5-Lipoxygenase Reaction Products Modulate Alveolar Macrophage Phagocytosis of Klebsiella pneumoniae

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The leukotrienes are potent lipid mediators of inflammation formed by the 5-lipoxygenase-catalyzed oxidation of arachidonic acid. Although the effects of leukotrienes on neutrophil chemotaxis and activation have been established, their role in modulating innate host defense mechanisms is poorly understood. In a previous study (M. Bailie, T. Standiford, L. Laichalk, M. Coffey, R. Strieter, and M. Peters-Golden, J. Immunol. 157: 5221–5224, 1996), we used 5-lipoxygenase knockout mice to establish a critical role for endogenous leukotrienes in pulmonary clearance and alveolar macrophage phagocytosis of Klebsiella pneumoniae. In the present study, we investigated the role of specific endogenous leukotrienes in phagocytosis of K. pneumoniae and explored the possibility that exogenous leukotrienes could restore phagocytosis in alveolar macrophages with endogenous leukotriene synthesis inhibition and enhance this process in leukotriene-competent cells. Rat alveolar macrophages produced leukotriene B4 (LTB4), LTC4, and 5-hydroxyeicosatetraenoic acid (5-HETE) during the process of phagocytosis, and the inhibition of endogenous leukotriene synthesis with zileuton and MK-886 dramatically attenuated phagocytosis. We also observed a reduction in phagocytosis when we treated alveolar macrophages with antagonists to the plasma membrane receptors for either LTB4, cysteinyl-leukotrienes, or both. In leukotriene-competent cells, LTC4 augmented phagocytosis to the greatest extent, followed by 5-HETE and LTB4. These 5-lipoxygenase reaction products demonstrated similar relative abilities to reconstitute phagocytosis in zileuton-treated rat alveolar macrophages and in alveolar macrophages from 5-lipoxygenase knockout mice. We conclude that endogenous synthesis of all major 5-lipoxygenase reaction products plays an essential role in phagocytosis. The restorative and pharmacologic effects of LTC4, LTB4, and 5-HETE may provide a basis for their exogenous administration as an adjunctive treatment for patients with gram-negative bacterial pneumonia.

Bacterial pneumonia is the leading cause of infectious death in industrialized nations (6), and its effective treatment grows increasingly more challenging due to the emergence of antibiotic-resistant strains and the increasing prevalence of immune suppression. The resident alveolar macrophage (AM) patrols the alveolar epithelial surface of the lung and maintains stability by phagocytosing and killing microorganisms (21). If the microbial burden in the alveolar space overwhelms the ability of the AM to clear invading pathogens or when encapsulated gram-negative bacteria reach the alveolar surface, these resident cells secrete substances such as leukotriene B4 (LTB4), complement, and cytokines, which recruit neutrophils from the peripheral circulation to the alveolar focus of infection (24).

Leukotrienes (LTs) are potent lipid mediators of inflammation derived via the 5-lipoxygenase (5-LO) pathway of arachidonic acid (AA) metabolism (Fig. 1). In particular, the enzyme 5-LO, in concert with its helper protein 5-LO-activating protein (FLAP), can oxygenate AA to 5-hydroperoxyeicosatetraenoic acid (5-HPETE). This intermediate can either be dehydrated to LTA4 or reduced to 5-hydroxyeicosatetraenoic acid (5-HPETE). 5-HETE can be further oxidized to 5-oxo-ETE, while LTA4 can be hydrolyzed to form LTB4, or conjugated with glutathione to form the cysteinyl-LTs (LTC4, LTD4, and LTE4). Alternative routes of metabolism found in certain cell types result in the synthesis of 12- and 15-HETE by 12- and 15-LO, respectively. In addition, metabolism via the cyclooxygenase pathway results in the formation of prostanoids and thromboxane.

