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Intrinsic 5-lipoxygenase activity is required for neutrophil responsivity

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ABSTRACT **We found that intrinsic neutrophil 5-lipoxygenase activity was necessary for human neutrophil adherence and chemotaxis *in vitro* and human neutrophil-mediated acute edematous injury in isolated perfused rat lungs given interleukin 8 intratracheally. Treatment with either Zileuton (a specific reversible competitive inhibitor of 5-lipoxygenase) or MK886 (a specific irreversible inhibitor of the 5-lipoxygenase activator protein) prevented stimulated neutrophil adherence and chemotaxis (but not superoxide anion production) *in vitro*. Zileuton- or MK886-inhibited neutrophil chemotaxis was not restored by adding leukotriene B_4 *in vitro*. Perfusion with neutrophils and either Zileuton or MK886, or with MK886-pretreated neutrophils (without adding MK886 to the perfusate), also prevented lung injury (reflected by lung weight gain and lung Ficoll retention) and perfusate leukotriene B_4 increases in isolated rat lungs given interleukin 8 intratracheally. Again, adding leukotriene B_4 to the perfusate did not damage interleukin 8-treated isolated lungs perfused with Zileuton-inhibited neutrophils. We conclude that intrinsic 5-lipoxygenase activity is required for neutrophil adherence and chemotaxis and neutrophil-mediated lung injury.**

Neutrophils participate in a vast array of acute and chronic inflammatory diseases. A prime example is the highly fatal (50% mortality) adult respiratory distress syndrome, which is an acute, oxidative edematous lung injury that for unknown reasons complicates a variety of pulmonary and extrapulmonary insults (1). Patients with this syndrome have increased bronchoalveolar lavage levels of interleukin 8 (IL-8), leukotriene B_4 (LTB_4), and neutrophils (2–5), but the relationships of these factors to lung injury are unclear. IL-8, a potent neutrophil chemoattractant (6), potentiates neutrophil 5-lipoxygenase enzyme activity *in vitro* (7, 20), but it is not known if the neutrophil "priming" effects of IL-8 *in vitro* are related to its biological effects *in vivo*, specifically if and how IL-8 causes tissue injury. The major 5-lipoxygenase product secreted by neutrophils is LTB_4, but a causal link between increased airway IL-8 levels and the recruitment of circulating neutrophils via LTB_4 production has not been identified. Furthermore, while LTB_4 is the predominant 5-lipoxygenase product secreted by neutrophils, LTB_4 precursors such as 5-hydroperoxyeicosatetraenoic acid and leukotriene A_4 (LTA_4), or other 5-lipoxygenase products such as lipoxins, may play important roles in inflammation (10). Finally, the contribution of intrinsic neutrophil 5-lipoxygenase activity to neutrophil function has not been determined.

To examine these potential relationships, we hypothesized that 5-lipoxygenase activation is required for neutrophils to respond to inflammatory stimuli and that elevated airway levels of IL-8 cause acute edematous lung injury via this pathway. To test this premise, we examined the effects of 5-lipoxygenase inhibition on neutrophil function *in vitro* and neutrophil-mediated injury in isolated lungs given IL-8 intratracheally.

MATERIALS AND METHODS

Purification of Human Neutrophils. Heparinized blood was obtained from healthy volunteers. Neutrophils were isolated by using a Percoll gradient and differential centrifugation. Each preparation contained highly purified (>99%) neutrophils that were suspended in Hanks' balanced salt solution (Sigma) at a concentration of 2×10^7/ml.

Assessment of Neutrophil Function *in Vitro*. Neutrophil chemotactic activity was determined by Boyden chamber assay quantitating activity as the number of neutrophils per five high-power fields (11) after stimulation with zymosan-activated serum or recombinant human IL-8 (200 ng/ml, Sandoz). Neutrophil adherence was assessed by quantitating the percentage of neutrophils adhering to nylon fibers (12) after stimulation with phorbol 12-myristate 13-acetate (PMA; 10^{-6} M, Sigma). Neutrophil superoxide production was determined by quantitating superoxide dismutase-inhibitable reduction of cytochrome *c* (13) in response to PMA (10^{-6} M). Zileuton [2.5 μM, Abbott, IC_{50} = 0.5 μM in rat basophil leukemia cells (14)], MK886 [1 μM, Merck, Quebec, Canada, IC_{50} = 102 nM in human neutrophils (15)], or LTB_4 (3 nM, Cayman Chemicals, Ann Arbor, MI) were added in some experiments. Both MK886 and Zileuton are highly specific inhibitors of 5-lipoxygenase and have no other identified effects on arachidonate metabolism. Finally, 5-lipoxygenase activity (calcium ionophore A23187-stimulated LTB_4 production) was measured in MK886-pretreated, Zileuton-treated, and control neutrophils.

