Effects of Neutrophil Elastase and Other Proteases on Porcine Aortic Endothelial Prostaglandin I_2 Production, Adenine Nucleotide Release, and Responses to Vasoactive Agents

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Abstract. The effects of neutrophil elastase on endothelial prostacyclin (PGI_2) production, nucleotide release, and responsiveness to vasoactive agents were compared with the effects of cathepsin G (the other major neutral protease of neutrophils), pancreatic elastase, trypsin, chymotrypsin, and thrombin. PGI_2 production by pig aortic endothelial cells cultured on microcarrier beads and perfused in columns was stimulated in a dose-dependent manner by trypsin, chymotrypsin, and cathepsin G (1–100 μg/ml for 3 min). Thrombin, while active at low concentrations (0.1–10 National Institutes of Health U/ml), induced smaller responses. Neutrophil and pancreatic elastase had little or no effect on PGI_2 production. Dose-dependent, selective release of adenine nucleotides was induced by neutrophil elastase (3–30 μg/ml). The other proteases were much less active; for example, trypsin (100 μg/ml) induced a response only ~5% as great as did 30 μg/ml neutrophil elastase. After exposure to 30 μg/ml neutrophil elastase, cells did not exhibit the characteristic burst of PGI_2 production in response to extracellular ATP; responsiveness gradually returned after 40–120 min. This effect was not seen with the other proteases. Elastase partly inhibited responses to bradykinin and had no effect on PGI_2 production that was stimulated by ionophore A23187. There was no evidence of cytotoxicity, as measured by release of lactate dehydrogenase. Neutrophil degranulation can generate concentrations of elastase and cathepsin G comparable with those tested in the present study, and the effects of these enzymes on endothelial function lead us to suggest that they may play a role in vasoregulation and vascular pathology.

Introduction

It is now widely recognized that the endothelium is not merely a passive vascular lining but plays an active role in physiological processes such as hemostasis, vasoregulation, and inflammation (1). Endothelial cells contribute to hemostasis by secreting prostacyclin (PGI_2)[1] (2, 3), a powerful inhibitor of platelet function, which is also a vasodilator, and to vasoregulation through prostacyclin and through the phenomenon of endothelium-dependent vasodilation (4), which is induced by several agents including ATP, bradykinin, and thrombin (5, 6) and involves mediator(s) other than PGI_2 (7). Endothelial contributions to inflammation include the regulation of leukocyte traffic (neutrophils have a particular affinity for endothelium) and the control of vascular permeability, which is mediated by agents acting either directly on endothelial cells or indirectly, by activating neutrophils, which then secrete constituents (as yet unidentified) that affect the endothelium (8).

The capacity of activated neutrophils to damage endothelial cells has been noted both in vitro and in vivo, and neutrophil proteases have been implicated in this endothelial cytotoxicity (9–11). The endothelial damage recorded in these studies resulted in cell death, whereas neutrophil-dependent increases

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1. *Abbreviations used in this paper:* DMEM, Dulbecco's modified Eagle's medium; LDH, lactate dehydrogenase; PGE_2 and $PGF_{1\alpha}$, prostaglandins E_2 and $F_{1\alpha}$, respectively; PGI_2, prostacyclin, prostaglandin I_2.

in vascular permeability involve more subtle (and, presumably, reversible) effects; as potentially lethal stimuli can, at lower concentrations, exert transient, nonlethal effects on endothelial functions (12), it seemed possible that neutrophil proteases might, under some conditions, reversibly affect endothelial functions involved in vasoregulation. Other proteases are known to exert such effects: Thrombin induces endothelium-dependent vasodilation (13), and both thrombin and trypsin stimulate prostacyclin production (14) and the selective release of nucleotides from endothelium (15, 16). Therefore, we set out to determine whether the major neutral proteases of human neutrophils (elastase and cathepsin G) affected PGI_2 production and/or nucleotide release from endothelial cells, and whether endothelial responses to stimuli such as ATP, bradykinin, and ionophore A23187 were altered after exposure to these enzymes. Possible cytotoxicity was assessed morphologically and biochemically, and the effects of the neutrophil enzymes were compared with those of other neutral proteases. Our results showed a distinctive spectrum of effects of neutrophil elastase on endothelial cell function, different from that of other proteases studied. These effects have implications for the in vivo modulation of inflammation and vasoregulation.