Although the roles of LTs in neutrophil recruitment and cell activation are well established (20), their role in host defense is poorly understood. In addition to their role in neutrophil recruitment, LTs might promote host defense by virtue of their abilities to augment microbial phagocytosis and killing both in vitro and in vivo (1, 12). Evidence that the 5-LO pathway is activated during the course of lower respiratory tract infection includes the presence of elevated LTB4 levels in the bronchoalveolar lavage fluid of patients with bacterial pneumonia and elevated LTB4 and LTC4 levels in lung homogenates in animal models of bacterial pneumonia (4, 16). An important role for endogenously produced LTs in the host response to pneumonia was established by our recent report that 5-LO knockout (KO) mice exhibited enhanced mortality and reduced bacterial clearance compared with their wild-type (WT) counterparts following intratracheal administration of the gram-negative bacterium Klebsiella pneumoniae (1). This in vivo defect in bacterial clearance was associated with reduced phagocytosis and killing of K. pneumoniae in vitro studies with AMs from 5-LO KO mice, compared with WT mice. Interestingly, phagocytosis could be enhanced in AMs from 5-LO KO mice by the addition of exogenous LTB4.

In the present study, we sought to extend our previous work on 5-LO products and AM phagocytosis. First, we wanted to explore the roles of both endogenously produced and exogenously added 5-LO metabolites in phagocytosis. Second, we wished to delineate the individual effects of all of the major
5-LO metabolites. Our data support an important role for endogenous 5-LO metabolites synthesized during the process of phagocytosis, as well as a pharmacologic effect of exogenously added products, in promoting AM phagocytosis of these relevant gram-negative bacteria.

MATERIALS AND METHODS

Cell isolation and culture. Animals included pathogen-free 125- to 150-g female Wistar rats (Charles River Laboratories, Portage, Mich.), 5-LO KO mice, and their WT controls (129/SvEv) (The Jackson Laboratory, Bar Harbor, Maine). Resident AMs were obtained by lung lavage as previously described (1, 29). Ninety-six percent of the cells obtained from lavage have been identified and their WT controls (129/SvEv) (The Jackson Laboratory, Bar Harbor, Maine), 5-LO KO mice, female Wistar rats (Charles River Laboratories, Portage, Mich.), and 5-LO KO mice, each diluted in HBSS. Where skin incisions were anesthetized with sodium pentobarbital and blood was collected and allowed to clot at room temperature for 4 h. The blood was refrigerated overnight to allow the clot to retract. The clotted blood was centrifuged at 1,900 g for 10 min at 37°C. The bacteria in culture were determined spectrophotometrically at 600 nm by using a standard curve to calculate the inoculum concentration (15). temporary immobilization injection. The formalin-killed bacterial suspension was then frozen at −80°C. At 10 days after the primary inoculation of K. pneumoniae cells, the inoculum was diluted in HBSS and quantified with an on-line radioactivity detector (Radiomatic model 515; Packard, Downers Grove, IL). The released disintegrations per minute correspond to a given eicosanoid were expressed as the percentage of radioactivity incorporated into the cell before incubation with or without K. pneumoniae.

Phagocytosis assay. AMs (106) were allowed to adhere to glass eight-well Labtek slides (Nunc, Naperville, Ill.) for 1 h in RPMI 1640. Following adherence, the RPMI 1640 was removed and replaced with fresh medium. In some experiments, cells were pretreated for 15 min with the 5-LO enzyme inhibitor zileuton (10 μM) (Abbott Laboratories, Chicago, Ill.), the FLAP inhibitor MK-886 (1 μM) (Merck-Frost, Montreal, Canada), the cysteinyI-LT receptor antagonist LY171883 (1 μM), or the LTbR receptor antagonist LY292476 (1 μM) (both from Eli Lilly, Indianapolis, Ind.), each diluted in HBSS. Where appropriate, LO metabolites (Cayman Chemical, Ann Arbor, Mich.) were added in HBSS to the AM monolayers 5 to 10 min before to the addition of 106 K. pneumoniae organisms opsonized with 1% specific immune serum. The culture contents were mixed for 1 min with a plate shaker (Hoefer Instruments, San Francisco, Calif.). The AM cultures were then incubated for 30 min at 37°C. After the incubation period, the extracellular bacteria were removed by three washes with HBSS. After being dried overnight, the plastic well chambers were removed and the slides were stained with Diff-Quik. For each slide, a standard pattern of high-power fields was examined by light microscopy (magnification, ×1,000) to enumerate 100 cells. The phagocytic index was determined by multiplying the percentage of cells containing at least one bacterium by the number of bacteria per positive cell. In rat AMs under control conditions, the phagocytic index was 38 ± 9 (mean ± standard error [SE]). The ability of this microscopic method to distinguish intracellular from surface-associated bacteria was verified by comparing the phagocytic index of untreated AM monolayers with that of monolayers with cytochalasin D (5 μg/ml), which has been shown to completely inhibit the internalization of adherent particles by inhibiting actin polymerization (25).

