Human Leukocyte Cathepsin G and Elastase Specifically Suppress Thrombin-Induced Prostacyclin Production in Human Endothelial Cells

By Babette B. Weksler, Eric A. Jaffe, Mark S. Brower, and Olatoun F. Cole

Polymorphonuclear leukocytes (PMN) when activated release products that can potentially injure endothelial cells or alter endothelial function. Exposure of cultured human umbilical vein endothelial cells to cathepsin G and elastase isolated from human PMN at concentrations reached in vivo (100 ng/mL to 10 μg/mL) selectively inhibited thrombin-induced prostacyclin production and the thrombin-induced rise in cytosolic free calcium ([Ca²⁺]i) concentration. These proteases also blocked thrombin-induced release of arachidonic acid from prelabeled endothelial cells (EC). In contrast, induction of prostacyclin (PGI₂) production by arachidonate, histamine, or the calcium ionophore A23187 was not altered by treatment of EC with these proteases. The effects of the proteases were concentration-dependent, were blocked by serum or serum protease inhibitors, and were reversed when the endothelial cells were further cultured for 24 hours in the absence of the proteases. Elastase, but not cathepsin G, also produced detachment of endothelial cells. Thus, the major leukocyte proteases selectively suppress thrombin-induced prostacyclin production by human vascular endothelial cells and may alter the hemostatic balance at sites of PMN activation.

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Materials. Tissue culture medium, penicillin, streptomycin, and L-glutamine were from M.A. Bioproducts (Walkersville, MD). Tissue culture plasticware was from Falcon (Oxnard, CA). Purified human thrombin was a kind gift from Dr John W. Fenton II (State Department of Health, Albany, NY). Arachidonic acid was purchased from Nuchem (Elysin, MN). Prostaglandin standards were purchased from Upjohn (Kalama, MI). Radiolabeled compounds were obtained from New England Nuclear (Boston). The ionophore A23187 was from Calbiochem (La Jolla, CA) and Quin2/AM from Molecular Probes (Eugene, OR). Other chemicals were from Sigma (St Louis).

Preparation of cathepsin G and elastase. Both cathepsin G and elastase were purified from the granule fraction of human leukocytes isolated from WBC concentrates using nitrogen cavitation. The enzymes were isolated by affinity chromatography on Trasylol-Sepharose CL-4B; the two enzymes were then separated by chromatography on CM-52 cellulose. The purified elastase used in our studies ran as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as did the cathepsin G. By active site titration with Ac-Ala-Anval-ONp as described by Powers and Gupton, the preparations of elastase used in these experiments were found to be 92% active. By active site titration with p-nitrophenyl-N'-acetyl-N-benzylcarbazate, the preparations of cathepsin G used in these studies were found to be 86% active. In addition, in the presence of a polyclonal antibody prepared against elastase, elastase preparations gave a single immunoprecipitin arc on double diffusion analysis in agarose gels. Similar findings were obtained using purified cathepsin G and a polyclonal anticathepsin G antibody.

Payment of α₂-macroglobulin and α₁-antitrypsin. α₂-Macroglobulin and α₁-antitrypsin were purified from human plasma as previously described.

Endothelial cell culture. HUVEC were isolated and cultured as previously described in gelatin coated 24-well Costar tissue culture plates, using Medium 199 containing L-glutamine, antibiotics, and 20% pooled, heat-inactivated human serum. HUVEC were plated at 40,000 cells/well and were used just before confluence (100,000 cells/well) in passages 1 through 3. PGI₂ production by these cells is

From the Division of Hematology-Oncology, Department of Medicine and Specialized Center of Research in Thrombosis, Cornell University Medical College, New York, NY.

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Address reprint requests to Babette B. Weksler, MD, Division of Hematology-Oncology, Cornell University Medical College, 1300 York Ave, New York, NY 10021.

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stable during these passages and is maximal just before the cells reach confluence. Following their use in experiments, HUVEC were detached from the tissue culture plates by brief treatment with 0.02% collagenase and 10 mmol/L EDTA and counted in a Coulter Counter model ZBI (Coulter Inc, Hialeah, FL).

