Lipoxygenase Products Induce Neutrophil Activation and Increase Endothelial Permeability After Thrombin-Induced Pulmonary Microembolism

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We examined the mechanism of the neutrophil (PMN)-dependent increase in pulmonary vascular permeability to protein after thrombin-induced pulmonary microembolism. Humoral factors that activate PMNs after thrombin-induced pulmonary microembolism were characterized in pulmonary lymph obtained from unanesthetized sheep challenged with intravenous infusion of α-thrombin. Time-dependent increases in PMN migration, aggregation, and superoxide anion ($O_2^-$) generation were induced by the pulmonary lymph obtained within 20 minutes after thrombin infusion. The pulmonary lymph neutrophil activating factors present in ether extracts of lymph had retention times of leukotriene B$_4$ (LTB$_4$) and monohydroxyeicosatetraenoic acids (HETEs) by high-performance liquid chromatography. The postthrombin lymph samples containing the LTB$_4$ and HETEs increased PMN $O_2^-$ generation and endothelial monolayer permeability to $^{125}$I-albumin in the presence of PMNs layered on the endothelial monolayers. Control lymph samples replete with LTB$_4$, 5-HETE, and 15-HETE induced increases in PMN $O_2^-$ generation and endothelial monolayer permeability to $^{125}$I-albumin in the presence of PMNs layered on the endothelial monolayers. Maximal increases in PMN $O_2^-$ production and endothelial permeability occurred when LTB$_4$, 5-HETE, and 15-HETE were coincubated with PMNs, indicating a synergistic action of these mediators in inducing PMN activation. Endothelial monolayer permeability to $^{125}$I-albumin did not increase with postthrombin lymph samples obtained after pretreatment with the 5-lipoxygenase inhibitor, L-651,392. The results indicate that lipoxygenase products generated in the lungs after thrombin-induced microembolism contribute to increased endothelial permeability secondary to PMN activation.

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vascular coagulation and the mechanisms by which neutrophils mediate the increase in lung vascular permeability remain unclear. In the present study, we examined the ability of the pulmonary lymph obtained after thrombin-induced microembolism to mediate neutrophil migration, aggregation, and superoxide anion (O$_2^-$) generation. We also assessed and characterized factors appearing in pulmonary lymph after thrombin challenge for their ability to induce a neutrophil-dependent increase in the albumin permeability across cultured pulmonary artery endothelial monolayers.

Materials and Methods

Animal Preparation

The experiments were made in unanesthetized sheep (n=9) weighing 20–24 kg prepared with chronic lung lymph fistulas. The sheep were fasted for 48 hours before surgical preparation. Anesthesia was induced with sodium thiopental (25 mg/kg body wt i.v.) with 1% halothane in an oxygen and nitrous oxide mixture delivered by a Harvard animal ventilator (South Natick, Massachusetts). The surgical site was prepared in a sterile manner. The efferent duct of the caudal mediastinal node was located through a right thoracotomy and cannulated with a heparin-coated silastic catheter. The node was ligated caudal to the distal margin of the inferior pulmonary ligament and was divided. The diaphragmatic, esophageal, and chest wall lymphatic afferents were cauterized to reduce contamination by nonpulmonary lymph. The chest wall and skin were closed and the catheter was exteriorized. During the same surgery, a 9-F polyvinylchloride catheter was introduced into the right carotid artery and secured, and a 7.5-F Swan-Ganz catheter was introduced into the main pulmonary artery from the right jugular vein. The animals were allowed to recover for at least 4 days postoperatively. The animals had blood-free pulmonary lymph and were afebrile.

Lymph flow was determined by collecting lung lymph in preweighed plastic test tubes containing approximately 150 mg sodium citrate. Lymph was collected during a 2 to 3 hour steady-state baseline period (defined as four consecutive 15-minute periods during which the lymph flow rate did not vary by more than 0.25 ml/hr). All animals then received 100 mg of intravenous tranexamic acid followed by a 15-minute infusion of 80 U/kg of human a-thrombin (the native enzyme) prepared by Dr. John Fenton, New York State Department of Health Laboratories, Albany, New York. This dosage of tranexamic acid depresses fibrinolysis and prolongs the retention of microthrombi in the pulmonary circulation resulting in sustained increases in pulmonary lymph flow and transvascular protein clearance after thrombin challenge. Tranexamic acid alone has no significant effect on lymph flow or protein clearance.

The lymph samples of equal volumes were collected during the baseline period and at 5, 10, 15, 20, 30, and 60 minutes after thrombin challenge. The lymph samples were placed on ice after collection and centrifuged for 10 minutes at 1,500g and 4°C to remove cells and undissolved sodium citrate crystals. Some aliquots collected at these times were stored at -70°C. The effects of these lymph samples on neutrophil migration, aggregation, and O$_2^-$ generation were examined.

Preparation of Pulmonary Lymph

Aliquots of pulmonary lymph were stored at -70°C after collection, and other aliquots were incubated for 30 minutes at either 0°C, 37°C, 60°C, or 100°C and centrifuged for 10 minutes at 1,500g and 4°C. The lymph samples were used to test their effect on neutrophil O$_2^-$ production and determine the temperature stability of generated factor(s) on neutrophil O$_2^-$ production.

