. Importantly, when the activation of NADPHox was abrogated, the ability of LTs to enhance bacterial killing was lost, strongly suggesting that NADPHox activity mediates the effects of LTs. Our study also documented that LTs can stimulate AM H$_2$O$_2$ production in the absence or presence of infection. This was shown using exogenous LTs, inhibitors of LT synthesis, and LT receptor antagonists.

The constitutive membrane component of NADPHox is the flavocytochrome b_{558}, which is formed by gp91phox and p22phox and which represents the catalytic core of the holoenzyme. Activation of flavocytochrome b_{558} requires translocation of the cytosolic components p47phox, p40phox, p67phox, and rac-1 or rac-2. NADPHox activation often follows the stimulation of cell-membrane G-protein–coupled receptors and involves multiple downstream signaling pathways that culminate in the phosphorylation of enzyme complex components by specific kinases such as PKC,[61,62] mitogen-activated protein (MAP) kinases,[63,64] and protein kinase B/Akt.[65,66]

To study NADPHox activation in vitro, we used immunoblot analysis to quantify the phosphorylation and translocation of p47phox to the cell membrane. We found that all 5-LO metabolites enhanced the translocation of p47phox. In most instances, the translocation of p47phox requires its phosphorylation, though precedent does exist for lipids such as AA, linoleic acid, and diacylglycerol eliciting translocation without phosphorylation.[67,68] We verified that LTB$_4$ did indeed cause the phosphorylation of p47phox. We similarly observed that both classes of LTs induced the phosphorylation and translocation of p40phox to the plasma membrane (data not shown). To our knowledge, this is the first report showing that LTB$_4$ can stimulate the phosphorylation and translocation of NADPHox components.

The NADPHox complex of monocytes/macrophages has in general been less studied than that of neutrophils. Few reports show the molecular mechanisms involved in the phosphorylation, translocation, and assembly of the phox proteins in macrophages, especially the AM. Yamamori et al[69] showed that PI3-kinase mediates the phosphorylation of p47phox in HL-60 cells stimulated by N-formyl-methionyl-leucyl-phenylalanine (fMLP) through activation of the classical PKC and PKC-δ. Zhou et al[70] reported that t-butyl hydroperoxide increased p47phox translocation to the AM membrane. However, our results are the first to demonstrate that LTs can regulate the phosphorylation and translocation of cytosolic components of NADPHox.

We investigated the effect of Ca^{++}-dependent PKC-α and Ca^{++}-independent PKC-δ in the phosphorylation of p47phox. We studied these isoforms of PKC because they are implicated in NADPHox activation and are present in AMs.[55,71] Using the cell-permeable PKC inhibitors rottlerin (PKC-δ) and Ro-32-0432 (PKC-α), we found that PKC-δ mediates the LTB$_4$-simulated phosphorylation and subsequent translocation of p47phox (Figure 6A-C). These results are supported by the findings of O'Flaherty et al[72] that LTB$_4$ can induce nonconventional PKC activation in human neutrophils. In addition, Bey et al[73] demonstrated that in human monocytes stimulated by opsonized zymosan, p47phox phosphorylation and membrane translocation is dependent on PKC-δ but not PKC-α.

Figure 7. Proposed model of NADPHox activation by LTB$_4$.
Either binding of IgG-coated targets with the FcγR or ligation of BLT1 by LTB$_4$ results in increased levels of intracellular Ca^{++} and diacylglycerol (DAG). Ca^{++} activates cPLA$_2$ activity, which results in enhanced AA release and subsequent generation of LTs. LTB$_4$ stimulates PKC-δ through an unknown mechanism, whereas DAG directly activates this enzyme. Once activated, PKC-δ induces phosphorylation and translocation of p47phox to the plasma membrane, generating ROIs. LTs may also act indirectly by increasing cPLA$_2$ activity (dashed line and question mark).
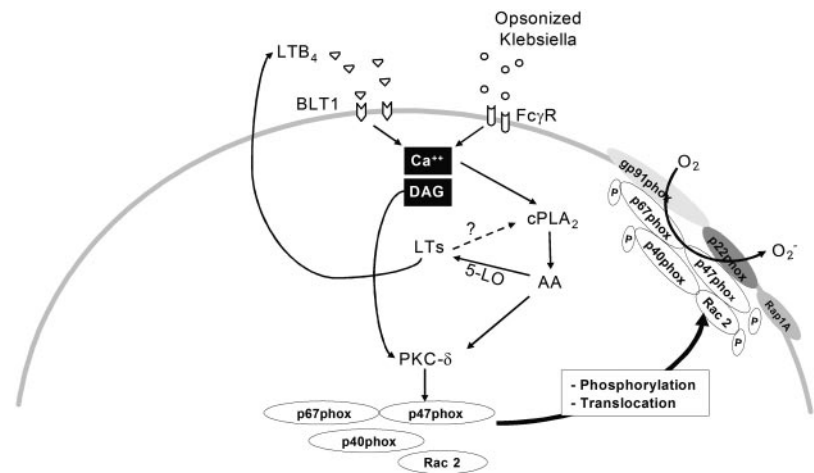
Once we determined the molecular mechanism of NADPHox activation by LTB$_4$, we sought to evaluate whether this mechanism is also involved in AM microbicidal activity. Our results indicated that PKC-δ is an upstream target of LTB$_4$ required for the amplification of AM microbicidal activity. That PKC is involved in macrophage microbicidal activity has been demonstrated. However, the role of PKCs in AM effector function is not well understood. Considering phagocytosis and microbicidal activity in macrophages, Heale and Speert[74] showed that PKC agonists increase phagocytosis of *Pseudomonas aeruginosa* by murine AMs. St-Denis et al[75] demonstrated that PKC-α is involved in the killing of *Leishmania donovani* and *Legionella pneumophila* in RAW 264.7. In addition, PKC-ϵ–deficient mice experience a decreased period of survival during Gram-negative bacterial infection.[76] However, the role of PKC-δ and microbicidal activity has never been assessed in macrophages.

We noted that optimal concentrations of LTB$_4$ and cysLTs exhibited no additive effects on bacterial killing. This can likely be explained by the convergence in signaling pathways downstream from BLT1 and cysLT1 because both are coupled to G$_q$ and to G$_i$ proteins[77,78] and both are known to activate PKC.[79] We previously reported that effects of both classes of LTs on phagocytosis were similarly dependent on PKC.[10] LTB$_4$ has been reported to activate cytosolic phospholipase A$_2$ (cPLA$_2$) and to release AA. It is well established that AA can itself activate PKC-δ[80] as well as p47phox and p67phox.[67] The possibility that AA is a downstream mediator of the effects of LTs was not explored in the present investigation.

In summary, this paper describes the important role that 5-LO metabolites play in amplifying AM microbicidal activity against bacterial pathogens. This activation follows the ligation of specific LT receptors and involves the subsequent activation of NADPHox. Furthermore, our results implicate PKC-δ in the activation of NADPHox and the subsequent increase in microbicidal activity stimulated by LTs (Figure 7). Although previous studies have established separately that LTs enhance the microbicidal activity of leukocytes and augment the generation of antimicrobial molecules by these cells, our study, for the first time, directly links these 2 phenomena.

Acknowledgments

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