

Phosphatidic Acid Signaling Mediates Lung Cytokine Expression and Lung Inflammatory Injury After Hemorrhage in Mice

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Summary

Because phosphatidic acid (PA) pathway signaling may mediate many basic reactions involving cytokine-dependent responses, we investigated the effects of CT1501R, a functional inhibitor of the enzyme lysophosphatidic acid acyltransferase (LPAAT) which converts lysophosphatidic acid (Lyso-PA) to PA. We found that CT1501R treatment not only prevented hypoxia-induced PA increases and lyso-PA consumption in human neutrophils, but also prevented neutrophil chemotaxis and adherence in vitro, and lung injury and lung neutrophil accumulation in mice subjected to hemorrhage and resuscitation. In addition, CT1501R treatment prevented increases in mRNA levels and protein production of a variety of proinflammatory cytokines in multiple lung cell populations after blood loss and resuscitation. Our results indicate the fundamental role of PA metabolism in the development of acute inflammatory lung injury after blood loss.

Acute edematous lung injury (ARDS)¹ is a highly fatal (mortality >50%) disorder, characterized by increased lung cytokine levels and massive neutrophil accumulation in the lung (1). Blood loss, such as occurs after severe injury, even if resuscitated, is associated with increased expression of proinflammatory cytokines, such as TNF- α and IL-1 β , and the frequent development of acute lung injury (2–5). Although ARDS often occurs after an ischemia (hypoxia)–reperfusion (reoxygenation) insult caused by hemorrhage and resuscitation, the relationship between hypoxia, cytokine activation, neutrophil recruitment, and lung injury is unknown.

Certain species of PA that do not contain arachidonate in either the sn-1 or sn-2 positions are produced from lyso-PA by the membrane associated enzyme, lysophosphatidic acid acyl transferase (LPAAT). Each system in which PA formation via LPAAT has previously been demonstrated (i.e., IL-1 β , lipid A or hydrophobic chemotherapeutic compounds) either involves activation of phospholipase A₂, or causes membrane perturbation or release of reactive oxygen intermediates which indirectly activate phospholipase A₂ (6, 7). These PA species are then rapidly converted to 1,2 diacylglycerol (DAG) by phosphatidate phosphohydrolase (PAPH) (7–9). Both PA

and DAG function as intracellular second messengers and can be distinguished from the end-products of the phosphatidyl inositol pathway by the absence of sn-2 arachidonate (7, 8). CT1501R, 1-(5-R-hydroxyhexyl)-3,7-dimethyl xanthine, is a functional inhibitor of LPAAT activity which reduces specific PA generation (8). For example, CT1501R suppresses the generation of PA induced by bacterial lipopolysaccharide with an IC₅₀ of 0.5–0.6 μ M in the murine monocytic leukemia cell line, P388, in vitro (8). Adding CT1501R to lipopolysaccharide-stimulated blood mononuclear cells inhibited TNF- α , IL-1 β and IL-6 release, as well as IL-1 β and TNF- α mRNA accumulation, without affecting IL-8 expression (10). Similarly, inhibiting PA formation with CT-1501R protected mice from endotoxin lethality (8). Because of the apparent role of PA as an intracellular second messenger in neutrophil activation (11) and cytokine dependent responses, we investigated the effects of inhibiting PA generation on neutrophil function in vitro as well as cytokine expression and the development of acute lung injury after hemorrhage and resuscitation in vivo.

Materials and Methods

Human Neutrophil Isolation. Human neutrophils (PMN) were prepared from heparinized blood obtained from consenting, healthy human volunteers. Neutrophils were isolated and highly purified (>99%) by Percoll gradient and differential centrifugation and resuspended in HBSS.

¹ Abbreviations used in this paper: AM, alveolar macrophages; ARDS, acute edematous lung injury; IPMC, intraparenchymal pulmonary mononuclear cells; LPAAT, lysophosphatidic acid acyltransferase; lyso-PA, lysophosphatidic acid; PA, phosphatidic acid.

