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Endothelin-like pulmonary vasoconstrictor peptide release by α-thrombin

(endothelial cells/hemostasis/pulmonary circulation/HPLC/endothelial-derived constrictor factor)

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ABSTRACT **The endothelial cells lining the vessel wall can modulate vasomotor tone by releasing vasoactive factors, such as endothelial-derived constricting factors. We observed that α-thrombin, but not catalytically inactivated α-thrombin, mediated the release of two pulmonary vasoconstrictor peptides into the venous effluent of guinea pig lungs. These peptides elicited a slow-onset, long-lasting pulmonary vasoconstriction similar to the effect of endothelin, an endothelial-derived 21-amino acid vasoconstrictor peptide previously isolated from cells in culture. One of the isolated peptides coelutes with endothelin upon reverse-phase HPLC with an acetonitrile gradient and has a molecular weight comparable to endothelin as determined by gel-permeation HPLC. The other vasoconstrictor peptide elutes earlier than endothelin on reverse-phase HPLC and exhibits a lower molecular weight. The studies show the release of endothelin-like pulmonary vasoconstrictor peptides in the intact lung by α-thrombin, a central regulatory enzyme in hemostasis.**

The vascular endothelium produces vasodilators, prostacyclin and the endothelial-derived relaxing factor (1, 2), and vasoconstrictors collectively termed endothelial-derived constrictor factors (EDCF) (3). One EDCF has been identified as endothelin, a 21-amino acid peptide initially isolated from cultured porcine aortic endothelial cells (4). Originally the human and porcine endothelins were found to have identical sequences (5), and the DNA sequence of the rat gene indicated 6 amino acid replacements in the amino-terminal half of the molecule (6). The two intrachain disulfide bonds and the hydrophobic carboxyl terminus [which appear necessary for the vasoconstrictor activity (7)] were conserved. More recently, three genes have been identified for endothelin in a human DNA library by Inoue *et al.* (8). These authors have suggested the nomenclature of ET-1 to designate the original porcine/human endothelin, ET-2, which has a Trp^6,Leu^7 substitution, and ET-3, the peptide initially designated as rat endothelin (8).

Endothelin is released constitutively by cultured aortic endothelial cells (4). The endothelin-induced vasoconstriction is not inhibitied by α-adrenergic, cholinergic, histaminergic, and serotonergic antagonists (3, 4), nor by cyclooxygenase or lipoxygenase inhibitors (4). The vasoconstrictor response requires extracellular Ca^{2+}, is associated with increased intracellular free Ca^{2+}, and may be inhibited by dihydropyridine Ca^{2+} channel antagonists (4, 9). A number of stimuli and vasoactive mediators such as thrombin, norepinephrine, or the Ca^{2+} ionophore A23187 stimulate endothelin mRNA transcription *in vitro* (4). Endothelin may participate in hypertensive responses (10–13) because of its vasoconstrictor properties. However, whether endothelin or endothelin-like peptides are released *in vivo* is not known. In this study, we examined whether endothelin or endothelin-like peptides are released in the intact circulation. Studies were made in perfused guinea pig lungs challenged with α-thrombin, which we have shown to induce pulmonary vasoconstriction (14).