The phagocytic index of AMs treated with cytochalasin D was determined to be 10% of the value obtained without the drug, indicating that 90% of the cell-associated bacteria in these experiments were actually internalized.

Statistical analysis. A minimum of four replicate wells per condition were studied in each experiment, and the number of individual experiments is indicated in the figure legends. Data are expressed as the mean ± SE. Where appropriate, values were analyzed by using a one-way analysis of variance (ANOVA)
HPLC chromatogram in Fig. 2A, control cultures contained a eicosanoids released into cell culture medium after incubation for 1 h. Eicosanoids were extracted from the medium before separation by reverse-phase HPLC. As indicated by the total radioactivity incorporated into cells. Peaks are designated on the basis of coelution with authentic standards. TXB₂, thromboxane B₂; HHT, 12-hydroxy-heptadecatrienoic acid.

To determine if AM phagocytosis of opsonized K. pneumoniae induces LT synthesis, we determined the pharmacologic effects on AM phagocytosis of K. pneumoniae. AMs were pretreated with medium alone, zileuton (10 μM), or MK-886 (1 μM) for 15 min before the addition of opsonized K. pneumoniae. As shown in Fig. 4, pre-incubation of AMs with MK-886 or zileuton reduced LT synthesis to approximately 60% of control level whereas the combination of the two receptor antagonists led to a further reduction in the phagocytic index to about 50% of the control level. These data indicate that both endogenously produced LTB₄ and cysteinyl-LTs enhance phagocytosis and do so, at least in part, by moving out of the cell and activating their plasma membrane receptors.

Exogenous LTs exert pharmacologic effects on AM phagocytosis. In the previous experiments, we established the importance of endogenous LTs as modulators of AM phagocytosis. We next addressed the possibility that 5-LO metabolites have pharmacologic effects on AM phagocytosis of K. pneumoniae. In these experiments, we used four products of the 5-LO pathway (LTB₄, LTC₄, 5-HETE, and 5-oxo-ETE) and, for comparison, the alternative lipoxygenase products 12-HETE and 15-HETE (Fig. 1). Each was used at 1 nM because preliminary dose-response experiments (results not shown) with 5-LO inhibitors: zileuton, which inhibits LT synthesis by interfering with its presentation of AA to 5-LO (33) (Fig. 1). In comparison to the control condition, both zileuton (10 μM) and MK-886 (1 μM), each at levels known to inhibit LT synthesis (3, 36), significantly attenuated AM phagocytosis of opsonized K. pneumoniae by approximately 50% (Fig. 3). The attenuation of phagocytosis by the inhibition of endogenous LT production indicates that LTs play a significant role in bacterial phagocytosis.

Inhibition of phagocytosis by antagonists to plasma membrane receptors for LTs. The preceding experiments established a role for a product(s) of the 5-LO pathway but did not identify the responsible endogenous metabolite. Since it is known that AMs express separate plasma membrane receptors for LTB₄ and the cysteinyl-LTs and since both were produced during phagocytosis (Fig. 2B), we used a strategy involving selective receptor antagonists to determine the separate contributions of these two specific 5-LO metabolites (Fig. 1). AMs were treated with medium alone or medium containing LTB₄ receptor antagonist (LY292476) (1 μM), cysteinyl-LT receptor antagonist (LY171883) (1 μM), or both for 15 min before the addition of opsonized K. pneumoniae. As shown by the HPLC chromatogram in Fig. 2B, radioactive metabolites of AA were detected. As shown by the HPLC chromatogram in Fig. 2B, LTB₄ was the principle eicosanoid produced, with lower levels of the other 5-LO products, LTC₄ and 5-HETE. The cyclooxygenase products thromboxane B₂ (a nonenzymatic metabolite of thromboxane A₂) and 12-hydroxyheptadecatrienoic acid were also detected. These results indicate that the 5-LO pathway in AMs is activated during phagocytosis of opsonized K. pneumoniae.