Isolation and Perfusion of Lungs. After adult male Sprague–Dawley rats (350 ± 50 g) were anesthetized with pentobarbital (60 mg/kg i.p.), the lungs were excised, buffer-perfused, and ventilated continuously with a tidal volume of 3 cc at a rate of 60 per min with 5% CO_2/21% O_2/74% N_2. Immediately, lungs were perfused essentially blood-free and then continuously perfused with Earle's balanced salt solution (Sigma) containing Ficoll-70 (Sigma) (40 g/liter), calcium chloride (0.265 g/liter), magnesium sulfate (0.09767 g/liter), potassium chloride (0.4 g/liter), sodium chloride (6.8 g/liter), monobasic sodium phosphate (0.122 g/liter), D-glucose (1 g/liter), and sodium bicarbonate (2.2 g/liter), with the final pH adjusted to 7.40. Perfusate (30 cc) was passed through the lungs to remove residual blood. The system was then closed, and 30 cc of perfusate was continuously recirculated at a rate of 40 cc/kg of body wt per min.

Isolated Lung Experimental Protocol. After evaluation of 50 ng, 250 ng, 500 ng, and 1 μg of IL-8, a dose of 500 ng was chosen for all subsequent experiments. IL-8, diluted in 0.5 cc of saline, was injected intratracheally immediately upon lung

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Abbreviations: IL-8, interleukin 8; LTB_4, leukotriene B_4; LTA_4, leukotriene A_4; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes.

Table 1. Effect of 5-lipoxygenase inhibitors on neutrophil function *in vitro*

Neutrophil test conditions	ZAS-stimulated chemotaxis,*† PMN/5HPF	IL-8-stimulated chemotaxis,*† PMN/5HPF	PMA-stimulated adherence,*‡ %	PMA-stimulated superoxide anion production,*† μM cytochrome *c* reduced/30 min
Control	33.3 ± 1.5 (5)	29.1 ± 3.1 (5)	77.6 ± 1.7 (5)	77.8 ± 5.5 (6)
MK886	10.8 ± 1.2 (6)‡	11.1 ± 0.6 (4)‡	29.4 ± 2.5 (5)‡	68.2 ± 2.7 (3)
Zileuton	12.2 ± 1.1 (4)‡	10.8 ± 1.2 (3)‡	41.2 ± 4.8 (6)‡	90.7 ± 3.0 (3)
Zileuton + LTB_4	11.7 ± 0.7 (4)‡			
MK886 + LTB_4	10.9 ± 0.7 (3)‡			

Numbers in parentheses represent *n*. ZAS, zymosan-activated serum; PMN, polymorphonuclear leukocytes; 5HPF, five high-power fields.
*Mean ± SEM (number of determinations).
†Unstimulated neutrophils had chemotactic activities of 10.6 ± 0.4 neutrophils per high-power field, adherence of 49.8 ± 5.6%, and superoxide anion production of 5.8 ± 1.4 μM cytochrome *c* reduced per 30 min.
‡Value significantly different ($P < 0.05$) from value for control (stimulated) neutrophils.

isolation. After a 20-min equilibration period during which Zileuton (2.5 μM) or MK886 (1 μM) was at times added to the perfusate, freshly purified human neutrophils (4×10^7) were added to the perfusate chamber (initial circulating concentration, ≈1300 neutrophils per μl). In other experiments, neutrophils were preincubated with MK886 (1 μM) for 20 min at 28°C, centrifuged at 1000 × *g* for 10 min, and then resuspended in fresh HBSS before addition to the perfusate. Finally, in some experiments, LTB_4 (final concentration, 3 nM) was added with neutrophils and Zileuton to perfusates of lungs given IL-8 intratracheally. Lung weights and pulmonary artery perfusion pressures were monitored continuously throughout the 60-min experimental protocol with force and pressure transducers, respectively. After each experiment, lungs were freeze-clamped in liquid N_2, homogenized, and centrifuged at 15,000 × *g* for 10 min. Ficoll concentrations were determined on the supernatants. Perfusate samples were centrifuged at 5000 × *g* for 5 min, and the supernatants were removed and stored at −70°C for LTB_4 determination.