MATERIALS AND METHODS

Lungs were obtained from healthy Hartley guinea pigs of either sex (Buckberg, Tomkins Cove, NY) weighing 500 ± 25 g (mean ± SEM), anesthetized with pentobarbital sodium (50 mg/kg, i.p., Abbott). The trachea was cannulated before opening the thorax. Heparin sodium (700 units/kg) was administered by intracardiac injection, and the animals were exsanguinated. The heart and lungs were carefully removed *en bloc* and suspended from one end of a beam balance (14). Catheters were placed in the pulmonary artery and left atrium. The lungs were covered with plastic wrap to reduce evaporative fluid loss. Perfusion of the system with a peristaltic pump (Harvard Apparatus, model 1215) was begun within 5 min of pneumothorax. The perfusate was a phosphate-buffered Ringer's solution containing 0.5% bovine albumin (fatty-acid-free, recrystallized, Sigma). The Ringer's solution contained 137 mM NaCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 2.68 mM KCl, 0.06 mM $NaHCO_3$, 0.120 mM NaH_2PO_4, 0.869 mM Na_2HPO_4, and 5.55 mM dextrose. The perfusion rate was set at 28 ml/min in all experiments. Recirculation of the perfusate was begun after the venous effluent was clear of blood. Light microscopy of sample lungs indicated that they were essentially free of residual leukocytes and platelets (H. Lum and A.B.M., unpublished observations). The total perfusate volume was 300 ml. The lungs were suspended from a counterweighted beam balance with left atrial and pulmonary arterial pressure continuously measured with pressure transducers (Gould P50 and P23, respectively, Cleveland). Continuous recordings of the weight changes were made on a three-channel recorder (Gould model 2400S) to ensure that there was no pulmonary edema during the course of the experiment. Left atrial and airway pressures were adjusted to 3 and 1 cmH_2O, respectively, for all experiments. The lungs were continuously inflated with 95% O_2/5% CO_2.

Three hundred milliliters of pulmonary venous effluent was collected into a reservoir containing hirudin (20 nM final concentration) from either control (saline injection) or thrombin-treated (1 nM) lungs. A fresh (naive) lung was then prepared as described (14) and perfused with collected effluent. Complete inhibition of any thrombin not cleared by the

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Abbreviations: EDCF, endothelial-derived constrictor factors; PVF, pulmonary vasoconstrictor factor(s).

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original lung was verified by testing an aliquot of the effluent for clotting activity using a fibrinometer (Becton Dickinson).

Pulmonary venous effluent was collected from thrombin-challenged lungs and extracted with 2 vol of HPLC-grade diethyl ether (Burdick and Jackson). Most of the pulmonary vasoconstrictor factor(s) (PVF) activity localized at the ether/water interface, which was decanted, dried overnight on a Speed Vac (Savant), rehydrated in HPLC starting solvent (for HPLC analysis) or in Ringer's/albumin solution for injection into lungs (100 μl).

Isocratic reverse-phase HPLC (15) was done on an Ultrex C_{18} (4.6 mm × 25 cm) purchased from Phenomenex (Belmont, CA). The mobile phase was 32% acetonitrile (Burdick and Jackson)/H_2O, pH 3.0 with phosphoric acid. Flow rate was 1 ml/min; ambient temperature and detection was at 203-nm absorbance.

Gradient-elution reverse-phase HPLC was performed using a Zorbax C_3 column (1.0 cm × 30 cm) (DuPont). The mobile phase was solvent A: 0.1% trifluoracetic acid (TFA)/H_2O; solvent B: 0.1% TFA/acetonitrile. A linear gradient (0–60% acetonitrile) was generated over 40 min. Flow rate was 2.0 ml/min; ambient temperature and detection was at 215 nm. Amino acid analysis of the material detected in the HPLC peaks was done (16). Erhlich's reagent (17) was used to detect the presence of tryptophan. The material in the peaks was collected, dried to remove acetonitrile, resuspended in Ringer's/albumin solution, and injected into the pulmonary artery of naive lungs.

Gel-permeation HPLC was performed by using a TSK-125 column with guard column (Bio-Rad) to determine molecular weights of the factors generated. The nominal M_r range of this column is 500–20,000 for proteins and peptides under denaturing conditions. The mobile phase was 0.10 M phosphate, pH 3.0 (with phosphoric acid) containing 5% dimethyl sulfoxide. The column was eluted isocratically at 0.7 ml/min; detection was at 259 nm. The column was calibrated, and a selectivity curve (distribution coefficient or K_{av} versus logarithm of M_r) was generated using cytochrome c, insulin, and glucagon (Sigma), and a series of peptides of known M_r values synthesized in our laboratory.