Inhibition of endogenous LT synthesis reduces AM phagocytosis of K. pneumoniae. To determine if the synthesis of endogenous LTs plays an important functional role in AM phagocytosis of opsonized K. pneumoniae, we determined the effect on K. pneumoniae phagocytosis of two different types of LT synthesis inhibitors: zileuton, which inhibits LT synthesis by complexing with an iron atom in the active site of the 5-LO enzyme (5), and MK-886, which inhibits LT synthesis by binding to FLAP and interfering with its presentation of AA to 5-LO (33) (Fig. 1). In comparison to the control condition, both zileuton (10 μM) and MK-886 (1 μM), each at levels known to inhibit LT synthesis (3, 36), significantly attenuated AM phagocytosis of opsonized K. pneumoniae by approximately 50% (Fig. 3). The attenuation of phagocytosis by the inhibition of endogenous LT production indicates that LTs play a significant role in bacterial phagocytosis.

RESULTS

AM phagocytosis of opsonized K. pneumoniae induces LT synthesis. To determine if AM phagocytosis of opsonized K. pneumoniae results in the synthesis of LTs, medium with or without opsonized K. pneumoniae was incubated with AMs that had been prelabeled with [³¹]AA for 1 h. Radioactive eicosanoids released into cell culture medium after incubation were separated by reverse-phase HPLC. As indicated by the HPLC chromatogram in Fig. 2A, control cultures contained a small amount of unmetabolized free [³¹]AA but no eicosanoid metabolites. However, when the AMs were incubated with opsonized K. pneumoniae, radioactive metabolites of AA were detected. As shown by the HPLC chromatogram in Fig. 2B, LTB₄ was the principle eicosanoid produced, with lower levels of the other 5-LO products, LTC₄ and 5-HETE. The cyclooxygenase products thromboxane B₂ (a nonenzymatic metabolite of thromboxane A₂) and 12-hydroxyheptadecatrienoic acid were also detected. These results indicate that the 5-LO pathway in AMs is activated during phagocytosis of opsonized K. pneumoniae.

In Fig. 2. Representative radioactive HPLC elution profiles from prelabeled AMs incubated in the absence (A) or presence (B) of opsonized K. pneumoniae for 1 h. Eicosanoids were extracted from the medium before separation by HPLC. The radioactivity in each fraction was expressed as a percentage of the total radioactivity incorporated into cells. Peaks are designated on the basis of coelution with authentic standards. TXB₂, thromboxane B₂; HHT, 12-hydroxy-heptadecatrienoic acid.

and the Bonferroni test for mean separation. In all cases, P < 0.05 was considered significant.

FIG. 3. Effect of endogenous LT synthesis inhibition on AM phagocytosis of K. pneumoniae. Data are expressed as the mean phagocytic index (percentage of control) ± SE (n = 4). *P < 0.05 with respect to the control by ANOVA.
be optimal. Exogenous lipids were coincubated with AMs 5 to 10 min before the addition of opsonized *K. pneumoniae*. As shown in Fig. 5, phagocytosis was significantly enhanced by exogenous LTC₄ (approximately 220% of the control level), 5-HETE (200% of the control level), and LTB₄ (175% of the control level). The effect of 5-oxo-ETE, which is known to have effects on neutrophils (30), was comparable to that of LTB₄, and was nearly statistically significant (*P* = 0.10). 12-HETE had no effect on phagocytosis, whereas 15-HETE, a known inhibitor of leukocyte function (18), reduced phagocytosis slightly but not significantly.

