Inactivation of Endothelin by Polymorphonuclear Leukocyte-Derived Lytic Enzymes

By Paola Patrignani, Aldo Del Maschio, Gianfranco Bazzoni, Luisa Daffonchio, Alicia Hernandez, Rossana Modica, Lili Montesanti, Daniele Volpi, Carlo Patrono, and Elisabetta Dejana

Cultured bovine aortic endothelial cells (BAEC) released endothelin-1 (ET-1) in the culture medium in a time-dependent fashion. Coincubation of fMLP-activated human polymorphonuclear leukocytes (PMN) with BAEC caused a fast (maximal activity was reached within 15 minutes) and cell number-dependent disappearance of ET-1 from the medium. This effect was direct to ET-1, because it was also present when PMN were incubated with the synthetic peptide in the absence of BAEC. PMN-dependent disappearance of ET-1 was associated with loss of constrictor activity on isolated rabbit aorta. PMN-released products were responsible for ET-1 degrading activity, because supernatants of activated PMN were equally effective as the intact cells. Resting PMN, in the same time frame, were ineffective. Eugin C, a potent blocker of PMN-derived elastase and cathepsin G, reversed the ET-1 inhibitory activity of fMLP-stimulated PMN and of their supernatant. Direct addition of elastase and cathepsin G to synthetic ET-1 destroyed its immunoreactivity and this effect was blocked by eugin C. High-performance liquid chromatography (HPLC) analysis supported the hypothesis that ET-1 degradation by PMN was due to enzymatic proteolysis. These data provide evidence that activated PMN are able to degrade ET-1 through the release of proteases. Because physiologic concentrations of PMN can destroy high amounts (up to 100 nmol/L) of ET-1 within a few minutes, we propose that this mechanism of ET-1 inactivation has biologic relevance.

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ENDOTHELIN-1 (ET-1), a 21-amino acid peptide recently isolated from the medium of cultured endothelial cells, represents one of the most potent vasoconstrictors known. Relatively high blood levels of this peptide have been found in different pathologies, including uremia, acute myocardial infarction, and acute renal failure.

ET-1 can also play a role in inflammatory conditions; indeed, interleukin-1 and tumor necrosis factor, two mediators of the acute inflammatory response, were reported to stimulate endothelial cell ET-1 production in vitro and in vivo. Despite being devoid of any direct stimulatory effect on leukocyte activation, ET-1 can exert an antiinflammatory action by suppressing enhanced vascular permeability induced by several inflammatory mediators.

The interaction of polymorphonuclear leukocytes (PMN) with endothelial cells, one of the first events in the acute inflammatory response and tissue ischemia, induces profound changes in the biosynthesis of potent endothelial modulators of vascular tone and permeability. The aim of this work was to assess the effect of PMN on ET-1 production by endothelial cells. We report that addition of fMLP-activated PMN dramatically reduces ET-1 content and biologic activity in endothelial cell culture media. This effect was apparently due to degradation of ET-1 by PMN-derived lytic enzymes. We suggest that this is a physiologic mechanism of ET-1 inactivation in vivo.

MATERIALS AND METHODS

Cells. PMN were isolated from venous blood of healthy volunteers according to the method of Boyum and resuspended in Hepes-Tyrode buffer supplemented with 0.1% bovine serum albumin (BSA). BAEC were cultured in Modified Eagle’s Medium (MEM) supplemented with 15% fetal bovine serum (GIBCO-Europe, Paisley, UK) as described, and used between three and 10 passages. For the experiments described, bovine aortic endothelial cells (BAEC) detached by brief exposure to trypsin (0.25%)-EDTA (0.022%), were plated and grown to confluence in 24-well plates.

Experimental procedures. Aliquots of PMN (0.37 to 3 x 10^6 cells in a final volume of 300 µL) were added to each BAEC well in the presence of fibronogen (Kabi Vitrum, Stockholm, Sweden) and cytochalasin B (0.38 mg/mL and 2.5 µg/mL, respectively). After addition of 10^{-7} mol/L fMLP (Sigma Chemical, St Louis, MO) or the equivalent volume of isotonic saline, plates were incubated at 37°C for the times indicated (15 to 180 minutes). At the end of the incubation, the assay medium was collected and centrifuged at 12,000g for 1 minute. Supernatants were then collected and stored at -20°C for ET-1 determination by radioimmunoassay.

