Thrombin-Mediated Release of Lipids From Pulmonary Artery Endothelial Cells Promotes Neutrophil Adherence

Marilyn A. Fisher, Peter J. Del Vecchio, Gerard P. Palace, Elizabeth M. Denholm, Linda Lai, and Asrar B. Malik

We previously have described the ability of α-thrombin (the native procoagulant enzyme) to stimulate adherence of neutrophils to pulmonary artery endothelial cells. In the present study, we observed that conditioned medium factors released by α-thrombin (10^{-8} M) treatment of cultured ovine pulmonary artery endothelial cells increased neutrophil adherence to naive pulmonary artery endothelial monolayers. This effect was independent of any residual α-thrombin present in the medium. In contrast to thrombin-induced neutrophil adherence, adherence of neutrophils mediated by the conditioned medium was not inhibited by the anti-CD18 monoclonal antibody 60.3, indicating a CD18-independent mechanism. The factors generated by the action of α-thrombin on endothelial cells also resulted in concentration-dependent neutrophil migration. The neutrophil adherence- and migration-promoting activities were isolated in the ether portion after extraction of the conditioned medium. Chromatographic analysis showed that the active components (which resolved into two peaks by reversed-phase high-performance liquid chromatography) were relatively hydrophilic low molecular weight lipids without phosphorus or amino acids. Reconstitution of these peaks indicated that they mediated neutrophil adhesion and migration responses. The results indicate that lipid factors promoting neutrophil adhesion and migration are generated by the action of thrombin on pulmonary artery endothelial cells. The generation of these factors may contribute to the amplification of the lung inflammatory response after pulmonary intravascular coagulation induced by thrombin. (Circulation Research 1991;68:930–939)

Thrombin-induced pulmonary intravascular coagulation increases pulmonary capillary permeability to protein.1–3 This response is characterized by formation of interendothelial junctional gaps and endothelial injury.1,4 The intravenous infusion of α-thrombin in animal models causes the uptake of neutrophils in the pulmonary vascular bed, which precedes the increase in capillary permeability.5–8 Neutrophil-derived oxidants and proteases have been implicated in mediating the thrombin-induced pulmonary microvascular injury.9

The basis of pulmonary vascular neutrophil uptake after pulmonary intravascular coagulation remains unclear. Thrombin can directly stimulate neutrophil adherence to endothelial cell monolayers by increasing endothelial adhesiveness10–12 through upregulation of the endothelial cell adhesion molecule GMP-140.13 Thrombin challenge can also lead to fibrin deposition in the pulmonary microcirculation and neutrophil attachment to fibrin by a CD18-dependent mechanism.14 In the present study, we examined the possibility that thrombin stimulates the release of factors from endothelial cells capable of mediating neutrophil adhesion.10 We observed that adherence- and migration-promoting lipid factors are released from thrombin-stimulated endothelial cells. The release of these factors may play a role in amplifying lung inflammatory responses after thrombin generation in vivo.

**Materials and Methods**

**Materials**

Neutrophil isolation medium was obtained from Los Alamos Diagnostics (Los Alamos, N.M.). Chromium-51 (\(^{51}\text{Cr}\)) was obtained from New England Nuclear (Boston). Dulbecco’s modified Eagle’s me-
dium (DMEM), RPMI culture medium, fetal bovine serum, and nonessential amino acids were obtained from Gibco (Grand Island, N.Y.). Hanks’ balanced salt solution (HBSS) was prepared in our laboratory. Gentamicin sulfate was obtained from Whittaker M.A. Bioproducts (Walkersville, Md.). All water used was obtained from a MilliQ water purification system (Millipore Corp., Bedford, Mass.). Acetonitrile and methanol were Burdick & Jackson brand (Baxter Healthcare Corp., Edison, N.J.), and certified ACS chloroform (Fisher Scientific Co., Fairlawn, N.J.) was used. Ether was obtained from J.T. Baker Inc. (Phillipsburg, N.J.).