**Incubation of HUVEC monolayers with leukocyte proteases.** Preconfluent monolayers of HUVEC were washed twice with warm (37°C) HEPES buffered saline solution (HBS; contains 140 mmol/L NaCl, 11 mmol/L glucose, 4.5 mmol/L KCl, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 10 mmol/L HEPES, pH 7.4) and 0.5 mL of this solution at 37°C was placed over each monolayer. Cathepsin G (0.1 g/mL to 10 μg/mL) or elastase (0.01 g/mL to 10 μg/mL) was added for five to 30 minutes; enzymatic activity was then inhibited by addition of 10% (vol/vol) serum. The buffer overlaying the cell monolayer was collected for measurement of PG₁ seen released by the EC during the incubation with protease. Further manipulations of the monolayers are described below.

**Stimulation of prostacyclin production by EC monolayers.** Following treatment with proteases, HUVEC monolayers were again washed and 0.5 mL of fresh HBS added to each well. PG₁ production was induced by addition of the following stimuli: thrombin (0.1 to 2 U/mL), sodium arachidonate (25 μmol/L), histamine (30 μmol/L), or ionophore A23187 (1 to 10 μmol/L), and the wells were incubated at 37°C for five minutes after gentle mixing. The buffer overlaying the stimulated cells was then collected for measurement of PG₁ released by the HUVEC. Control wells for these experiments were treated similarly to experimental wells, except that the buffer used to dissolve the proteases was substituted for the protease solutions.

PG₁ production varies from culture to culture in response to stimulation with various agonists though PG₁ production is consistent within a given experiment using cells from the same culture and the same agonist. To summarize the results from multiple separate experiments each performed in triplicate and present our data in a single figure, we have expressed the data from each experiment as percent of control and then averaged the data and presented the mean ± SEM. Within a given experiment, the absolute amount of PG₁ release induced by thrombin or arachidonate, in the absence of proteases, was similar. In ten experiments in which replicate monolayers of cells were stimulated with either thrombin or arachidonate and PG₁ production compared, PG₁ synthesis induced by thrombin was 84.5 ± 13.5% (mean ± SEM) of that induced by arachidonate. In addition, the production of PG₁ induced by either histamine or A23187 was also similar in amount to that induced by thrombin or arachidonate. Stimulation with any of the agonists elicited a three to 20-fold increase in PG₁ synthesis over baseline, unstimulated PG₁ production.

**Effects of cathepsin G and elastase on intracellular calcium mobilization in EC.** Confluent monolayers of HUVEC were detached from culture dishes by treatment with 0.01% EDTA or 0.05% collagenase and 0.05% EDTA in HBS containing 2.5 mg/mL of bovine serum albumin. The HUVEC were washed twice by centrifugation in fresh HBS, loaded with Quin 2/AM (final concentration, 5 μmol/L), treated with cathepsin or elastase for ten minutes at 37°C, and then washed. Treated HUVEC were then maintained at room temperature until used. Aliquots of the HUVEC suspensions (4 × 10⁶ cells/mL) were incubated in a Perkin-Elmer fluorescence spectrophotometer (Model 650-10S) with excitation set at 340 nm (2 nm slit) and emission set at 494 nm (5 nm slit) and fluorescence recorded during a five-minute exposure to buffer or thrombin. The change in intracellular calcium concentration ([Ca²⁺]i) following stimulation with thrombin was calculated by using the formula: [Ca²⁺]i = Kᵦ(F - Fₘᵦ)/(Fₘᵦ - F), where Kᵦ = 115 mmol/L, F = fluorescence of the unknown, Fₘᵦ = fluorescence of Triton-lysed cell suspensions in the presence of HBS containing 1.5 mmol/L Ca²⁺, and Fₘᵦ = fluorescence of the Triton-lysed cell suspension in the presence of 4 mmol/L EGTA, 20 mmol/L Tris base, pH 7.4. Intrinsic fluorescence of HUVEC suspensions was low and did not change with the above additions. A detailed description of this method has been recently published by our laboratory.³⁶