Sulfitolysis was used to break disulfide bonds to obtain better estimates of molecular weights (18). Peptides were incubated for 18 hr at 25°C in dimethyl sulfoxide/0.10 M phosphate, pH 3.0. The pH was then increased to 8.3 with NaOH, and 0.020 M $CuSO_4$ and 0.140 M Na_2SO_4 (final concentrations) were added. The peptide was incubated for 2 hr at room temperature and then injected onto the gel-permeation HPLC column.

RESULTS

Injection of α-thrombin (1–10 nM) into the pulmonary artery of guinea pig lungs perfused at constant flow caused a rapid, transient (lasting 4 min) increase in perfusion pressure (phase I) as shown (14), followed by a smaller sustained elevation in the pressure (phase II) (Fig. 1 *Left*). The increase in perfusion pressure under constant flow conditions indicated pulmonary vasoconstriction (13). Infusion of 1 nM diisopropylphospho-α-thrombin, an enzymatically inactive form (19), did not increase the pulmonary perfusion pressure (Fig. 1 *Left*). Pretreatment of lungs with 10 μM indomethacin (a cyclooxygenase inhibitor) blocked the initial response (phase I) but not the sustained phase II vasoconstriction (Fig. 1 *Left*).

Pulmonary venous effluent samples were collected from control and α-thrombin-challenged lungs perfused with Ringer's/albumin solution to determine whether the sustained response resulted from α-thrombin or generation of stable transferable factor(s). The effluent samples were treated with excess hirudin [a specific high-affinity thrombin inhibitor (20)] and infused into naive perfused lungs. Control effluent samples (i.e., fluid obtained from saline-challenged lungs or fluid obtained before thrombin challenge) did not elicit increases in pulmonary perfusion pressure, whereas effluent from thrombin-challenged lungs displayed a slow-onset, long-lasting pressure rise comparable to the second-phase response seen with α-thrombin (Fig. 1 *Right*). Lungs challenged with hirudin-inhibited α-thrombin showed no increase in pulmonary perfusion pressure.

The pulmonary vasoconstrictor factor(s) (PVF) present in pulmonary venous effluent was retained on C_{18} reverse-phase HPLC cartridges and could be eluted with methanol.

