Human Neutrophils Release Serine Proteases Capable of Activating Prorenin

Victor J. Dzau, Debra Gonzalez, Carol Kaempfer, Daniel Dubin, and Bruce U. Wintroub

Proteases from human neutrophils can generate angiotensin II directly from angiotensin I or angiotensinogen. We examined whether neutrophil protease also influences angiotensin formation by activating human prorenin (also called inactive renin). When incubated with partially purified plasma and amniotic prorenin, sonicates from 10^6 neutrophils resulted in 120 ± 30% and 1,240 ± 290% increase in renin activity, respectively. The pH optimum of neutrophil prorenin-activating enzyme(s) is 6.5–7.0, and the activity of the enzyme(s) is inhibited by a mixture of serine protease inhibitors but not by inhibitors of other proteases, suggesting that prorenin-activating enzyme(s) is a neutral serine protease(s). Stimulation of neutrophils by f-met-leu-phe in the presence of cytochalasin B resulted in release of prorenin-activating enzyme(s) in a dose-dependent fashion. We attempted to isolate prorenin-activating enzyme(s) from neutrophil granules using aprotinin-affinity and carboxymethyl cellulose chromatographies. Prorenin-activating enzyme(s) coeluted with cathepsin G and elastase activities. Prorenin activation was greatly inhibited by anticathepsin G antisera. Purified cathepsin G activated prorenin in a dose-dependent fashion. Elastase probably also contributes to prorenin activation since purified elastase also activated human prorenin. We speculate that this neutrophilic angiotensin-generating system may play a role in the local generation and concentration of angiotensins by influencing multiple steps of the renin-angiotensin system. (Circulation Research 1987;60:595–601)

Up to 90% of human plasma renin exists in an inactive form that is believed to be the biosynthetic precursor of the active enzyme.1,2 The identification of plasma and amniotic fluid inactive renins as prorenin has been recently made possible by studies of prosegment antibody12 and of expressed prorenin, using recombinant DNA technology.6 Plasma prorenin can be activated by cold or acid treatment and by limited proteolysis with serine proteases such as trypsin and urinary kallikrein.1,2 Although plasma contains large quantities of this putative renin precursor, the fate of plasma prorenin has not been defined. One possible role of plasma prorenin is to serve as a source for the rapid production of active enzyme at specific local vascular sites.3 Thus, like other vasoactive molecules, such as histamine, bradykinin, and serotonin, angiotensin II may be formed in the vicinity of tissue targets so as to modulate local vascular events.3 The peripheral conversion of plasma prorenin to the active enzyme at a tissue site or cell surface may result in a high local concentration of active renin and accelerated generation of angiotensin II.

Among the possible candidates responsible for enzymatic activation of plasma prorenin in tissue sites, neutrophil-dependent mechanisms are particularly attractive. The human neutrophil contains the lysosomal serine protease cathepsin G, which is capable of generating angiotensin II (AII) from angiotensinogen or angiotensin I (AI).3,4 Since prorenin can be activated by other serine proteases, the present study investigated whether the neutrophilic serine protease can activate human prorenin. The data indicate that human neutrophil granules contain cathepsin G and elastase that can activate prorenin.

Materials and Methods

The following materials were used: bovine pancreatic α-chymotrypsin; cytochalasin B; angiotensins I and II; N-formyl-L-methionyl-L-leucyl-L-phenylalanine (f-met-leu-phe); benzoyl-L-tyrosine ethyl ester (BTEE); phenylmethl sulfonyl fluoride (PMFS); soybean trypsin inhibitor (SBTI); dissoypropylfluorophosphate (DFP); aprotinin; disodium ethylenediamine tetraacetate (EDTA); N-ethylenediamine (NEM); iodoacetate (Sigma Chemical Co., St. Louis, Mo.); porcine pancreatic elastase (Worthington, Malvern, Penn.); N-succinyl-(L-alanyl)-p-nitroanilide [suc-(ala)-pNA] (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.); Ficoll-Paque dextran T-500 (Pharmacia Fine Chemicals, Division of Pharmacia, Inc., Piscataway, N.J.); sodium tetrathionate (ICN Biomedicals, Plainview, N.Y.); dithiothreitol (DTT) (Calbiochem, San Diego, Calif.); 2,3, dimercapto-1-propanol (BAL) (Aldrich, Milwaukee, Wis.); captoprill (Squibb, Princeton, N.J.).
Isolation of Neutrophils and Formation of Neutrophil Sonicates

