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Research Article

A human neutrophil protease, initially termed neutral peptide-generating protease, has been shown to cleave angiotensin II directly from angiotensinogen and has been identified as leukocyte cathepsin G. When purified neutrophils were disrupted by nitrogen cavitation and fractionated by differential centrifugation, 44 and 24% of the angiotensin II-generating activity was in the lysosomal and undisrupted cell fractions, respectively. Cytochalasin B-treated human neutrophils stimulated with N-formyl-L-methionyl-L-leucyl-L-phenylalanine released beta-glucuronidase, lysozyme, and angiotensin II-generating protease in a dose-dependent fashion, consistent with localization of this protease to the neutrophil granule. Individually purified angiotensin II-generating protease and cathepsin G had similar proteolytic and esterolytic activity for angiotensinogen and N-benzoyl-L-tyrosine ethyl ester on a weight basis, exhibited identical mobilities by SDS-gradient polyacrylamide gel electrophoresis and pH 4.3 disc-gel electrophoresis, and gave precipitin lines of antigenic identity on Ouchterlony analysis with goat antibody to the angiotensin II-generating protease. Thus, the angiotensin II-generating protease of human neutrophils has been identified as cathepsin G on the basis of subcellular localization, substrate specificity, physicochemical characteristics, and antigenic identity.

Identification of a Human Neutrophil Angiotensin II-generating Protease as Cathepsin G

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ABSTRACT A human neutrophil protease, initially termed neutral peptide-generating protease, has been shown to cleave angiotensin II directly from angiotensinogen and has been identified as leukocyte cathepsin G. When purified neutrophils were disrupted by nitrogen cavitation and fractionated by differential centrifugation, 44 and 24% of the angiotensin II-generating activity was in the lysosomal and undisrupted cell fractions, respectively. Cytochalasin B-treated human neutrophils stimulated with *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine released β-glucuronidase, lysozyme, and angiotensin II-generating protease in a dose-dependent fashion, consistent with localization of this protease to the neutrophil granule. Individually purified angiotensin II-generating protease and cathepsin G had similar proteolytic and esterolytic activity for angiotensinogen and *N*-benzoyl-L-tyrosine ethyl ester on a weight basis, exhibited identical mobilities by SDS-gradient polyacrylamide gel electrophoresis and pH 4.3 disc-gel electrophoresis, and gave precipitin lines of antigenic identity on Ouchterlony analysis with goat antibody to the angiotensin II-generating protease. Thus, the angiotensin II-generating protease of human neutrophils has been identified as cathepsin G on the basis of subcellular localization, substrate specificity, physicochemical characteristics, and antigenic identity.

INTRODUCTION

Human neutrophils were previously found to possess an enzyme that generates a vasoactive and spasmogenic peptide from heat-inactivated and genetically null α_1-antitrypsin-deficient plasma (1). The peptide, designated neutral peptide, is ~1,000 mol wt and is distinguished from kinins by its nearly neutral isoelectric point and its inactivation by trypsin as well as by chymotrypsin (1). The neutrophil neutral peptide-generating protease was purified to homogeneity and is a 29,000–30,000-mol wt single polypeptide chain, serine, neutral protease, inhibitable by diisopropylfluorophosphate, by α_1-antitrypsin, and by soybean and lima bean trypsin inhibitors (1, 2). This protease is distinguished from collagenase by its lower molecular weight and from elastase by its inability to digest orcein-elastin, to hydrolyze *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide (suc-[ala]$_3$-*p*NA),[1] or to be inhibited by the elastase inhibitor acetyl-(L-alanine)$_2$-proline-valine chloromethyl ketone (2). The plasma protein substrate for neutral peptide generation was recently purified to homogeneity and shown to be a 62,000–67,000-mol wt single polypeptide chain glycoprotein, with an isoelectric point of pH 4.6–5.0 (3), and an amino-terminal animo acid sequence identical to the covalent structure of angiotensin I (4). Furthermore, the peptide product of the interaction of purified neutrophil protease and angiotensinogen is angiotensin II on the basis of its antigenic and physicochemical characteristics, and amino acid composition and sequence (5). The angiotensin II-generating human neutrophil protease, which is also fibrinogenolytic and fibrinolytic (6), is now identified as cathepsin G on the basis of subcellular localization, substrate specificity, and physicochemical and antigenic characteristics.

