

The two-step conversion of big endothelin 1 to endothelin 1 and degradation of endothelin 1 by subcellular fractions from human polymorphonuclear leukocytes

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ABSTRACT The metabolism of big endothelin 1 (bET) and endothelin 1 (ET-1) by subcellular fractions from human polymorphonuclear leukocytes (PMNs) was investigated by bioassay and reversed-phase high-performance liquid chromatography. More than 80% of endothelin-converting activity was recovered from the cytosolic fraction, which in addition to ET-1 generated other peptides from bET. The processing of bET to all its metabolites including ET-1 was prevented by the serine protease inhibitor 3,4-dichloroisocoumarin (DCI; 50 μ M) or the elastase inhibitor ONO-5046 (100 μ M) but not by phenylmethylsulfonyl fluoride (PMSF; 143 μ M), another serine protease inhibitor. Paradoxically, human leukocyte elastase, despite generating a bET fragmentation pattern similar to that of PMN cytosol, produced very little ET-1. However, subsequent treatment of the elastase-derived metabolites of bET with PMN cytosol in the presence of ONO-5046 dramatically increased the amount of ET-1 formed. The generation of ET-1 following this intervention was inhibited by DCI. The PMN membrane preparation degraded ET-1 to a major metabolite, similar to that produced from ET-1 by elastase, and several minor products, paralleled by a loss of its smooth muscle contracting activity. The degradation of ET-1 by PMN microsomes was prevented by DCI, PMSF, or ONO-5046. Our results suggest that an elastase-initiated serine protease cascade is responsible for the sequential conversion of bET to ET-1 by the PMN cytosol. Elastase also partly accounts for the ET-metabolizing properties of PMN microsomes.

The 21-amino acid peptide endothelin 1 (ET-1), initially isolated from endothelial cells in culture, is a powerful vasoconstrictor *in vitro* and *in vivo* (1). The pharmacological properties of ET-1, however, are not confined to vascular smooth muscle and include diverse effects on cardiac, renal, pulmonary, and nervous functions (2). Human ET-1 is generated from its 38-amino acid precursor big endothelin 1 (bET) through cleavage of the Trp²¹-Val²² bond by an as yet unidentified endothelin-converting enzyme (ECE). Yanagisawa *et al.* (1) initially suggested that ECE was a chymotrypsin-like serine protease. It was subsequently demonstrated that *N*-tosyl-L-phenylalanine chloromethyl ketone, an inhibitor of chymotrypsin-like serine proteases or isatoic anhydride, an inactivator of chymotrypsin, significantly reduced the secretion of ET by bovine cultured pulmonary artery endothelial cells (3). Indeed, chymotrypsin can generate ET-1 in addition to bET₁₋₃₁ from bET (4), and chymase, a serine protease from lung mast cells, also converts bET to ET-1 (5). However, most of the recent studies have focused on the ECE activities of aspartic (6, 7) and neutral metalloproteases (8, 9).

The existence of ET-metabolizing enzymes has also been demonstrated. These include a phosphoramidon (PHA)-

sensitive neutral endopeptidase 24.11 purified from rat kidney (10) and a similar endopeptidase present in aortic smooth muscle cells from rat fetus (11). Cathepsin D, which converts bET to ET-1 at acidic pH, can also degrade ET-1 by removing the C-terminal tripeptide Ile-Ile-Trp (12). Although a physiological role for ET-1 remains to be established, elevated plasma levels of the peptide have been reported in pathological states such as acute myocardial infarction (13), pre-eclampsia (14), or endotoxic shock (15), reflecting either an increased production or a decreased inactivation of the peptide. It is not yet known whether the increased ET-1 concentration actually contributes to the etiopathogenesis of these diseases or is simply a consequence of the underlying pathology. However, a common feature of all these disorders is the accumulation and activation of polymorphonuclear leukocytes (PMNs) within discrete sites in the circulation, suggesting a possible link between ET-1 formation and PMN function. Indeed, Sessa *et al.* (16) have demonstrated that freshly isolated human PMNs rapidly convert exogenous bET to ET-1. This conversion was more evident in nonactivated cells, as activation of PMNs with the chemotactic peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe) resulted in a substantial degradation of the newly formed ET-1, probably due to serine proteases, in particular cathepsin G (17). These findings were confirmed by another study showing that coincubation of fMet-Leu-Phe-activated PMNs with cultured endothelial cells decreased the formation of ET-1 (18). This concomitant degradation of ET-1, however, hampers the characterization of the ECE activity in intact PMNs. We have, therefore, attempted to localize and characterize further the converting and degrading activities in subcellular fractions from human PMNs.