Methods

Materials. Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), trypsin (EC 3.4.21.4 bovine pancreatic, 10,000–13,000 *N*-α-benzoyl-L-arginine ethyl ester U/mg protein), thrombin (human plasma, 3,000 National Institutes of Health (NIH) U/mg protein), α-chymotrypsin (EC 3.4.21.1, type I-S from bovine pancreas), bradykinin triacetate, ATP (disodium salt from equine muscle), *N*-α-benzoyl-DL-arginine *p*-nitroanilide HCl, pyruvate, and α-nicotinamide adenine dinucleotide (NADH) were all purchased from Sigma Chemical Co., Poole, United Kingdom. Trypsin (1:250) was also obtained from Difco Laboratories, Detroit, MI. Calcium ionophore A23187, from Calbiochem-Behring, Cambridge, United Kingdom was stored at −20°C in dimethyl sulfoxide (stock concentration was 10^{-2} M).

Neutrophil elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) were prepared by Drs. J. Saklatvala and A. J. Barrett from human leukocytes obtained by leukophoresis from a patient with chronic myelogenous leukemia (17). Pancreatic elastase was prepared from pig pancreas by Dr. James Travis, University of Georgia, Athens. A specific inhibitor of neutrophil elastase of the chloromethylketone class, CH_3O-Suc-Ala-Ala-Pro-Val-CH_2Cl, was provided by Dr. James C. Powers, Georgia Institute of Technology, Atlanta (18). Furoyl saccharin, a structurally unrelated inhibitor of elastase (19), was provided by Dr. Morris Zimmerman, Merck Institute, Rahway, NJ.

Endothelial cell columns were perfused either with Dulbecco's modified Eagle's medium (DMEM) to which 20 mM Hepes had been added to maintain the pH at 7.4 or with Krebs' solution of the following composition (in millimolar): NaCl, 119; KCl, 3.1; $MgSO_4$, 0.6; $NaHCO_3$, 25; KH_2PO_4, 1; $CaCl_2$, 1.3; and glucose, 11.1; pH was maintained at 7.4 by gassing the solution with 95% O_2:5% CO_2.

Agonists were diluted, at least 100-fold, in the appropriate perfusion medium immediately before use. Unless otherwise stated, cells were exposed to ATP and bradykinin for 2 min, to neutral proteases for 3 min, and to A23187 for 5 min.

Endothelial cell culture. Endothelial cells were isolated from porcine thoracic aorta, essentially as previously described (20), plated in 25-cm^2 tissue culture flasks, and grown to confluence at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured using 0.1% trypsin (Difco Laboratories, 1:250)/0.25% EDTA, seeded onto 2.0-ml microcarrier beads (5×10^5 beads; Biosilon, A/S Nunc, Roskilde, Denmark) in 70 ml of growth medium, and stirred intermittently (21) until >50% of bead surface was covered (~$5–10 \times 10^6$ cells; up to 10 d). To visualize endothelial cells, an aliquot of beads was fixed in ethanol and stained with 0.1% methyl violet in water.

Some experiments measuring ^{3}H-purine release were performed with cells grown for 6–12 passages (1:2 split when confluent) before seeding onto microcarrier beads. Experiments measuring PGI_2 production were performed only with cells in first passage, since PGI_2 production has previously been shown to decline during multiple subpassages (21). No differences in purine release were noted in subcultured cells.

Endothelial cell columns. 0.4–0.5 ml of microcarrier beads covered with endothelial cells ($1–2 \times 10^6$ cells) was packed into a 1-ml plastic syringe plugged with glass wool; the syringe was closed with a rubber-tipped plunger through which plastic tubing had been threaded, as previously described (7, 21). Columns were continuously perfused from below with either Hepes-buffered DMEM or Krebs' solution at 0.35 ml/min in a 37°C chamber, and fractions were collected at 1-min intervals. All columns were preperfused for at least 60 min without agitation before starting experiments. Pump tubing fluid volume was <0.3 ml.

Release of prostaglandins. Samples of column fractions (5–20 μl) were assayed directly for the stable product of PGI_2 (6-oxo-prostaglandin $F_{1\alpha}$ [$PGF_{1\alpha}$]) and for prostaglandin E_2 (PGE_2) by radioimmunoassay (22).

Release of ^{3}H-purines. To determine purine release, cells were prelabeled with [^{3}H]adenosine for 60 min as follows: columns were washed for 5–10 min with serum-free DMEM, and then 5 μCi/ml [2-^{3}H]adenosine (22 Ci/mmole, Amersham International, Amersham, United Kingdom) in the same medium was recirculated at 0.35 ml/min through the column for 60 min at 37°C from a 5-ml reservoir, using a closed circuit system. Approximately 55% of the label was taken up by the cells after 60 min, and >90% of this intracellular label is in the form of adenine nucleotides (mainly ATP) (20). The closed circuit was then disconnected and the column was perfused for 15 min with medium or buffer to remove unincorporated radioactivity. Fractions were then collected every minute onto trichloracetic acid (TCA) (final concentration, 5%) and 50-μl aliquots were counted to determine total ^{3}H-purine release. At the end of the experiment, cell-associated radioactivity on the column was measured after solubilizing cells in 0.5 ml 0.2% Triton X-100. The profile of ^{3}H-purines released was determined by thin-layer chromatography of 40-μl aliquots after neutralizing the samples with 5 M K_2CO_3 (20). Columns ($1–2 \times 10^6$ cells) usually contained $3–10 \times 10^6$ cpm and released ~0.15% min^{-1} of this total when perfused with DMEM alone.