Platelet-activating factor 16:0 (PAF) standard was purchased from Calbiochem Corp. (La Jolla, Calif.), and arachidonate metabolite standards (stored at −80°C) were obtained from Cayman Chemical Co. (Ann Arbor, Mich.). Crystalline, lyophilized bovine serum albumin (BSA) and phorbol 12-myristate 13-acetate were obtained from Sigma Chemical Co. (St. Louis). Low endotoxin-containing BSA was obtained from Intergen Co. (Purchase, N.Y.). N-Formyl-methionyl-leucylphenylalanine (f-MLP) was obtained from Peninsula Laboratories, Inc. (Belmont, Calif.). α-Thrombin was a generous gift from Dr. John W. Fenton, II, New York State Department of Health, Albany, N.Y. Monoclonal antibody (MAb) IB415 (anti-CD18) was a generous gift from Dr. Samuel Wright, Rockefeller University, New York. MAb 60.316,17 (anti-CD18) and MAb OKM-135.31 (anti-CD11b) were generous gifts from Dr. John M. Harlan, University of Washington, Seattle. RR1/118,19 (anti–ICAM-1) was a gift from Dr. Robert Rothlein, Boehringer-Ingelheim Pharmaceuticals, Ridgefield, Conn. The PAF receptor antagonist WEB 208620 was obtained from Boehringer-Ingelheim. Culture plates with 24 wells were obtained from Corning Glass Inc. (Corning, N.Y.). The modified Boyden chamber and the 3-μm pore size polyvinylpyrrolidone-free Nuclepore polycarbonate filters used in the migration assays were obtained from Neuroprobe, Inc. (Cabin John, Md.).

Endothelial Conditioned Medium

Each roller bottle (850 cm²) containing approximately 1×10⁶ sheep pulmonary artery endothelial cells was washed three times with 60 ml HBSS at 37°C. Then, 54 ml HBSS was added along with 6 ml 1×10⁻²⁷ M α-thrombin mixed in phosphate buffered saline and 0.1% BSA to yield a final concentration of 1×10⁻⁸ M α-thrombin. The bottles were degassed with nitrogen gas for 10 seconds. The endothelial cells were incubated for 1 hour with α-thrombin, the medium was removed, and 60 ml fresh HBSS was added to each roller bottle. The roller bottles were incubated for an additional 1 hour at 37°C, and then the conditioned medium was transferred into centrifuge tubes on ice. This medium was either used without further treatment or was purified by ether extraction.

The ether extraction procedure involved adding 13.5 ml ice-cold ether to 20 ml ice-cold conditioned medium in capped centrifuge tubes. The tubes were shaken vigorously for 10 minutes and then centrifuged for 20 minutes at 900g (4°C). The ether and interface layers (which is referred to as the ether extract) were transferred into collection flasks using disposable glass Pasteur pipettes. The ether was removed using a rotary evaporator in an ice-water bath, and the remaining fluid was frozen in a dry-ice and methanol bath and lyophilized. Ether extraction of the conditioned medium for the bioassays was carried out at neutral pH to avoid possible decomposition of certain lipid components.

Neutrophil Isolation

Human venous blood was collected into heparinized plastic syringes, layered onto neutrophil isolation medium, and centrifuged at 250g for 30 minutes. The neutrophil layer was aspirated and transferred to a separate centrifuge tube. The neutrophil layer then was mixed with an equal volume of HBSS (pH 7.4, 20 mM EDTA) and centrifuged to a pellet. Red blood cells were hypotonically lysed with distilled water for 30 seconds, and the osmolality was restored with 1.2% NaCl. The washed neutrophil layer was centrifuged to a pellet at 200g, resuspended in HBSS (pH 7.4, 20 mM EDTA, 25 mM HEPES), and counted before labeling with ⁵¹Cr. Sheep neutrophils were prepared as described previously. The ⁵¹Cr neutrophil labeling was carried out as described previously.

Preparation of Endothelial Cell Monolayers

Sheep pulmonary artery endothelial cells were obtained and prepared as described previously. The cells were used at population doublings of 17 to 25; no discernible differences were noted in the thrombin-induced neutrophil adhesion responses in cells with different population doublings.

Adherence Assay

The ⁵¹Cr-labeled neutrophil adherence assay was carried out as described previously using 24-well culture plates. ⁵¹Cr-labeled neutrophils (2×10⁶) suspended in 1 ml DMEM were placed on a confluent layer of endothelial cells growing on the bottom of each well of the 24-well plate. Incubation occurred for 60 minutes at 37°C in 5% CO₂, after which the monolayer was gently washed three times with warmed DMEM to remove nonadherent neutrophils. The cells were lysed with 1N NaOH and counted in an 1193 Gamma Trac gamma counter, TM Analytic, Inc., Brandon, Fla.