MATERIALS AND METHODS

Materials. Synthetic bET-1 and ET-1 were purchased from the Peptide Institute (Osaka). Human leukocyte elastase, human leukocyte cathepsin G, pepstatin A, leupeptin, soybean trypsin inhibitor, PHA, phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (DCI), and indomethacin were obtained from Sigma. ONO-5046 [*N*-[2-[4-(2,2-dimethylpropionyloxy)phenylsulfonylamino]benzoyl]aminoacetic acid] was the kind gift of H. Iwamura from Ono Pharmaceutical (Osaka).

Bioassay. Male New Zealand White rabbits (2.0–2.5 kg) were killed by intravenous injection of sodium pentobarbi-

Abbreviations: bET, big endothelin 1; ET-1, endothelin 1; PMNs, polymorphonuclear leukocytes; DCI, 3,4-dichloroisocoumarin; PMSF, phenylmethylsulfonyl fluoride; PHA, phosphoramidon; ECE, endothelin-converting enzyme; RbJV, rabbit jugular vein; RP-HPLC, reversed-phase HPLC.

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tone (60 mg/kg). The jugular vein (RbJV) was removed, trimmed of periadventitial fat, cut into helical strips, and superfused as described (16).

Subcellular Fractionation of Human PMNs. The entire procedure for preparing subcellular fractions was carried out at 0°C–4°C. Approximately 1×10^9 frozen human PMNs prepared as described for rabbit PMNs (19) were thawed, subjected to three additional cycles of freeze–thawing, and suspended in 50 mM Tris·HCl buffer containing 10 mM EDTA, 0.1 mM dithiothreitol, 1.15% KCl, and the protease inhibitors leupeptin (2 µg/ml), pepstatin A (2 µg/ml), soybean trypsin inhibitor (10 µg/ml), and PMSF (44 µg/ml). The cells were homogenized further with an ultrasonic processor (Sonic and Materials, Danbury, CT, model GE 375; five cycles of 10 sec each), and the homogenate was centrifuged at $10,000 \times g$ for 20 min. The resulting supernatant was centrifuged at $200,000 \times g$ for 30 min and the pellet, comprising the mixed membrane or microsomal fraction, was washed and resuspended in 50 mM Tris·HCl containing 10% (vol/vol) glycerol. The supernatant representing the cytosol was concentrated further by using Centricon-10 microconcentrators (from Amicon) with a M_r cut-off of 10,000. Protein concentrations were determined by Peterson's modification of the micro-Lowry assay with bovine serum albumin as a standard (20). Aliquots of both fractions were stored at –80°C until further use.

Incubations for Bioassay and HPLC. Protein (60 µg) of either the membrane or cytosolic fraction was incubated with 10 µM bET for 30 min (membrane) or 5–60 min (cytosol) in phosphate-buffered saline (PBS; composition, 137 mM NaCl/2.7 mM KCl/7.4 mM Na₂HPO₄/1.5 mM KH₂PO₄) containing 10 mM CaCl₂ and 5 mM MgCl₂ in a total vol of 300 µl. Alternatively, 10 µM bET was incubated with human leukocyte-derived serine proteases, cathepsin G, or elastase (each at 5 µg/ml) for 30 min. In a separate series of experiments 5 µM ET-1 was incubated with either the PMN subcellular fractions, cathepsin G, or elastase in 300 µl of PBS containing 1 mM CaCl₂ for 30 min. Some of the incubation mixtures contained the following inhibitors: DCI (50 µM), PMSF (143 µM), PHA (100 µM), or the elastase inhibitor ONO-5046 (100 µM) (21). At the end of each incubation period, 50 µl of the mixture was removed and 5- to 20-µl aliquots (equivalent to 50–200 pmol of bET) of the bET or 1–2 µl of the ET-1 incubation mixtures were assayed for contractile activity. The remaining 250 µl was processed and analyzed by reversed-phase HPLC (RP-HPLC) using a binary mixed linear gradient, as reported by Hecker *et al.* (22). In some experiments, fractions were collected at relevant time intervals, freeze-dried, and subjected to electrospray mass spectrometry analysis.