HPLC Analysis of PA and Lyso-PA. Human neutrophils were placed in hypoxia (95% N₂ + 5% CO₂) and then reoxygenated (21% O₂ + 5% CO₂). After neutrophils were fixed in ice-cold methanol, lipids were extracted and separated by HPLC with a Waters μ -Poracil[®] silica column, using an anisocratic gradient (7–9). Lipids in the column effluent were monitored at 206–224 nm. HPLC fractions were also analyzed by thin layer chromatography, amine content, acyl content, and mass spectrometry to confirm peak identities. Fast-atom bombardment mass spectrometry spectra were acquired using a VG 70 SEQ tandem hybrid instrument of EBQ geometry (VG Analytical, Altrincham, UK) as previously described (7, 8). Mass of total PA varied from 0.65–0.75% in unstimulated neutrophils, to 2.7–3.6% of total detectable lipids in stimulated neutrophils (increased by 3.5–5-fold); lyso-PA mass in neutrophils pretreated with CT1501R and subjected to hypoxia–reoxygenation could range as high as 7–8% of total detectable lipids.

Neutrophil Function Studies. Neutrophil chemotactic activity was determined by Boyden chamber assay and quantitated as the number of migrating PMN/5HPF using zymosan-activated serum as the chemoattractant (12). Neutrophil adherence was assessed by quantitating the percentage of neutrophils adhering to nylon fibers after addition of phorbol myristate acetate (PMA 10⁻⁶ M; Sigma Chem. Co., St. Louis, MO) (13). Neutrophil superoxide production was determined by quantitating superoxide dismutase inhibitable reduction of cytochrome c in response to PMA (10⁻⁶ M) (14).

Hemorrhage and Resuscitation Evaluation. After methoxyflurane anesthesia, male BALB/c mice (Harlan-Sprague-Dawley, Indianapolis, IN) 8–14 wk old, had 30% of their calculated blood volume (~0.55 ml for a 20-gm mouse) withdrawn via cardiac puncture over 60 s (2, 15). Blood was collected in a heparinized (5 U) syringe, kept at 37°C for 1 h, and then reinfused into the retroorbital plexus of the briefly (<1 min) reanesthetized mouse. Resuscitated survivors did not develop hemothorax, bleeding into the pericardial space, lung or cardiac contusion. Control mice were subjected similarly to anesthesia and cardiac puncture without blood withdrawal, and then injected retroorbitally with heparin (5 U) in 0.2 ml PBS.

Semiquantitative PCR Analysis of Cytokine mRNA Levels. Relative cytokine mRNA levels were determined by semiquantitative PCR on alveolar macrophages (AM), peripheral blood monocytes (PBMC), and intraparenchymal pulmonary mononuclear cells (IPMC) collected from mice 3 d after hemorrhage–resuscitation (2, 4). AM were isolated by centrifugation of bronchoalveolar lavage (BAL) obtained by injecting and then aspirating 1.0 ml PBS intratracheally. PBMC were isolated from heparinized blood diluted 2:1 with PBS, pH 7.3, and layered onto a lympholyte-m gradient. After centrifugation at 600 g for 20 min at 15°C, cells at the interface were collected, washed in RPMI 1640, counted, and assessed for viability which was consistently greater than 98% by trypan blue exclusion. IPMC were isolated by collagenase digestion and Percoll gradient purification (16). mRNA was extracted from isolated cells using oligo dT columns (Micro-FastTrack; Invitrogen, San Diego, CA). cDNA was then synthesized from the mRNA of 20,000 AM, 100,000 PBMC, or 100,000 IPMC using Maloney murine leukemia virus reverse transcriptase and random hexamer oligonucleotides (2, 17). After a 2-min, 94°C denaturation step, between 34 and 38 cycles of PCR were conducted (45 s, 94°C denaturation; 45 s, 60°C annealing; and 2 min, 72°C extension) on cDNA from 1000 AM, 10,000 IPMC, or 10,000 PBMC. All cDNA samples were mixed with aliquots of the same PCR master mix using appropriate cytokine MIMICs (Clontech, Palo Alto, CA) as internal controls for standardization of PCR product (2, 18). Cytokine primers (Clontech) were used at 0.4 μ M. To detect

amplified cDNA, the PCR product was analyzed by agarose gel electrophoresis. The number of PCR cycles were selected for the cytokine product from each cellular population so that the ethidium bromide stained amplified DNA products were between barely detectable and below saturation. The gel was then captured for computer integration using a UVP System (UVP Inc., San Gabriel, CA). Densitometry results were normalized to those for the cytokine MIMIC.

ELISA Assay for Cytokines. After centrifugation, supernatants from BAL were stored at –70°C. ELISA for TNF- α and IFN- γ content in BAL supernatants used paired monoclonal anti-mouse TNF- α (MP6-XT3 and biotinylated MP6-XT22) and IFN- γ (R4-6A2 and biotinylated XMG1.2) antibodies (PharMingen, San Diego, CA), and alkaline phosphatase-conjugated streptavidin (Southern Biotechnology, Birmingham, AL), with recombinant mouse TNF- α and IFN- γ as standards. The sensitivity of the ELISA was 10 pg/ml.