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[1] *Abbreviations used in this paper*: BTEE, *N*-benzoyl-L-tyrosine ethyl ester; f-Met-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; Na^+K^+ATPase, Na^+K^+ adenosine triphosphatase; SDS-gradient PAGE, sodium dodecyl sulfate-gradient polyacrylamide gel electrophoresis; suc-(ala)$_3$-*p*NA, *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide.

METHODS

Bovine pancreatic α-chymotrypsin, cytochalasin B, phorbol myristate acetate, angiotensin II, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-Met-Leu-Phe), porcine pancreatic elastase (Sigma Chemical Co., St. Louis, Mo.); Affi-Gel Blue, Bio Gel P300 (Bio-Rad Laboratories, Richmond, Calif.); quaternary aminoethyl Sephadex A-25, phenyl-Sepharose CL-4B, Ficoll-Paque, Dextran T-500 (Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, N. J.); gelatin (Difco Laboratories, Detroit, Mich.); *N*-succinyl-(L-ala)$_3$-*p*-nitroanilide [suc-(ala)$_3$-*p*NA] (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.); and carboxymethyl cellulose (Whatman Ltd., Kent, England) were purchased as idicated. Purified human neutrophil cathepsin G and elastase were supplied by James Travis, Athens, Ga. (7).

Cytochalasin B was dissolved in dimethylsulfoxide at a stock concentration of 1 mg/ml; f-Met-Leu-Phe was dissolved in Hanks' balanced salt solution at a stock concentration of 0.25 mM. Stock solutions were divided into portions and frozen until use. Protein concentration was determined by OD_{280} with human serum albumin as standard. Polyacrylamide disc-gel electrophoresis was performed at pH 4.3 in 7.5% gels in a Buchler apparatus (Buchler Instruments, Inc., Fort Lee, N. J.) (8), SDS-gradient polyacrylamide gel electrophoresis (SDS-gradient PAGE) was carried out in 4–30% acrylamide gels (Pharmacia Fine Chemicals) as described (6). Immune and nonimmune goat IgG were purified as described (9).

Assay of angiotensin II-generating protease activity. Samples to be assessed for angiotensin II-generating protease activity were incubated in duplicate with partially purified or purified angiotensinogen (3) for 30 min at 37°C. The reactions were stopped by immersion of the tubes in ice, and each reaction mixture was bioassayed directly for angiotensin II contractile activity on isolated, atropinized, guinea pig terminal ileum suspended in Tyrode's solution and standardized with synthetic angiotensin II (10). The results are expressed as the mean contractile activity in angiotensin II equivalents. Generation of contractile activity was linear for the first 60 min of incubation.

Angiotensinogen was purified to homogeneity by a five step procedure that included ammonium sulfate precipitation, Affi-Gel Blue affinity chromatography, hydroxylapatite chromatography, phenyl-Sepharose hydrophobic chromatography, and Sephacryl S-200 gel filtration (3). Alternatively, angiotensinogen was partially purified by a procedure that included three of the purification steps. Partially purified material was heated to 61°C for 2 h, divided into aliquots and stored at −70°C at a final protein concentration of 3.4 mg/ml.

Isolation of human neutrophil angiotensin II-generating protease and cathepsin G. Human angiotensin II-generating protease was purified to homogeneity from the low speed sediment of homogenized neutrophils by elution in 1.0 M NaCl, aprotinin-Sepharose affinity chromatography, precipitation in low ionic strength buffer at pH 8.0, and Sephadex G-100 gel filtration (2).

Human neutrophil cathepsin G was purified from peripheral blood neutrophils and assayed by hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester (BTEE) in an assay standardized with bovine pancreatic α-chymotrypsin (11, 12). The granule fraction was obtained by disruption of 4.2×10^9 human neutrophils by nitrogen cavitation (400 lb/in^2 for 30 min at 4°C). The resultant suspension was centrifuged at 400 *g* for 20 min at 4°C to sediment nuclei and broken cells and at 10,000 *g* for 30 min at 4°C to pellet the granules. The cathepsin G was isolated from the granule pellet by the method of Baugh and Travis (7, 13). 5 μg purified cathepsin G yielded three stained protein bands of 26,000, 27,000, and 29,000 mol wt when analyzed by reduced SDS-gradient PAGE; 10 μg cathepsin G contained no detectable elastase activity as measured by the hydrolysis of suc-(ala)$_3$-*p*NA (14) in an assay standardized with porcine pancreatic elastase.