Chemokinesis Assay

The lower well of a modified Boyden chamber was filled with 29 μl endothelial conditioned medium/RPMI/0.2% low endotoxin BSA solution. Neutrophils, suspended in RPMI with 0.2% (wt/vol) low endotoxin BSA to a final volume of 50 μl, were placed in the upper well of the chamber in a final concentration of 1×10⁶ neutrophils per milliliter. The neutrophils were separated from the test agents by a 3-μm pore size polyvinylpyrrolidone-free Nucle-
pore polycarbonate filter in a 48-microwell chamber. The chamber was incubated for 1 hour at 37°C in 5% CO₂. The top side of the filter was washed with phosphate buffered solution three times, and the non-migrated neutrophils were scraped off. The filters then were fixed in methanol for 15 minutes, stained in toluidine blue for 15 minutes, washed with two 3-minute rinses of distilled water, air-dried, and mounted on glass slides. Cells migrating to the lower side of the filters were counted by counting 10 consecutive fields per filter, observed with a ×50 objective as previously described. All groups were tested in triplicate. RPMI/BSA with no test agent added served as the negative control. Results of neutrophil (PMN) migration induced with the test medium (TM) were expressed as a mean percentage of maximum migration occurring with 10⁻¹⁰ M f-MLP (which was considered to be 100%), corrected for the amount of migration occurring in the RPMI/BSA negative control group such that % PMN migration = (No. migrated PMN<sub>TM</sub>− No. migrated PMN<sub>RPMI/BSA</sub>)/(No. migrated PMN<sub>f-MLP</sub>− No. migrated PMN<sub>RPMI/BSA</sub>)×100

Amino Acid Analysis

Amino acid analysis was performed using a Picotag system (Waters Chromatography, Division of Millipore, Milford, Mass.) and the procedure developed by Bidlingmeyer et al. Dehydrolyzed samples were hydrolyzed with 6N HCl at 108°C for 24 hours and derivated with ethanol: triethylamine: water: phenylisothiocyanate (7:1:1:1, vol/vol). Neutralized, dehydrated residue was reconstituted in 5% acetonitrile in sodium phosphate (0.005 M, pH 7.4) for analysis. Amino acid standards (250 pM; from Waters) were processed and analyzed along with samples. Phenylthiocarbamyl amino acid derivatives were separated by reversed-phase high-performance liquid chromatography (RP-HPLC) at 38°C on a Picotag column (300×3.9 mm). A convex gradient from 6% acetonitrile in sodium acetate (0.14 M, pH 6.4, 0.5% triethylamine) to 60% acetonitrile in water was run in 10 minutes on the chromatograph (Waters model 730 data module, model 721 programmable system controller, model 6000A solvent delivery system, model 440 absorbance detector, and model U6K injector).

Molecular Weight Determination

Molecular weight estimates were obtained by aqueous gel permeation chromatography using a Bio-Gel TSK125 and TSK10 column (300×7.5 mm) (Bio-Rad Laboratories, Richmond, Calif.) in separate analyses. The nominal range was 500–20,000 and 100–1000 Da, respectively, under the conditions used. The column was eluted at 1.2 ml/min with sodium chloride (0.1 M)/trifluoroacetic acid (0.001%) and sodium phosphate (0.1 M, pH 3)/dimethyl sulfoxide (5%) in separate analyses. The chromatographic system is described below. Selectivity curves were constructed using cytochrome C, NAD, insulin, vitamin B<sub>2</sub>, thiamine, and synthetic in-house peptides.

Reversed-Phase High-Performance Liquid Chromatographic Analysis

Ether-extracted conditioned medium was analyzed by direct-injection RP-HPLC by incubating the sample with acetonitrile and centrifuging at 15,000g to precipitate salts. Lipids also were selectively extracted into water-saturated chloroform from water and methanol using the Bligh-Dyer extraction technique. The Bligh-Dyer procedure was applied to both the ether extract of conditioned medium and unextracted conditioned medium and gave similar results. This procedure was performed with sodium citrate (0.5 M, pH 3.5)/sodium chloride (1.0 M) /ethanol (0.05%) or at pH 3.0 with phosphoric acid alone. The final residue was reconstituted in mobile phase for analysis. Using precolumn techniques, functional group reactivity of chromatographic peaks was assessed from changes in peak area (absorption characteristics) and retention times. To determine the presence of amine/imine functionalities, the sample was incubated with buffered ninhydrin at 70–80°C for 20 minutes. Carboxylic groups were determined by esterification with phenacyl bromide and triethylamine at 50°C for 2 hours or with ethereal diazomethane.