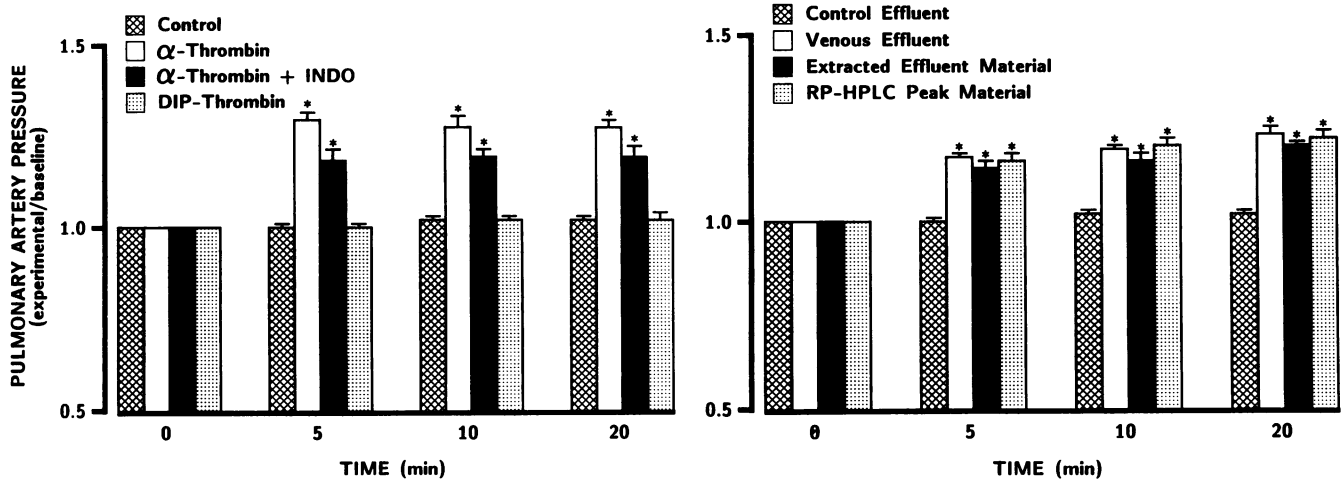

FIG. 1. (*Left*) Time course of pulmonary vasoconstriction. Changes in guinea pig-isolated lung pulmonary artery pressure (expressed as experimental value/baseline value) versus time after addition of either control saline, 10 nM α-thrombin, or 10 nM α-thrombin into lungs pretreated with 10 μM indomethacin (INDO), or 10 nM diisopropylphospho-α-thrombin (DIP-Thrombin) (enzymatically inactivated). Note that phase I constriction (0–4 min) is not included in this graph for clarity. Data are expressed as mean ± SEM. An asterisk indicates significantly ($P < 0.05$) different from time-matched control values. The thrombin plus indomethacin group was not significantly different from the thrombin alone group past the 5-min time point. Data were analyzed by two-way repeated measures ANOVA followed by multiple comparison tests to determine specific group significance. These studies represent six-to-eight lungs per group. (*Right*) Changes in pulmonary artery pressure during perfusion with saline control venous effluent, venous effluent from thrombin-treated lungs (hirudin was added to both effluents), ether-extracted material from the effluent of thrombin-treated lungs, or the material collected under the isocratic elution reverse-phase HPLC peak shown in Fig. 2. Statistical analysis and significance as above.

Furthermore, PVF were concentrated at the aqueous–organic interface during ether extraction. The extracted PVF induced pulmonary vasoconstriction (Fig. 1 *Right*). Isocratic reverse-phase HPLC (15) of the interface material after ether extraction revealed a single peak, which increased following α-thrombin challenge (Fig. 2). Injection of the reconstituted peak into the pulmonary artery of naive guinea pig lungs perfused with Ringer's/albumin elicited the slow-onset, phase II pulmonary vasoconstriction (Fig. 1 *Left*).

Comparison of the reverse-phase HPLC retention time of PVF indicated that the material was none of the following lipids: thromboxane B_2, 6-keto prostaglandin $F_{1\alpha}$, 6-keto prostaglandin E_1, leukotriene B_4, 20-OH-leukotriene B_4, 20-COOH leukotriene B_4, 5-hydroxy-5,8,11,13-icosatetraenoic acid (5-HETE), 12-HETE, 15-HETE, arachidonic acid, 11β-prostaglandin $F_{2\alpha}$, prostaglandin E_1, prostaglandin E_2, prostaglandin D_2, and platelet activating factor. The appearance of PVF was not inhibited by pretreatment of lungs with indomethacin (10 μM) or nordihydroguanidic acid (10 μM), further demonstrating that PVF were not arachidonic acid-derived metabolites. PVF reacted with ninhydrin (21) and exhibited a UV absorption spectrum consistent with a peptide.

When the isocratically eluted material was rechromatographed using gradient-elution reverse-phase HPLC optimized for peptide separation, we detected two ninhydrin-positive peaks (peaks I and II) (Fig. 3 *Top*) displaying pulmonary vasoconstrictor activities (Fig. 4). Peak II eluted at a retention time coincident with synthetic porcine/human endothelin or ET-1 (Fig. 3 *Bottom*). Because the isocratic reverse-phase HPLC peak contained two vasoconstrictor peptides, we evaluated which of these was more abundant in the lung effluent by application of the interface material to gradient elution reverse-phase HPLC without prior isocratic reverse-phase HPLC separation. Peak II was the predominant vasoconstrictor peptide in the lung effluent as based on UV absorbance and quantitative amino acid analysis (Fig. 3 *Middle*).