Histologic Analyses. At 3 d after hemorrhage–resuscitation, mice were killed and the pulmonary circulation flushed by injecting PBS into the right ventricle. Lungs were formalin injected through the trachea, then removed en bloc with the heart and placed into formalin. Hematoxylin and eosin sections were prepared and examined blindly by a pulmonary pathologist (RT). Grading of the histopathologic changes (HE stain \times 92 magnification) in the lungs (neutrophil infiltration, interstitial edema, and intraalveolar hemorrhage) was performed on a 0 to 3 scale (with 0 being normal and 3 being the most severe abnormality).

Statistical Analyses. Data are presented as mean \pm SEM. Groups were compared using one way analysis of variance and either the Student-Newman-Keuls or the Tukey-Kramer Multiple Comparison tests for differences between groups. A *p* value of <0.05 was considered to be statistically significant.

Results and Discussion

To test the inhibition of LPAAT by CT1501R, we exposed CT1501R-treated human neutrophils to hypoxia and then reoxygenation in vitro. After exposure to hypoxia for 60 min and then reoxygenation for 20 min, human neutrophils had increased PA concentrations with a retention time (R_f) characteristic of linoleate-enriched PA species (Fig. 1). Hypoxia–reoxygenated neutrophils preincubated with 10–100 μ M CT-1501R had decreased PA (Fig. 2 a) and increased lyso-PA (Fig. 2 b) concentrations compared to untreated neutrophils. CT1501R treatment also reduced stimulated human neutrophil adherence (Fig. 3 a) and chemotaxis (Fig. 3 b), but not neutrophil superoxide anion generation (Fig. 3 c), in vitro.

Severe hemorrhage causes a systemic hypoxia and then replacement of blood reoxygenates ischemic tissues (19). A frequent consequence of these events is the development of ARDS which appears to derive from an oxidative insult involving neutrophils and/or xanthine oxidase (1). Because our findings indicated that CT1501R treatment not only inhibited the generation of PA species during hypoxia–reoxygenation but also neutrophil function in vitro, we suspected that PA participates in inflammatory lung injury after hemorrhage–resuscitation. Mice treated 1 h after severe hemorrhage with CT-1501R given along with the previously removed blood during the resuscitation phase had decreased (*p* < 0.05) interstitial lung edema (Fig. 4 a) and intraalveolar hemorrhage (Fig. 4 b) compared to untreated mice. Moreover, after

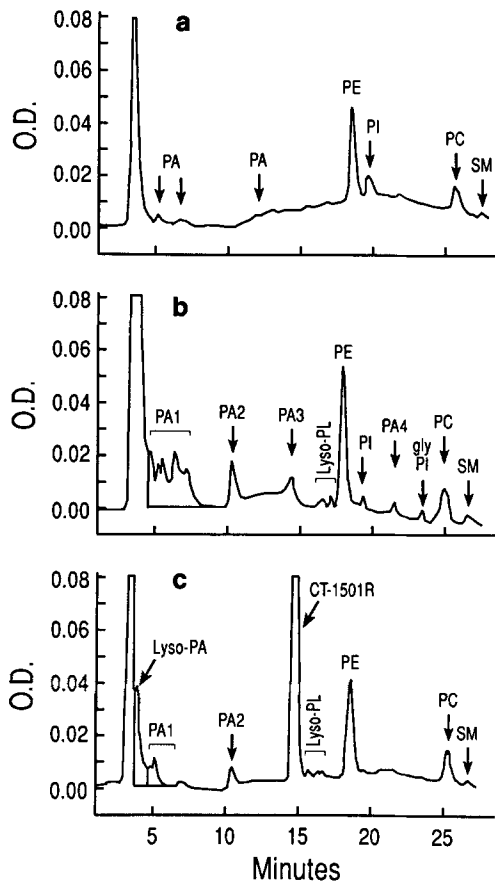


Figure 1. Effect of hypoxia reoxygenation on neutrophil PA metabolism. Representative experimental HPLC tracings for untreated, control neutrophils (a), untreated neutrophils exposed to hypoxic conditions for 60 min and then reoxygenation for 20 min (b), and neutrophils incubated with 10 μM CT1501R, then exposed to hypoxia-reoxygenation (c). Control neutrophils synthesized little PA or lyso-PA, whereas untreated, hypoxia-reoxygenated neutrophils synthesized a variety of PA species, particularly Rf 4–8 min PA. In contrast to PA, neutrophils treated with CT1501R accumulated lyso-PA species, with attenuation in synthesis of PA species, particularly Rf 4–8 min. Consistent with published results (9), phosphatidyl inositol (PI) mass was diminished by hypoxia-reoxygenation (b), and this process was not inhibited by adding CT1501R (c).