Endothelial cell cytotoxicity. The effects of proteases on cell morphology, vital dye exclusion, and release of lactate dehydrogenase were determined to detect possible cytotoxicity. Endothelial cells were plated at confluent densities (~10^5 cells/well) in 16-mm diameter wells of tissue culture plates and used within 2 d. Cells were rinsed once with phosphate-buffered saline (PBS) to remove serum and incubated with proteases for up to 15 min in Krebs' solution at 37°C. Cell morphology and the percentage of cells excluding trypan blue (0.1% in PBS) were assessed visually.

The release of lactate dehydrogenase (LDH) from cells in static culture and from columns was assayed according to standard techniques (23) as follows: cells were solubilized in 0.2% Triton X-100 or column fractions were made up to 1.0 ml in 0.2% Triton X-100. 40 μl each of pyruvate and NADH, both at 2.5 mg/ml in PBS, were added and the reaction was followed at room temperature in a double-beam spectrophotometer. The reference cuvette lacked only NADH.

Protease assays. Two assays of protease activity were used, one for trypsinlike activity and another specific for neutrophil elastase activity. The trypsinlike protease assay was a modification of method II of Erlanger et al. (24); it measured the yellow product, *p*-nitroanilide (E_{410} nm) cleaved from the synthetic substrate benzoyl-D-L-arginine *p*-nitroanilide HCl by trypsin and related serine proteases. 5 M sodium formate, pH 3.0, was used to stop the reaction. The neutrophil elastase assay measured the fluorescent product, 7-amino-4-methylcoumarin (NMec), which is cleaved from the synthetic substrate CH_3O-Suc-Ala-Ala-Pro-Val-NMec (provided by Dr. A. J. Barrett). Trypsin, chymotrypsin, and cathepsin G do not cleave this substrate under the conditions of the assay (25).

Protease assays were used routinely to standardize enzyme activity in all preparations used, to monitor the recovery of enzymes in the column effluent, and to check the effects of inhibitors.

Analysis of results. To compare results obtained in different columns, responses were expressed as multiples of the base line value, defined as the ratio of peak response to the mean of five values immediately before exposure to the stimulus. To compare the effects of repeated stimuli on the same column, each net response (peak minus base line) was expressed as a percentage of the net response to the initial stimulus.

Results

Effects of proteases on endothelial cell functions

Stimulation of prostacyclin production. Trypsin, chymotrypsin, and cathepsin G were all potent stimulators of PGI_2 production, each with a threshold active concentration <1.0 μg/ml. Fig. 1 shows the dose-response in one experiment with trypsin (1–100 μg/ml). Stimulated PGI_2 production values (mean±SEM) for 1, 10, and 100 μg/ml were 3.2±0.6-fold (n = 6), 12.8±2.5-fold (n = 5), and 16.2±4.5-fold (n = 5), respectively, over base-line value. Stimulation of PGI_2 production was immediate in onset, reached a peak ~ 2 min after addition of the stimulus, and returned towards base line while the stimulus was still present. Patterns of responses to chymotrypsin and cathepsin G were similar to that seen with trypsin: For example, mean responses obtained were 7.4-fold stimulation with 10 μg/ml chymotrypsin and 30-fold stimulation with 100 μg/ml of cathepsin G.

Of the other proteases tested, thrombin and pancreatic elastase stimulated PGI_2 production with a threshold active concentration of 3–10 μg/ml, but maximal stimulation was only 2.8-fold with thrombin (10 NIH U/ml) and 4.6-fold with pancreatic elastase (100 μg/ml). Neutrophil elastase was also a weak stimulator of PGI_2 production, with a threshold active concentration of 10–30 μg/ml and little effect even at 100 μg/ml (Fig. 1).