Determination of PAF, leukotrienes C<sub>4</sub> and D<sub>4</sub> (LTC<sub>4</sub> and LTD<sub>4</sub>), and other lipids was performed using RP-HPLC at ambient temperature. The liquid chromatograph consisted of two Model 2300 pumps (ISCO, Lincoln, Neb.), a Valco injection valve, and a V4 variable-wavelength absorbance detector (ISCO) with a 3.5-μl analytical flow cell. Analysis was at 192–203 nm after separation on an Ultrasphere 5μ C18 column (250×4.6 mm) (Phenomenex, Rancho Palos Verdes, Calif.) with a Vydac guard column (50×3.2 mm, C18) (Rainin Instrument Co., Inc., Woburn, Mass.).

A mobile phase consisting of 2.5 mM phosphoric acid in 60% aqueous acetonitrile at 2 ml/min was used for the analysis of LTC<sub>4</sub> and LTD<sub>4</sub>. Because of the phosphoric acid, long retention times were obtained for the LTC<sub>4</sub> (10 minutes) and LTD<sub>4</sub> (28 minutes). Individual hydroxyeicosanoids (HETEs) and hydroperoxyeicosanoids (HPETEs) also were resolved using this solvent system. PAF was analyzed as modified from a described method using 2.5 mM phosphoric acid in 70% aqueous acetonitrile at 2 ml/min, with a retention time of 5 minutes. The retention time for PAF was confirmed by including standards of sphingomyelin and L-δ-lecithin in the analysis. Sample reanalysis using 0 and 0.25–1.0 mM trifluoroacetic acid in the mobile phase instead of 2.5 mM phosphoric acid was used to confirm the chromatographic peaks, which changed the retention times for LTC<sub>4</sub> and LTD<sub>4</sub> but not for PAF. Resolution of relatively hydrophilic lipids such as the prostaglandins (PGs) and thromboxane B<sub>2</sub> was achieved using 32% acetonitrile in 2.5 mM phosphoric acid.
Phosphorus Determination

Total phosphorus was determined spectrophotometrically using the Fiske-Subbarow procedure after sulfuric acid hydrolysis at 160°C for 6 hours. Standards were prepared using phosphoric acid (0 and 5–100 nM, analytical reagent grade, Mallinckrodt, Paris, Ky.).

Statistics

Results were calculated as mean±SEM. Eight replicate samples were used for each experimental group. Differences between groups were compared by the unpaired Student’s t test.

Results

The endothelial cell–derived medium obtained by incubating $1 \times 10^{-8}$ M α-thrombin with sheep pulmonary artery endothelial cells for 1 hour contained transferable factors that caused adhesion of neutrophils to naive ovine pulmonary artery endothelial cell monolayers (an increase from a basal adhesion of 3.2±0.4% to 25.8±1.8% with the conditioned medium). The proadherent activity was localized in the ether phase, that is, 26.0±1.3% neutrophil adherence induced by the ether phase of extracted conditioned medium versus 7.5±0.6% adherence induced by the postextraction aqueous phase (Figure 1). In contrast, the ether extract of conditioned medium obtained from endothelial cells incubated in HBSS alone (controls) caused 8.9±0.8% neutrophil adherence, and the postextraction aqueous phase gave a response of 6.6±0.5% (Figure 1). The ether extraction procedure proved effective in concentrating the active components even with a solvent-to-medium ratio of 0.65, because no bioactivity was present in the postextraction aqueous phase and no RP-HPLC chromatographic constituents were detected in the aqueous phase.

The adhesion-promoting activity was sustained over a relatively long period of time. When fresh medium and neutrophils were added to endothelial monolayers that had been stimulated with α-thrombin ($10^{-8}$ M) for either 1 or 3 hours, similar neutrophil adhesion values of 28.5±1.4% and 31.7±1.8%, respectively, were observed (Figure 2), indicating that maximal activity was generated by the 1-hour thrombin challenge period. The factors released by endothelial cells continued to be generated into fresh medium for up to an hour after the thrombin-containing medium had been washed off (Figure 3). The ether extract of the conditioned medium produced by the thrombin-stimulated endothelial cells caused neutrophil adherence to naive endothelial cells in a dose-dependent fashion (Figure 4). The activity present in 1 unit was defined as the material produced by 2.5×105 endothelial cells during a 1-hour collection period after thrombin stimulation.