Assessment of Lung Ficoll Retention. Samples of lung homogenate supernatants were added to a solution of 0.05% anthrone (Sigma) in sulfuric acid, mixed well, and allowed to equilibrate for 20 min. Ficoll concentrations were determined by measuring absorbance spectrophotometrically at 627 nm and represent the total Ficoll retained per lung (16).

Assessment of Perfusate LTB_4 Concentrations. LTB_4 levels were determined by enzyme linked immunoassay using antibody to LTB_4 (Advanced Magnetics, Cambridge, MA) and acetylcholinesterase conjugated to LTB_4 (17, 18).

Statistical Analysis. Values were compared by analysis of variance and corrected by the Student–Newman–Keuls test for differences between groups; a *P* value of <0.05 was considered significant.

RESULTS

Effect of 5-Lipoxygenase Inhibitors on Neutrophil Function *in Vitro*. Neutrophils stimulated with zymosan-activated serum or IL-8 had increased ($P < 0.05$) chemotactic activity compared with unstimulated neutrophils *in vitro* (Table 1). In contrast, in the presence of MK886 or Zileuton, stimulated neutrophils had decreased ($P < 0.05$) chemotactic activity compared with untreated, stimulated neutrophils (Table 1). Furthermore, adding LTB_4 did not increase ($P > 0.05$) the chemotactic activity of Zileuton- or MK886-inhibited neutrophils (Table 1). In parallel, PMA-stimulated neutrophils had increased ($P < 0.05$) adherence to nylon fibers compared with unstimulated neutrophils *in vitro* (Table 1). Similarly to chemotaxis inhibition, stimulated neutrophils treated with Zileuton or MK886 had decreased ($P < 0.05$) adherence compared with untreated, stimulated neutrophils (Table 1). Likewise, PMA-stimulated neutrophils (but not IL-8-stimulated neutrophils; data not shown) had increased ($P < 0.05$) superoxide anion production compared with unstimulated neutrophils *in vitro* (Table 1). However, in contradistinction to the adherence and chemotaxis inhibition, Zileuton- or MK886-treated neutrophils stimulated with PMA had the same ($P > 0.05$) superoxide anion production as control neutrophils stimulated with PMA (Table 1). In response to the calcium ionophore A23187, Zileuton-treated neutrophils released 14.6 ± 6% and MK886-treated neutrophils released 13.8 ± 2.7% as much LTB_4 as untreated, A23187-stimulated control neutrophils *in vitro* ($n = 4$).

Effect of Intratracheal IL-8 and Neutrophil Perfusion on Isolated Rat Lungs. Isolated rat lungs given 50 ng, 250 ng, or 500 ng of IL-8 intratracheally and then perfused with neutrophils gained progressively more weight (Fig. 1). Isolated rat lungs given 500 ng of IL-8 intratracheally and then perfused with neutrophils had increased ($P < 0.05$) lung weights compared with lungs given only IL-8, lungs given only neutrophils, or control lungs (lungs not given IL-8 or neutrophils; Fig. 2*A*). In parallel, isolated rat lungs given IL-8 intratracheally and then perfused with neutrophils had increased ($P < 0.05$) lung Ficoll retention compared with lungs given only IL-8, lungs given only neutrophils, or control lungs (Fig. 2*B*). Along with weight increases and Ficoll retention, lungs given IL-8 and then perfused with neutrophils had increased ($P < 0.05$) perfusate LTB_4 levels compared with lungs given only IL-8, lungs given only neutrophils, or control lungs (Fig. 2*C*). Pulmonary artery perfusion pressures increased (1–2 mmHg; 1 mmHg = 133 Pa) transiently (5–10 min) after adding neutrophils in *all* isolated perfused lungs.

Effect of 5-Lipoxygenase Inhibitors on Lung Injury and Perfusate LTB_4 Levels. Isolated rat lungs given IL-8 and then