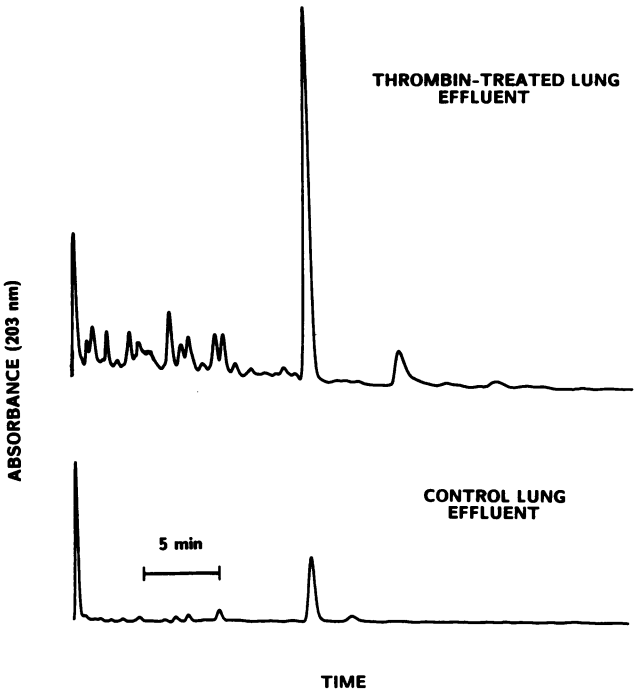

FIG. 2. Thrombin-stimulated release of vasoconstrictor peptide. Isocratic reverse-phase HPLC chromatograms of control or baseline pulmonary venous effluent extract samples (*Bottom*) and thrombin-challenged pulmonary venous effluent extract (*Top*). Pulmonary venous effluent was collected from thrombin-challenged lungs and extracted with 2 vol of HPLC-grade diethyl ether. Most PVF activity localized at the ether/water interface that was decanted, dried overnight, and rehydrated in HPLC starting solvent (for HPLC analysis) or in Ringer's/albumin solution for injection into lungs. A C_{18} column (4.6 mm × 25 cm) eluted isocratically with 32% acetonitrile/water at pH 3.0 was used in these separations.