**Inhibitor studies.** HUVEC monolayers were treated with either elastase or cathepsin G (1 μg/mL) or with elastase or cathepsin G (1 μg/mL) that had been pre-incubated with α₁-antitrypsin (1:5.1 molar ratio of inhibitor:enzyme) or α₁-macroglobulin (1:2 molar ratio of inhibitor:enzyme), respectively. After washing with buffer, the HUVEC monolayers were stimulated with thrombin (0.5 U/mL) to induce PG₁ production and the monolayers were observed by phase microscopy for morphologic changes. Similar HUVEC monolayers were exposed to the PMN proteases, which had been preincubated with medium containing 10% pooled human serum (a source of both α₁-antitrypsin and α₁-macroglobulin), or the serum-containing medium was added after incubations with PMN proteases. Capacity to respond to thrombin by PG₁ production was tested immediately afterward.

**Recovery from the effects of protease treatment.** To ascertain if HUVEC treated with human PMN cathepsin G or elastase were damaged or permanently altered by the enzyme treatment, residual enzyme was inactivated by briefly incubating the monolayer of HUVEC with fresh medium containing 20% human serum, washing again with buffer, and covering the HUVEC monolayer with fresh medium containing 10% human serum. The monolayers of enzyme-treated HUVEC were then further cultured for five or 24 hours following enzyme treatment. The HUVEC monolayers were tested at these intervals during the "recovery" period for their capacity to produce PG₁, when stimulated by thrombin.

**Release of ³¹C-arachidonate from prelabeled EC.** Preconfluent HUVEC plated in T-25 tissue culture flasks were washed with HBS. Fresh medium containing 20% pooled human serum and 100 μmol/L ibuprofen (final concentration) was added to the HUVEC monolayers for ten minutes at 37°C. Ibuprofen, a reversible inhibitor of cyclooxygenase, was used to prevent activation of that enzyme during incubation of the HUVEC with labeled arachidonate and subsequent manipulations.³⁷ Next, 0.2 μCi of ['³¹C]-arachidonic acid was added to the incubation medium in a final volume of 4 mL/flask and the cells were incubated overnight at 37°C. The medium was removed, and the cell monolayers were washed with 1 mL aliquots of HBS containing 2 mg/mL of fatty-acid free bovine serum albumin (BSA) until minimal radioactivity remained in the washes. All washes to this point contained ibuprofen. The cells were covered with HBS and incubated with cathepsin G (1 to 5 μg/mL) for ten minutes. The buffer was removed and replaced with fresh HBS containing fatty-acid free BSA. The BSA was included as an arachidonate trap. Arachidonate release was stimulated by thrombin (2 U/mL) or the ionophore A23187 (10 μmol/L). The incubation fluids were collected and aliquots counted for released ['³¹C] by liquid scintillation counting.

**Prostacyclin measurement.** PG₁ released into the medium or into incubation buffers were measured by radioimmunoassay of its stable hydrolysis product 6-keto-PGF₁α in unextracted fluid as previously described.³¹ Each experimental variant was run in triplicate and replicates were assayed in duplicate.

**Evaluation of cell detachment.** All cultures of HUVEC were inspected by phase microscopy before and after protease treatment. Cell detachment was quantified by counting the cells present in the supernatant medium and, after washing the HUVEC monolayers, treating adherent HUVEC with 0.05% collagenase and 0.02% EDTA and counting the released cells in a Coulter Counter model ZBI.

**Statistical evaluation.** Data are presented as means ± SEM. Statistical calculations were performed using the SYSTAT statistics.
package (Evanston, IL). A value of \( P < .05 \) was considered significant.