The anti-CD18 MAbs 60.3 (10 μg/ml) and IB4 (10 μg/ml) reduced the thrombin-mediated sheep neutrophil adherence to sheep pulmonary artery endothelial cells, whereas thrombin-induced adhesion was unaffected using the control MAb OKM-1 (10 μg/ml) directed against an epitope on CD11b that does not mediate neutrophil adhesion (Figure 5). Neutrophil adhesion to naive endothelial cells mediated by the conditioned medium was not altered by MAb 60.3 (Figure 5). The anti-ICAM-1 MAb, RR1/1 (10 μg/ml), used to examine the role of ICAM-1 in the response, was shown to cross-react with sheep pulmonary artery endothelial cells challenged with thrombin. Addition of RR1/1 to the thrombin-treated endothelial cells before applying the 31Cr-labeled neutrophils resulted in a decrease of the thrombin-induced neutrophil adherence from 21.6±1.7% to 9.5±0.7% (Figure 6). In contrast, RR1/1 did not affect neutrophil adherence induced by endothelial conditioned medium (Figure 6).

Ninhydrin studies and PicoTag analysis indicated that there were no amino acids present (<1 μM per 500 μg sample) in ether-extracted conditioned medium obtained from thrombin-stimulated endothelial cells (Figure 7). Neutrophil adherence mediated by the conditioned medium was unaffected by storing the ether extract at −15°C for 24 hours; however, activity was abolished if the conditioned medium was stored at 60°C for 30 minutes or at 4°C for 48 hours (Figure 8), indicating that active components were unstable lipids.

![Figure 1. Effects of conditioned medium released from thrombin-treated ovine pulmonary artery endothelial cells and its ether and aqueous phases after ether extraction on neutrophil (PMN) adherence to naive pulmonary artery endothelial cell monolayers. Bars indicate mean±SEM; n=8 in each group. *p<0.05 from other groups.](http://circres.ahajournals.org/
Addition of endothelial conditioned medium to endothelial cells pretreated for 10 minutes with the PAF receptor antagonist WEB 2086 (1×10⁻⁴ M) resulted in a significant neutrophil adherence response of 22.9±0.8% compared with baseline adherence of 8.3±0.4% (Figure 9). This adherence response was similar to that induced by conditioned medium added to endothelial cells that had not been pretreated with WEB 2086 (26.2±1.5%). In another study, neutrophils were pretreated with WEB 2086 (10⁻⁴ M), and then these treated cells were added to endothelial cell monolayers; in this case, the conditioned medium resulted in the same adhesion response as controls that had not been pretreated with WEB 2086 (19.2±1.6%).

Neutrophil migration also occurred in response to increasing concentrations of endothelial conditioned medium produced by thrombin stimulation. The migration response occurred at a threshold concentration of 5 units (Table 1). At a higher concentration of 50 units, the neutrophil migration response reached a maximal value but decreased at 100 units (Table 1).

Components of the conditioned medium were resolved using RP-HPLC, and collected fractions were concentrated through lyophilization for activity determinations. Two chromatographic peaks increased in size with α-thrombin treatment. These are shown separated using 60% (Figure 10A) and 32% (Figure 10B) acetonitrile in 2.5 mM phosphoric acid. These constituents possessed proadherent activity. No other chromatographic peaks consistently increased in size with α-thrombin treatment as assessed through linear gradient separations from 0% to 100% acetonitrile in 0.1% trifluoracetic acid or 2.5 mM phosphoric acid.
The thrombin-mediated proadherent peaks extracted essentially completely into chloroform from acid water and methanol using the Bligh-Dyer technique, indicating that the active components were lipids. The lipid components were concentrated 20-fold using the Bligh-Dyer procedure before HPLC analysis. A single extraction gave almost complete recovery as determined by comparing peak heights of lipid extracts to direct injections of conditioned medium after buffer salt precipitation. The active lipids were determined by gel permeation chromatography to have molecular weights in the region of 275–975 Da.