Other aliquots of pulmonary lymph obtained before and after the thrombin challenge were extracted with ice-cold anhydrous diethyl ether (Mallinckrodt, Paris, Kentucky). The pulmonary lymph and diethyl ether were combined in a 1:4 ratio (vol/vol) with 1% halothane in an oxygen and nitrous oxide mixture delivered by a Harvard animal ventilator. The mixtures were agitated in the cold and the ether layers were removed. This process was repeated four times and the ether fractions were combined in acid-washed, N$_2$-flushed glass round-bottomed flasks. The ether was removed by directing a stream of dry N$_2$ gas over the surface of the ice-cold ether solution until the ether evaporated. Aliquots of ether-extracted lymph used in assays of neutrophil migration, aggregation, and O$_2^-$ generation and in studies of albumin permeability across endothelial monolayers were resuspended in Hank's Balanced Salt Solution (HBSS; GIBCO Laboratories, Grand Island, New York) containing 25 mM HEPES adjusted to pH 7.4; each ether extract was resuspended in a volume of HBSS-HEPES equal to the original volume of lymph. Aliquots of pulmonary lymph extract, which were analyzed by reverse phase high-performance liquid chromatography (RP-HPLC), were resuspended in 500 μl of a solution containing 75 parts methanol and 25 parts water (vol/vol) and 0.01% acetic acid.

In other studies, aliquots of control pulmonary lymph obtained from chronically prepared sheep during the baseline period (i.e., before the thrombin challenge) were prepared by adding leukotriene B$_4$ (LTB$_4$; 10$^{-9}$ M to 10$^{-6}$ M; Merck-Frosst, Montreal, Canada), 5-hydroxyeicosatetraenoic acid (5-HETE; 10$^{-9}$ M to 10$^{-6}$ M; The Upjohn Co., Kalamazoo, Michigan), and 15-HETE (10$^{-9}$ M to 10$^{-6}$ M; Upjohn). The reconstituted lymph samples were extracted with diethyl ether and resuspended in aqueous medium in the same manner as the pulmonary lymph samples obtained from thrombin-challenged sheep.

Neutrophil Isolation Method

Human neutrophils were prepared from theuffy coats of whole blood obtained within 4 hours of the
experiment from unpaid volunteer American Red Cross donors. Purified neutrophils were prepared from theuffy coat by dextran sedimentation and Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, New Jersey) gradient centrifugation and hypotonic lysis with distilled water of contaminating erythrocytes. The final preparation had a neutrophil purity of greater than 98% and neutrophil viability as assessed by Trypan blue dye exclusion of 99.0±0.04% (mean±SEM). The studies of the effects of neutrophil stimulation on transendothelial albumin permeability employed a heterologous system of human neutrophils and cultured bovine pulmonary artery endothelial cells. The other studies described below in Neutrophil Function Assays were done using sheep neutrophils.

Healthy awake sheep (20–25 kg weight) were used as blood donors for neutrophil isolation. Blood (90 ml) was drawn via jugular venipuncture with siliconized needles and plastic syringes. Plastic or siliconized labware was used throughout the experimental procedures involving leukocytes. The whole blood was mixed with 10 ml 0.1 M of EDTA (pH 7.4) immediately upon collection. The neutrophils were isolated using a Percoll-isotonic saline gradient centrifugation technique. The final preparation had neutrophil purity of 81% with lymphocytes comprising the large portion of remaining cells (16%).

Neutrophil Function Assays

Neutrophil migration was determined by measurement under low-power light microscopy of Wright’s stained plastic plates in which isolated sheep neutrophils had migrated under agarose. The neutrophil migration in millimeters was measured after a 3-hour incubation at 37°C in a humidified atmosphere of 95% air-5% CO2 as the difference between the maximum migration of the neutrophils toward the presumed chemoattractant (postthrombin lymph extracts) and maximum migration of the neutrophils away from the chemoattractant (control lymph extract).

Neutrophil O2− generation was determined by the reduction of ferricytochrome C. Isolated neutrophils were suspended in a HEPES buffer containing calcium and magnesium at pH 7.4 at a concentration of 106 neutrophils/ml. Ferricytochrome C (type III, horse heart; Sigma Chemical Co., St. Louis, Missouri) was dissolved in HBSS at a concentration of 3,000 U/ml. Phorbol myristate acetate (PMA) was dissolved in dimethyl sulfoxide and then dissolved in HBSS at a concentration of 200 ng/ml. The final volume of all test samples was 2.5 ml, and all samples contained 83 μl of ferricytochrome C solution, 0.3–0.5×106 neutrophils/ml, and either 17 μl of PMA, α-thrombin (10−6 to 10−10M), or 150 μl of pulmonary lymph or a fraction of pulmonary lymph. Paired test samples were prepared with 50 U/ml superoxide dismutase in one sample of each pair; all assays were incubated 10 minutes at 37°C with gentle rocking, and the reaction was stopped by placing the sample into an ice bath. The samples were centrifuged for 10 minutes at 1,500g at 4°C, and the optical absorbance was determined at 550 nm of the supernatants. Superoxide anion generation production (in nmol/105 neutrophils/10 minutes) was calculated with the extinction coefficient for ferricytochrome C at 550 nm, E550=21.0×103 M−1 cm−1.