Isolation, subcellular fractionation, and lysosomal release of neutrophils. Normal human peripheral blood neutrophils were isolated by Ficoll-Hypaque density centrifugation, followed by dextran sedimentation and hypotonic lysis of residual erythrocytes (15). The 94–98% pure neutrophils were washed twice and suspended at a concentration of 10^7 neutrophils/ml in Hanks' balanced salt solution with 0.4% gelatin for enzyme release experiments and in modified Hanks' balanced salt solution containing 2.5 mM $MgCl_2$, lacking calcium (disruption buffer) for subcellular fractionations.

Neutrophils were disrupted by nitrogen cavitation and subcellular fractions were isolated by differential centrifugation (16). Each subcellular fraction was disrupted by sonication and assayed for angiotensin II-generating protease and marker enzyme activities. Enzyme activity was expressed as percentage of total enzyme activity available in the postcavitation starting material.

Duplicate 1-ml neutrophil suspensions were incubated with or without cytochalasin B (5 μg/ml) for 10 min at 37°C before incubation with a degranulating stimulus for various time intervals at 37°C. The release reaction was stopped by immediate centrifugation at 400 *g* for 10 min at 4°C. Supernatants and pellets, resuspended to 1 ml, were sonicated and assayed for angiotensin II-generating protease, β-glucuronidase, lysozyme, and lactic dehydrogenase. Enzyme activity released into the supernatant was expressed as percentage of the total enzyme activity available after sonication of replicate unstimulated neutrophil suspension. Total recoverable enzyme activities from supernatants and pellets of stimulated cells were consistently >90% of those in unstimulated cells. Lactic dehydrogenase (17), β-glucuronidase (18), lysozyme (19), ouabain-sensitive Na^+K^+ adenosine triphosphatase (Na^+K^+ATPase) (20), and 5′-nucleotidase (21) were assayed as described.

RESULTS

Lysosomal localization of the angiotensin II-generating protease. The location of the angiotensin II-generating protease in human neutrophils was determined by subcellular fractionation and by lysosomal enzyme release. The distribution of the marker enzymes in four separate experiments with $3.0–5.5 \times 10^8$ neutrophils (Fig. 1) was, as previously reported (16), based upon biochemical and ultrastructural analysis of the subcellular fractions: pellet 1 contained some of each marker, reflecting the presence of unbroken cells and nuclei; pellet 2 was enriched for lysosomal granules as indicated by the presence of β-glucoronidase and lysozyme; pellet 3 contained plasma membrane as indicated by the display of only ouabain-sensitive Na^+K^+ATPase; and supernatant three represented cytosol based on the selective distribution of lactic

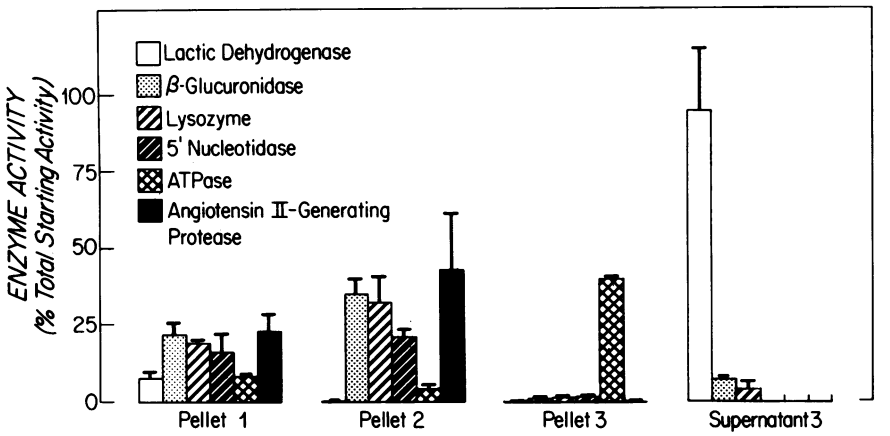

FIGURE 1 Distribution of enzyme activities among subcellular fractions of nitrogen-cavitated human neutrophils. Results shown are mean values with standard deviations from four separate experiments except for 5'-nucleotidase and Na^+K^+ATPase, which were determined in only two.