Samples of ether or Bligh-Dyer-extracted conditioned medium as assayed by PicoTag amino acid analysis were shown to contain no detectable amino acid moieties (<1 nM per 500 µg sample), indicating LTC₄ and LTD₄ were not released. Also, the retention times of the thrombin-generated bioactive components (2.5–3.0 minutes; Figure 10A) were dissimilar to those of LTC₄ (10 minutes) and LTD₄ (28 minutes) under identical conditions (not shown). Additionally, the RP-HPLC peaks were insensitive to ninhydrin treatment, indicating that no amine/imine functionality was present in the components. Analysis of phosphorus using the Fiske-Subbarow procedure showed the absence of detectable phosphorus (<3 nM per 500 µg sample), indicating that the thrombin-generated lipids were dissimilar to phospholipids as PAF. The active lipids did not coelute with PAF 16:0 (nor PAF 18:0, not shown), HETEs or HPETEs (Figure 10A), and PGD₂, PGE₂, PGE₁, 6-keto-PGF₁α, thromboxane B₂, or LTB₄ (Figure 10B). Ultraviolet absorbance spectra of the bioactive lipids, using a DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) showed a wavelength maximum at 200 nm in acidic water but no characteristic bands of HETEs, HPETEs, or of the leukotriene series.

Discussion
We have shown previously that α-thrombin challenge of pulmonary artery endothelial monolayers results in an increase in neutrophil adhesion to pulmonary artery endothelial cells and that this response lasts 3–4 hours. Moreover, the effect of thrombin on the endothelial cell is the result, in part,
of expression of endothelial adhesive sites. In the present study, we have extended these observations by showing that thrombin challenge of pulmonary artery endothelial cells releases endothelial-derived factors that promote neutrophil adhesion to naive endothelial monolayers as well as neutrophil migration. These endothelial-derived factors could not be attributed to residual thrombin or thrombin-generated peptides that may be present in the medium fluid, because the ether-extracted conditioned medium contained no amino acids. Bligh-Dyer extraction of the conditioned medium indicated that the generated bioactive factors were lipids.

The neutrophil adherence mediated by the conditioned medium was different from that induced by α-thrombin. The primary difference is that adhesion mediated by conditioned medium obtained from thrombin-treated endothelial cells was independent of the neutrophil CD18 integrin because it could not be inhibited by anti-CD18 MAbs, whereas thrombin-induced adhesion was partially inhibited by the anti-CD18 antibodies. Moreover, thrombin-mediated neutrophil adhesion was dependent, in part, on the endothelial ICAM-1 molecule, because it could be partially inhibited by the MAb RR1/1 directed against ICAM-1. In contrast, RR1/1 did not reduce neutrophil adhesion induced by the conditioned medium, suggesting that this response was ICAM-1 independent.

These results agree with the hypothesis that thrombin-mediated neutrophil adhesion is partly CD18-dependent and, intrinsically, is to a similar degree ICAM-1 dependent because the neutrophil CD18 integrin binds with ICAM-1 ligand on endothelial cells. The basis of the adhesion response mediated by the endothelial conditioned medium is

**FIGURE 6.** Neutrophil (PMN) adherence induced by α-thrombin (α-T) is inhibited by addition of anti-ICAM-1 monoclonal antibody RR1/1 (10 μg/ml), whereas PMN adherence induced by conditioned medium is not inhibited by RR1/1. Bars indicate mean±SEM; n=8 for each group. * Significant reduction (p<0.05).

**FIGURE 7.** PicoTag analysis of the conditioned medium (panel A) compared with analysis of known standards (panel B). Panel A was obtained by processing 500 μg (dry wt) of ether-extracted conditioned medium (which included the interface), and panel B was from analysis of 250 pm phenylthiocarbamyl amino acid standards. The limit of detection was estimated to be 1 mM per sample. The first peak in panel A is the solvent front, and the second peak is due to reagent amine groups, but there were no amino acids present in the conditioned medium.
not clear, because neither anti-CD18 nor anti-ICAM-1 MAb inhibited the neutrophil adhesion response. The adhesion mediated by the conditioned medium may involve activation of other adhesion molecules, which promote neutrophil binding to endothelial cells, such as MEL-14\textsuperscript{15} on neutrophils and ELAM\textsuperscript{36} and GMP-140\textsuperscript{13} on endothelial cells.