**RESULTS**

*Effects of leukocyte proteases on prostacyclin production.* Both cathepsin G and elastase derived from human PMN selectively inhibited the capacity of HUVEC to produce PGI\(_2\) in response to stimulation with thrombin. Treatment of HUVEC with cathepsin G or elastase over the concentration range of 0.01 to 10 \( \mu \)g/mL inhibited thrombin-induced PGI\(_2\) production in a concentration-dependent manner (Fig 1); cathepsin G and elastase (10 \( \mu \)g/mL) inhibited PGI\(_2\) production by 85% and 79%, respectively. In contrast, arachidonic acid-induced synthesis of PGI\(_2\) by HUVEC exposed to these enzymes was not decreased by treatment with either enzyme (Fig 1) and a slight increase in arachidonic acid-induced PGI\(_2\) synthesis was observed after treatment with cathepsin G at 10 \( \mu \)g/mL (Fig 1A). The specificity of the enzyme effects on PGI\(_2\) responsiveness was further demonstrated by the fact that histamine-induced PGI\(_2\) synthesis was unchanged after treatment with cathepsin G (Fig 2) and was only minimally decreased (21%) after treatment with elastase. Moreover, neither cathepsin G nor elastase altered the PGI\(_2\) response to the calcium ionophore A23187 (data not shown).

The inhibitory effect of the leukocyte proteases on PGI\(_2\) production induced by thrombin was rapid, mainly occurring within one to five minutes; the effect increased very slightly over time.

The direct effects of these enzymes on PGI\(_2\) production by unstimulated HUVEC, in contrast, ranged from undetectable to slightly stimulatory at the concentrations used. Cathepsin G only modestly stimulated basal PGI\(_2\) production by HUVEC monolayers (18 ± 6% above control basal levels at 1 \( \mu \)g/mL and 46 ± 12% above basal levels at 10 \( \mu \)g/mL; N = 50 and 21, respectively; mean ± SEM), while exposure of HUVEC to elastase stimulated basal PGI\(_2\) production only at a concentration of 10 \( \mu \)g/mL and then to a variable degree (data not shown).

The concentrations of elastase that inhibited subsequent thrombin-induced PGI\(_2\) synthesis tended to cause detachment of the HUVEC from the culture dish (see below). We therefore designed experiments in which the buffer layer was not removed between treatment of the cell monolayer with elastase or cathepsin G and addition of thrombin, so that at harvest of the buffer after incubation with thrombin, both adherent and detached cells remained in the culture dishes. Results of such studies similarly showed that both enzymes inhibited thrombin-induced PGI\(_2\) synthesis in a concentration-dependent manner (data not shown). In addition, suspensions of HUVEC also showed decreased thrombin-induced PGI\(_2\) production after elastase treatment.

Under certain conditions, cathepsin G and elastase inter-
act in a synergistic fashion, such as in the degradation of elastin.\(^2\) The mechanism for such synergy appears to be enhanced cleavage of the initial substrate by elastase, which renders the fragments released more susceptible to cathepsin G digestion. However, a combination of the two proteases used at both subinhibitory and inhibitory concentrations did not increase the degree of inhibition of thrombin-induced PGI\(_2\) synthesis compared with the effects of either enzyme alone.

**Recovery of HUVEC function after protease treatment.** When HUVEC monolayers exposed to cathepsin G were then recultured in fresh medium after inactivation of cathepsin G, thrombin-inducible PGI\(_2\) production was restored in a time-dependent fashion (Fig 3). This recovery of thrombin-responsiveness could be delayed or prevented by addition of cycloheximide to the medium during the recovery period. The concentrations of cycloheximide used in these experiments (0.1 to 0.5 \(\mu\)g/mL) did not decrease cell viability as measured by trypan blue dye exclusion.

**Effects of protease inhibitors on HUVEC detachment.** Elastase rapidly induced morphologic changes in HUVEC monolayers; cell contraction was the first response to this enzyme, even at low concentrations. At 10 \(\mu\)g/mL elastase, immediate cell contraction was followed by cell detachment with marked disruption of the monolayer within two to three minutes. In contrast, cathepsin G, which also markedly altered PGI\(_2\) production induced by thrombin, did not cause HUVEC detachment, even at 10 \(\mu\)g/mL although it also induced transient contraction of the cells. The cells that detached with elastase remained viable by trypan blue staining, released PGI\(_2\) when stimulated with arachidonate, and could be replated with normal growth thereafter, suggesting that exposure to elastase did not injure the cells during their detachment from the extracellular matrix.