Human peripheral blood neutrophils from normal volunteers were isolated by dextran sedimentation, hypotonic lysis of erythrocytes, and Ficol-Hyphaque density centrifugation as described. Neutrophils, 10^7/ml in Hank's balanced salt solution (HBSS), were broken by sonication, 30 W at 20 kHz (Sonicator Model W220F, Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) for 60 seconds at 0°C and retained as neutrophil sonicates.

Release of Neutrophil Lysosomal Enzyme

Neutrophils were suspended in HBSS in 0.1% bovine serum albumin, and 1-ml suspensions (1 x 10^6 cells/ml) were incubated with or without cytochalasin B (5 µg/ml) for 15 minutes at 37°C before incubation with f-met-leu-phe (2.5 x 10^-6 to 2.5 x 10^-10 M) for 15 minutes at 37°C. The release reaction was stopped by centrifugation at 400g for 10 minutes at 4°C. Supernatants were retained, pellets were washed and resuspended to 1 ml and sonicated as described earlier, and 200 µl of neutrophil supernatants and cell pellets were assayed for prorenin activation (see section on enzyme assays), β-glucuronidase, and lactic dehydrogenase activity. Total recoverable enzyme activity from supernatants and pellets of stimulated cells was consistently >90% of that in unstimulated cells.

Enzyme Assays

Renin activity was measured by incubation of 50 µl of renin source with 250 µl of renin-free plasma from nephrectomized sheep (810 ng of angiotensin I equivalent/ml) at 37°C, pH 7.4, for 1 hour in the presence of angiotensinase inhibitors in final concentrations of 3.4 mM 8-hydroxyquinoline, 1.6 mM dimercapto propaneol, and 2.5 mM sodium EDTA. Renin-free plasma was obtained by passage through antirenin affinity chromatography. The final volume of reaction mixture was 300 µl. Incubates were boiled and centrifuged in an Eppendorf micro-centrifuge (13,000g x 5 minutes), and the supernatants were assayed for angiotensin I generation. The stability of angiotensin I was assessed in each experiment by examining the recovery of exogenously added angiotensin I in the incubation mixture. A recovery of <90% was considered unacceptable.

The extent of prorenin activation was measured in HBSS, pH 7.4, by incubating 100 µl of a prorenin source in the presence of 100 µl of trypsin (final concentrations 0.1 to 5 mg/ml in 0.1% bovine serum albumin), or sonicates from 10^6 neutrophils, or an enzyme solution such as cathepsin G or elastase for 30 minutes at 37°C. The final volume of the assay was 200 µl. These experimental conditions were determined to be optimal for activation on the basis of a series of pilot studies. Human amniotic fluid with little or no measurable active renin served as the source of prorenin. The concentration of trypsin-activatable renin was 98 ± 12 ng Al/ml/hr (n = 13). In selected experiments, partially purified human prorenin from plasma was employed (see below). The activation was stopped by the addition of 33 µl of protease inhibitors: 0.44 trypsin inhibition units (TIU) aprotinin, 1 mg/ml SBTI, and 1 mg/ml PMSF. Under the above condition, our studies have determined that this combination of inhibitors completely inhibited the BTEE hydrolytic activity of our samples or 10^-5 M cathepsin G (data not shown). Total renin activity in the incubate was then assayed as described. Activation of prorenin was determined by subtraction of renin activity of untreated samples from that measured after enzyme treatment.

To demonstrate that neutrophil sonicates generated renin from human plasma or amniotic fluid, 100 µl neutrophil sonicates were incubated with 100 µl amniotic fluid or plasma, and reaction mixtures were exposed to 30 µl of 1:50 diluted preimmune serum or antihuman renin antiserum (R1723) for 60 minutes at 37°C prior to assessment of renin activity.