In some experiments, either PMN (in the presence or absence of 10^{-7} mol/L fMLP) or PMN-derived supernatants were incubated (15 minutes at 37°C) with different concentrations of synthetic ET-1 (1 to 100 nmol/L). Samples were then centrifuged and the supernatants collected for measuring ET-1 recovery. PMN-derived supernatants were prepared by centrifuging 1.5 x 10^6 cells/mL that had been previously incubated (37°C for 15 minutes) with 3 x 10^{-7} mol/L fMLP or isotonic saline. In some experiments, supernatants were obtained following PMN stimulation with platelet-activating factor (PAF), leukotriene B4 (LTB4), or zymosan-activated serum (ZAS). ET-1 degrading activity of PMN and PMN-derived supernatants was evaluated in presence of the following protease inhibitors: leupeptin (Sigma), phosphoramidon (Boehringer, Mannheim, Germany), euglin C (a generous gift of Dr H.P. Schnebli, Ciba-Geigy, Basel, Switzerland), and EDTA (Merck, Darmstadt, Germany). These compounds were added to PMN or PMN-derived supernatants before addition of synthetic ET-1 (100 nmol/L). Samples were then incubated at 37°C for 15 minutes, centrifuged, and subsequently assayed for ET-1 recovery.

Studies with purified proteases. In a set of experiments, ET-1 (100 nmol/L) was incubated (15 to 180 minutes at 37°C) in the absence or presence of 10 to 200 nmol/L purified human elastase or cathepsin G, respectively. Coincubation of fMLP-activated PMN dramatically reduces ET-1 content and biologic activity in endothelial cell culture media. When ET-1 was added to PMN or PMN-derived supernatants, coinoculation with either elastase or cathepsin G caused a further decrease in ET-1 content. ET-1 degrading activity of PMN and PMN-derived supernatants was evaluated in presence of the following protease inhibitors: leupeptin (Sigma), phosphoramidon (Boehringer, Mannheim, Germany), euglin C (a generous gift of Dr H.P. Schnebli, Ciba-Geigy, Basel, Switzerland), and EDTA (Merck, Darmstadt, Germany). These compounds were added to PMN or PMN-derived supernatants before addition of synthetic ET-1 (100 nmol/L). Samples were then incubated at 37°C for 15 minutes, centrifuged, and subsequently assayed for ET-1 recovery.

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From the University of Chieti “G. D’Annunzio” School of Medicine, Chieti; Laboratory of Vascular Biology, Istituto di Ricerche Farmacologiche “Mario Negri,” Milan; Institute of Pharmacological Sciences, University of Milan; and Farmatrua Carlo Erba, Milan, Italy.

Submitted December 24, 1990; accepted July 18, 1991.

Supported by the Italian National Research Council (CNR) (Project CNR No. 89.01277.04).

Address reprint requests to Dr Aldo Del Maschio, Istituto di Ricerche Farmacologiche “Mario Negri,” Via Eritrea 62-20157, Milan, Italy.

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0006-4971/91/7810-0021$3.00/0
(Elastin Products, Owensville, MO) or cathepsin G (a generous gift of Dr M. Chignard, Institut Pasteur, Paris, France) in 1 mL of Hepes-Tyrode buffer or human whole blood. At the end of the incubation, samples in buffer were immediately frozen; samples in blood were centrifuged at 4°C (3,000 rpm, 10 minutes), and plasma was collected and frozen. Samples were then assayed for ET-1 content by radioimmunoassay.

Radioimmunoassay technique. A radioimmunoassay technique for endothelin was developed. The assay used 2,000 dpm of \(^{3}^{5}^{1^{-}}\)ET-1 (Novabiochem, Laufelfingen, Switzerland) and an anti-ET-1 serum (Peninsula, Belmont, CA) diluted 1:100,000 that showed 100% of cross-reactivity with both ET-1 and ET-2, 9% with ET-3, 1.5% with big ET-1 (1-36), and 0.83% with big ET-1 (1-39). Aliquots (100 μL) of unextracted incubation media were incubated at 4°C for 72 hours in 2.5 mL of 0.025 mol/L Tris-phosphate-HCl (pH 9). Separation of the antibody-bound ET-1 from free ligand was achieved by rapidly adding 50 μL of charcoal-treated human plasma and 100 μL of a charcoal suspension (100 mg/mL) and subsequent centrifugation at 3,000 rpm for 10 minutes at 4°C. Supernatant solutions (antibody-bound ET-1) and pellets (charcoal-adsorbed ET-1) were counted in a Beckman gamma counter (Fullerton, CA). The IC\(_{50}\) value (concentration of unlabeled ET-1 that reduced by 50% the bound to free ratio of the homologous tracer) was 2.4 fmol/mL. The least detectable concentration that could be measured with 95% confidence (ie, 2 SD at zero) was 0.2 fmol/mL. Validation of ET-1 measurements was obtained by dilution and recovery studies.