Stimulation of ^{3}H-purine release. Basal release of purines was 0.15±0.02% min^{-1} of the labeled nucleotide pool (mean±SEM, n = 13, range 0.05–0.29%). Of the proteases studied, neutrophil elastase was the most potent stimulus of ^{3}H-purine release. The lowest effective concentration was ~3 μg/ml, and mean responses for 3, 10, and 30 μg/ml were 2.9±0.2-fold (n = 3), 9.8±2.0-fold (n = 5), and 92.4±7.1-fold, respectively, (n = 3) over base-line values. Fig. 2 shows the effect of 1–30 μg/ml of neutrophil elastase on ^{3}H-purine release in a representative experiment. The response was immediate in onset, peaks occurred ~4min after addition of the stimulus and, in contrast to PGI_2 production, returned toward base line only after removal of the stimulus. Trypsin and pancreatic elastase induced maximal responses of only 3.5±0.5-fold (n = 3) and 1.7-fold over base line, respectively, at 100 μg/ml (Fig. 2), and thrombin, chymotrypsin, and cathepsin G were also much less effective than neutrophil elastase (e.g., thrombin, at 10 NIH U/ml, stimulated release by 2.4±0.2-fold; n = 3).

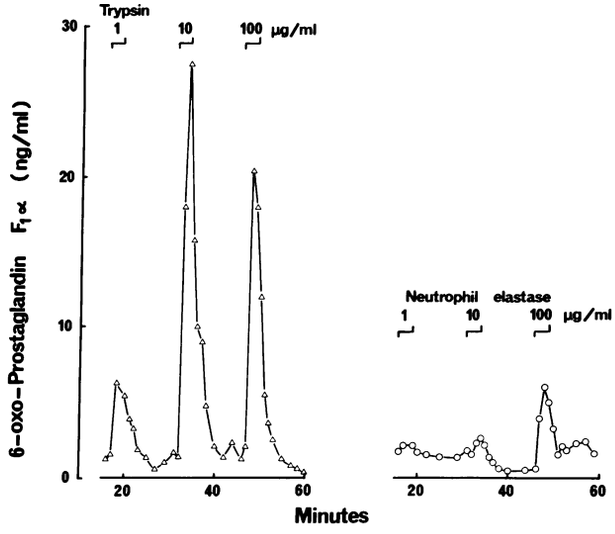

Figure 1. Prostacyclin production by endothelial cells on microcarrier bead columns in response to trypsin or to neutrophil elastase. Preperfusion was for 60 min at 0.35 ml/min before collecting fractions every minute. Column volume was 0.4 ml, estimated to contain 1–2 × 10^6 cells. Enzymes were dissolved in DMEM, brought to 37°C immediately before testing, and perfused for 3 min; in this and in all subsequent Figures, the duration of infusion of each stimulus is indicated by the length of the horizontal bars. Prostacyclin was assayed in 20-μl aliquots of eluate as 6-oxo-$PGF_{1\alpha}$ (see Methods).

Thin-layer chromatography of the released ^{3}H-purines showed that neutrophil elastase selectively stimulated the release of nucleotides (ATP, ADP, AMP) rather than nucleosides (inosine, hypoxanthine, adenosine). Basal release of ^{3}H-nucleotides from unstimulated cell columns was <0.05% per min; this rose to a maximum of 0.9% per min after 100 μg/ml trypsin and 6.4% per min after 30 μg/ml elastase. These values were obtained by chromatography of samples using a solvent system (26) that resolves individual nucleosides but not nucleotides. Nucleoside release in these stimulated responses was small (<25% of all ^{3}H-purines) and comprised mainly adenosine, with very little inosine and hypoxanthine; in contrast,

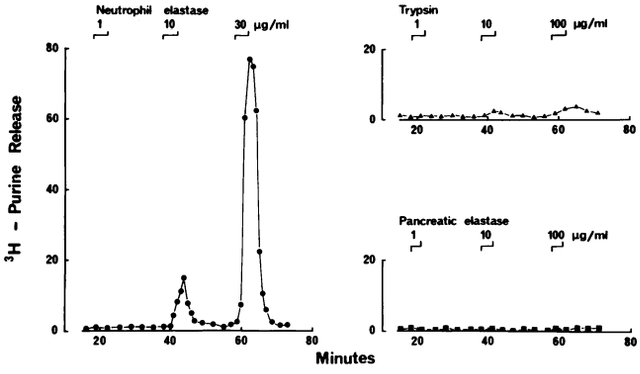

Figure 2. Release of ^{3}H-purines from endothelial cells on microcarrier bead columns in response to neutrophil elastase, trypsin or pancreatic elastase. Cells and columns were as described in Methods and in Fig. 1 legend. Endothelial cell purines were prelabeled by incubating the cells with [^{3}H]adenosine for 60 min (see Methods). Results are expressed as multiples of base-line ^{3}H-release (see Methods). Fractions were collected every minute. Effluent (0.35 ml/min) was collected into 60 μl of 50% TCA to inhibit nucleotide breakdown.

the small amount of purine release in unstimulated samples comprised up to 85% nucleosides. Chromatography under conditions that resolve nucleotides (20) revealed that substantial amounts of ADP and AMP were present in addition to ATP; the proportion varied in different samples. As ATP is by far the most abundant nucleotide within endothelial cells (20), the presence of the AMP and ADP suggests that released ATP may be metabolized by endothelial ectonucleotidases, as found in previous studies (15).