**The ability of exogenous LTs to enhance phagocytosis is mediated largely via interactions with plasma membrane receptors.** We next wished to determine if the positive effects of exogenous LTB₄ and LTC₄ (Fig. 5) were mediated by their interaction with surface receptors. As shown in Fig. 6, receptor antagonists completely prevented the increase in phagocytosis over baseline caused by exogenous LTB₄ and LTC₄ (1 nM) alone. However, the phagocytic index for exogenous LTB₄ plus antagonist still slightly exceeded the value for the antagonist alone.

**The inhibitory effects on phagocytosis caused by inhibition of endogenous LT synthesis are overcome by the addition of exogenous LTB₄.** In the next set of experiments, we investigated whether it was possible to restore phagocytosis with exogenous LTB₄ in AMs treated with inhibitors of endogenous LT synthesis. In these experiments, AMs were not treated or were treated with zileuton for 15 min before the addition of medium or exogenous LTs (1 nM), or 5-HETE (1 nM). As shown in Fig. 7, when exogenous LTB₄, LTC₄, or 5-HETE was added to AMs treated with zileuton, the phagocytic response was increased to 125% (LTB₄ and LTC₄) and 110% (5-HETE) of control levels. These results indicate that exogenous LTB₄, LTC₄, and 5-HETE were each capable of completely overcoming the inhibitory effects of endogenous LT inhibition on phagocytosis.

**Exogenous LTB₄ partially restore phagocytosis in AMs from 5-LO KO mice.** We previously reported (1) that AMs from 5-LO KO mice showed reduced phagocytosis of *K. pneumoniae* compared with cells from their WT counterparts. Furthermore, phagocytosis in these cells could be enhanced by the addition of exogenous LTB₄. Since the experiments presented above indicated that exogenous LTC₄ and 5-HETE, in addi-
The principal 5-LO reaction product recovered from the medium of AMs cultured with opsonized *K. pneumoniae* was LTB4, followed by LTC4 and 5-HETE. The LT profile observed in this experiment was similar to that seen with other phagocytic stimuli such as zymosan, in which the ratio of LTB4 to LTC4 is about 10:1 (29). In the present experiments, we opsonized the bacteria with specific immune serum, which would be expected to contain both anti-*K. pneumoniae*-specific antibodies and complement. Either whether or both of these opsonins are required for the synthesis of LTs is an issue for future investigation. However, other reports demonstrate LT generation by leukocytes incubated with both unopsonized (8) and serum-opsonized (7) bacteria.

In this study, we used a pharmacologic approach to demonstrate that endogenously generated LTs enhance phagocytosis. This conclusion is based on the observations that inhibition of LT synthesis by two inhibitors acting via distinct molecular mechanisms reduced phagocytosis. This finding (confirmed in Fig. 8) is in agreement with our previous report, which demonstrated that AMs from 5-LO KO mice exhibited defective phagocytosis compared with AMs from WT mice. Likewise, blocking the action of LT with receptor antagonists also attenuated phagocytosis. When antagonists to receptors for both LTB4 and cysteinyI-LTs were combined, phagocytosis was inhibited to a greater extent than with either alone and to an extent similar to that observed with synthesis inhibitors. Taken together, these results suggest that the modulatory actions of the two groups of LTs are not redundant.

Previously, we observed the ability of exogenous LTB4 to increase phagocytosis in AMs from 5-LO KO mice. We verified the role of LTB4 in phagocytosis in the present experiments by using both specific LT receptor antagonists and exogenous addition of LTB4. The positive effect of LTB4 on phagocytosis was not surprising, since there is ample evidence demonstrating that LTB4 stimulates many leukocyte functions, including the upregulation of complement receptors and increased calcium mobilization in human peripheral blood neutrophils.

**DISCUSSION**

The AM provides the first line of antibacterial defense in the distal lung and maintains the sterility of the alveolar surface by ingesting and killing bacteria (21). Among the various substances released by the AM during its encounter with bacteria are the LTs. We previously demonstrated that 5-LO KO mice exhibited impaired bacterial clearance of *K. pneumoniae* in vivo and an associated defect in phagocytosis in AMs in vitro (1). The present study was designed to further delineate the role of LTs in AM phagocytosis by distinguishing the contribution of individual endogenous 5-LO reaction products and determining whether exogenous LTs exert a pharmacologic effect on LT-competent cells.