**Effects of protease inhibitors.** \(\alpha\)-Macroglobulin, one of the main plasma inhibitors of leukocyte proteases, suppressed the inhibitory effect of cathepsin G on thrombin-induced PGI\(_2\) synthesis (Fig 4A). Similarly, \(\alpha\)-antitrypsin suppressed the inhibitory effect of elastase (Fig 4B). To demonstrate that these protease inhibitors at the concentrations present in blood could block the inhibitory effects of these enzymes on PGI\(_2\) production by HUVEC, cathepsin G was preincubated with medium containing 10% serum and the mixture then added to HUVEC monolayers. Subsequent thrombin-induced PGI\(_2\) production was equivalent to that in control cultures (Fig 5). In contrast, if the serum was added to the cells after incubation with cathepsin G, subsequent thrombin-induced 6-keto-PGF\(_{1\alpha}\) production was significantly suppressed.

Preincubation of elastase with \(\alpha\)-antitrypsin or with serum, which contains \(\alpha\)-antitrypsin, also prevented the effects of elastase on cell detachment.

**Effect of cathepsin G or elastase treatment on the capacity of HUVEC to mobilize intracellular calcium.** Since thrombin induces rapid rises in cytosolic free calcium ion concentration ([Ca\(^{++}\)]) in HUVEC,\(^2\) the effect of enzyme pretreatment on calcium mobilization was evaluated. Suspensions of HUVEC preloaded with the calcium fluorescent indicator Quin 2 were first exposed to cathepsin G or elastase and then stimulated with thrombin. In these protease-treated HUVEC, the usual rise in [Ca\(^{++}\)], induced by thrombin was inhibited in a concentration-dependent manner (Fig 6). At the concentrations tested, cathepsin G and elastase had similar inhibitory effects, which paralleled their inhibition of PGI\(_2\) synthesis. However, in the absence of thrombin, neither cathepsin G nor elastase induced a rise in [Ca\(^{++}\)], (data not shown).

**Inhibition by cathepsin G of \(^{14}\)C-arachidonic acid release from prelabeled EC.** The effect of cathepsin G treatment of HUVEC on thrombin-induced activation of phospholipase was then tested. EC were prelabeled with \(^{14}\)C-arachidonic acid in the presence of ibuprofen to inhibit cyclooxygenase during arachidonate-loading. When these cells were stimulated by thrombin or ionophore, they released significant amounts of \(^{14}\)C-arachidonate into the medium compared with HUVEC stimulated only with buffer. Exposure of the prelabeled HUVEC to cathepsin G before the cells were stimulated with thrombin or ionophore resulted in total inhibition of \(^{14}\)C-arachidonate release induced by thrombin; the suppression of ionophore-induced \(^{14}\)C-arachidonate release was not significantly different from the control.
PMN PROTEASES SUPPRESS THROMBIN-INDUCED PG\textsubscript{I2}

Fig 4. Plasma protease inhibitors block the effects of cathepsin G and elastase on thrombin-induced PG\textsubscript{I2} production by HUVEC. (A) \(\alpha\)-Macroglobulin (\(\alpha\)-M) suppresses cathepsin G inhibition of thrombin-induced PG\textsubscript{I2} production. (B) \(\alpha\)-Antitrypsin (\(\alpha\)-AT) suppresses elastase inhibition of thrombin-induced PG\textsubscript{I2} production. In both A and B, HUVEC were treated with either buffer, the active enzyme (1 \(\mu\)g/mL), or the enzyme (1 \(\mu\)g/mL) inhibited by premixing with protease inhibitor at a 2:1 (\(\alpha\)-M) or 1.5:1 (\(\alpha\)-AT) molar excess. The cells were then stimulated with thrombin (0.5 U/mL). Data are expressed as percent of control after correction for unstimulated PG\textsubscript{I2} production (mean \pm SEM). Tukey's multiple comparison procedure at \(p < 0.05\) confirms that thrombin-induced PG\textsubscript{I2} production by HUVEC exposed to either enzyme plus the appropriate inhibitor is no different from that of control HUVEC and significantly different from HUVEC exposed to enzyme alone.