Statistical and Data Analysis. The recoveries of bET and ET-1 were evaluated from their respective integrated peak areas on the RP-HPLC chromatograms. Data were expressed as means \pm SEM of n separate experiments and comparison between means was made by a one-way analysis of variance followed by a Bonferroni t test with $P < 0.05$ considered statistically significant. The bioassay results were expressed and tabulated as a percentage of control contraction (taken as 100%) induced by either bET incubated with cytosol for the conversion data or ET-1 incubated with buffer alone for the degradation experiments.

RESULTS

Activation of bET by PMN Cytosol. Aliquots of bET incubated with the PMN cytosol for 30 min elicited ET-1-like contractions of the RbJV (Fig. 1*b*; $n = 13$), in contrast to bET incubated with buffer alone, which was inactive up to doses of 200 pmol. Incubation of bET with the microsomal fraction, on the other hand, produced only negligible contractile

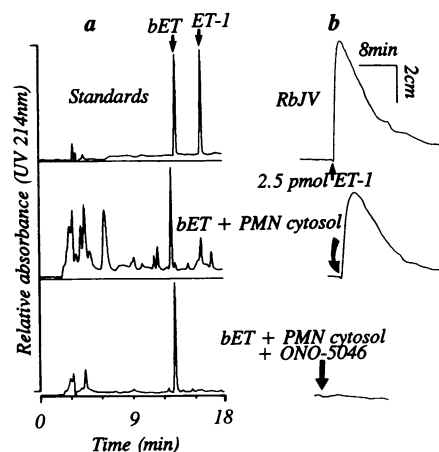


FIG. 1. Representative RP-HPLC chromatograms (*a*) and corresponding RbJV contractions (*b*) of bET incubated with PMN cytosol for 30 min at 37°C. Note the extensive metabolism of bET by the PMN cytosol, resulting in generation of biologically active ET-1 and other peptides ($n = 13$). This activation is prevented by coincubation with ONO-5046 (100 µM; $n = 9$).

activity (results not shown). The activation of bET by the cytosol was inhibited by coincubation with the serine protease inhibitor DCI (Table 1) or the selective elastase inhibitor ONO-5046 (Fig. 1*b*; Table 1). Interestingly, the serine protease inhibitor PMSF paradoxically potentiated the contractile response, whereas the metalloprotease inhibitor PHA did not prevent the production of vasoconstrictor activity (Table 1).

Metabolism of bET by PMN Cytosol. The retention time of synthetic bET on RP-HPLC was 13.2 ± 0.04 min, whereas that of ET-1 was 15.5 ± 0.04 min ($n = 17$; Fig. 1*a*). RP-HPLC analysis revealed that, upon incubation with the PMN cytosol >98% of bET was metabolized to several peptides, of which ET-1 (retention time, 15.5 ± 0.07 min) comprised $11.3\% \pm 0.5\%$ ($n = 13$; Figs. 1*a* and 2). The microsomal fraction also processed bET in a manner similar to the cytosol (results not shown); however, the amount of ET-1 generated was negligible and the converting activity was therefore characterized in the cytosol. The identity of bET and ET-1 was confirmed by electrospray mass spectrometry analysis with the molecular weights of the relevant fractions being 4282 and 2492, respectively. Conversion of bET to ET-1 was maximum after 30 min of incubation with the PMN cytosol (280 ± 13 pmol; $n = 13$), 46% of maximum (130 ± 47 pmol; $n = 3$) after 5 min, and 87% of maximum (244 ± 34 pmol; $n = 3$) after 15 min. The proteolytic processing of bET to ET-1 and other peptides was prevented by the serine protease inhibitors DCI or ONO-5046 ($n = 9$; Fig. 2), whereas PMSF, while partly attenuating the formation of some bET-derived peptides, had no effect on the production of ET-1 ($n = 6$; Fig. 2). The metalloprotease inhibitor PHA had no effect on the conversion of bET to ET-1 or to any of the other products seen on HPLC ($n = 6$; Fig. 2).

