Arachidonic acid in postshock mesenteric lymph induces pulmonary synthesis of leukotriene B₄

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INTESINAL ISCHEMIA/REPERFUSION (I/R) is central in the pathogenesis of post-injury multiple organ failure; however, identification of the underlying mechanistic link remains elusive. Earlier investigation focused on translocation of intestinal flora to the systemic circulation following gut I/R with the portal vein as the conduit. Although initial animal models offered supportive evidence (5, 29), clinical corroboration in the critically injured patient was lacking (21). Attention shifted from the bacterial translocation hypothesis when Deitch and colleagues (19) began investigating the role of the mesenteric lymphatics following trauma/hemorrhagic shock (T/H/S). Cytotoxicity associated with postshock mesenteric lymph (PSML) was not seen in corresponding portal venous blood (20). However, lymphatic diversion before T/H/S abrogated polymorphonuclear neutrophil (PMN)-mediated lung injury (4, 35). Recently, work in our laboratory has demonstrated that the non-ionic lipid fraction extracted from PSML primed PMNs for increased superoxide (O₂⁻) production, increased adhesion molecule surface expression, and also inhibited PMN apoptosis (9, 25).

Phospholipase A₂ (PLA₂), a proximal enzyme in the arachidonic acid (AA) cascade found in the gut, contributes in the generation of proinflammatory lipids via the lipoxygenase and cyclooxygenase pathways (22, 23). Increased activity of this enzyme has been established following gut I/R (24). Clinically, mortality related to multiple organ failure correlates with increased plasma levels of the secretory isoform of PLA₂ (27, 32, 33). Additionally, the administration of a PLA₂ inhibitor prevents lung injury related to splanchic hypoperfusion following T/H/S, and PLA₂ blockade abrogates PMN priming activity associated with the lipid fraction of PSML (16). AA is a biologically active lipid released from the cellular membrane by PLA₂ that can engage the leukotriene B₄ (LTB₄) receptor and initiate LTB₄ production with autocrine effects (11, 31). AA also promotes 5-lipoxygenase translocation to the nucleus, a key step in LTB₄ production (22, 23). LTB₄ is a non-ionic lipid recognized as a potent mediator of PMN chemotaxis and endothelial adherence as well as PMN priming and O₂⁻ production (22, 25). Additionally, LTB₄ has been invoked as a key proinflammatory mediator in an array of clinical disorders (3, 6). Our previous work in vitro has confirmed that LTB₄ induces robust priming of human PMNs (25). Animal studies in other laboratories have verified the important role of LTB₄ in the pathogenesis of acute lung injury (ALI) following splanchnic I/R (15, 18, 30). Consequently, we hypothesized that LTB₄ is the key mediator in PSML responsible for PMN-mediated ALI. However, mass spectrometric analysis of PSML failed to detect LTB₄ but rather indicated an increase in AA that can provide a stimulus for increased LTB₄ production (11.56 ± 0.29 nmol O₂⁻/min; 3.75 × 10⁵ cells/ml; P < 0.01) that was attenuated by LTB₄ receptor antagonism. Collectively, these data indicate that splanchic ischemia/reperfusion activates gut PLA₂-dependent release of AA into the lymph where it is delivered to the lungs, provoking LTB₄ production and subsequent PMN-mediated lung injury.

Methods

Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at the University of Colorado Denver. All materials were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise specified. Heparin was purchased from Elkins-Sinn (Cherry Hill, NJ) and Ficoll-Paque from Pharmacia Biotech (Uppsala, Sweden). AA was provided by Cayman Chemical (Ann Arbor, MI). The LTB₄ receptor antagonist CP-105,696...
nitrogen and stored at −80°C until processing. The right lung was infused with 3 ml of OCT (Fischer Scientific, Pittsburg, PA) mixed 1:1 with PBS. A section of the lower lobe was fast frozen and stored at −80°C until processed. Tissue sections (5 μm) were air-dried on slides and fixed with acetone/methanol (70:30, vol/vol) at −20°C for 10 min, then air dried and blocked for 1 h at room temperature in 10% normal donkey serum in PBS. Rabbit anti-rat PMN IgG (Accurate Chemical, Westbury, NY) diluted 1:50 in PBS with 1% BSA was then added to the slide and incubated overnight at 4°C. Consecutive sections of the tissue were incubated with isotype to assess nonspecific binding. After slides were washed with PBS to remove excess antibody, 1:100 Alexa 488-conjugated donkey-antirabbit IgG (Molecular Probes, Eugene, OR) and 1:1,000 Alexa 633 Wheat Germ Agglutinin (Molecular Probes) in PBS with 1% BSA were incubated at room temperature for 1 h. After excess secondary antibody was washed, the sections were mounted and sealed. Images were taken on Zeiss Axiovert 100 with motorized stage control using the program Slidebook (III Intelligent Imaging Innovations, Denver, CO).