hemorrhage, mice resuscitated with CT1501R had fewer ($p < 0.05$) pulmonary interstitial neutrophils than untreated mice (Fig. 4 c) and the percentage of BAL leukocytes that were neutrophils in lungs of CT1501R treated mice ($6 \pm 2\%$) was less ($p < 0.05$) than the percentage of neutrophils in untreated mice ($14 \pm 2\%$). The decreased injury and inflammation in lungs of CT1501R treated mice was corroborated by histological analysis (Fig. 5).

For unknown reasons, increased lung cytokine levels are a prominent feature in patients with ARDS and in mice subjected to hemorrhage-resuscitation (2, 4, 20, 21). We found that alveolar macrophages (Fig. 6 a), blood monocytes (Fig. 6 b) and intraparenchymal lung mononuclear cells (Fig. 6 c) from hemorrhaged mice resuscitated with CT1501R had decreased ($p < 0.05$) mRNA levels for TNF- α compared to untreated, hemorrhaged and resuscitated mice. Similarly, in-

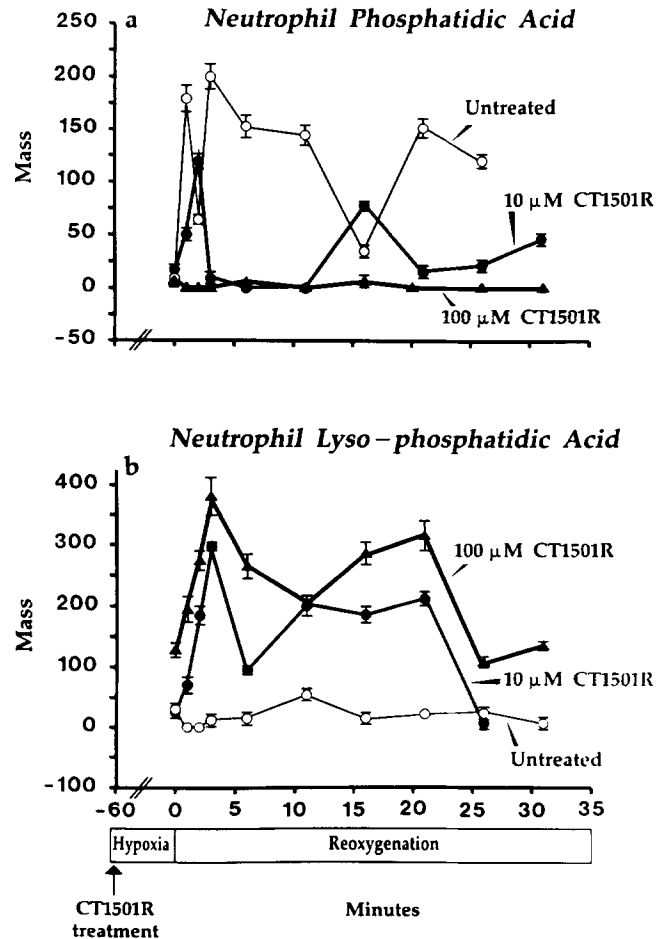


Figure 2. Effect of hypoxia-reoxygenation on neutrophil PA metabolism. CT1501R treatment inhibited PA increases and lyso-PA decreases in human neutrophils subjected to hypoxia-reoxygenation in vitro. (a) After hypoxia-reoxygenation, untreated neutrophils had a rapid rise in Rf 4–8 min PA (enriched in sn-2 linoleoyl moieties and sn-1 acyl C18 saturated and unsaturated moieties) and essentially a partially sustained or oscillatory production of PA. Adding 10 or 100 μM CT1501R to neutrophils at the onset of hypoxia blunted PA production. (b) Hypoxia-reoxygenated, untreated neutrophils had little Rf 2–3 min lyso-PA (enriched in sn-1 acyl C18 saturated and unsaturated moieties), whereas CT1501R treated neutrophils accumulated lyso-PA. Lipid masses are determined by absorption at 217–222 nm, and then normalized to total mass. The masses shown are averaged from five experiments where each time point was performed in triplicate, with error bars showing SEM.