Neutrophil aggregation was determined using the electrical impedance mode on an electronic aggregometer with full deflection indicating 100% aggregation. Neutrophils were suspended in HBSS at a concentration of 107 cells per milliliter and were incubated for 10 minutes at 37°C with constant stirring. Zymosan-treated sheep serum (ZTS) or sheep pulmonary lymph was added to induce aggregation. The samples were maintained at 37°C with constant stirring until a steady-state was attained (defined at no further changes in impedance). The percent aggregation was measured as the percentage of the full scale change in impedance due to the addition of ZTS to the neutrophils.

Transendothelial O2−-Albumin Permeability

Polycarbonate micropore membranes (13 mm diameter, 0.8 μm pore size; Nucleopore, Pleasanton, California) were gelatinized as described previously. The micropore membranes were inverted on the base of plastic cylinders (11 mm inner diameter; Adaps, Inc., Dedham, Massachusetts) and were sterilized by exposure to ultraviolet light for 24 hours. The established bovine pulmonary artery endothelial cell line (CCL-209) of Del Vecchio and Smith was obtained at 16 serial passages from the American Type Tissue Collection, Rockville, Maryland. Endothelial cells (4×105) were plated onto each gelatinized membrane and allowed to grow to confluent monolayer. The endothelium was characterized as having factor VIII antigen and angiotensin converting enzyme activity. The endothelium had the typical cobblestone morphology with tight interendothelial junctions. The gelatinized membrane with the endothelial cell monolayer was assembled into a system for the study of transendothelial transport of albumin in the absence of a hydrostatic gradient across the endothelial cell monolayer and filter. The system consisted of two fluid-filled compartments connected via the Nucleopore membrane, with one side covered by the endothelial cell monolayer. The upper compartment contained a total volume of 700 μl of Dulbecco’s Modified Eagle’s Medium (DMEM; GIBCO Labs, Wheaton, Illinois) with 4% bovine serum albumin. The upper compartment was held within styrofoam flotation rings and floated in the 25 ml of DMEM with 4% albumin contained in the lower compartment. The lower compartment was stirred constantly to ensure complete mixing, and the entire
system was maintained at 37°C in a water bath. For each endothelial monolayer, the 125I-albumin permeability was measured before and 60 minutes after adding the stimulus to the monolayer. Control wells were run for each experiment. Control wells contained DMEM and 125I-albumin in the upper well. Each monolayer system served as its own control.

The theoretical basis for this method and the method for measurement of endothelial permeability have been described by us previously. The 125I-albumin permeability was determined by obtaining two 25 μl samples from the upper compartment only at the beginning of each experiment. The upper well activity did not change significantly over the assay period. Samples of 400 μl were taken from the lower compartment before stimulus of the monolayer and every five minutes for 60 minutes after the addition of the stimulus. The radioactive contents of the samples were measured with a gamma counter (Mirax Gamma Count, 5000 Series, Packard, Downers Grove, Illinois). Free iodine measured at the conclusion of each experiment was less than 1%. The clearance of 125I-albumin (Clab) was calculated as

\[ \text{Cl}_{ab} = \frac{V_A [A]}{[L_a]} \]

where \( V_A \) is the lower compartment volume, \( A \) is the concentration of tracer in the lower compartment, and \( [L_a] \) is the concentration of tracer in the upper compartment. The average clearance rate in microliters per minute was calculated by fitting the measured clearances for each portion of a single experiment to a linear function using the least-squares linear regression.

The transendothelial 125I-albumin permeability studies were made using human neutrophils and bovine pulmonary arterial endothelial cells. Neutrophils (10⁶ cells) were layered on the endothelial monolayer for 5–10 minutes before the test stimulus was added. The ratio of neutrophils and endothelial cells in each well was 10 to 1. The 125I-albumin clearances were determined for 60 minutes thereafter. The test stimuli, ether extracts of pulmonary lymph, were dried and dissolved in DMEM. The dissolved extract (50 μl) was added to the upper 700 μl well of the permeability chamber, which also held the 125I-albumin tracer.

Some sheep (n=3) were treated with the 5-lipoxygenase inhibitor L-651,392, provided by Dr. Anthony Ford-Hutchinson of Merck-Frosst, Canada, Montreal, Quebec. L-651,392 (5 mg/kg) was dissolved in 100 ml of methylcellulose in Tween-80. The drug was administered via a stomach tube, and the sheep were studied 24 hours later. Previous studies have indicated that L-651,392 is an effective 5-lipoxygenase inhibitor in sheep. The postthrombin lymph obtained from these animals was assessed to determine its effects on endothelial monolayer albumin permeability, and these results were compared with paired untreated animals challenged with thrombin 7 to 10 days before treatment with L-651,392.