In the experiments reported here, the recovery of synthetic ET-1 after 15 minutes of incubation averaged 80%. This was considered the maximal recovery (100%) and the results are referred to this value. This reduction of ET-1 recovery did not further decrease with time (up to 180 minutes) and probably reflected an aspecific value. This reduction of ET-1 recovery did not further decrease to ET-1 (final concentration, 1 nmol/L) was recorded during 20 hours under a load of 2 g. Samples (1 mL) containing synthetic ET-1 methanol and 10 mL water. After washing with 10 mL methanol and 10 mL water. After washing with 10 mL of trifluoroacetic acid (TFA; 0.1%), the peptides were eluted with 3 mL of acetonitrile (60%) in 0.1% TFA. The eluted samples were concentrated by evaporation under vacuum and reconstituted in 0.15 mL of 30% acetonitrile in 0.1% TFA and subjected to reverse-phase HPLC analysis by using a \(\mu\)-bondapack C18 column (0.39 × 30 cm; Waters). Elution was performed by using a binary gradient consisting of 10% acetonitrile in 0.1% TFA (solvent A) and 90% acetonitrile in 0.1% TFA (solvent B). The elution profile incorporated a linear gradient from 10% to 80% solvent B in 60 minutes at a flow rate of 1 mL/min. Eluates were monitored by absorbance at 220 nm. Synthetic ET-1 eluted as a single peak with a retention time of 28.2 ± 0.2 minutes (mean ± SD, n = 4). ET-1 extraction by SEP-PAK C18 cartridges caused the appearance of an additional peak eluting at 26.6 ± 0.2 minutes. Since both peaks were recognized by ET-1 antiserum, the peptide eluting at 26.6 minutes is likely to represent an aggregated form of ET-1, as recently reported.

**RESULTS**

**Effect of PMN on endothelin immunoreactivity (ET-ir) released by cultured BAEC.** BAEC released ET-ir in a time-dependent fashion (Fig 1). When increasing concentrations of fMLP-activated PMN were added, the amount of ET-ir measured in the medium was dramatically reduced (Fig 1). With 1.5 to 3 × 10\(^{6}\) PMN, ET-ir was barely detectable at 15 minutes. fMLP (10\(^{-7}\) mol/L) added per se to BAEC did not significantly alter ET-ir content in the medium (not shown). The addition of unstimulated PMN (3 × 10\(^{6}\)) to BAEC did not significantly reduce the amount of ET-ir measured at 15 minutes and 2 hours of incubation (115% ± 16% and 90% ± 17% of the amount of ET-ir measured in the absence of PMN, respectively; means ± SEM of three separate experiments).

Superoxide dismutase (10 to 100 U/mL), a superoxide anion scavenger, did not affect the reduction of BAEC-derived ET-ir by stimulated PMN (3 × 10\(^{6}\) cells/well) up to 3 hours of incubation (data not shown).

**Effect of PMN on synthetic ET-1.** We next studied whether ET-1 reduction in BAEC culture medium was due to a direct effect of PMN on this peptide. For this purpose, synthetic ET-1 was incubated with fMLP-activated PMN. The synthetic peptide was degraded to an extent dependent on PMN number (Fig 2). At a physiological concentration (3 × 10\(^{6}\)), activated PMN were able to destroy up to 100 nmol/L ET-1 within 15 minutes. In the same time frame,
unstimulated PMN (3 x 10^6) did not significantly change ET-1 recovery (1 to 100 nmol/L; data not shown).

Effect of PMN on ET-1 biologic activity. Functional studies performed on isolated rabbit aorta confirmed that PMN-induced reduction of ET-ir corresponded to a loss of biologic activity. ET-1 (100 nmol/L) was incubated (15 minutes at 37°C) with fMLP (10^-7 mol/L) in the presence or absence of PMN (3 x 10^6); samples were centrifuged and supernatants collected. Supernatants that had been incubated in the absence of PMN induced a slow and long-lasting contraction; conversely, no response was observed following ET-1 preincubation with activated PMN (Fig 3).

Furthermore, the contracting activity of ET-1 was unaffected when ET-1 and supernatants of activated PMN were added simultaneously to the vessel. The supernatant of activated PMN did not modify per se the basal tone (data not shown).