traparenchymal lung mononuclear cells from hemorrhaged mice resuscitated with CT1501R had decreased ($p < 0.05$) amounts of mRNA for IL-1 β (Fig. 6 d), IL-6 (Fig. 6 e), and IFN- γ (Fig. 6 f) compared to untreated, hemorrhaged and resuscitated controls. In parallel, BAL recovered from hemorrhaged and CT1501R resuscitated mice had less ($p < 0.05$) TNF- α (Fig. 6 g) and IFN- γ (Fig. 6 h) activity than BAL from untreated, hemorrhaged, and resuscitated mice. Thus, resuscitation with CT1501R after hemorrhage decreased the expression and activity of a number of different cytokines in circulating mononuclear cells and resident lung cells.

Our results show that PA may fundamentally mediate multiple events responsible for the development of acute lung

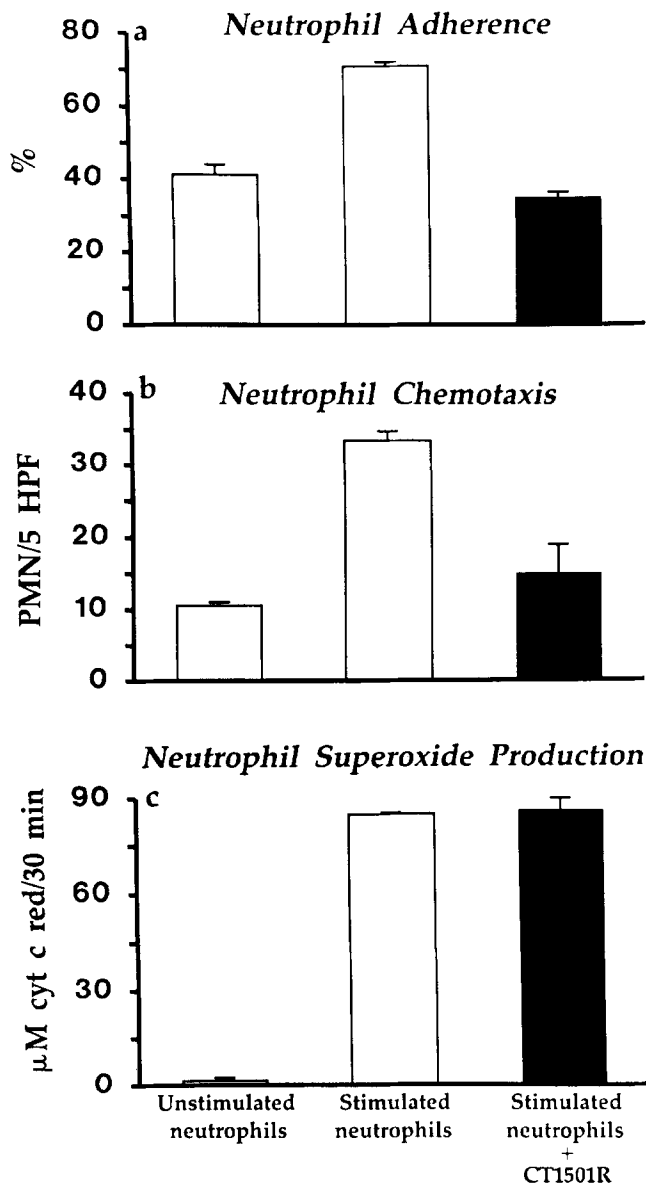


Figure 3. Effect of LPAAT inhibition of neutrophil function in vitro. Adding CT1501R ($7 \mu\text{M}$) decreased ($p < 0.05$) stimulated human neutrophil adherence (a) and chemotaxis (b), but not superoxide anion generation (c), in vitro. Each value is the mean \pm SE of six or more determinations compared by analysis of variance and corrected by Student-Newman-Keuls test for differences between groups using a p value of < 0.05 as significant.

injury after hemorrhage and resuscitation. CT1501R treatment after blood loss decreased lung injury and lung neutrophil accumulation in mice subjected to hemorrhage-resuscitation—a finding paralleled by CT1501R treatment decreasing neutrophil function in vitro. Our results also suggest that systemic hypoxia followed by tissue reoxygenation, such as occurs in hemorrhage-resuscitation, induces the formation of PA in neutrophils as well as in lung tissue. Increased pro-