**Mass spectrometry.** A portion of each left lung was homogenized in HBSS, and protein content was determined using microbicinchoninic acid assay with a BSA standard. Samples containing 5 mg of protein were aliquotted, and ice-cold methanol was added to comprise 50% total volume. The samples were then centrifuged at 500 g for 10 min and the supernatant was stored at −80°C until processing. Human PMN isolation. Human PMNs were isolated from heparinized blood obtained from healthy donors using dextran sedimentation, Ficoll gradient density centrifugation, and hypotonic lysis of contaminating red blood cells (35). The PMNs were resuspended in Kreb’s Ringer phosphate with dextrose at pH 7.35 to a final concentration of 3.75 × 10^6 cells/ml. The final cell population was >99% PMNs by differential staining and was >99% viable by trypan blue exclusion.

PMN **O₂⁻** anion assay and LTB₄ inhibition. O₂⁻ anion generation by PMNs was measured by O₂⁻ dismutase-inhibitable cytochrome c reduction in 96-well microplates as described previously (35). The isolated PMNs were incubated with either buffer, preshock lymph, or postshock lymph alone or with the LTB₄ receptor antagonist CP-105,696 (100 nM) for 5 min at 37°C. Isolated PMNs were also incubated with 3 μM AA alone or AA with 100 nM LY-255283 (LTB₄ receptor antagonist) for 10 min. All priming assays were completed at 37°C in duplicate with a separate O₂⁻ dismutase blank and unstimulated control. In human PMNs, the respiratory burst was initiated with the addition of 1 μmol N-formyl-methionyl-leucyl-phenylalanine (fMLP) to experimental wells, and the maximal rate of O₂⁻ production (Vmax) was determined using the slope of the absorbance curve (35). Variability in PMN priming ability was standardized to priming with 2 μmol platelet activating factor (Paf), then stimulation with fMLP for each experiment (28). Agents were considered “priming” if comparable to the PMN-Paf response following addition of fMLP.

**Fig. 1.** Postshock mesenteric lymph (PSML) primes human polymorphonuclear neutrophils (PMNs). PSML increased PMN (3.75 × 10^6 cells/ml) superoxide (O₂⁻) production, compared with lymph collected before hemorrhagic shock, when stimulated by 1 μmol fMLP [maximal rate of O₂⁻ production (Vmax) of 11.56 ± 1.25 vs. 3.95 ± 0.29 nmol O₂⁻/min; P < 0.01].

**Fig. 2.** Leukotriene B₄ (LTB₄) receptor inhibition decreased PMN priming caused by PSML. PMSL added to PMNs pretreated with a LTB₄ receptor antagonist (CP-105,696) had attenuated priming (Vmax of 2.64 ± 0.58 vs. 11.56 ± 1.25 nmol O₂⁻/min; P < 0.01).
to precipitate proteins. The internal standards (2 ng each of [d₄]LTB₄ and [d₈]5-HETE) were added to the supernatants, samples were diluted with water to a final concentration of methanol of <15%, then lipid extraction was performed using a solid-phase extraction cartridge [Oasis HLB 1cc (30 mg), Waters], which was preactivated with 1 ml of MeOH and then 1 ml of H₂O. The metabolites were eluted from the cartridge with 1 ml of methanol, then dried and reconstituted in 40 μl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with NH₄OH) + 20μl solvent B (AcCN/MeOH; 65/35; vol/vol). Aliquots (25 μl) of plasma, lymph, and lung samples were injected into a HPLC system, and separation of metabolites was conducted using a C18 column (Gemini 150 × 2 mm, 5 μm, Phenomenex) eluted at a flow rate of 200 μl/min with a linear gradient from 45 to 98% of mobile phase B. Solvent B was increased from 45 to 75% in

13 min, to 98% in 2 min, and held at 98% for another 11 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer where analyses were performed in the negative ion mode using multiple reaction monitoring of the specific transitions: m/z 335 → 195 for LTB₄, m/z 339 → 197 for [d₄]LTB₄, m/z 303 → 205 for AA, and m/z 327 → 116 for [d₈]5-HETE. Quantitations were determined using a standard isotope dilution curve ([d₄]-LTB₄ for LTB₄ and [d₈]5-HETE for AA) (13). An additional experiment was performed employing 1 × 10⁷ quiescent human PMNs, which were incubated with 3 μM AA for 10 min then stimulated with 1 μmol fMLP. Ice-cold methanol was added to comprise 50% of the total volume; then the samples were then stored at −80°C until processed by reverse-phase high-pressure liquid chromatography with triple quadrupole mass spectrometry (LC/MS/MS) for LTB₄ production.