Effect of PMN-derived supernatants on synthetic ET-1. We then investigated whether intact PMN were required for ET-1 degrading activity. When supernatants of fMLP-activated PMN (3 x 10^6) were incubated for 15 minutes with 100 nmol/L ET-1, the peptide was degraded (3% ± 2% of recovery) as effectively as when the same number of intact cells was used (5% ± 1% of recovery). Supernatants of PMN stimulated by fMLP (10^-7 mol/L) in the absence of cytochalasin B did not reduce ET-1 (100 nmol/L) recovery (95% ± 3% vs 5% ± 2% in absence or presence of cytochalasin B, respectively), suggesting that extracellular enzymatic release was necessary to cause ET-1 disappearance.

Supernatants of PMN stimulated by other agonists were tested for their ability to degrade ET-1. When PAF (10^-5 mol/L), LTB_4 (3 x 10^-7 mol/L), and ZAS (5%) were used, PMN supernatants reduced ET-1 (100 nmol/L) recovery to, respectively, 60% ± 4%, 62% ± 3%, and 75% ± 3% of the total amount of ET-1 added, at 15 minutes of incubation; longer incubation times (up to 60 minutes) did not modify the recovery. None of these compounds was effective in the absence of cytochalasin B (not shown).

Effect of PMN-derived proteolytic enzymes on synthetic ET-1. In an attempt to define whether PMN derived proteases were responsible for ET-1 degradation, different protease inhibitors were used. Leupeptin (2 x 10^-4 mol/L), phosphoramidon (10^-4 mol/L), and EDTA (2 x 10^-3 mol/L) did not affect ET-1 (100 nmol/L) degradation induced by fMLP-activated PMN (3 x 10^6) or their supernatants. In contrast, eglin C (25 μg/mL), a serine protease inhibitor, reversed the effect of both intact PMN and supernatants in the same experimental conditions (Table 1).

Incubation of 100 nmol/L ET-1 with two PMN-derived serine proteases, elastase and cathepsin G (10 to 200 nmol/L), for different times (15 to 180 minutes), resulted in a dose-dependent reduction of ET-1 recovery (Fig 4), which was reversed by 25 μg/mL eglin C (Fig 5).

Table 1. Effect of Protease Inhibitors on PMN-Dependent ET-1 Degradation

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ET-1 Recovery (%)</th>
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<tbody>
<tr>
<td></td>
<td>PMN</td>
</tr>
<tr>
<td>Eglin C</td>
<td>58.2 ± 4.2</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>3.9 ± 1.5</td>
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<tr>
<td>Phosphoramidon</td>
<td>4.9 ± 2.1</td>
</tr>
<tr>
<td>EDTA</td>
<td>4.5 ± 1.7</td>
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NOTE. ET-1 (100 nmol/L) was incubated (15 minutes at 37°C) with fMLP (10^-7 mol/L)-stimulated PMN or supernatants derived from fMLP (3 x 10^-7 mol/L)-stimulated PMN in the absence or presence of eglin C, leupeptin, phosphoramidon and EDTA (25 μg/mL, 2 x 10^-4 mol/L, 10^-4 mol/L and 2 x 10^-2 mol/L, respectively). ET-1 recovery was determined by radioimmunoassay. Data (mean ± SEM) are derived from three different experiments.
Effect of cathepsin G and elastase on ET-1 in whole blood.
To assess whether PMN-derived proteolytic enzymes were able to degrade ET-1 in the presence of naturally occurring plasma antiproteases, elastase and cathepsin G (100 nmol/L) were incubated in whole blood with 100 nmol/L synthetic ET-1 for different times. Cathepsin G induced consistent and time-dependent ET-1 degradation, while elastase had no effect (Fig 6). Only a slight decrease of ET-1 recovery occurred during the incubation in whole blood (Fig 6).

Analysis of ET-1 degradation by HPLC. To collect further evidence of ET-1 degradation by PMN, synthetic ET-1 was incubated with purified cathepsin G and elastase and ET-1 recovery was evaluated by HPLC analysis. Extraction of synthetic ET-1 by SEP-PAK C18 cartridges resulted in the appearance of two different peaks (Fig 7A), both of which reacted with ET-1 antiserum (not shown). After incubation of 400 pmol ET-1 with 100 nmol/L cathepsin G (15 minutes at 37°C), the two peaks disappeared, while a major peak, with a retention time of 5.5 minutes, was observed. Eglin C (25 μg/mL) prevented ET-1 degradation by cathepsin G (Fig 7B). Similar results were obtained with 100 nmol/L elastase (not shown).