Processing of bET by Human Leukocyte-Derived Serine Proteases, Elastase, or Cathepsin G. Elastase cleaved bET to

Table 1. Effect of protease inhibitors on activation of bET by PMN cytosol

Sample	n	Contraction of RbJV, % of control
bET in buffer	7	0
bET + PMN cytosol (control)	13	100
bET + PMN cytosol + DCI	9	0
bET + PMN cytosol + PMSF	6	181 \pm 21
bET + PMN cytosol + PHA	6	91 \pm 21
bET + PMN cytosol + ONO-5046	9	18 \pm 4

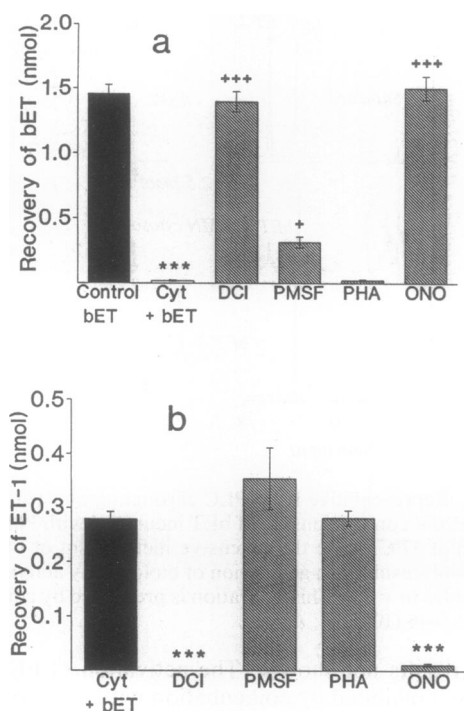


FIG. 2. Effect of protease inhibitors on the metabolism of bET (a) and formation of ET-1 by the PMN cytosol (b) after incubation of bET with PMN cytosol. More than 98% (***, $P < 0.001$ vs. control) of bET was metabolized by the PMN cytosol and this metabolism was prevented by DCI or ONO-5046 (+++, $P < 0.001$ vs. cytosol), and to a lesser extent by PMSF (+, $P < 0.05$). Consequently, DCI or ONO-5046 inhibited the formation of ET-1 (***, $P < 0.001$ vs. cytosol), but PMSF or PHA had no effect. Note that in the presence of DCI no ET-1 was detected. cyt, Cytosol.

several peptides ($n = 7$; Fig. 3a) and this metabolism was inhibited by DCI or ONO-5046 and to a lesser extent by PMSF ($n = 4$; results not shown). The fragmentation pattern of bET metabolism by elastase was similar to that observed with the PMN cytosol, but the pure enzyme generated negligible amounts of ET-1 (<1% conversion), as shown by the relative lack of contractile activity on the RbJV (Fig. 3b). However, the selective elastase inhibitor ONO-5046 prevented the conversion of bET to ET-1 by the PMN cytosol (Figs. 1 and 2), suggesting that elastase is involved in the generation of ET-1, possibly via the formation of intermediates. To test this hypothesis, elastase-treated bET was incubated with the PMN cytosol in the presence of ONO-5046, and this intervention produced a 17-fold increase in the generation of ET-1 as assessed by bioassay and RP-HPLC (Fig. 3). The formation of ET-1 under these conditions was prevented by coincubation with DCI (Fig. 3c).

bET incubated with cathepsin G, when assayed for activity, elicited small contractions of the RbJV ($n = 4$; results not shown). This activity was due to the formation of a metabolite chromatographically distinct from ET-1, which was identified as bET₁₋₃₁ by electrospray mass spectrometry analysis (M_r , 3628).