High-Performance Liquid Chromatography Assay

Diethyl ether extracts of pulmonary lymph obtained before and 10, 20, 30, 40, and 60 minutes after the thrombin challenge were analyzed by RP-HPLC. Immediately before analysis, the ether extracts were evaporated to dryness and resuspended in 1 ml of a 75% solution of methanol and 25% water (vol/vol) with 0.01% glacial acetic acid. The liquid chromatographic analysis was performed in a Waters HPLC equipped with a model U6K injector and a model M-45 solvent delivery system, which was connected to a model 450 variable wavelength detector and an Omnichrome B-500 recorder (Houston Instruments, Austin, Texas).

Leukotrienes and HETEs were eluted from a reversed phase Radial-Pak Cartridge (C-18, 8 mm i.d.×10 cm) held in a Z-module system. The methanol-water:acetic acid solvent was delivered at 500 psi and 1.5 ml/min. The effluent was scanned at 270 nm and 235 nm and was then collected in sequential 4 ml aliquots with a fraction collector. Chromatographic standards were LTB₄ and 5-HETE acid. The standards were dissolved in a solution of 75% methanol and 25% water (vol/vol) with 0.01% glacial acetic acid. The final concentration of each standard was 50 ng/500 μl.

The collected fractions were stored at 0°C. Each fraction was evaporated to dryness with a rotary evaporator. During the evaporation process, the samples were maintained under a reduced atmospheric pressure by means of an oil diffusion vacuum pump and were incubated at 37°C in a water bath. All samples were immediately resuspended in 25 μl of absolute methanol followed by 475 μl of distilled water, and they were frozen at −70°C. Aliquots containing 150 μl of the resuspended material were tested for the ability to stimulate neutrophil O₂⁻ generation in vitro.

Statistics

Differences from baseline values within groups were determined by the two-way analysis of variance with least significant differences for multiple comparisons. Differences among two or more sets of samples of pulmonary lymph obtained at different times after the thrombin challenge were determined with the two-way analysis for repeated measures. When a significant difference among the groups was evident by the analysis of variance, the t test was used to determine the significance. Results were considered significant at the p<0.05 level.

Results

Sheep Experiments (n=6)

The mean baseline and 1 hour postthrombin (n=6 sheep) values for the pulmonary lymph flow (Qlym) and pulmonary hemodynamic responses to throm-
TABLE 1. Pulmonary Lymph and Hemodynamic Responses to the Thrombin Challenge (80 U/kg) in Awake Sheep. Pretreated With Tranexamic Acid to Inhibit Fibrinolysis

<table>
<thead>
<tr>
<th></th>
<th>Qlym (ml/hr)</th>
<th>Ppa (mm Hg)</th>
<th>PVR (mm Hg/l/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>8.1±2.5</td>
<td>15.1±1.5</td>
<td>3.8±0.5</td>
</tr>
<tr>
<td>Postthrombin</td>
<td>32.5±2.8*</td>
<td>36.2±2.8*</td>
<td>11.2±1.8*</td>
</tr>
</tbody>
</table>

Qlym, pulmonary lymph flow; Ppa, mean pulmonary artery pressure; PVR, pulmonary vascular resistance calculated as pulmonary perfusion pressure (pulmonary arterial pressure - left atrial pressure) divided by cardiac output.

*Significant from baseline (p<0.05).

All values are shown as mean±SEM.

Effects of Pulmonary Lymph on Neutrophil Function

Neutrophil migration, aggregation, and O$_2^-$ generation in response to pulmonary lymph obtained before and after the thrombin challenge are shown in Figure 1. There was a time-dependent increase (p<0.05) in migration of isolated donor sheep neutrophils toward the pulmonary lymph obtained after thrombin, which was maximum with the lymph sample obtained 15 minutes after the thrombin challenge (Figure 1). The neutrophil migration observed with the lymph sample was comparable to that observed with ZTS. A time-dependent increase in neutrophil aggregation also occurred in response to pulmonary lymph (Figure 1); maximal neutrophil aggregatory activity in lymph occurred with lymph obtained 15 to 30 minutes (p<0.05) after the thrombin challenge and decreased toward baseline by 60 minutes. The degree of aggregation was comparable to that produced by ZTS. Sheep neutrophil O$_2^-$ generation in response to pulmonary lymph is shown in Figure 1. Addition of pulmonary lymph to neutrophils resulted in a time-dependent O$_2^-$ generation (p<0.05), with a maximum response observed with the lymph obtained 15 minutes after thrombin. O$_2^-$ production decreased toward baseline with the 60-minute lymph sample.

Neutrophil O$_2^-$ production persisted after the lymph was incubated for 30 minutes at 0°C and 37°C but not when the samples were heated to 60°C or 100°C (Figure 2). Ether extracts of lymph retained the capacity to stimulate neutrophil O$_2^-$ generation whereas the aqueous phase had no activity (Figure 2). When a volume of ether equal to that used for extraction of the lymph was evaporated to

FIGURE 1. Neutrophil migration under agarose, neutrophil aggregation, and O$_2^-$ generation induced with samples obtained before and after the thrombin challenge. Bars indicate mean±SEM; BL, baseline. *Significant difference as determined by the two-way analysis of variance (p<0.05).