The major findings of this study were as follows: (i) AM phagocytosis of *K. pneumoniae* induced LT synthesis; (ii) inhibition of endogenous LT synthesis reduced AM phagocytosis of *K. pneumoniae*; (iii) exogenous LTC4, LTB4, and 5-HETE have pharmacologic effects, since they enhanced the AM phagocytosis of *K. pneumoniae* in LT-competent AMs; (iv) endogenous and exogenous LTB4 and LTC4 augmented AM phagocytosis primarily via their plasma membrane receptors; and (v) the impairment in phagocytosis observed in cells rendered LT deficient by either pharmacologic or genetic means could be overcome by the addition of exogenous LTC4, LTB4, or 5-HETE.

If endogenous LTs played a physiologic role in supporting phagocytosis, one would expect their synthesis to be demonstrable during the encounter of the phagocyte with bacteria.
trophils (23) and phagocytosis in murine peritoneal macrophages (12). Likewise, the effects of exogenous 5-HETE and 5-oxo-ETE on phagocytosis were not surprising, since these eicosanoids activate certain leukocyte functions in a manner similar to that of LTBR (30).

Since the cysteinyl-LTs are best known for their ability to elicit smooth muscle contraction (20), the effects of LTC4 on phagocytosis were somewhat surprising. These data suggest that cysteinyl-LTs possess important leukocyte-activating properties that have not been generally recognized. One mechanism by which they might augment phagocytosis relates to their ability to promote actin polymerization (26). Although we observed cysteinyl-LTs to augment phagocytosis within 30 min, it is possible that in vivo cysteinyl-LTs further enhance phagocytosis by also upregulating Fc receptor expression via de novo synthesis (31).

It is well known that macrophages express LTBR (32) and cysteinyl-LT receptors (11) on their plasma membrane. We used LY2942476 (a LTBR receptor antagonist) and LY171883 (a cysteinyl-LT receptor antagonist) in our experiments to determine if LTs were acting through these receptors to enhance phagocytosis. Based on the data presented in Fig. 6, it appears that most, but perhaps not all, of the actions of LTBR and LTC4 on phagocytosis are mediated via classical plasma membrane receptors. A precedent for LT actions via a novel intranuclear receptor has recently been presented (13). Further studies are necessary to elucidate the mechanism underlying any receptor-independent actions of LTs in this experimental system.

In addition to the 5-LO products mentioned above, 12- and 15-HETE, which are formed by 12- and 15-LO, respectively, were used as negative controls. The fact that these LO products failed to augment phagocytosis demonstrates that the positive effect of 5-LO products on phagocytosis was not due to a nonspecific physical interaction of lipids with K. pneumoniae and AMs. The inability of 12-HETE to influence phagocytosis is consistent with the work of Sun and Funk, who showed that Fc receptor-mediated phagocytosis was not significantly altered in macrophages from 12-LO KO mice (35). That 15-HETE tended to decrease phagocytosis fits well with the fact that it generally has suppressive effects on leukocyte functions (18). This may actually reflect the ability of 15-HETE to inhibit 5-LO metabolism (14).

These results raise the possibility that the inhibitory effects of zileuton or MK-886 on AM phagocytosis reflect increased 5-LO metabolism (14). Since the cysteinyl-LTs are best known for their ability to promote actin polymerization (26). Although we observed cysteinyl-LTs to augment phagocytosis within 30 min, it is possible that in vivo cysteinyl-LTs further enhance phagocytosis by also upregulating Fc receptor expression via de novo synthesis (31).

In summary, we have shown that endogenously produced LTs play an essential role in AM phagocytosis of K. pneumoniae. We have also provided evidence that LTC4, LTBr, and 5-HETE, in addition to enhancing phagocytosis in AMs with absent LT synthetic capacity, possess the ability to enhance phagocytosis in normal AMs. Future investigations will determine whether these effects extend to other microbes and to other populations of phagocytic cells and will explore the molecular mechanisms by which LTs augment phagocytosis.

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