DISCUSSION
In this report, we demonstrate that short-term (15-minute) coincubation of BAEC with fMLP-activated PMN dramatically reduced ET-ir in the culture medium. This effect was not due to inhibition of ET-1 synthesis and release by endothelial cells, since it was also apparent when incubating synthetic ET-1 with activated PMN. Furthermore, synthetic ET-1 disappearance was also present when supernatants of activated PMN were used, thus indicating that ET-1 degrading activity (or at least a large part of it) was not cell-associated or due to PMN uptake of the peptide. fMLP was the most potent inducer of ET-1 degrading activity by PMN, but also PAF, LTB₄, and ZAS were partially effective. The observation that eglin C, a powerful serine protease inhibitor, was able to reverse the inhibitory action of PMN supernatants and that purified elastase and cathepsin G could effectively destroy ET-1,

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**Fig 4.** Effect of (A) elastase and (B) cathepsin G on ET-1 degradation. ET-1 (100 nmol/L) was incubated for 15 minutes at 37°C in the absence (●) or presence of different doses of the enzymes (10, ●; 100, ●; 200 nmol/L, ◦). Data (mean ± SEM) are derived from three different experiments.

**Fig 5.** Effect of eglin C on ET-1 degradation induced by purified elastase and cathepsin G. ET-1 (100 nmol/L) was incubated with elastase or cathepsin G (100 nmol/L) either in the absence (■) or presence (●) of eglin C (25 μg/mL) for 15 minutes at 37°C. Data are mean ± SD of three different experiments. **P < .01 vs control (in the absence of eglin C). Student’s t test.

**Fig 6.** Effect of elastase and cathepsin G on ET-1 in whole blood. ET-1 (100 nmol/L) was incubated in whole blood for the indicated times at 37°C in absence (▲) or presence of 100 nmol/L elastase (●) or cathepsin G (◆). Data are mean ± SEM of three different experiments.
strongly suggests that PMN-derived serine proteases are responsible for ET-1 degrading activity. Noteworthy, supernatants of PMN stimulated in the absence of cytochalasin B, a condition associated with low levels of PMN enzymatic release in vitro, did not cause ET-1 degradation. However, the release by PMN of proteolytic enzymes in inflammatory sites may play an active role in ET-1 degradation in vivo.

Other investigators recently reported that ET-1 is particularly sensitive to the hydrolysis induced by the kidney neutral endopeptidase. This enzyme is also present on PMN membrane. However, we found that phosphoramidon, a specific neutral endopeptidase inhibitor, did not affect PMN-derived ET-1 degrading activity. Therefore, it is unlikely that this enzyme (either cell-associated or shedded in the medium) could be responsible of ET-1 destruction, at least under the present experimental conditions.

ET-1 degradation by PMN-derived proteolytic enzymes evaluated by radioimmunoassay was confirmed by the results showing loss of ET-1 vasoconstrictor activity following exposure of the peptide to activated PMN (Fig 3). Moreover, HPLC analysis indicated that proteolytic cleavage products may be generated on PMN activation (Fig 7), as recently reported.

The observation reported here that activated PMN could destroy ET-1 activity might have biologic relevance. Indeed, physiologic concentrations of PMN are able to degrade a large quantity of this peptide within a few minutes (Figs 1 and 2).

Although naturally occurring protease inhibitors might limit the ability of PMN-derived proteases to degrade ET-1, data reported here indicate that cathepsin G-dependent degradation of ET-1 is also evident in whole blood, ie, in the presence of plasma protease inhibitors. At variance with cathepsin G, elastase produced only little, if any, ET-1 degradation. This is likely to reflect the fact that the plasma protease inhibitors α₁-protease inhibitor and α₂-macroglobulin bind with higher affinity to elastase than to cathepsin G. Furthermore, cathepsin G ability to degrade ET-1 might be scarcely impaired in whole blood, since its specific inhibitor, α₁-antichymotrypsin, is present at relatively low concentrations. Experiments performed in whole blood indicated that ET-1 recovery did not significantly decrease with time (up to 180 minutes); these results support the in vivo findings that ET-1 is cleared from the circulation mainly by removal into parenchymal tissues.

The results of this work suggest that under conditions associated with the release of proteolytic enzymes by activated PMN, ET-1 may be degraded with loss of biologic activity. In the case of acute inflammatory reaction and in tissue ischemia, PMN adhere to the endothelium in response to chemotactic stimuli. The local release of ET-1 induced by inflammatory mediators and the consequent vasoconstriction and inhibition of vascular permeability could therefore be reduced or abolished by the action of PMN-derived proteases. This effect could be further amplified by the release of PGI₂ from endothelial cells in response to the interaction with activated PMN.

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