Endothelial cell cytotoxicity. Incubation with 30 μg/ml neutrophil elastase for 5 min at 37°C had no effect on cell morphology, or on trypan blue exclusion (~99% in control and treated cultures). Some uptake of trypan blue was noted after 15 min incubation with 30 μg/ml neutrophil elastase (though not with 3 or 10 μg/ml), as well as detachment of some cells from the culture vessel.

Basal release of LDH from columns was 0.78±1.16%/min (n = 11) of total column activity and varied up to 2.5-fold during individual experiments. There was no increase in LDH release during a 3-min infusion of neutrophil elastase at concentrations up to 30 μg/ml (mean, 0.67% min), nor over the 20-min period after the elastase infusions. This indicates that under these conditions elastase neither induced leakage of LDH nor detached cells from the beads. Infusion of 100 μM ATP, either before or after elastase, had no effect on LDH release (0.36%/min), and the other proteases tested were also without effect on LDH release.

Effects of proteases on endothelial cell responses to vasoactive agents

ATP-induced PGI_2 production. ATP (100 μM) stimulated PGI_2 production by 18.2±3.2-fold over basal production (n = 11). When cells were challenged a second time with ATP (100 μM) 20–40 min after the first challenge, the stimulation of PGI_2 production was similar to the initial response (96.0±1.7%, n = 5). Since the proteases tested had variable effects on PGI_2 production and on purine release, the effects of exposure to these proteases on subsequent PGI_2 responses to ATP were studied. As shown in Fig. 3, neutrophil elastase (30 μg/ml), itself a weak stimulator of PGI_2 production but a potent stimulator of ^{3}H-purine release, completely eliminated the PGI_2 response to a second challenge with ATP; the second response was 1.3±0.3% of the initial response to ATP (n = 5). In contrast, PGI_2 responses to a second challenge with ATP after exposure to pancreatic elastase, thrombin, and chymotrypsin (all at 30 μg/ml) averaged 95% of initial ATP responses (n = 3); these three proteases behaved similarly in not affecting PGI_2 responses to a second challenge with ATP, despite substantial differences in their potency as direct stimulators of PGI_2 production (Fig. 3). Trypsin, another potent stimulus to PGI_2 production, also had no effect on subsequent responses

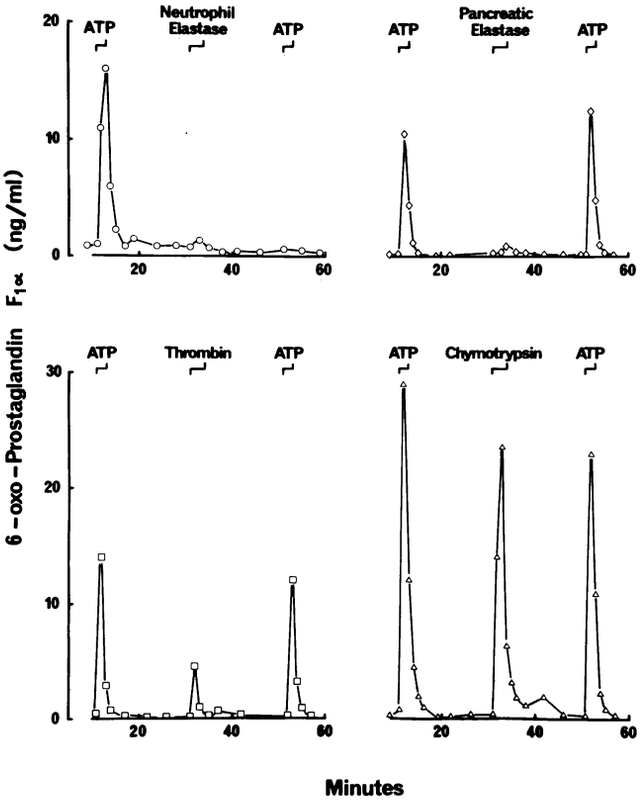

Figure 3. Prostacyclin production by endothelial cells on microcarrier beads in response to ATP before and after exposure to neutrophil elastase, pancreatic elastase, thrombin, or chymotrypsin. Enzymes (30 μg/ml), cell columns, and aliquots for assay were prepared as described in Fig. 2 legend.

to ATP. The minimum concentration of neutrophil elastase required to inhibit responses to ATP was ~10 μg/ml, and prolonged (15 min) exposure to a subthreshold concentration of elastase (1 μg/ml) did not affect subsequent responses to ATP (Fig. 4). ATP also stimulates the production of PGE_2 by endothelial cells (27), and in some samples the effect of elastase on ATP-induced PGE_2 production was determined; PGE_2 was inhibited in parallel with PGI_2. In separate experiments, we found that the ability of ATP to release [^{3}H]arachidonate from prelabeled endothelial cells (22) was also inhibited after exposure to elastase.