FIGURE 2. Temperature stability of the factors contained in postthrombin pulmonary lymph which stimulate neutrophil superoxide anion (O$_2^-$) generation (top). The relative contributions of the ether-soluble fraction of postthrombin lymph and the aqueous fraction in inducing neutrophil superoxide anion (O$_2^-$) (bottom).
TABLE 2. Neutrophil Superoxide Anion Generation Induced by Fractions of Pulmonary Lymph Ether Extract Separated by HPLC

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Retention time (min)</th>
<th>Neutrophil superoxide anion generation (nmol/10⁶ cells/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.3</td>
<td>Baseline lymph 0.4±0.2</td>
</tr>
<tr>
<td>2</td>
<td>8.0</td>
<td>0.5±0.6</td>
</tr>
<tr>
<td>3</td>
<td>10.7</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>4</td>
<td>13.3</td>
<td>0.9±0.6</td>
</tr>
<tr>
<td>5</td>
<td>16.0</td>
<td>2.5±2.1</td>
</tr>
<tr>
<td>6</td>
<td>18.7</td>
<td>5.3±1.8</td>
</tr>
<tr>
<td>7</td>
<td>21.3</td>
<td>1.5±1.8</td>
</tr>
<tr>
<td>8</td>
<td>23.9</td>
<td>1.5±1.0</td>
</tr>
<tr>
<td>9</td>
<td>26.7</td>
<td>1.3±1.3</td>
</tr>
<tr>
<td>10</td>
<td>29.3</td>
<td>1.9±1.1</td>
</tr>
<tr>
<td>11</td>
<td>31.9</td>
<td>5.2±7.1</td>
</tr>
<tr>
<td>12</td>
<td>34.7</td>
<td>5.3±0.8</td>
</tr>
<tr>
<td>13</td>
<td>37.3</td>
<td>4.3±2.9</td>
</tr>
<tr>
<td>14</td>
<td>39.9</td>
<td>3.8±1.0</td>
</tr>
<tr>
<td>15</td>
<td>42.7</td>
<td>1.2±1.0</td>
</tr>
</tbody>
</table>

For comparison purposes, phorbol myristate acetate (10⁻⁷ M) resulted in O₂⁻ generation of 24.2±2.6 nmol/10⁶ neutrophils/10 min.

*Value differs significantly from the corresponding baseline lymph values (p<0.05). The retention time for each fraction is the time required to collect half the volume of the corresponding fraction plus the time required to collect all preceding fractions. HPLC, high-performance liquid chromatography.

n=6 sheep experiments.

All values are shown as the mean±SEM.

dryness, the resuspended ether residue had no effect on neutrophil O₂⁻ generation.

Analysis of Diethyl Ether Extract of Pulmonary Lymph

The absorbances at 270 nm and 235 nm of the effluent from the RP-HPLC column for representative samples of the diethyl ether extracts of pulmonary lymph obtained before and after the thrombin challenge are shown in Figure 3 (top). The chromatographic analysis of samples obtained after thrombin differs from that of the baseline lymph samples by the appearance of two absorbance peaks with retention times of 13.3 minutes and 34.7 minutes, corresponding to LTB₄ and HETEs (Figure 3 top and bottom). In addition, a peak with the retention time of the cyclooxygenase product 12-hydroxyheptadecatrienoic acid was present in postthrombin lymph.

The results of O₂⁻ generation by neutrophils incubated with the material obtained after drying and resuspending the individual fractions collected after RP-HPLC of diethyl-ether extracts of prethrombin and postthrombin lymph are shown in Table 2. Since whole lymph obtained between 10 and 20 minutes after the thrombin challenge was the most potent with respect to its ability to stimulate neutrophil O₂⁻ generation, we compared the results of HPLC-fractionated lymph obtained at baseline and at 10 to 20 minutes after the thrombin challenge. None of the fractions obtained from baseline lymph significantly increased neutrophil O₂⁻ generation.

The maximal increases in neutrophil O₂⁻ generation were induced by RP-HPLC fractions of 10–20-minute postthrombin lymph having the chromatographic appearance of LTB₄ and HETEs (Fractions 4 and 12 in Table 2). The fraction (Fraction 5 in Table 2) having the chromatographic appearance of 12-hydroxyheptadecatrienoic acid did not significantly increase neutrophil O₂⁻ generation (Table 2).

The results of O₂⁻ generation by neutrophils incubated with pulmonary lymph reconstituted with LTB₄, 5-HETE, and 15-HETE are shown in Table 3. The greatest increases in neutrophil O₂⁻ generation were observed when neutrophils were incubated with equimolar concentrations (10⁻⁷ M and 10⁻⁸ M) of LTB₄, 5-HETE, and 15-HETE added together (p<0.05). The combination of LTB₄ and 15-HETE (10⁻⁷ M and 10⁻⁸ M) also resulted in significant O₂⁻ generation (p<0.05); however, the response was not significant with LTB₄ alone and with LTB₄ plus 5-HETE (Table 3).