Fig 5. Serum blocks the effect of cathepsin G on thrombin-induced PG\textsubscript{I2} production. HUVEC were exposed either to cathepsin G in the absence of serum, cathepsin G premixed with 10% serum, or cathepsin G only during incubation and 10% serum added after the incubation. The cells were then washed and stimulated with thrombin (0.5 U/mL for five minutes) and release of 6-keto-PGF\textsubscript{1\alpha} assayed. \(P\) values (one way ANOVA) are indicated on the figure.

Fig 6. The inhibitory effects of cathepsin G and elastase on thrombin-induced rises in \([\text{Ca}^{2+}]\). Suspensions of Quin 2 loaded HUVEC, first treated with cathepsin G or elastase for ten minutes, were stimulated for five minutes with 2 U/mL thrombin and the change in \([\text{Ca}^{2+}]\), was measured by change in fluorescence as detailed in the Materials and Methods section. Both PMN proteases inhibited the rise in \([\text{Ca}^{2+}]\) in a dose-dependent manner (\(P < 0.008\), two-way ANOVA). There was no significant difference in the effects of the two PMN proteases on thrombin-induced rises in \([\text{Ca}^{2+}]\) over the concentration range tested (\(P = 0.448\), two-way ANOVA). The baseline \([\text{Ca}^{2+}]\) was 59.6 \pm 3.3 nmol/L and \([\text{Ca}^{2+}]\) after thrombin stimulation was 236.8 \pm 47.9 nmol/L (mean \pm SEM, \(N = 6\)); thrombin elevated \([\text{Ca}^{2+}]\) 3.97-fold over control values.

release was of much lesser degree and was not statistically significant (Fig 7). In contrast, the basal release of \(^{14}\text{C}\)-arachidonate induced by buffer was not altered by treatment with cathepsin G.

DISCUSSION

PMN can alter EC function during inflammation by a variety of mechanisms involving the release of enzymes, arachidonic acid products, and toxic oxygen species at the EC surface. Among the pathologic responses of EC to such assault are the acquisition of thrombogenic properties, alteration of prostaglandin production, cell contraction, cell detachment, and cell lysis.

Our studies demonstrate that the neutral proteases cathepsin G and elastase from human PMN, at concentrations that fall well within levels achievable in vivo (0.1 to 10 \(\mu\)g/mL equivalent to 4 nmol/L to 0.4 \(\mu\)mol/L), suppress thrombin-induced PG\textsubscript{I2} production by HUVEC in a manner consistent with cleavage of an EC membrane receptor for
following exposure to PMN neutral proteases suggests that phospholipase activity is likewise unaffected, since these stimuli activate endogenous phospholipases. This conclusion is further supported by the observation that only thrombin-induced release of 14C-arachidonate, but not ionophore A23187-induced arachidonate release, is inhibited by leukocyte protease treatment.

The parallel inhibition by PMN proteases of thrombin-inducible increases in \([\text{Ca}^{++}]\), and in PG\(_I_2\) supports the concept that a PMN protease-sensitive binding site for thrombin is necessary for thrombin-mediated activation of phospholipases A\(_2\) and C. Since we have previously shown that the thrombin-induced rise in inosine triphosphate (IP\(_3\)) is necessary for both the thrombin-induced rises in \([\text{Ca}^{++}]\), and in PG\(_I_2\) synthesis, our findings suggest that the same protease-sensitive binding site mediates both the rise in \([\text{Ca}^{++}]\), and in PG\(_I_2\) synthesis.

In their capacity to alter thrombin-mediated responses of HUVEC, cathepsin G and elastase are similar to chymotrypsin and platelet calcium-activated protease, other proteases that we have previously demonstrated inactivate or possibly cleave the thrombin receptor from the EC surface. Consistent with this concept, elastase was recently shown to diminish the number of high affinity thrombin binding sites on the surface of human platelets\(^{39}\) and cathepsin G has chymotryptic activity.\(^{12}\) Similarly, treatment of porcine aortic EC with PMN elastase blocks ATP- and bradykinin-induced arachidonate release and PG\(_I_2\) synthesis but not that induced by A23187, although chymotrypsin was ineffective.\(^{4}\) This differential susceptibility to chymotrypsin might relate to species and anatomic differences in the cells used or to the agonists tested (and hence the receptors affected).