Inactivation and Metabolism of ET-1 by PMN Microsomes. Incubation of ET-1 with PMN microsomes virtually abolished its contractile activity ($6\% \pm 2\%$ of control; $n = 8$; Fig. 4b), implying a degradation of the peptide. Most of the ET-degrading activity was recovered from the microsomal fraction and the inactivation was prevented by DCI, PMSF, or ONO-5046 but not by PHA ($n = 4$; Table 2). RP-HPLC analysis confirmed that ET-1 was metabolized by PMN microsomes, producing a major peak (retention time, 14.3 ± 0.04 min) and several minor metabolites ($n = 5$; Fig. 4a). In addition, a component of the microsomal fraction, presum-

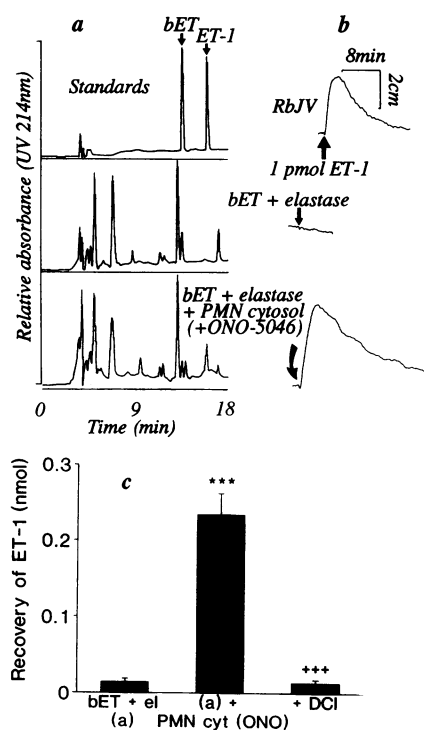


FIG. 3. Processing of bET by elastase. Incubation of bET (10 μ M) with elastase (5 μ g/ml) for 30 min produced negligible amounts of ET-1 despite a fragmentation pattern similar to that observed with the PMN cytosol. Subsequent treatment of this incubation mixture with the PMN cytosol in the presence of ONO-5046 resulted in a substantial increase (***, $P < 0.001$ vs. bET + elastase) in the formation of ET-1 ($n = 6$) as assessed by HPLC (a) and bioassay (b). (c) Recovery of ET-1 from this series of experiments. Generation of ET-1 under these conditions was inhibited by DCI (+++, $P < 0.001$ vs. cytosol treated; $n = 4$). el, Elastase; cyt, cytosol.

ably a peptide, unrelated to ET-1 metabolism, eluted at 9.0 ± 0.06 min. The metabolism of ET-1 was prevented by DCI, PMSF, or ONO-5046 but not by PHA ($n = 4$; Fig. 5).

Processing of ET-1 by Cathepsin G or Elastase. ET-1 was almost completely metabolized by cathepsin G to at least one major and two minor products, resulting in a loss of its

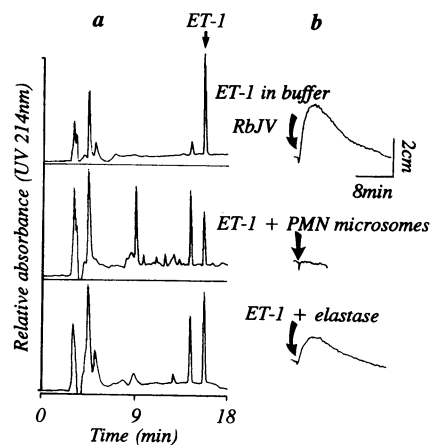


FIG. 4. RP-HPLC chromatograms (a) and corresponding contractions of the RbJV (b) of ET-1 incubated with either PMN microsomes or elastase for 30 min at 37°C (representative of five to eight experiments). Elastase partially degraded ET-1 to a peptide with a retention time of 14.3 ± 0.04 min, with a corresponding loss of biological activity. PMN microsomes inactivated ET-1 by metabolizing it to a major fragment with a retention time similar to that generated from ET-1 by elastase and several minor products. Note the microsomal component eluting at 9 min.