The duration of the inhibition of ATP-stimulated prostaglandin production by neutrophil elastase was studied by repeated additions of 100 μM ATP at varying intervals after exposure to elastase (30 μg/ml). PGI_2 production in response to ATP was abolished 20–30 min after elastase but increased progressively from ~40 min onwards, returning to normal after ~2 h.

Effects of neutrophil elastase on stimulation of PGI_2 production by bradykinin and ionophore A23187

To determine whether neutrophil elastase inhibited PGI_2 production induced by agents other than ATP, responses to bradykinin and ionophore A23187 were studied. The PGI_2 response to 3×10^{-8} M bradykinin was reduced by ~90%, 20 min after exposure to elastase (30 μg/ml). Desensitization was seen with repeated exposure to bradykinin alone (second responses were about half of initial responses), which made elastase effects difficult to quantify, but in all experiments responses after elastase, when compared with responses to bradykinin alone, were consistently reduced although not abolished.

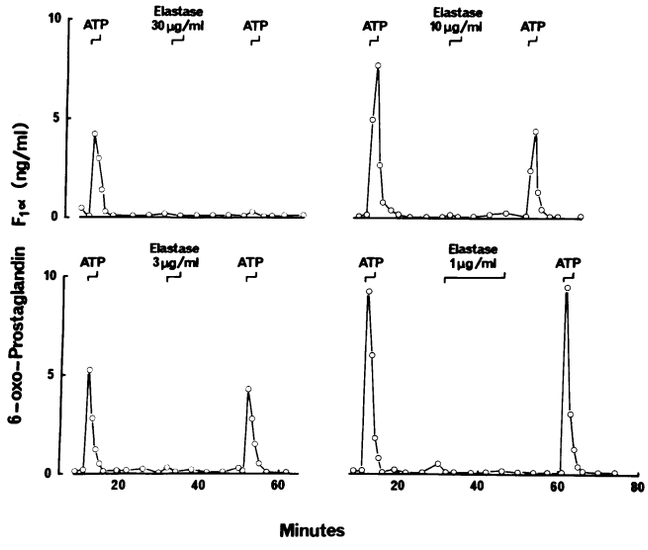

Figure 4. Prostacyclin production by endothelial cells on microcarrier bead columns stimulated by ATP (100 μM) before and after varying concentrations (1, 3, 10, 30 μg/ml) of neutrophil elastase for 3 or 15 min as indicated. Details are outlined in Fig. 1 legend.

The calcium ionophore A23187 was a potent stimulus of PGI_2 production but endothelial cells do not respond to successive challenges. Therefore, to test the effects of granulocyte elastase on responses to A23187, two cell columns prepared from the same microcarrier culture, only one of which was exposed to neutrophil elastase, were both exposed to A23187 at identical times from the onset of perfusion. Cells not exposed to elastase showed a 96.4±19.6-fold increase in PGI_2 production to A23187 (10 μM, 5 min); columns exposed to elastase (30 μg/ml, 3 min exposure 40 min before A23187) showed a 91.2±13.3-fold increase over basal PGI_2 production ($n = 4$). Thus, endothelial PGI_2 production in response to A23187 is unaffected by prior exposure to neutrophil elastase.

Effects of neutrophil elastase after enzyme inhibition

To determine the role of the catalytic site in the effects of neutrophil elastase on endothelial cells, concentrations of elastase known to abolish subsequent PGI_2 responses to ATP were incubated for 1 h with fivefold molar excess concentrations of the specific chloromethylketone inhibitor of neutrophil elastase, CH_3O-Suc-Ala-Ala-Pro-Val-CH_2Cl (12). This abolished elastase activity against a specific substrate (see Methods). As shown in Fig. 5, neutrophil elastase (30 μg/ml) previously incubated with inhibitor had virtually the same effect on PGI_2 responses to ATP as the enzyme alone; similar results were obtained with furoyl saccharin, a structurally unrelated elastase inhibitor (19). Furthermore, neutrophil elastase-inhibitor mixtures were as powerful stimuli of ^{3}H-purine release as were comparable concentrations of neutrophil elastase alone. The inhibitor alone had no effect on ^{3}H-purine release or on responses to ATP. Heat treatment of the elastase preparation (100°C for 5 min) did not block its effect on endothelial cells, but preincubation with serum inhibited its effect.