To assess the pH optimum of neutrophil activity, 65 µl neutrophil sonicates were incubated with 100 µl amniotic fluid titrated with sodium citrate, sodium acetate, potassium phosphate, or Tris-HCl buffers to yield final pH varying by 0.5 units from pH 3.0—9.0, and the activation of amniotic prorenin was determined. At the end of the activation period, the reaction mixture was titrated to neutral pH, and angiotensin I generation was assayed as described.

Neutrophil cathepsin G was measured by hydrolysis of BTEE or conversion of angiotensin I to angiotensin II by high performance liquid chromatography (HPLC) assay as described. Neutrophil elastase activity was determined by a spectrophotometric assay, which measured hydrolysis of succinyl (ala3) p-nitroanilide.

Enzyme inhibition experiments were carried out by incubation of 50 µl of an enzyme source with 50 µl of an appropriate inhibitor combination in 50 µl HBSS for 60 minutes at 37°C prior to assessment of the activation of amniotic fluid inactive renin. The inhibitors employed were: 1) EDTA (0.25 mM) plus capto-pretrol (10^-4 M); 2) sodium tetrathionate (0.25 mM), N-ethylendiamine (1 mM) plus iodoacetate (1 mM); 3) PMSF (0.5 mg/ml), DFP (5 mM), SBTI (1 mg/ml) plus aprotinin (0.44 TIU); 4) DTT (2 mM) plus BAL (1.6 mM); or 5) a combination of all of the above.

Purification of Enzymes

Amniotic fluid or plasma inactive renin (prorenin) was partially purified by affigel blue chromatography. Amniotic fluid or plasma (50 ml) was applied to a 20-ml affigel blue column under previously described conditions. Prorenin was eluted by 0.5 NaCl. A tenfold purification and 80% recovery were obtained. The eluate contained no detectable renin substrate or antitrypsin activity as described. The fractions containing prorenin were pooled and concentrated fivefold by ultrafiltration (Amicon, Danvers, Mass.).

The human neutrophil prorenin-activating enzyme was purified from fresh human neutrophils by a modification of a method originally designed for isolation of neutrophil elastase and cathepsin G. Briefly,
the granule fraction was first obtained by disruption of
4.2 X 10^9 human neutrophils by nitrogen cavitation
(400 psi for 30 minutes at 4° C) in HBSS buffer. The
resultant material was centrifuged at 400g for 20
minutes at 4° C to separate nuclei and unbroken cells
and at 10,000g for 30 minutes at 4° C to pellet the
granules. The granule pellet was suspended in 0.01 M
Tris-HCl, pH 7.4, 1 M NaCl, dispersed by sonication,
and centrifuged as described. The supernate was sub-
jected to aprotinin-Sepharose affinity chromatography
(2.5 x 12 cm), eluted with 0.1 M sodium acetate and 2
M NaCl, pH 4.5. Fractions containing prorenin-acti-
vating activity were pooled, dialyzed with 0.1 M
sodium acetate, pH 5.0, and applied to carboxy-
methyl-cellulose (CM) chromatography (2.5 x 12 cm)
equilibrated with the same buffer. Elution of enzymes
was carried out by stepwise application of 20 ml of 0.5
M NaCl and 1.0 M NaCl. Pools were concentrated to
20 ml and assessed for appropriate enzymes.

Antiserum to Renin
Antirenin antibody (R1723) was obtained from a
rabbit immunized with pure human renal renin. Antiserum R1723 has a 50% inhibitory titer of
1:30,000 against 1 x 10^4 Goldblatt units of Medical
Research Council human renal renin. The antiserum is
specific for renin as demonstrated by immunodiffusion
and immunoelectrophoresis analysis. Furthermore, it
did not cross-react with cathepsins D, G, or B, elas-
tase, renal kallikrein, or pepsin. This antiserum binds
both renin and prorenin.

Antiserum to Cathepsin G
Antiserum to purified cathepsin G was obtained
from a goat as described. This antiserum yielded a
single precipitin arc and a reaction of complete identify
when diffused against a 2 M NaCl extract of 2 x 10^6
human neutrophils and 7.5 /g of cathepsin G. The
antiserum failed to yield precipitin arc when diffused
against 10 /g of leukocyte elastase. The IgG fraction
(linal concentration 90 mg/ml) inhibited 100% of the
ability of 500 ng of cathepsin G to generate angiotensin
II from angiotensin I.