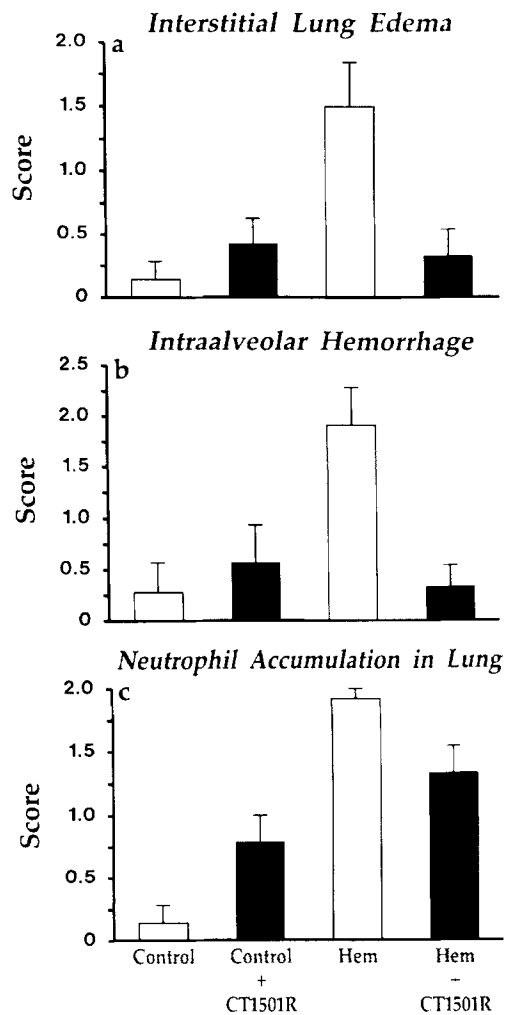
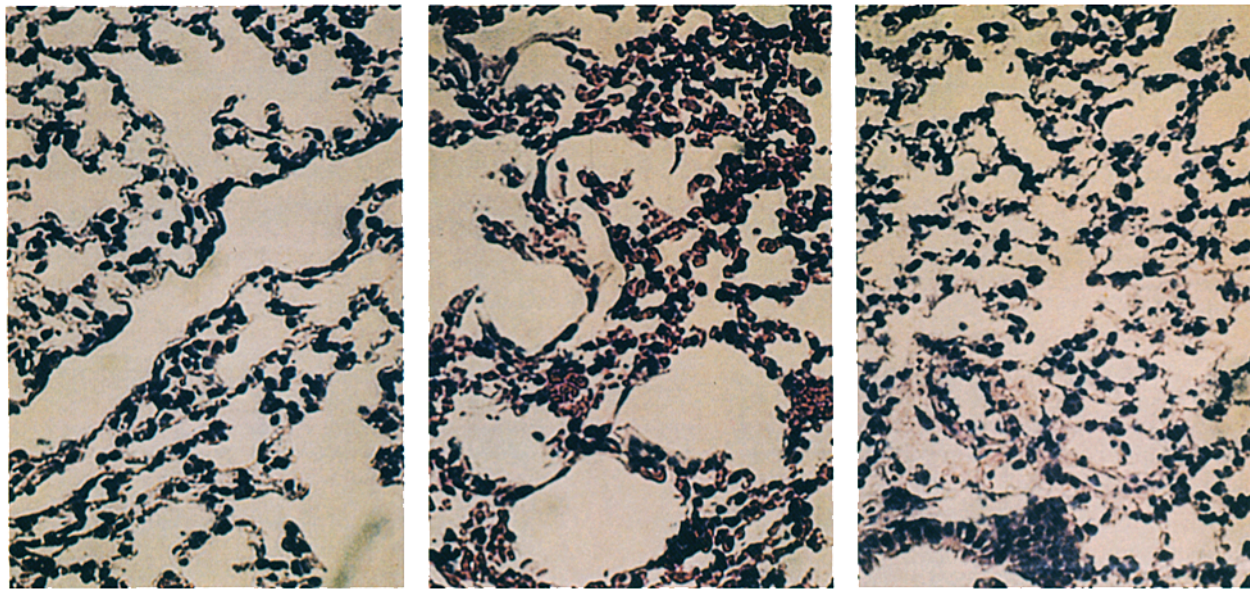


Figure 4. Effect of LPAAT inhibition of lung injury in mice subjected to hemorrhage and resuscitation. Hemorrhaged mice treated with CT-1501R during resuscitation had reduced ($p < 0.05$) interstitial lung edema (a), intraalveolar hemorrhage (b), and neutrophil accumulation (c), compared to untreated mice subjected to hemorrhage-resuscitation. Treatment with CT 1501R (100 mg/kg given in 0.2 ml PBS) or 0.2 ml PBS i.v. was started 1 h after hemorrhage or cardiac puncture, and then was given i.p. every 8 h for a total of 9 doses over 72 h. Each mean \pm SE for 6–8 mice was analyzed by one way analysis of variance with the Tukey-Kramer multiple comparison test using a p value of < 0.05 as significant.