The resistance of this elastase effect to enzyme inactivation contrasts with stimulation of endothelial PGI_2 production by trypsin (which we found was abolished by heat inactivation or soybean trypsin inhibitor) or by thrombin (which is abolished by treatment with diisopropylfluorophosphonate; see reference 16).

Discussion

The results of our present study demonstrate that neutrophil elastase, although a weak stimulator of PGI_2 production compared with some other neutral proteases, inhibited PGI_2 production in response to other stimuli, especially ATP. Furthermore, in contrast to the other neutral proteases studied, neutrophil elastase was a powerful stimulator of adenine nucleotide release. Responses to neutrophil elastase were immediate, of brief duration, and were not associated with endothelial cell injury as measured by LDH release. Accurate evaluation of such effects can be made only with the perfused

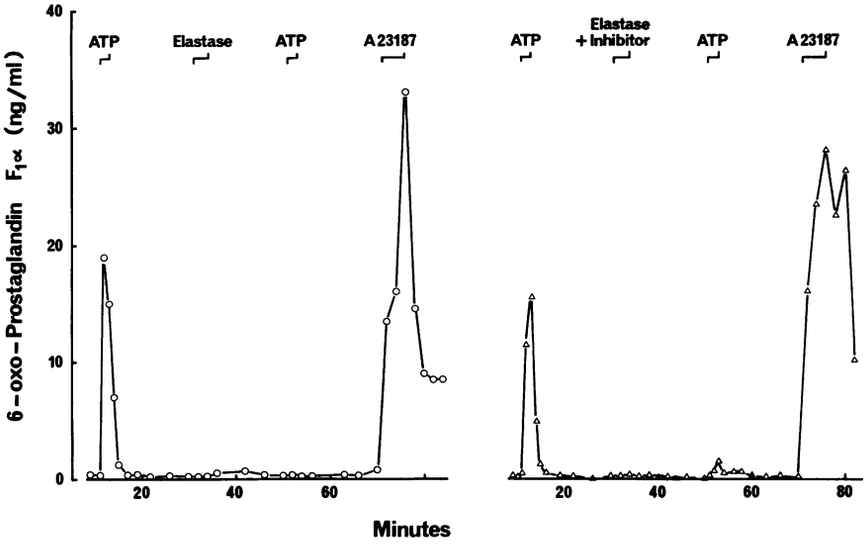

Figure 5. Prostacyclin production by endothelial cell columns stimulated by ATP (100 μM) before and after either neutrophil elastase (30 μg/ml, 1 μM) or neutrophil elastase plus inhibitor (see Methods, 5 μM) followed by stimulation with ionophore A23187 10 μM). Details are outlined in Fig. 1 legend.

bead-column technique, which enhances stimulator effects and permits kinetic analysis of individual responses to repeated stimuli and of the interactions between different stimuli without medium change or mechanical intervention.

Stimulation of endothelial PGI_2 production by thrombin, trypsin, and chymotrypsin (14) was confirmed, and the kinetics of responses explored. Cathepsin G, another powerful stimulant, has not been tested previously. This enzyme is one of the two most abundant neutral proteases in human neutrophils (the other being elastase) and is also released when neutrophils degranulate. The concentrations of 6-oxo-$PGF_{1\alpha}$ measured in column eluates after stimulation with cathepsin G indicate that similar responses in vivo would have profound biological effects: peak concentrations were 5–30 ng/ml, and PGI_2 at concentrations around 0.1 ng/ml inhibits platelet aggregation (2).

Neutrophil elastase (3–30 μg/ml) stimulated purine release, whereas other proteases tested at comparable concentrations had little or no effect. Previous reports of trypsin-induced nucleotide release used 1 mg/ml, the concentration routinely used to subculture cells (15); the present study showed that trypsin (100 μg/ml) stimulated nucleotide release (as distinct from total ^{3}H-purine release) by sixfold. This can be compared with >200-fold stimulation of nucleotide release by 30 μg/ml neutrophil elastase.