Table 2. Effect of protease inhibitors on inactivation of ET-1 by PMN microsomes

Sample	<i>n</i>	Contraction of RbJV, % of control
ET-1 in buffer (control)	8	100
ET-1 + PMN mic	8	6 ± 2
ET-1 + PMN mic + DCI	4	69 ± 18
ET-1 + PMN mic + PMSF	4	53 ± 9
ET-1 + PMN mic + PHA	4	15 ± 2
ET-1 + PMN mic + ONO-5046	4	100 ± 3

mic, Microsomes.

contractile activity (results not shown). Elastase, on the other hand, metabolized ET-1 to a lesser extent, producing a single peak with a retention time (14.3 ± 0.04 min) similar to the major peak generated from ET-1 by the microsomal fraction ($n = 5$; Fig. 4a). Consequently, elastase inhibited the biological activity of ET-1 only partially (Fig. 4b).

DISCUSSION

The present investigation confirms our earlier findings that PMNs convert bET to ET-1 and degrade ET-1 (16, 17). The combination of the techniques of bioassay and RP-HPLC enabled us to gain further insight into the nature of ECE activity in PMNs. Moreover, the use of gradient elution, as opposed to the isocratic HPLC system used in our earlier experiments, has allowed us to visualize an entire spectrum of metabolites. Based on the fragmentation pattern observed on RP-HPLC, we found that elastase accounts for a large percentage of the bET-metabolizing potential of the PMN cytosol. However, despite the similarity between elastase and the PMN cytosol in processing bET, the amount of ET-1 generated by the synthetic enzyme was negligible. Nevertheless, the serine protease inhibitor DCI or the elastase inhibitor ONO-5046 prevented the conversion by the cytosol of bET to all its various metabolites including ET-1, indicating that a serine protease(s), most likely elastase, generates an intermediate that is subsequently cleaved by another protease(s) to yield mature ET-1. This hypothesis was confirmed when incubation of the elastase-derived metabolites of bET with the ONO-5046-treated cytosol greatly enhanced the generation of ET-1. Since DCI inhibited the formation of ET-1 under these conditions, the second step must therefore also be serine protease dependent. Thus, a serine protease cascade, initiated by elastase, accounts for ECE activity in the PMN cytosol. Despite a small but significant reduction in the formation of some bET-derived metabolites, PMSF did not prevent its conversion to ET-1, suggesting that this

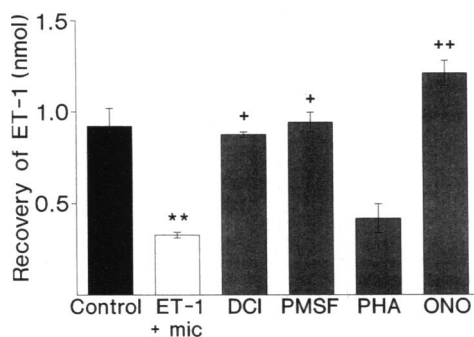


FIG. 5. Effect of protease inhibitors on PMN microsome-induced degradation of ET-1 ($n = 4-7$ experiments). PMN microsomes metabolized ET-1 (**, $P < 0.01$ vs. control), and this degradation was prevented by DCI or PMSF (+, $P < 0.05$ vs. ET-1 + microsomes) or the selective elastase inhibitor ONO-5046 (**, $P < 0.01$ vs. ET-1 + microsomes). mic, Microsomes.

general serine protease inhibitor was unable to block formation of the putative intermediate involved in the production of ET-1.

Most of the ET-1-degrading activity was present in the microsomal fraction, and the major metabolite formed had a retention time similar to that generated from ET-1 by elastase. This finding is at variance with our earlier data showing that degradation of ^{125}I -labeled ET-1 by activated PMNs produced a chromatographic profile virtually indistinguishable from that seen with purified human leukocyte cathepsin G (17). Degradation of ET-1 by the microsomal fraction, however, also appears to involve more than one enzyme, for there was no major breakdown of ET-1 by the cytosol despite the elastase-like activity present there. Moreover, elastase alone does not metabolize ET-1 effectively. Therefore, elastase may initiate the metabolism of ET-1, with further cleavage of the elastase-derived fragments by other specific or nonspecific proteases.