Effects of Pulmonary Lymph on In Vitro Transendothelial ¹²⁵I-Albumin Permeability

The addition of resuspended ether extract of baseline lymph resulted in transendothelial ¹²⁵I-albumin clearance rate of 0.415±0.050 µl/min (Table 4). The clearance rate of endothelial monolayers (0.505±0.073 µl/min) incubated with baseline lymph extract and neutrophils was not significantly different from the value obtained by adding neutrophils to the endothelium without the lymph extract (0.469±0.022 µl/min) (Table 4). The ¹²⁵I-albumin clearance rate obtained for endothelial monolayers incubated with
Figure 3. Top: Reverse-phase high-performance liquid chromatography of the dried and resuspended residue of the ether-soluble fraction of pulmonary lymph obtained before (left-hand panel) and at 10–20 minutes after thrombin (right-hand panel). The ultraviolet absorbance of the collected fractions was measured at 270 nanometers (270 λ) during the first portion of the tracing, then at 235 λ. Decimal values indicate relative sensitivity of the recorder. A peak with a retention time corresponding to leukotriene B4 (LTB4) and an unresolved peak of several hydroxyeicosatetraenoic acids (HETEs) appears after the thrombin challenge. Prostaglandin (PG) metabolite is the cyclooxygenase product 12-hydroxyheptadecatrienoic acid. Bottom: Reverse-phase high-performance liquid chromatographic appearance of LTB4 and 5-HETE standards.

Postthrombin lymph extract of 0.510±0.030 was not different from the value obtained after incubation of endothelial monolayers with prethrombin lymph extract. However, the transendothelial albumin clearance rate increased (p<0.05) with the addition of postthrombin lymph extract to the neutrophil-endothelial cell coculture (Table 4). This increase was comparable to that produced by PMA-
TABLE 3. Effects of Ether Extract of Pulmonary Lymph Replete With LTB₄, 5-HETE, and 15-HETE on Neutrophil Superoxide Anion Generation

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Concentration (M)</th>
<th>Superoxide generation (nM/10 min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td></td>
<td>0.9±0.5</td>
</tr>
<tr>
<td>LTB₄</td>
<td>10⁻⁶</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td></td>
<td>10⁻⁹</td>
<td>0.1±0.1</td>
</tr>
<tr>
<td>LTB₄ + 5-HETE</td>
<td>10⁻⁶</td>
<td>1.1±0.8</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>3.4±1.6</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸</td>
<td>3.5±1.4</td>
</tr>
<tr>
<td></td>
<td>10⁻⁹</td>
<td>0.7±0.6</td>
</tr>
<tr>
<td>LTB₄ + 15-HETE</td>
<td>10⁻⁶</td>
<td>5.1±0.7*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁷</td>
<td>6.4±1.0*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>LTB₄ + 5-HETE + 15-HETE</td>
<td>10⁻⁷</td>
<td>8.0±1.2*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁸</td>
<td>8.4±1.4*</td>
</tr>
<tr>
<td></td>
<td>10⁻⁹</td>
<td>4.8±1.3</td>
</tr>
</tbody>
</table>

LTB₄, leukotriene B₄; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid.

*Differs from the values for HBSS (p<0.05).

n=3 and all studies were performed in triplicate. All values are shown as the mean±SEM.

Effect of Pulmonary Lymph Obtained from L-651,392-Treated Sheep on Transendothelial ¹²⁵I-Albumin Permeability

Addition of ether extract of postthrombin pulmonary lymph to the neutrophil-endothelial cell cocultures induced an increase in transendothelial ¹²⁵I-albumin permeability, which did not occur with the prethrombin lymph (Table 6). Postthrombin lymph obtained from L-651,392 pretreated sheep did not result in an increase in endothelial ¹²⁵I-albumin permeability (Table 6).

TABLE 4. Effects of Pulmonary Lymph Obtained From Thrombin-Challenged Sheep on Clearance Rates of ¹²⁵I-Albumin Across Pulmonary Artery Endothelial Monolayers

<table>
<thead>
<tr>
<th>Upper well content</th>
<th>¹²⁵I-Albumin clearance rate (µl/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstimulated neutrophils</td>
<td>0.469±0.022</td>
</tr>
<tr>
<td>Ether extract of baseline lymph</td>
<td>0.415±0.051</td>
</tr>
<tr>
<td>Ether extract of baseline lymph</td>
<td>0.505±0.073</td>
</tr>
<tr>
<td>Ether extract of postthrombin lymph + neutrophils</td>
<td>0.510±0.030</td>
</tr>
<tr>
<td>Ether extract of postthrombin lymph + neutrophils</td>
<td>0.710±0.040*</td>
</tr>
<tr>
<td>PMA (10⁻⁷ M) + neutrophils</td>
<td>0.661±0.035*</td>
</tr>
</tbody>
</table>

PMA, phorbol myristate acetate.

*Differs from the values for unstimulated cells and for the ether extract of baseline lymph (p<0.05).

n=8 in each test group.

All values are expressed as the mean±SEM.

Effect of α-Thrombin on Neutrophil O₂⁻ Generation

The effect of α-thrombin (10⁻⁶ to 10⁻¹⁰ M) on neutrophil O₂⁻ generation is shown in Table 7. Addition of thrombin of increasing concentrations did not result in neutrophil O₂⁻ generation.