Statistical analysis. All data are represented as means ± SE. Statistical differences were determined among groups by paired or
independent (when appropriate) ANOVA followed by a Bonferroni post hoc analysis. Significance is reported as $P < 0.05$.

RESULTS

PMN priming by PSML is LTB$_4$ dependent. PSML increased PMN O$_2$ production when stimulated by 1 μmol fMLP with a $V_{\text{max}}$ of 11.56 ± 1.25 vs. 3.95 ± 0.29 nmol O$_2$/min (with 3.75 × 10$^7$ cells/ml) (Fig. 1; $n = 5$; $P < 0.01$). PSML added to PMNs pretreated with a LTB$_4$ receptor antagonist (CP-105,696, 100nM) had attenuated priming 2.64 ± 0.58 vs. 11.56 ± 1.25 nmol O$_2$/min (Fig. 2; $n = 5$; $P < 0.01$). Furthermore, PMN accumulation in the lung was decreased by 40% with lymph diversion during hemorrhagic shock (Fig. 3; $n = 3$). The PMNs are displayed in green (Alexa 488), and the cellular membranes are displayed in blue (Alexa 633 Wheat Germ Agglutinin). These data indicate that PSML bioactivity is attenuated by LTB$_4$ receptor blockade, and diversion of the PSML decreases PMN infiltration and subsequent lung injury.

LTB$_4$ is present in the lung following T/HS but not lymph. LTB$_4$ was evaluated by LC/MS/MS in both lung or preshock and postshock lymph. LTB$_4$ levels were increased nearly eightfold in the lungs following T/HS compared with control (104.5 ± 18.7 vs. 13.4 ± 4.7 pmol; $n = 4$; $P < 0.01$). Diversion of the lymph by cannulation during shock reduced the amount of LTB$_4$ produced in the lung to 27.5 ± 17.0 pmol (Fig. 4; $n = 4$; $P < 0.05$). Surprisingly, LTB$_4$ was not detected in the lymph (Fig. 5; traces represent labeled isotope control in blue and lymph in red; $n = 3$; $P < 0.01$). These data suggest that, although LTB$_4$ is not present in the lymph, PSML contains a mediator capable of increasing LTB$_4$ in the lung.

Fig. 6. Arachidonic acid (AA) is increased in PSML. LC/MS/MS analysis revealed that AA, the precursor for leukotrienes, was found to increase modestly in PSML ($P < 0.05$). Red trace demonstrates AA found in the lymph compared with [d8]-HETE, the added internal standard, which is displayed as the blue trace ($P < 0.05$).

Fig. 7. Delivery of AA to the systemic circulation by PSML. Although the actual concentrations in the PSML were moderately elevated compared with preshock values, we have previously shown that lymph flow following shock and resuscitation consistently increases over threefold in the first hour of resuscitation alone (9). Therefore, the minimum amount of AA in PSML delivered to the lungs increased from −129.5 to 749.8 ng/h postshock ($P = 0.08$).

Fig. 8. LTB$_4$ production by AA-stimulated PMNs. In vitro, 3 μM AA induced a ninefold increase in LTB$_4$ production in PMNs (1 × 10$^7$ cells/ml) when stimulated with 1 μmol fMLP compared with control ($P < 0.05$).

Fig. 9. AA primes PMNs that is attenuated with LTB$_4$ receptor inhibition. AA (3 μM) primed PMNs (3.75 × 10$^7$ cells/ml) for increased O$_2$ release when stimulated by 1 μmol fMLP ($V_{\text{max}}$ of 5.74 ± 2.2 vs. 1.33 ± 0.22 nmol O$_2$/min). The addition of 100 nM LTB$_4$ receptor antagonist (LY-255283) abrogated the AA-stimulated PMN priming to 2.67 ± 1.5 nmol O$_2$/min ($P < 0.05$).
AA is increased in PSML and stimulates PMN LTB₄ production. LC/MS/MS analysis revealed that AA, the precursor for leukotrienes, was found to increase in PSML (Fig. 6; lymph in red and internal standard in blue; n = 3; P < 0.05). The concentrations in the PSML were elevated compared with preshock values. However, considering that lymph flow consistently increases greater than threefold following shock and resuscitation (9), then the minimum the amount of AA in PSML delivered to the lungs increased from ~129.5 to 749.8 ng/h postshock (Fig. 7; n = 3; P = 0.08). Moreover, the AA concentration in the plasma following T/HS increased by 38% in undiverted animals compared with 12% in diverted animals. In vitro, 3 μM AA induced a ninefold increase in LTB₄ production in quiescent PMNs (1 × 10⁷ cells/ml) compared with control (Fig. 8; P < 0.05) and primed PMNs (3.75 × 10⁷ cells/ml) for increased O₂⁻ release when stimulated by 1 μmol fMLP (Vₘᵡᵢₙ of 5.74 ± 2.2 vs. 1.33 ± 0.22 nmol O₂⁻/min). The addition of 100 nM LTB₄ receptor antagonist (LY-255283; Biomol, Plymouth Meeting, PA) abrogated the AA-stimulated PMN priming to 2.67 ± 1.5 nmol O₂⁻/min (Fig. 9, n = 3; P < 0.05). AA has been shown to act as a ligand for the LTB₄ receptor, which, once activated, stimulates leukotriene production (31). These data indicate that AA is increased in the PSML and is capable of PMN priming and inducing LTB₄ production by PMNs.