The formation of a biologically active metabolite (bET₁₋₃₁) from bET by human leukocyte-derived cathepsin G is consistent with an earlier study showing that bET is cleaved by cathepsin G to a fragment of 27-31 amino acids, which is 5 times less potent than ET-1 as a vasoconstrictor (23).

The metalloprotease inhibitor PHA has been reported to prevent the conversion of bET to ET-1 *in vitro* in cultured endothelial (9) or smooth muscle (24) cells and to reduce the pressor effects of bET *in vivo* (25). PHA-sensitive endopeptidases have also been shown to possess ET-metabolizing activity (10, 11). In the present study, PHA had no effect on either bET conversion by the cytosol or ET-1 metabolism by PMN microsomes. However, in earlier experiments with intact PMNs, PHA partly inhibited the generation of ET-like activity, whereas the formation of ET-1, as assessed by RP-HPLC analysis remained unaffected (17). Assuming that the pressor effect of bET is almost entirely due to its conversion to ET-1, measurable levels of the mature peptide should be detected in the plasma. Indeed, a rapid increase in immunoreactive ET-1 has been demonstrated after bET injection into rabbits (26), but, in this study, the antiserum used had a high cross-reactivity with bET. More recently, it has been shown that the increase in ET-1-like immunoreactivity following intravenous administration of bET in the pig was not affected by PHA (27). In the same study, PHA, while inhibiting the pressor response to bET, did not influence the accompanying increase in plasma ET-1. PHA also produced an incomplete and variable inhibition of the conversion of bET to ET-1 by mast cell chymase in the perfused rat lung (5), which was shown to be related to the inhibition of mast cell degranulation. These findings suggest that PHA may be acting by other mechanisms to reduce the bET-induced pressor responses, and therefore its mode of action needs to be reevaluated.

Serine proteases have been implicated in the conversion of bET to ET-1 (3-5). We have now demonstrated that an elastase-initiated serine protease cascade in the PMN cytosol converts bET to ET-1. These findings provide a model for elucidating the fate of parenterally administered bET, which may also be activated in a sequential manner by different proteases at discrete sites within the circulation. However, it should be noted that the metabolic profile observed with subcellular fractions does not necessarily reflect the metabolism of bET or ET-1 by intact PMNs, where a selective release of serine proteases may occur upon activation of these cells. Human leukocyte elastase is stored along with cathepsin G in the azurophilic granules of PMNs (28). Both proteases avidly degrade connective tissue proteins such as elastin, collagen, or proteoglycan, although elastase is the more active of the two in this respect (29). The proteolytic activity of cathepsin G and elastase is controlled by the plasma inhibitors α_1 -antichymotrypsin and α_1 -antitrypsin,

respectively (28, 29). More recently, α_1 -antitrypsin has been found colocalized with elastase in the primary granules of PMNs (30). The presence of endogenous inhibitors makes it less likely for PMN-derived serine proteases to be involved in the physiological processing of bET or ET-1. But an inherited or acquired deficiency (28, 29) of these inhibitors can permit an unrestrained action of such enzymes. Moreover, in pathological conditions in which PMNs are activated, even in the presence of functional endogenous inhibitors, sufficiently high concentrations of these proteases can overcome the activity of inhibitors and thereby actively participate in the generation and degradation of ET-1. In this context, a recent study demonstrated an elevation of plasma ET-1 as well as elastase in a group of women suffering from pre-eclampsia (14), suggesting a possible link between elastase and ET-1. A separate report showed that a factor from the serum of pre-eclamptic women suppressed ET-1 production in primary cultures of human umbilical vein endothelial cells (31). The authors suggested that pre-eclamptic serum contains an endopeptidase responsible for the observed degradation of ET-1. As PMN activation is a feature of pre-eclampsia (32), the excessive release of serine proteases may well account for this effect.

The existence of similar correlations in other disorders associated with PMN activation may yield important information regarding their etiopathogenesis and, therefore, merit investigation.

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