Discussion

In the present study, we have analyzed the neutrophil activating properties of pulmonary lymph obtained before and at specific time intervals after thrombin-induced pulmonary microembolism in chronically prepared unanesthetized sheep. The purpose of the study was to determine the time course of generation of humoral mediators, to characterize these mediators, and to examine their poten-
TABLE 7. Effects of a-Thrombin on Neutrophil Superoxide Anion Generation

<table>
<thead>
<tr>
<th>a-Thrombin concentration (M)</th>
<th>Superoxide anion generators (nmol/10^6 cells/10 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^{-10}</td>
<td>0.8±0.4</td>
</tr>
<tr>
<td>10^{-9}</td>
<td>1.1±0.4</td>
</tr>
<tr>
<td>10^{-8}</td>
<td>1.6±0.6</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>1.0±0.02</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>1.4±0.05</td>
</tr>
</tbody>
</table>

n=3 at each concentration.
Values are expressed as the mean±SEM.

Our earlier studies indicated that thrombin-induced pulmonary intravascular coagulation results in sustained increases in pulmonary lymph flow and vascular permeability. The increase in lung vascular permeability was associated with a decrease in circulating neutrophils and with an increase in pulmonary uptake of radiolabeled autologous neutrophils. The increase in permeability after thrombin was shown to be neutrophil-dependent since prior neutrophil depletion prevented the response. In the present study, humoral factors with the ability to activate neutrophils (i.e., to induce superoxide generation, aggregation, and directed migration) were sought in pulmonary lymph since the lymph reflects events occurring in the lung microvasculature and the lung interstitium.

Previous studies suggest that lipoxygenase products are involved in the mediation of lung vascular injury after thrombin. The increase in pulmonary transvascular protein flux after thrombin-induced pulmonary microembolism was attenuated in sheep pretreated with BW755C (an inhibitor of both cyclooxygenase and lipoxygenase pathways) compared to sheep treated only with the cyclooxygenase inhibitor indomethacin and meclofenamate, indicating that generation of products of the lipoxygenase pathway contributes to the increased pulmonary vascular permeability after thrombin. In another study in which sheep were challenged with E. coli endotoxin, the appearance in pulmonary lymph of 5-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) preceded the increase in lung vascular permeability. It was unclear whether 5-HETE and the other lipoxygenase products participate in the pulmonary microvascular injury or appear coincidentally with the onset of the injury.

In the present study, we observed that the pulmonary lymph obtained from unanesthetized sheep after thrombin-induced pulmonary microembolism contained substance(s) that caused neutrophil migration, aggregation, and O_2^- generation in vitro. The maximum alterations in neutrophil function occurred with the pulmonary lymph samples obtained between 10 and 20 minutes after the end of the thrombin infusion (i.e., within 25 to 35 minutes after the beginning of the infusion). Maximal increases in pulmonary lymph flow and transvascular protein clearance were evident at 15 minutes following the end of thrombin infusion, indicating that pulmonary microvascular injury occurred rapidly and in conjunction with the generation for neutrophil activating factors in a pulmonary lymph. The intrapulmonary generation of factors causing neutrophil activation was transient since migration, aggregation, and O_2^- generation stimulated by post-thrombin lymph decreased to baseline values within 60 minutes postthrombin. This may reflect short-lived generation of these factors by pulmonary inflammatory cells. It is unlikely that thrombin alone is the mediator of the response since thrombin alone at concentrations as high as 10^{-6} M did not stimulate neutrophil O_2^- generation. Moreover, all lymph samples contained hirudin to inactivate any residual thrombin.

The finding that the diethyl ether extract of whole lymph (but not the aqueous phase) induced neutrophil O_2^- generation suggests that lipids are the putative factors in lung lymph responsible for these changes. Resuspension of the lipid containing fractions of pulmonary lymph obtained between 10 and 20 minutes after thrombin challenge increased the permeability of 125I-albumin across bovine pulmonary artery endothelial monolayers. The most significant increases in endothelial 125I-albumin permeability occurred in the presence of neutrophils, suggesting that the factors contained in the ether extract mediate the increase in permeability secondary to neutrophil activation. Endothelial albumin permeability did not increase by the addition of extracts of baseline (i.e., prethrombin) lymph to the neutrophil-endothelial cell cocultures, indicating that the factors inducing the increase in endothelial permeability are generated in vivo as a consequence of the thrombin challenge.

It is unlikely that residual ether in the resuspended ether extracts mediates the response since 125I-albumin permeability after incubation of the monolayer with the resuspended ether extract of postthrombin lymph was greater than after incuba-
tion with similarly prepared prethrombin lymph. The putative lipids released into lymph after thrombin may have direct effects on the albumin permeability of endothelial cell monolayers; however, the greatest effect was observed in the presence of neutrophils. The cellular pathways responsible for the increase in albumin permeability are not known. Increases in the size of interendothelial junctions and/or transcellular vesicular transport and active transport may contribute to the permeability characteristics of the endothelium.