We previously showed that elastase can alter the function of human \(\alpha\)-thrombin by limited proteolysis near the \(\gamma\)-cleavage site resulting in decreased fibrinogen clotting and platelet-stimulatory activity.\(^{41}\) However, the effects on EC we observed here were unlikely to reflect direct effects of cathepsin G or elastase on thrombin or their competition for thrombin binding sites. First, thrombin-induced PG\(_I_2\) release was suppressed by elastase in experiments in which elastase was neutralized and removed by washing before the EC were exposed to thrombin (Fig 1). Second, elastase-induced proteolysis of thrombin is much slower (\(\leq 10%\) within three minutes)\(^{41}\) than the induction of HUVEC PG\(_I_2\) synthesis by thrombin (\(\geq 90%\) of maximal PG\(_I_2\) synthesis within one minute).\(^{20}\) Third, thrombin pre-incubated with elastase induced as much PG\(_I_2\) synthesis as did thrombin pre-incubated with buffer.\(^2\) Last, it is unlikely that elastase competes for the thrombin binding site since active site-blocked elastase fails to interfere with the biologic activity of thrombin.\(^{41}\)

Since HUVEC bind thrombin by several independent mechanisms, eg, via heparan sulfate and thrombomodulin, and only a small amount of the thrombin bound probably participates in induction of PG\(_I_2\) synthesis, isotopic methods of measuring thrombin binding on HUVEC have not been useful in characterizing such sites.\(^{41}\) The demonstration of specific alterations of thrombin-responsiveness of HUVEC after PMN protease treatment may provide a sensitive

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![Graph](https://example.com/graph.png)

**Fig 7.** Cathepsin G suppresses thrombin-induced release of 14C-arachidonate from HUVEC. HUVEC monolayers were pre-loaded with 14C-arachidonate, exposed to cathepsin G for ten minutes, and then stimulated for five minutes with thrombin (2 U/mL), buffer, or the calcium ionophore A23187 (10 \(\mu\)mol/L). Thrombin stimulated cells showed a significant decrease in release of 14C-arachidonate compared with control cultures not exposed to cathepsin G \((P < .001\), one-way ANOVA). Buffer did not release 14C-arachidonate above basal levels \((P = .396)\). The decrease in ionophore A23187-induced 14C-arachidonate release after treatment with cathepsin G was not significant \((P = .628)\).

thrombin. It has been clearly demonstrated that human leukocyte extracts contain 1 to 12 \(\mu\)g elastase/10\(^7\) PMN.\(^{14,33-36}\) Assuming 5,000 PMN/\(\mu\)L, normal PMN in blood contain 0.5 to 6 \(\mu\)g elastase/\(\mu\)L, release 1.7 to 3.9 \(\mu\)g/mL elastase into blood during coagulation,\(^{37}\) and if stimulated, release up to 40 \(\mu\)g of elastase/10\(^7\) PMN or 20 \(\mu\)g/mL.\(^{39}\) If we assume that one PMN secretes its elastase (using a mid-level estimate of 6 \(\mu\)g elastase/10\(^7\) PMN or 20.3 \(\times 10^{-18}\) mol/PMN for a molecular weight of 29,500) while located in a capillary 6 \(\mu\)m in diameter and over the length occupied by one endothelial cell (\(\approx 50 \mu\)m yielding a volume \(\approx 1.4 \times 10^{-12}\) L), the elastase concentration in that localized space will be 14.4 \(\mu\)mol/L or 424 \(\mu\)g/mL. Thus in a localized area, only one PMN per endothelial cell could supply elastase far in excess of the concentrations that we have shown inhibit thrombin-induced PG\(_I_2\) production, although blood flow would tend to rapidly wash the released elastase away and bring plasma protease inhibitors into the area. However, if elastase is liberated locally into a space between apposed PMN and the surface of an EC, then its local concentration could be even higher and enzyme released into such a space would not be washed away nor exposed to protease inhibitors. The amount of cathepsin G in PMN is, on a weight basis, similar to that of elastase\(^{20}\) and thus the same reasoning applies to cathepsin G.