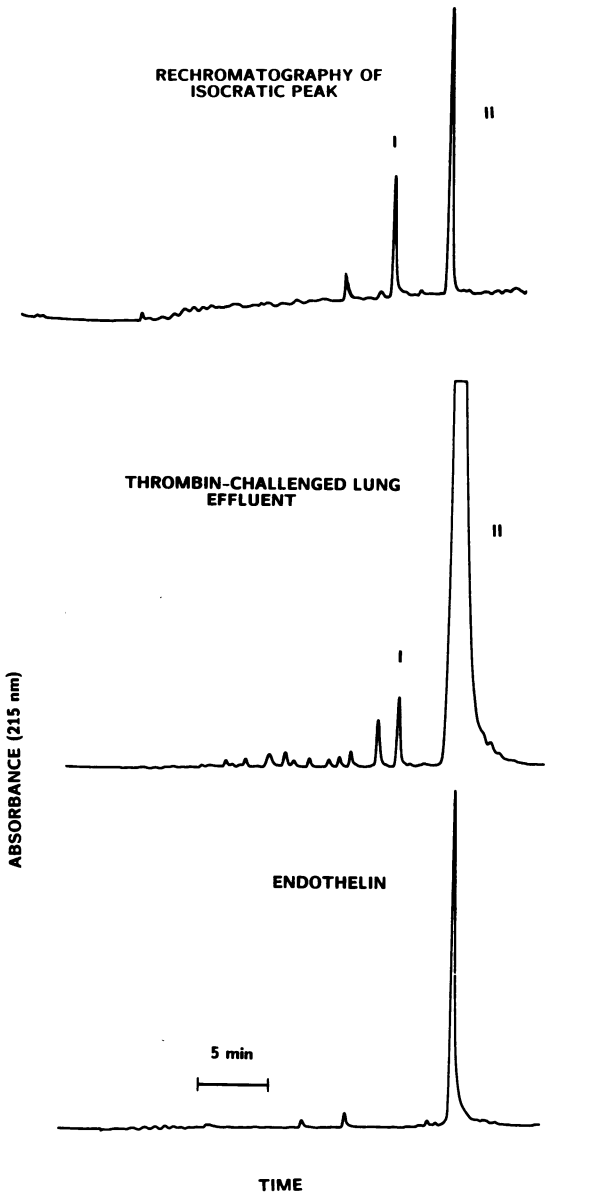

FIG. 3. Gradient-elution HPLC of vasoconstrictor peptides. (*Top*) Rechromatography of the vasoconstrictor material collected under the isocratic HPLC peak (Fig. 2). (*Middle*) Material extracted from thrombin-challenged lungs without prior isocratic reverse-phase HPLC. (*Bottom*) Synthetic porcine/human endothelin (synthesized in this laboratory or commercially obtained) chromatographed under the same conditions. Gradient-elution reverse-phase HPLC was done by using a Zorbax C_3 column (1.0 cm × 30 cm). The mobile phase was solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/acetonitrile. A linear gradient (0–60%) of acetonitrile was generated over 40 min. Flow rate of 2.0 ml/min, ambient temperature.

Time-course measurements after thrombin challenge indicated complex kinetics. Peak I increased within 2 min and returned to baseline within 10 min, whereas peak II increased within 2 min but remained elevated above baseline for 60 min. We also examined whether injection of a second thrombin dose at 60 min after the first thrombin challenge would result in a second release of peaks I and II. The second challenge with thrombin did not elicit a further release of peaks I and II, nor an increase in perfusion pressure.

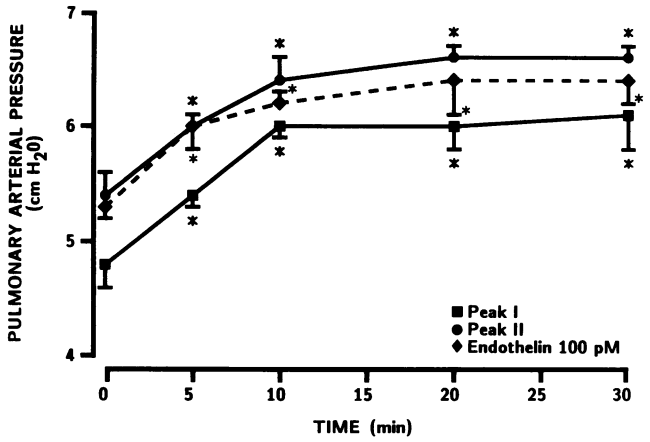

FIG. 4. Comparison of vasoconstrictor activity of isolated peptides with endothelin. Pulmonary artery pressure is shown as a function of time after addition of peak I material, peak II, or 100 pM synthetic endothelin. The material representing the peaks shown in Fig. 3 was collected, dried to remove acetonitrile and TFA and rehydrated in Ringer's/albumin, pH 7.4. The rehydrated peak material or endothelin was added directly to the fluid reservoir of the recirculating perfusion system. All three peptides caused a slow-onset long-acting vasoconstriction as indicated by increases in pulmonary perfusion pressure. Data are expressed as mean ± SEM. Data were analyzed by two-way repeated measures ANOVA followed by multiple comparison tests. *, Values different ($P < 0.05$) from t_0. $n = 3$ or 4. The pulmonary vasoconstrictor responses mediated by peak I, peak II, and endothelin (100 pM) were not significantly ($P > 0.5$) different from one another.