Analysis of the diethyl ether extracts of baseline and postthrombin lymph by RP-HPLC revealed that compounds which coelute with LTβ, and HETEs were present in postthrombin lymph and appeared in a time-dependent manner after thrombin challenge. LTβ, and the HETEs individually are weak stimulators of neutrophil O2•− generation in vitro in the absence of cytochalasin B. In the present study, fractions of ether extract of postthrombin lymph containing HETEs and LTβ (as identified by ultraviolet absorbance after RP-HPLC) resulted in O2•− generation by neutrophils in the absence of cytochalasin B. Other lipid mediators such as platelet activating factor may also be generated in pulmonary lymph after thrombin challenge, which could act synergistically with LTβ, and HETEs to induce neutrophil activation observed with the fractions of postthrombin lymph containing LTβ, or HETEs.

Since both LTβ, and HETEs appear in pulmonary lymph after thrombin challenge, there is the possibility that LTβ, and HETEs in combination enhance neutrophil activation and synergistically contribute to the development of lung vascular injury. O’Flaherty et al. have demonstrated a potentiating action of LTβ, and HETEs on neutrophil degranulation. We observed that the pulmonary lymph samples replete with 5-HETE, 15-HETE, and LTβ, resulted in both neutrophil O2•− generation and neutrophil-dependent increase in transendothelial 125I-albumin permeability. The control pulmonary lymph replete with only LTβ, or 5-HETE or 15-HETE, however, did not significantly increase O2•− generation or transendothelial albumin permeability. Therefore, a synergism between LTβ, and 5- or 15-HETEs may contribute to neutrophil activation and thereby to the neutrophil-dependent endothelial permeability observed with postthrombin lymph. The concentrations of LTβ, 5-HETE, and 15-HETE in the ether extracts of postthrombin lymph may have been greater than the amounts of LTβ, 5-HETE, and 15-HETE added to the lymph, which may not allow quantitative comparisons; however, the results of lymph repletion studies support the notion that lipoxygenation products act synergistically to increase endothelial permeability. The role of lipoxygenase products in mediating the increase in lung vascular permeability after thrombin is further supported by studies in sheep treated with the 5-lipoxygenase inhibitor L-651,392. The postthrombin pulmonary lymph did not induce a neutrophil-dependent direct increase in endothelial albumin permeability, indicating that lipoxygenase products are important mediators by inducing neutrophil activation.

The results of this study do not permit identification of the cellular source of the lipoxygenase products. The predominant cell type in pulmonary lymph is the lymphocyte, and it is possible that the lymphocytes are a source of these compounds. LTβ, synthesis by T-lymphocytes has been described in the presence of mitogenic stimuli; however, the rapid appearance of LTβ, and HETEs in lung lymph, within 15 minutes after thrombin, precludes lymphocyte proliferation as a source of LTβ, and HETEs. Activated neutrophils sequesstered in the pulmonary microcirculation and lung extravascular spaces and resident pulmonary macrophages are more likely sources of the lipoxygenase products. Since LTβ, is the predominant neutrophil chemotactic factor produced by the resident alveolar macrophages, we have observed generation of lipoxygenase products in bronchoalveolar lavage fluid after thrombin challenge. Their generation paralleled the development of neutrophil alveolitis and activation of macrophages, indicating that pulmonary inflammatory cells are a major source of the lipoxygenase products.

Although the present results point to an important role of lipoxygenase products, LTβ, and HETEs, in mediating the neutrophil-dependent lung vascular injury after thrombin, these findings do not preclude other important mediators of lung injury. For example, the hydroperoxyacids are precursors of HETEs and may be mediators of inflammation. Since the HETEs are short-lived intermediate metabolites, we were unable to assay for their presence in pulmonary lymph samples. There is also the possibility that nonlipids, such as the complement-derived peptide C5a, are the mediators of neutrophil activation and contribute to the neutrophil-dependent increase in endothelial permeability. However, the observation that nearly all of the neutrophil activating factors partitioned into the ether fraction indicates lipid mediators have a major role in producing neutrophil activation and the neutrophil-dependent increase in endothelial permeability to albumin. Complement-derived peptides partition into the aqueous phase (authors’ unpublished observation). Moreover, we have shown that complement activation in sheep does not increase lung vascular permeability.

In summary, we have demonstrated time-dependent intrapulmonary generation of lipoxygenase products (LTβ, and HETEs) after thrombin-induced pulmonary microembolism in sheep. Fractions of pulmonary lymph obtained after the thrombin challenge containing LTβ, and HETEs stimulated neutrophil O2•− generation in vitro and caused a neutrophil-dependent increase in the 125I-albumin permeability across cultured pulmonary artery endothelial cell monolayers. Pulmonary lymph obtained from thrombin-challenged sheep treated with the 5-
lipoxygenase inhibitor L-651,392 did not induce an increase in endothelial albumin permeability. The reconstitution of LTC$_4$, 5-HETE, and 15-HETE in pulmonary lymph induced neutrophil activation and a neutrophil-dependent increase in endothelial albumin permeability. We conclude that lipoxygenase products generated in the lungs after thrombin-induced pulmonary coagulation are important mediators of the neutrophil-dependent lung vascular injury.

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References

KEY WORDS • α-thrombin • lipoygenase products • HETE leukotriene B4 • pulmonary microembolism • neutrophil activation • lung vascular injury • endothelial monolayer
Lipoxygenase products induce neutrophil activation and increase endothelial permeability after thrombin-induced pulmonary microembolism.
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