duction of PA then induces neutrophil chemotaxis, proinflammatory cytokine generation and lung injury. Although the present experiments revealed that inhibition of LPAAT with CT1501R decreased in vitro neutrophil chemotaxis and adherence induced by zymosan and PMA, additional studies will be necessary to determine whether inhibition of neutrophil functions occurs when other signaling pathways are activated, particularly in the in vivo setting.

CT1501R also abrogated the induction of mRNA for several proinflammatory cytokines in multiple cell types in the



Control

Hemorrhage

Hemorrhage + CT-1501R

Figure 5. Effect of LPAAT inhibition on lung histology in mice subjected to hemorrhage and resuscitation. Lungs from hemorrhaged mice treated with CT1501R during resuscitation had reduced histologic abnormalities compared to lungs from untreated mice subjected to hemorrhage-resuscitation. Representative hematoxylin and eosin stained pulmonary sections obtained 3 d after cardiac puncture in mice subjected to methoxyflurane anesthesia and cardiac puncture, without blood withdrawal, followed 1 h later by retroorbital injection of 5 U heparin in 0.1 ml PBS (*Control*), in mice subjected to 30% blood volume hemorrhage and then resuscitated with the shed blood 1 h after (*Hemorrhage*) and in mice subjected to 30% blood volume hemorrhage and then resuscitated with the shed blood 1 h later, but treated with CT1501R every 8 h starting 1 h after hemorrhage (*Hemorrhage + CT1501R*). The pulmonary histology in control mice is not different from that seen in normal, unmanipulated and unhemorrhaged mice.

lungs of mice subjected to hemorrhage-resuscitation. The protean effects and extensive protection which occurred after CT1501R treatment is most likely explained by the basic signaling nature of PA on diverse components of the inflammatory cascade. Inflammatory stimuli translocate LPAAT to the plasma membrane (7); therefore, it is possible that translocation and activation of LPAAT also occurs after oxidative stress. PA produced by LPAAT may also directly activate atypical protein kinase C species or alternative kinases and may alter GTPase activating protein/ras interactions (22–24). Adding PA to cells also produces potent mitogenic effects, stimulates calcium flux and phospholipase C activity, and induces expression of several protooncogenes and growth factors (25–27). At our dose of CT1501R, inhibition of LPAAT appears to be the primary intracellular signaling pathway responsible for the observed effects on neutrophil function and lung injury, although it is possible that diacylglycerols formed from PA by phosphatidate phosphohydrolase rather than PA itself are the relevant signaling molecules affected by CT1501R. The IC_{50} for inhibiting phosphodiesterase by CT1501R is between 100 and 500 μM in vitro systems, significantly above concentrations (i.e., 10–30 μM) at which CT1501R effectively inhibits PA synthesis (6, 8). It is notable that PI hy-

drolisis and DG/IP3 formation, as well as Ca^{+2} flux, are not inhibited by CT1501R (8). Similarly, sphingomyelin hydrolysis to ceramide and TNF- α induced apoptosis via NF- κB are not inhibited by CT1501R (28).

Lyso-PA is a potent mitogenic stimulus through ras/MAPK and rho activation via an external receptor which functions to activate G proteins (29). However, intracellular injection of lyso-PA appears to lack the activating effects induced by interactions of lyso-PA with its external receptor (7, 30). Therefore, it is unlikely that intracellular accumulation of lyso-PA through LPAAT inhibition by CT1501R plays an important counter-regulatory role in intracellular signaling.

Our findings suggest that inhibiting PA may have potential in the treatment and prevention of conditions, such as ARDS and septic shock, which are associated with activation of a proinflammatory cytokine cascade (2, 8, 10, 20, 21, 31). Because PA proximally mediates a basic intracellular signaling mechanism, inhibiting PA with CT1501R may be more effective therapeutically than agents which have more discrete effects. For example, the interleukin-1 receptor antagonist decreases only IL-1 effects and has no direct effect on neutrophil function in vitro (32).