We have suggested that thrombin induces PG\(_I_2\) synthesis in HUVEC by activating phospholipase C thus forming inositol triphosphate, which elevates \([\text{Ca}^{++}]\).\(^{20}\) In turn, elevations in \([\text{Ca}^{++}]\), stimulate phospholipase A\(_2\) activity\(^{18}\) and release arachidonate that is used to synthesize PG\(_I_2\).\(^{17,39}\) The observation that arachidonate-induced PG\(_I_2\) synthesis is unaltered following exposure of HUVEC to cathepsin G and elastase suggests that PGH synthase (cyclooxygenase) and PG\(_I_2\) synthase are not affected. Similarly, normal induction of PG\(_I_2\) synthesis by histamine and the ionophore A23187

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The effects of cathepsin G and elastase on HUVEC are not completely parallel. Elastase, which degrades human fibronectin, causes contraction and detachment of HUVEC and thus appears to be the component released by activated PMN responsible for detaching EC from the subendothelium. In contrast, cathepsin G detaches HUVEC at similar concentrations despite the absence of elastase. Elastase interferes with the effects of elastase by inactivating plasma antiproteases. We found that HUVEC detached by elastase remained viable under the conditions of our studies in agreement with the studies of Harlan et al.

We observed little direct stimulation of PG12 synthesis when HUVEC were exposed to cathepsin G or elastase at concentrations that strongly inhibited thrombin-induced PG12 synthesis. Species and concentration differences probably account for the differences between our results and those reported by LeRoy et al. for porcine aortic EC, where PG12 production was strongly stimulated by cathepsin G (30-fold at 100 μg/mL) and 4.6-fold by pancreatic elastase (100 μg/mL) but only weakly so by PMN elastase. It is also possible that human microvascular EC might respond differently to these enzymes, as it has been found that HUVEC and microvascular EC respond very differently to H2O2 induced injury.

The effects of cathepsin G on HUVEC PG12 synthesis were reversible after removing the enzyme, indicating that the PMN-protease sensitive receptors responsible for thrombin-stimulated PG12 production are regenerated over time if protein synthesis is permitted. This situation is analogous to that following thrombin stimulation, when HUVEC become temporarily refractory to thrombin induction of PG12 synthesis, thrombin-responsiveness returning after some hours of reculture.

Plasma antiproteases that inactivate cathepsin G and elastase interfered with the effects of the PMN proteases on EC, as did serum. It has been proposed that in vivo, PMN injure vascular EC in a highly localized manner, perhaps best when tightly apposed to the EC surface, a setting that excludes plasma components such as protease inhibitors. We found that serum blocked the effects of cathepsin G and elastase observed under serum-free conditions, consistent with the concept that these enzymes are easily inactivated in the fluid phase. However, our results are at variance with those of LeRoy et al. who found that inhibition of elastase enzyme activity with CH2-O-Suc-Ala-Ala-Pro-Val-CH2Cl or furoyl saccharin did not ablate the inhibitory effect of elastase on ATP-induced PG12 production by porcine aortic EC. The reasons for this discrepancy are unknown.

Our studies indicate that isolated neutral proteases from human PMN specifically interfere with thrombin-induced PG12 production by HUVEC, and that these alterations occur rapidly at enzyme concentrations that do not induce severe cellular injury, since the enzyme-treated cells remain viable. These findings clarify and extend the studies of others with intact PMN or lysosomal extracts that implicated leukocyte elastase as the mediator of EC detachment, since only purified elastase, not cathepsin G, caused detachment. Because plasma antiproteases at physiological concentrations readily block these PMN proteases, close proximity between PMN and EC is most likely necessary for secreted cathepsin G and elastase to affect EC in vivo. Our experiments using purified enzymes serve to indicate that individual PMN neutral proteases have specific injurious effects on EC independent of the effects of toxic oxygen products.

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BB Weksler, EA Jaffe, MS Brower and OF Cole