dehydrogenase. Of the initial angiotensin II-generating activity, 44% was detected in the granule pellet, 24% in the unbroken cells, 0.4% in the plasma membrane fraction, and none in the cytosol fraction, with a mean overall recovery of ~68%. Recoveries of the other marker enzymes ranged from 40 to 103%. Incubation of cytocholasian B-treated human neutrophils for 15 min at 37°C with 0.25 nM–2.5 μM f-Met-Leu-Phe resulted in a dose-related release of angiotensin II-generating protease from 2 to 67%, β-glucuronidase from 2 to 50%, and lysozyme from 0 to 44%. Release of lactic dehydrogenase was <5% at all doses.

Identification of angiotensin II-generating protease as cathepsin G. The subcellular localization of the angiotensin II-generating protease to the neutrophil lysosomal granule and the chymotryptic nature of the cleavage of angiotensin II from angiotensinogen (3, 5) prompted an analysis of the relationship of the angiotensin II-generating protease to cathepsin G with respect to substrate cleavage, physicochemical properties, and antigenic characteristics. 50, 100, and 200 ng of angiotensin II-generating protease and cathepsin G were each incubated with 40 μg (612 pmol) of purified angiotensinogen in 200 μl of 0.01 M Tris, pH 7.4, 0.15 M NaCl for 15 min at 37°C. The reactions were stopped by cooling the tubes on ice and angiotensin-II generation was bioassayed directly on the guinea pig ileum. Angiotensin II generation was dose-related and similar at the same input of each enzyme (Fig. 2). The angiotensin II-generating activity was 594±100 and 617±68 pmol angiotensin II/μg protease (mean±SD) for the angiotensin II-generating protease and cathepsin G, respectively. The BTEE-hydrolyzing activity of 500–5,000 ng of each protease was also dose-related and similar at the same input of each enzyme (Fig. 2). The BTEE-hydrolyzing activity was 0.24±0.05 and 0.27±0.04 units/μg protease (mean±SD) for the angiotensin II-generating protease and cathepsin G, respectively.

The angiotensin II-generating protease and cathep-

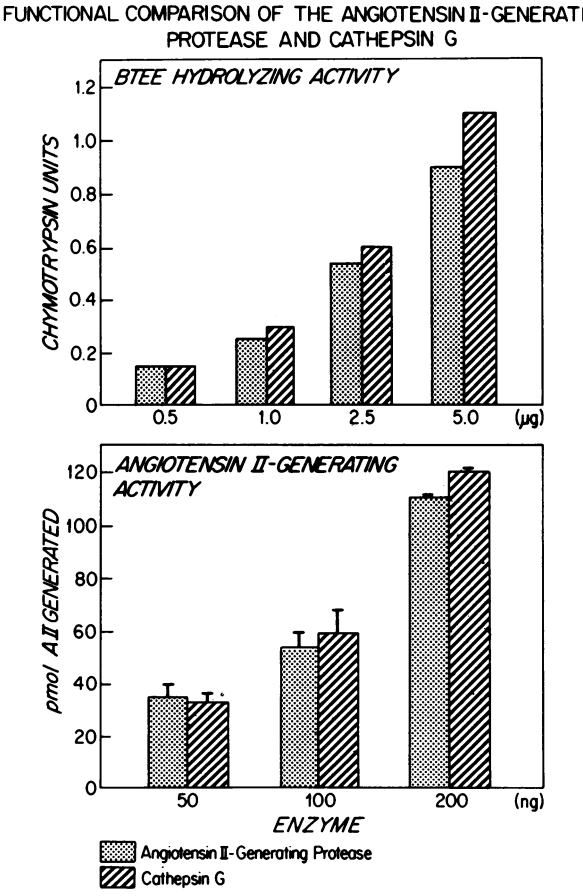

FIGURE 2 Dose-response of angiotensin II generation and BTEE hydrolysis by the angiotensin II-generating protease and cathepsin G.