What are possible candidates for endothelial-derived adhesion factors released by α-thrombin treatment of endothelial cells? The molecular masses of the factors mediating adhesion were less than 1,000 Da. The data ruled out PAF, LTB\textsubscript{4}, LTC\textsubscript{4}, LTD\textsubscript{4}, HETEs, and HPETEs as possible mediators because of the inability to detect these factors using RPHPLC analysis of the conditioned medium. PAF has been shown to be involved in the thrombin-induced neutrophil adhesion to human umbilical vein endothelial cells, although these data indicate that PAF remains associated with the endothelial cell membrane.\textsuperscript{37} In the present study, treatment of neutrophils with the PAF receptor antagonist WEB 2086 did not prevent a response mediated by the conditioned medium, suggesting that PAF expression did not mediate the response. The present study does not rule out the possibility that other arachidonic acid metabolites such as epoxyeicosatetraenoic acid, which may promote neutrophil adhesion and migration,\textsuperscript{38} are released by α-thrombin stimulation of endothelial cells. Studies by Farber et al\textsuperscript{39} have indicated that release of unresolved lipoxygenase metabolites mediated neutrophil migration in response to histamine and other mediators, which may be similar to the factors released by thrombin-challenged endothelial cells. Further investigation using lipoxygenase and cyclooxygenase inhibitors may help to define the role of various arachidonic acid metabolites in mediating neutrophil adhesion to endothelial cells.

The results of the present study explain the differences in the observations of Bizios et al\textsuperscript{10} and Zimmerman et al.\textsuperscript{11} Bizios et al\textsuperscript{10} observed that thrombin-induced neutrophil adhesion in ovine pulmonary artery endothelial cells was sustained over a period of hours, whereas Zimmerman et al\textsuperscript{11} showed that the neutrophil adhesion to human umbilical vein endothelial cells mediated by thrombin lasted only 15–60 minutes. The release of endothelial-derived factors into the medium from thrombin-challenged pulmonary arterial endothelial cells may then mediate this sustained neutrophil adhesion.

**Figure 8.** Neutrophil (PMN) adherence induced by conditioned medium stored at 60°C for 30 minutes, at 4°C for 48 hours, or at −15°C for 24 hours. Bars indicate mean±SEM; n=8 for each group. *p<0.05 from other groups.

**Figure 9.** Neutrophil (PMN) adherence induced by conditioned medium, conditioned medium in the presence of PMN pretreated with platelet activating factor receptor antagonist WEB 2086 (10\textsuperscript{-4} M), or conditioned medium in the presence of endothelial cells pretreated with WEB 2086 (10\textsuperscript{-4} M). Bars indicate mean±SEM; n=8 for each group. *p<0.05 from basal.
nary artery endothelial cells could account for the long-lived response observed by Bizios et al. The transient response observed with human umbilical vein endothelial cells may reflect differences in species and the site of vascular origin of these endothelial cells.

Neutrophils migrated toward the conditioned medium in a modified Boyden chamber, indicating that the medium contained neutrophil migration–promoting activity. The factors mediating the migration may be similar to those mediating adhesion. The basis of neutrophil migration may involve upregulation by the conditioned medium of neutrophil CD18 integrin and downregulation of MEL-14, both of which also participate in the neutrophil migration responses.

In conclusion, the results indicate that thrombin-induced neutrophil adhesion to pulmonary arterial endothelial monolayers is secondary to release of endothelial-derived lipid mediators and to expression of endothelial adhesion sites. The increase in neutrophil adhesion mediated by the endothelial-derived mediators is independent of neutrophil CD18 integrin, whereas the thrombin-mediated response is partly CD18 dependent. The release of endothelial-derived mediators may contribute to pulmonary vascular neutrophil uptake after intravascular coagulation, which may have critical implications in the pathogenesis of neutrophil-mediated lung vascular injury after thrombin-induced pulmonary microembolism.

**References**


**TABLE 1. Neutrophil Migration Mediated by Endothelial Medium Obtained After Treatment of Ovine Pulmonary Artery Endothelial Monolayers With α-Thrombin (10⁻⁸ M for 1 Hour)**

<table>
<thead>
<tr>
<th>Medium concentration*</th>
<th>Neutrophil migration (%)†</th>
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<tr>
<td>0</td>
<td>0±5</td>
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<tr>
<td>1</td>
<td>0±13</td>
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<td>5</td>
<td>84±14</td>
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<td>50</td>
<td>97±6</td>
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<td>60±9</td>
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Values are mean±SEM. All points were run in triplicate.

*Amount of biologically active factors generated by 2.5×10⁷ endothelial cells per milliliter of buffer per hour.

†Represented as percent migration induced by N-formyl-methionyleucylphenylalanine.


**KEY WORDS** - α-thrombin - conditioned medium - neutrophil adherence - neutrophil migration - endothelial cells - lipid factors - CD18
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