The material in peaks I and II was collected, dried to remove acetonitrile, resuspended in Ringer's/albumin solution, and injected into the pulmonary artery of naive lungs. Both peaks I and II displayed slow-onset and prolonged pulmonary vasoconstriction, comparable to the response of 100 pM synthetic human/porcine endothelin (Fig. 4). Amino acid analysis (15) of the material in peaks I and II suggested similarities in composition between the peptide in peak II and endothelin.‖ Ehrlich's reagent (17) indicated the presence of tryptophan in peak II, which was not detected in peak I. Amino acid compositions also indicated that peaks I and II were not angiotensin, neuropeptide Y, substance P, or other previously recognized vasoconstrictor peptides.

Molecular weights of the two peptides were determined by gel-permeation HPLC on a TSK-125 column. Sulfitolysis was used to break disulfide bonds to obtain better estimates of molecular weights (18). Peak II eluted at an apparent M_r of 2500, equivalent to that of human/porcine endothelin that had also been subjected to sulfitolysis (Fig. 5). Peak I eluted at an apparent M_r of 2100.

DISCUSSION

Injection of α-thrombin, the bioactive enzyme, into the pulmonary artery of guinea pig lungs perfused at constant flow resulted in an increase in the pulmonary perfusion pressure. The increase in perfusion pressure under constant flow conditions is indicative of pulmonary vasoconstriction or vasoocclusion. Vasoocclusion by aggregated platelets or leukocytes is unlikely because the isolated lungs were perfused with an acellular medium, and light microscopy of lungs indicated very few trapped residual platelets or leukocytes. Additionally, the original porcine/human endothelin (ET-1) and analogous peptides reportedly do not induce platelet aggregation (D.G.M. and J. E. Kaplan, unpublished observations; ref. 22). The intact catalytic site of α-thrombin was required for thrombin-induced pulmonary vasoconstriction because infusion of an enzymatically inactive form of thrombin (diisopropylphospho-α-thrombin) (19) did not induce vasoconstriction. Sustained (phase II) thrombin-induced pulmonary vasoconstriction was independent of arachidonic acid metabolites generated by cyclooxygenase and lipoxygenase pathways because cyclooxygenase and lipoxygenase inhibitors did not inhibit the response. The initial transient increase in pulmonary vasoconstriction, however, was blocked by these inhibitors, suggesting a role for thromboxane A_2 in the phase I response (14).

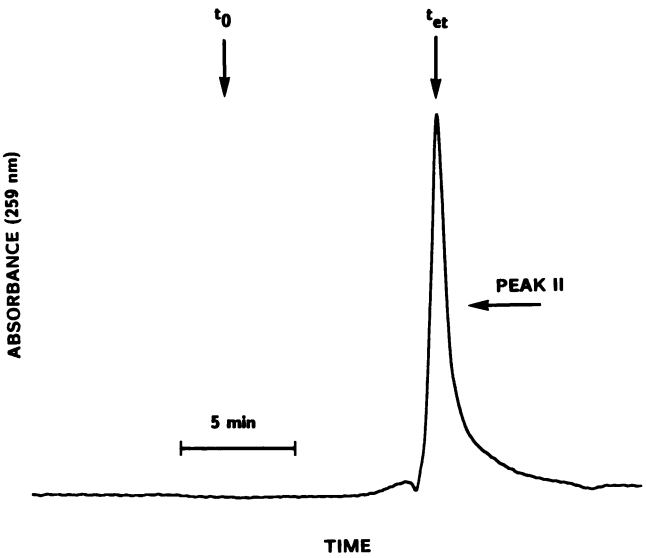

FIG. 5. Molecular weight estimation. After sulfitolysis (17), gel-permeation HPLC was used to estimate a M_r of 2500. Arrows indicate the retention times for the void (t_0) and synthetic endothelin (t_{et}; 2.46 kDa). The peak II peptide elutes at a molecular weight equivalent to synthetic endothelin.