DISCUSSION

Splanchnic hypoperfusion plays a critical role in the pathogenesis of multiple organ failure (17, 20). Initial rodent models invoked gut bacterial translocation as the unifying pathophysiological concept (5, 29). However, the inability to confirm translocation from the gut in the setting of major torso trauma (21) coupled with contradictory results from subsequent hemorrhagic shock models (14, 17) challenged the veracity of this theory. Recent work demonstrating that ALI following T/HS can be eliminated by diversion of the mesenteric lymphatics has redirected the investigative focus (4, 19). Subsequently, PSML has been shown to prime PMNs for the release of O₂⁻ (35), and this priming ability was contained in the lipid fraction of the lymph (10). Furthermore, this PSML lipid fraction delays PMN apoptosis, thereby enhancing cytotoxic potential, and this effect is neither endotoxin nor cytokine dependent (9, 10, 17).

PLA₂ is a large group of enzymes responsible for hydrolyzing cell membrane phospholipids producing lysophospholipids and free fatty acids (22). Of the two main types, secretory and intracellular, secretory PLA₂ (sPLA₂) represents the major culprit in inflammation and, as such, is considered a key target for pharmacological intervention (34). Of particular interest among the liberated free fatty acids is AA and its multiple products, the eicosanoids. These molecules are synthesized de novo following PLA₂ activity and include leukotrienes, pros-taglandins, thromboxanes, and lipoxins. The leukotrienes represent major mediators of inflammation, with LTB₄ recognized as a remarkably potent effector of PMN chemotaxis and aggregation as well as enhanced adherence to the endothelium (8, 22). Targeted disruption of LTB₄ synthesis or activity confers a distinct survival advantage in response to PLA₂-derived lipids (1, 12). Additionally, LTB₄ is a consistent mediator of ALI in animal models (7), and pretreatment with the PLA₂ inhibitor quinacrine attenuates lung injury in a rodent model of splanchnic I/R (10). The effect of this inhibition alters the PSML lipid profile as observed by normal-phase HPLC (10), implying that regulation of the lipid component(s) within PSML may be an important means of mediating gut-derived cytotoxicity following T/HS. An extension of this work, the present study sought to identify a specific lipid and its relationship to PSML-associated cytotoxicity. Therefore, we inhibited the PMN receptor in vitro to block the effects of LTB₄ presumably carried in the PSML.

However, our inability to identify LTB₄ in the PSML by mass spectrometry was unexpected. Instead, the PSML contained high concentrations of AA, a LTB₄ precursor. Interestingly, AA has been shown to activate the LTB₄ receptor or independently initiate LTB₄ production with further autocrine effects, including priming of the fMLP-activated respiratory burst (25, 31). AA has also been noted to participate in the initiation of 5-lipoxygenase translocation, a key step in LTB₄ production (22, 30). Therefore, we now believe that it is the release of AA into the PSML by the action of PLA₂ that ultimately results in distant organ injury such as ALI following T/HS. In the distal tissues, AA may stimulate the production of inflammatory mediators, but it is possible that AA is also processed to metabolites such as LTB₄ via transcellular metabolism that promotes PMN and granulocyte chemotaxis and activation. Previous work has shown that ischemic organs have the ability to produce AA that is delivered to PMNs for processing and transcellular metabolism (2, 26). The present study supports this hypothesis and, in fact, represents a new and unique circumstance whereby a substance produced by an ischemic organ is transported and processed in another distant organ to produce inflammation.

In conclusion, we propose that splanchnic I/R activates gut PLA₂ to release AA into PSML where it is conveyed to the lungs, presumably bound to carrier proteins. AA may then initiate inflammation and attract additional PMNs to infiltrate locally. These stimulated PMNs produce LTB₄ but also produce and release LTA₄, which is substrate for the lung endothelium and epithelium via transcellular metabolism to amplify further LTB₄ production and, therefore, incite subsequent lung injury.

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