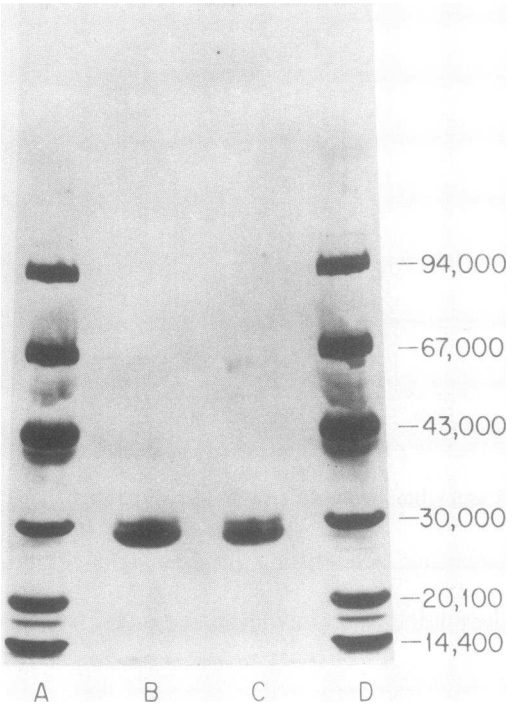

FIGURE 3 Reduced SDS-gradient PAGE of the angiotensin II-generating protease and cathepsin G. A and D, protein standards, B, 3.0 μg angiotensin II-generating protease, C, 3.0 μg cathepsin G.

sin G were compared for size and charge. On reduced SDS-gradient PAGE, 3 μg each of the angiotensin II-generating protease and cathepsin G showed major stained protein bands at 26,000, and minor bands at 27,000 and 29,000 mol wt (Fig. 3). 5 μg of each enzyme were subjected to replicate disc-gel electrophoresis at pH 4.3; each gave a major and two minor stained bands of identical electrophoretic mobilities in the region of 5 μg of lysozyme, which was electrophoresed in a parallel gel.

An antiserum to the angiotensin II-generating protease was obtained by injecting a goat with 100 μg of purified protein in complete Freund's adjuvant and boosting twice, 2 wk apart, with 75 μg of purified protein in incomplete Freund's adjuvant. The antiserum yielded a single precipitin arc when diffused against a 2 M NaCl extract of 2×10^6 human neutrophils. The IgG fractions from immune and preimmune goat serum were adjusted to a final protein concentration of 90 mg/ml. 1, 0.1, and 0.01 mg IgG were incubated with 500 ng angiotensin II-generating protease, and the mixtures were assayed for angiotensin II-generating activity. The doses of immune IgG inhibited angiotensin II-generating activity by 100%, 100%, and 30%, respectively, while no inhibition was detected with preimmune IgG. The antiserum failed to yield a precipitin arc when diffused against 10 μg of purified leukocyte elastase, and purified immune IgG did not inhibit the activity of 10 μg of the same enzyme on suc(ala)$_3$-*p*NA. The antiserum elicited a reaction of complete identity when diffused against 7.5 μg of the angiotensin II-generating protease, 7.5 μg of cathepsin G obtained from Dr. James Travis (7, 13), and 7.5 μg of purified cathepsin G (Fig. 4).

DISCUSSION

A human neutrophil neutral protease, previously termed the neutral peptide-generating protease (1) has recently been characterized as directly cleaving angiotensin II from angiotensinogen (3–5). This angiotensin II-generating protease has now been identified as leukocyte cathepsin G on the basis of lysosomal localization and evidence of functional, physicochemical, and antigenic identity. The protease was localized to the human neutrophil lysosomal granule by subcellular fractionation (Fig. 1) and noncytotoxic enzyme release. Although angiotensin II-generating protease distributed in a pattern similar to the lysosomal markers β-glucuronidase and lysozyme, the partial recovery of most markers necessitated confirmation of the protease's apparent lysosomal location by the demonstration of release with degranulation. Stimulation of cytochalasin B-treated human neutrophils with the chemotactic peptide f-Met-Leu-Phe resulted in the noncytotoxic release of the angiotensin II-generating protease with a dose-response similar to that of the lysosomal enzymes β-glucuronidase and lysozyme, indicating that angiotensin II-generating protease, like cathepsin G (22, 23), resides in the human neutrophil granule.