Neutrophil elastase also inhibited ATP-stimulated endothelial cell PGI_2 production; the mechanisms responsible are not yet known, but the possibilities are: (*a*) nonspecific cytotoxic effects; (*b*) receptor desensitization through release of ATP; (*c*) a direct effect of elastase on the receptor for ATP, either by proteolysis or via nonenzymic binding to the receptor. Relevant arguments include: (*a*) elastase did not have conventional cytotoxic effects under the conditions of our experiments, and did not disrupt the prostaglandin synthetic machinery (stimulation of PGI_2 production by ionophore A23187 was unaffected); (*b*) stimulation of PGI_2 production by repeated additions of ATP (100 μM) showed no desensitization; (*c*) inhibition of the catalytic site with a specific inhibitor did not block the effects of neutrophil elastase on endothelial cells. Thus, neutrophil elastase probably exerts its effects on endothelial cells by nonenzymic binding to the cell surface, as has been described for mononuclear cells (28). The concept that proteins can have multiple domains with biological activity is not unique to elastase: Several such examples are known, and some of these activities (like the ability of elastase to release purines from endothelial cells) are resistant to heat treatment; for example, the routine procedure for purification of platelet-derived growth factor involves boiling the preparation, and heating granulocyte cationic proteins increases their bactericidal and cytotoxic activity while destroying their proteolytic activity (29, 30).

The question remains as to which domain(s) on the elastase molecule might be involved in functional interactions with the endothelial surface. Recent studies in our laboratory (Needham, L. A., and J. L. Gordon, unpublished results) have shown that cationic (but not uncharged) polymers such as polylysine, polyarginine, and polyornithine induce ^{3}H-purine release from endothelial cells, and that their effectiveness increases with molecular size. This suggests that the elastase domain responsible for stimulating endothelial cells contains an assembly of basic amino acids. The possibility that the effects observed with elastase were caused by some contaminant introduced during preparation of the enzyme seems unlikely, because three different enzyme preparations were tested, in addition to two unpurified preparations of neutrophil granule extracts, which also stimulated purine release.

There is evidence that the effects of neutrophil granule proteins on endothelial cells are important in the inflammatory

process: Endothelial damage by activated neutrophils in vitro has been attributed to the release of proteases (11), and endothelial injury in vivo has been observed after neutrophil activation and degranulation but not after granulocyte-endothelial cell contact alone (9, 10). It should be noted that although previous studies in vitro (like our own experiments) used endothelial cells cultured from large vessels, most neutrophil-endothelial interactions in vivo take place in the microcirculation; whether endothelial cells from these different vascular sites respond similarly to neutrophil products remains to be determined. Previous studies of neutrophil-endothelial interactions in vitro usually determined cell death as the end point of injury, whereas the present study deals mainly with more subtle and reversible effects of neutrophil elastase on endothelial functions; however, we also noted that increasing the concentrations and/or exposure time resulted in cytotoxic effects, consistent with the concept that a potentially damaging stimulus can reversibly affect cell functions at lower concentrations than those that are cytotoxic (12). The concentrations of neutrophil elastase to which endothelial cells might be exposed in vivo are difficult to predict accurately but, on the basis of the extent of neutrophil-endothelial interaction that has been observed (8, 10, 31) and the content of elastase in human neutrophils (32), we can calculate that degranulation of neutrophils in the microcirculation could produce local concentrations of elastase in the range of 10–100 μg/ml, with concentrations in the microenvironment at the endothelial surface transiently even higher. The effects of this released elastase on endothelium would depend largely on the efficiency with which plasma inhibitors acted: We found that incubation with serum or plasma blocked the effects of elastase on endothelial cells but it is known that where there is intimate cell-substrate contact (as occurs in neutrophil-endothelial adhesion; see references 8, 10, 31) plasma inhibitors are, at least temporarily, ineffective (33).

The reversible effects of neutrophil elastase on endothelial cells may be important in the context of vasoregulation, because ATP is a potent vasoactive agent (34) that stimulates the release of vasodilator prostaglandins (including PGI_2) from vascular beds (35–36) and from endothelial cells in culture (reference 27 and Fig. 3), and also induces endothelium-dependent vasodilation (5, 6) in some blood vessels (13) by an unknown mechanism not involving prostaglandins (5, 7). Thus, the ability of neutrophil elastase to release nucleotides from endothelial cells could be expected to result in vasodilation, were it not for the fact that elastase temporarily blocks ATP-induced PGI_2 production. Whether elastase also blocks ATP-induced endothelium-dependent relaxation has not yet been established, and therefore it is not clear at present if exposure of endothelium to elastase in vivo would compromise all ATP-induced vasodilation, or only in those vessels where the effect of PGI_2 predominated. This topic merits investigation, because loss of endothelium-dependent vasodilation results in exaggerated vasoconstrictor responses (37), which might contribute to the paradoxical vasospasm seen in some conditions of peripheral vascular disease, such as those associated with Raynaud's phenomenon (38).

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