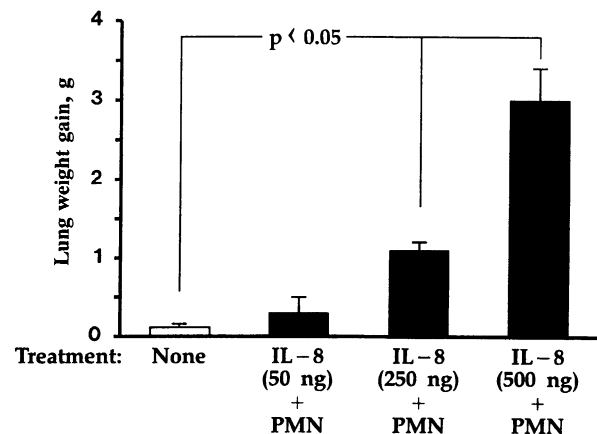

FIG. 1. Isolated rat lungs given 250 ng or 500 ng, but not 50 ng, of IL-8 intratracheally and then perfused with freshly purified human neutrophils for 60 min had increased ($P < 0.05$) weight gains compared with control lungs (not given IL-8) perfused with buffer alone for 60 min. Each value represents the mean ± SEM of three or more determinations.

perfused with neutrophils and Zileuton or MK886 had reduced ($P < 0.05$) lung weights (Fig. 2*A*), lung Ficoll retention (Fig. 2*B*), and perfusate LTB_4 levels (Fig. 2*C*) compared with lungs given IL-8 and then perfused with neutrophils. In addition, lungs given IL-8 and then perfused with MK886-pretreated neutrophils had decreased ($P < 0.05$) lung weights (Fig. 2*C*), lung Ficoll retention (Fig. 2*B*), and perfusate LTB_4 levels (Fig. 2*C*) compared with lungs given IL-8 and then perfused with neutrophils. Lungs given IL-8 and then perfused with neutrophils, Zileuton, and added LTB_4 had the same ($P > 0.05$) lung weights (Fig. 2*A*) and lung Ficoll retention (Fig. 2*B*) as lungs given IL-8 and then perfused with neutrophils and Zileuton.

DISCUSSION

We observed recently that an acute edematous injury occurred in human neutrophil-perfused isolated rat lungs given IL-8 intratracheally and that injury depended on neutrophil-derived oxygen radicals.* In the present investigation, we found that inhibiting 5-lipoxygenase activity prevented lung injury and perfusate LTB_4 increases in lungs given IL-8 intratracheally. Inhibiting 5-lipoxygenase activity also prevented IL-8-stimulated neutrophil chemotaxis *in vitro*. These findings suggest a mechanism that links the increased IL-8, LTB_4, neutrophils, and oxidative injury that occurs in lungs of adult respiratory distress syndrome patients (1). Specifically, it appears that increased airway IL-8 levels recruit neutrophils via 5-lipoxygenase stimulation. After recruitment, neutrophils, via the oxidant generator NADPH oxidase, release oxygen radicals that injure the lung. Thus, both 5-lipoxygenase and NADPH-oxidase activity are required, but neither alone is sufficient, for neutrophil-mediated lung injury after IL-8 administration.

More importantly, we found that *intrinsic* neutrophil 5-lipoxygenase activity—as opposed to 5-lipoxygenase activity of other lung cells—is pivotal in IL-8-mediated neutrophil responsivity *in vitro* and neutrophil-dependent lung injury. (*i*) Neutrophils pretreated with MK886, which irreversibly blocked neutrophil 5-lipoxygenase activity, did not injure lungs given IL-8, despite the intact 5-lipoxygenase function in other lung cells. (*ii*) Adding LTB_4 to the perfusate did not injure lungs given IL-8 and then perfused with neutrophils and Zileuton. (*iii*) Adding LTB_4 did not restore chemotaxis or adherence activity to 5-lipoxygenase-inhibited neutrophils *in vitro*. These results suggest an additional, previously unidentified, intracellular signaling role for 5-lipoxygenase products that is different than their well-known function as secretory products (10). In contrast to secreted LTB_4, which increases endothelial adhesiveness and permeability and activates neutrophils, intracellularly generated LTB_4 and/or other 5-lipoxygenase products appear to mediate intracellular mechanisms necessary for neutrophil adherence and chemotaxis in response to inflammatory signals. Interestingly, although some neutrophil–ligand interactions appear to activate 5-lipoxygenase, the response is not always LTB_4 release, suggesting in another way that intracellular 5-lipoxygenase products participate in neutrophil responsivity (19). If LTB_4 were solely generated for extracellular functions, then adding LTB_4 would have restored chemotaxis activity to Zileuton- or MK886-treated neutrophils *in vitro* and injury in IL-8-treated lungs perfused with neutrophils and Zileuton.