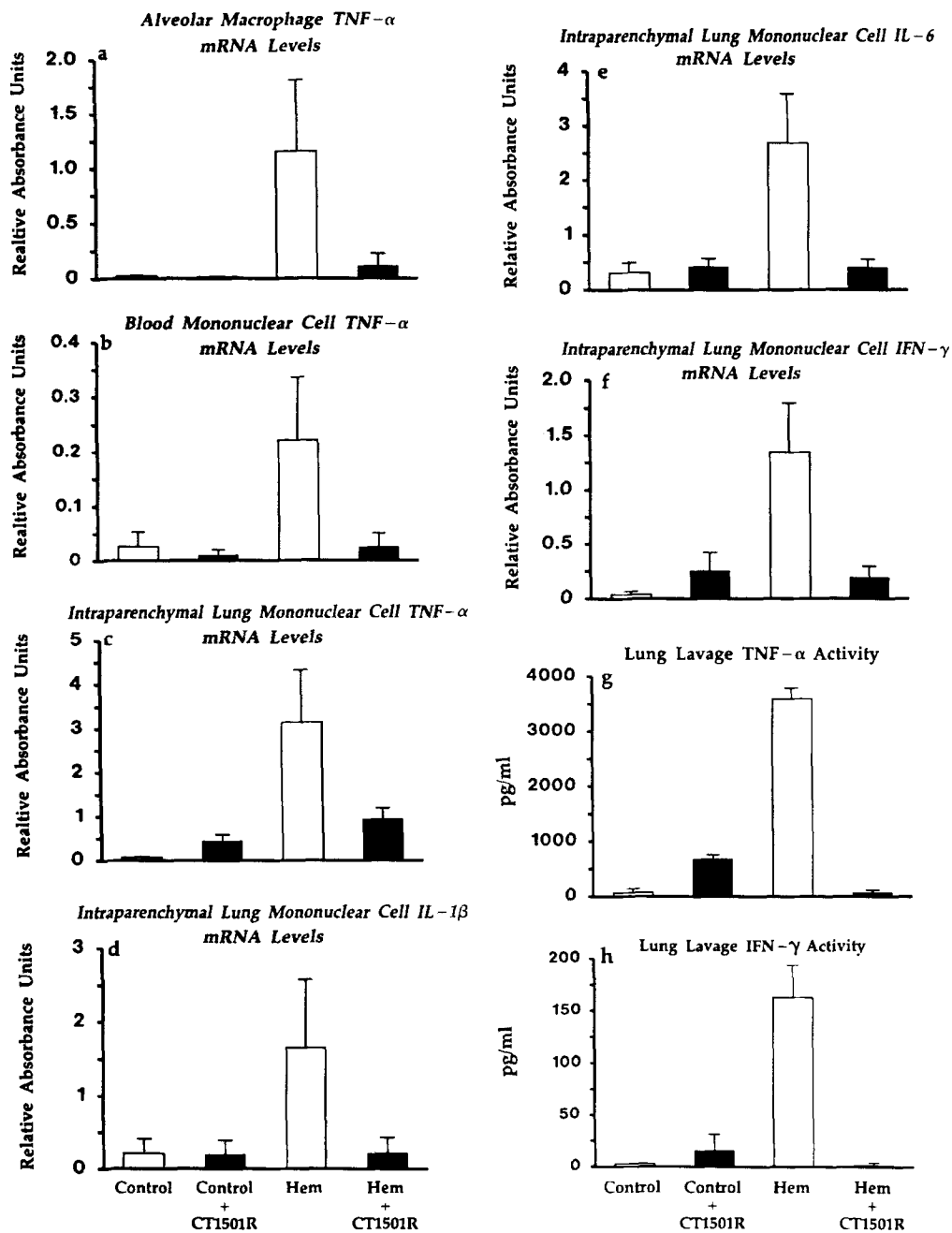


Figure 6. Effect of LPAAT inhibition on lung cytokines in mice subjected to hemorrhage and resuscitation. Hemorrhaged mice which were resuscitated with CT1501R had decreased ($p < 0.05$) amounts of mRNA for TNF- α in their alveolar macrophages (a) and blood mononuclear cells (b), decreased mRNA levels for TNF- α (c), IL-1 β (d), IL-6 (e), and IFN- γ (f) in their intraparenchymal lung mononuclear cells, and decreased TNF- α (g) and IFN- γ (h) activity in their bronchoalveolar lavage (BAL) compared to untreated hemorrhaged-resuscitated mice. The mean \pm SE for 6-8 mice was analyzed by one way analysis of variance with the Tukey-Kramer multiple comparison test using $p < 0.05$ as significant.

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