The pulmonary venous effluent samples collected from thrombin-challenged lungs perfused with Ringer's/albumin solution contained stable, transferrable factor(s) that induced pulmonary vasoconstriction in naive lungs. The vasoconstrictor activity was not the result of transferred thrombin because hirudin was added to inhibit any free thrombin in the effluent (20). The pulmonary vasoconstriction induced by the effluent samples was slow in onset and long-lasting, a response similar to the second phase of the response seen with α-thrombin (14). These results indicate that active α-thrombin releases transferrable factor(s), which induce sustained pulmonary vasoconstrictor response.

The vasoconstrictor activity was concentrated in the aqueous–organic interface during ether extraction, indicating the factor(s) generated were hydrophobic or amphipathic (23). Isocratic reverse-phase HPLC revealed a single peak that increased following α-thrombin challenge. Moreover, the injection of the reconstituted peak into the pulmonary artery of isolated-perfused guinea pig lungs produced a sustained pulmonary vasoconstrictor response similar to that occurring

‖Amino acid analysis of peak I: aspartic acid 0.59, glutamic acid 1.64, serine 1.06, glycine 1.38, arginine 0.89, threonine 0.69, alanine 0.83, tyrosine 0.71, valine 0.78, cysteine 0.47, isoleucine 1.73, leucine 0.33, phenylalanine 1.89, tryptophan not detected by Ehrlich's reagent. Amino acid analysis of peak II (values in parentheses are "expected values" for human endothelin): aspartic acid 1.08 (2), glutamic acid 2.16 (1), serine 0.61 (3), glycine 0.47 (0), histidine 0.55 (1), arginine 0.57 (0), threonine 0.77 (0), alanine 1.31 (0), tyrosine 0.70 (1), valine 0.98 (1), cysteine 0.46 (4), isoleucine 0.56 (2), leucine 2.05 (2), phenylalanine 0.74 (1), lysine 1.63 (1). Tryptophan was detected by Ehrlich's reagent. Since isoleucine–isoleucine in porcine/human endothelin should resist hydrolysis, the isoleucine values may be underestimated, cysteine is consistently underestimated by this method.

with thrombin challenge and injection of the material in the aqueous–organic interface.

The factor(s) generated were peptidic because the factor(s) were ninhydrin-positive, and the UV absorption spectrum was consistent with a peptide. Two ninhydrin-positive peaks were detected when the isocratically eluted material was rechromatographed using gradient-elution reverse-phase HPLC optimized for peptide separation. The material present in peak II had a retention time coincident with synthetic porcine/human endothelin (ET-1). Moreover, the material in peak II was the predominant vasoconstrictor peptide based on UV absorbance and amino acid analysis.

Injection of reconstituted peaks I and II displayed a slow-onset sustained pulmonary vasoconstrictor response similar to that of 100 pmol of synthetic porcine/human endothelin. Moreover, the molecular weight of material in peak II was in the range of endothelin (apparent M_r of 2500). The material in peak I eluted at an apparent M_r of 2100. Peak I may be a degradation product of peak II or it may be a different endothelin-like molecule.

The time course of generation of peaks I and II was different. Peak I increased rapidly and returned to baseline within 10 min, whereas peak II increased rapidly but was sustained and did not return to baseline by 60 min. Moreover, a second injection of thrombin at 60 min after the first injection resulted in no increased release of peaks I and II. The second injection also did not elicit an increase in perfusion pressure (vasoconstriction). Thus, the release of these peptides is consistent with the slow-onset sustained vasoconstrictor activity of thrombin. Sustained vasoconstrictor activity may play an important role in hemostasis and the reduction in perfusion in response to vascular injury.

In conclusion, we have isolated two pulmonary vasoconstrictor peptides from thrombin-challenged guinea pig lungs. One of these (peak II) has properties in common with endothelin, a recently described long-acting vasoconstrictor peptide. The other peptide (peak I) may be a degradation product of peak II or an unrelated, endothelin-like peptide. Both peptides trigger a slow-onset long-lasting pulmonary vasoconstriction. Our findings demonstrate the release of endothelin-like vasoconstrictor peptides in the intact lung circulation by α-thrombin.

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