Although neutrophil binding to a variety of ligands is associated with 5-lipoxygenase activation (20), a direct link between intracellular 5-lipoxygenase activity and neutrophil responsivity has not previously been identified. In leukotriene synthesis, 5-lipoxygenase sequentially converts arachidonic acid to 5-hydroperoxyeicosatetraenoic acid and LTA_4, and LTA_4 is then converted to LTB_4 by the enzyme LTA_4 hydrolase (10). LTA_4 is a volatile molecule, and relatively little is known of its biological function. However, LTA_4 participates cooperatively in leukotriene synthesis involving neutrophils and erythrocytes (21), endothelial cells (8, 22), and probably platelets as well (23). Neutrophil-derived LTA_4 may also regulate leukotriene metabolism by alveolar macrophages and alveolar epithelial cells (8). Consistent with our findings, cytoskeletal rearrangement in neutrophils may be linked to leukotriene synthesis (9). Consequently, intracellular LTB_4 and/or LTA_4 synthesis appears to be required for neutrophils to respond to an external stimulus, such as IL-8. The individual role of LTA_4 could not, however, be directly tested because of its extremely short half-life, which did not permit measurement in this system. In addition to leukotriene production, 5-lipoxygenase also catalyzes lipoxin formation. Lipoxins are formed by sequential 15-lipoxygenase and 5-lipoxygenase action on arachidonic acid. Relatively less is known of their biological actions, although a role in intracellular signaling has been postulated (10). Thus, while LTB_4 is the primary secretory product of neutrophil 5-lipoxygenase activation, one or more proximal products in this pathway

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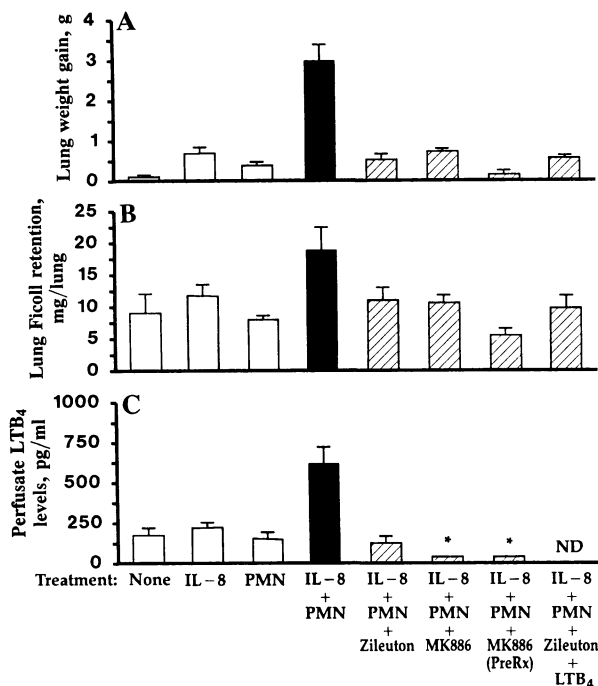

FIG. 2. Isolated rat lungs given IL-8 intratracheally and then perfused with PMN had increased ($P < 0.05$) lung weights (*A*), lung Ficoll retention (*B*), and perfusate LTB_4 levels (*C*) compared with lungs given only IL-8, lungs given only neutrophils, or lungs not given IL-8 or neutrophils (control lungs). Isolated rat lungs given IL-8 and then perfused with neutrophils and Zileuton or MK886 had decreased ($P < 0.05$) lung weights (*A*), lung Ficoll retention (*B*), and perfusate LTB_4 levels (*C*) compared with lungs given IL-8 and then perfused with neutrophils. Lungs given IL-8 and then perfused with MK886-pretreated neutrophils also had decreased ($P < 0.05$) lung weights (*A*), lung Ficoll retention (*B*), and perfusate LTB_4 levels (*C*) compared with lungs given IL-8 and then perfused with control neutrophils. Lungs given IL-8 and then perfused with neutrophils, Zileuton, and LTB_4 had the same ($P > 0.05$) lung weights (*A*) and lung Ficoll retention (*B*) as lungs given IL-8 and then perfused with neutrophils and Zileuton. Each value represents the mean ± SEM of four or more determinations. ND, not determined. *, Each determination was <40 pg/ml, which was the lower limit of sensitivity for the assay.

may transduce intracellular responsivity to inflammatory stimuli.

We found that intrinsic neutrophil 5-lipoxygenase activity is required for human neutrophil adherence and chemotaxis *in vitro* and IL-8-dependent lung injury in isolated rat lungs perfused with human neutrophils. Recognition of the integral role of 5-lipoxygenase activity in basic neutrophil responsivity suggests therapeutic possibilities for acute lung injury and other neutrophil-mediated disorders.

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