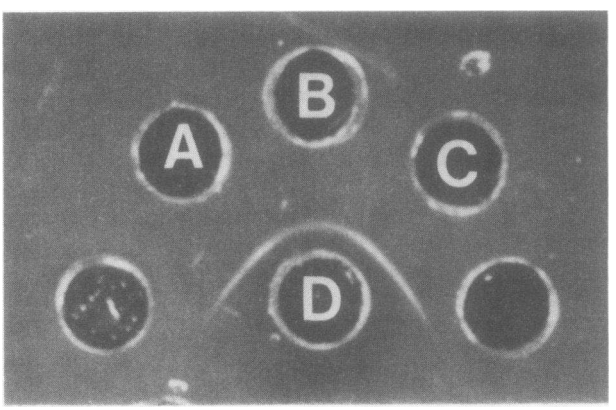

FIGURE 4 Antigenic analysis of the angiotensin II-generating protease and cathepsin G. A, 7.5 μg of angiotensin II-generating protease; B, 7.5 μg of cathepsin G from Dr. James Travis; C, 7.5 μg of purified cathepsin G; and D, undiluted antiserum to the angiotensin II-generating protease.

Functional similarity of the angiotensin II-generating protease and cathepsin G was established by demonstration of the ability of the angiotensin II-generating protease to cleave BTEE, a synthetic substrate of cathepsin G, and the ability of cathepsin G to generate angiotensin II from angiotensinogen. Both proteases cleaved each substrate with the same specific activities (Fig. 2). Further, the angiotensin II-generating protease, like cathepsin G (22, 24), was comprised of three cationic isoenzymes that corresponded to 26,000, 27,000, and 29,000 mol wt (Fig. 3). Finally, Ouchterlony analysis with a monospecific goat antibody to the angiotensin II-generating protease gave immunoprecipitates that fused in a line of antigenic identity between two preparations of cathepsin G and angiotensin II-generating protease (Fig. 4).

In the initial studies with heat-inactivated plasma used as substrate (1), ~90% of the angiotensin II-generating protease, then designated the neutral peptide-generating protease, was detected in the 100 *g* sediment of neutrophils, which had been broken by homogenization in 0.34 M sucrose and <5% was detected in the granule fraction sedimenting at 17,000 *g* (1). In a subsequent analysis (25) in which neutrophils were broken by hypotonic lysis in EDTA and fractionated on a sucrose gradient, the angiotensin II-generating activity was detected in association with 5′-nucleotidase and 25–40% of the angiotensin II-generating activity was detected in intact cell preparations, suggesting a membrane localization. Although 5′-nucleotidase is a plasma membrane ectoenzyme in guinea pig neutrophils (26, 27), it is now known not to be localized exclusively to the human neutrophil plasma membrane (28, 29). The failure to detect the angiotensin II-generating activity in association with the lysosomal marker β-glucuronidase during the earlier (1, 25) subcellular fractionation studies might have been due to lysosomal disruption and subsequent redistribution of the protease during homogenization of the neutrophils in low ionic strength medium. The apparent detection of 25–40% of the enzyme activity on the surface of intact cells, based on the inhibition of activity by the high molecular weight inhibitors α_1-antitrypsin and lima bean trypsin inhibitor (25), may have been the result of enzyme release by degranulation due to the heat-inactivated plasma used for substrate or the high neutrophil concentrations of 10^8/ml.

Angiotensin II is the principal biologically active peptide derived from the plasma protein angiotensinogen. Angiotensin II mediates a variety of biologic activities that include vasoconstriction (30), elevation of blood pressure (30), augmentation of venular permeability (31), and regulation of aldosterone production (32). Two pathways have been described for the generation of angiotensin II. Renin, a proteolytic enzyme of 40,000 mol wt (33), synthesized in the kidney, cleaves human angiotensinogen at a leucyl^{10}-valyl^{11} (Leu^{10}-Val^{11}) bond to release the decapeptide angiotensin I (34). Angiotensin I is then rapidly converted to angiotensin II by converting enzyme (35), a dipeptidyl carboxypeptidase found in high concentrations in the pulmonary vascular bed (36). A second pathway for angiotensin II-generation is initiated by a 28,700-mol wt rat salivary gland proteolytic enzyme, tonin (37), which directly cleaves angiotensin II from angiotensinogen and converts angiotensin I to angiotensin II (38). Leukocyte cathepsin G is functionally similar to tonin because it generates angiotensin II from angiotensinogen (3, 5) and converts angiotensin I to angiotensin II (39). The proteolytic function of cathepsin G that generates vasoactive angiotensin II (3, 5) and degrades fibrinogen and fibrin (6) may facilitate neutrophil egress from venules and movement into extravascular tissues, or it may provide a mechanism for local control of blood flow during the inflammatory response.

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