Results
Neutrophil Activation of Prorenin
Neutrophil sonicates increased the renin activity of
plasma and amniotic fluid by 120 ± 30% (n = 6, p <
0.001) and 1,243 ± 290% (n = 5, p < 0.001), respec-
tively. The amniotic fluid renin activity generated by
human neutrophils was fully neutralized by antirenin
antiserum (Figure 1). A similar result was observed for
plasma (data not shown). The pH optimum for the
neutrophil activation of prorenin was 6.5-7.0. PMSF,
DFP, SBTI, and aprotinin completely inhibited neu-
rophil-dependent activation of prorenin, whereas a
variety of other inhibitors had no demonstrable effect
(Figure 2). Taken together, these experiments suggest
that the neutrophil factor is a neutral serine pro-
peases(s).
Table 1. Prorenin Activation by Enzyme Released by Neutrophils

<table>
<thead>
<tr>
<th>Experimental conditions*</th>
<th>% Prorenin activation (n = 4)</th>
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<tbody>
<tr>
<td>PMN + buffer (HBSS)</td>
<td>0</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻⁶ M fMLP + cyto B</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻⁸ M fMLP + cyto B</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻¹⁰ M fMLP + cyto B</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻⁶ M fMLP + buffer</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻⁸ M fMLP + buffer</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>PMN + 2.5 × 10⁻¹⁰ M fMLP + buffer</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>PMN + buffer + cyto B</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + 2.5 × 10⁻⁶ M fMLP + buffer</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + 2.5 × 10⁻⁸ M fMLP + buffer</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + 2.5 × 10⁻¹⁰ M fMLP + buffer</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + buffer + cyto B</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + buffer + buffer</td>
<td>0</td>
</tr>
</tbody>
</table>

*200 μl supernatant from each above condition was incubated with 100 μl of amniotic fluid prorenin as described. PMN = 10⁶ neutrophils in 1 ml of reaction volume; fMLP = f-met-leu-phe (2.5 × 10⁻⁸ to 10⁻¹⁰ M final concentration); cyto B = cytochalasin B (5 μg/ml final concentration).

% activation = final renin activity - initial renin activity/initial renin activity in amniotic fluid prior to activation × 100.

n = number of experiments, results expressed as mean ± SEM.

HBSS = Hank’s balanced salt solution.

15, 25, and 42%, respectively. This result is consistent with localization of the prorenin-activating enzyme(s) to releasable subcellular compartment.

Identification of Neutrophil Prorenin-Activating Enzyme

The major releasable neutrophil neutral proteases are the azurophil granule enzymes cathepsin G and elastase. Since the prorenin-activating enzyme was a releasable serine protease, we subjected the activity to a protocol designed for purification of cathepsin G and elastase from the granule fraction of neutrophils broken by nitrogen cavitation. Neutrophil granules from 4.2 × 10⁶ cells were suspended in 0.01 M Tris, pH 7.4, containing 1.0 M NaCl and sonicated for 1 minute at 4°C to solubilize granule enzymes. The sonicate, which contained the prorenin-activating activity, was subjected to aprotinin-affinity chromatography. All detectable activity was absorbed to aprotinin sepharose and eluted by application of 4 volumes of 0.1 M acetate, pH 4.5, 2.0 M NaCl. Prorenin-activating activity coeluted with cathepsin G and elastase activities as determined by BTEE hydrolysis and angiotensin I conversion to angiotensin II (Figure 4). Following concentration, the prorenin-activating was applied to CM-cellulose and eluted with 0.5 and 1.0 NaCl. The starting material and each pool were assayed for prorenin activation and cathepsin G, and elastase activities (Table 2). The prorenin-activating activity increased approximately 3.5 times in the 1 M NaCl eluate parallel with that of cathepsin G (Table 2). To further examine the role of cathepsin G, the 0.5 and 1.0 M NaCl pools obtained after the CM-cellulose step.

Figure 4. Elution profile of aprotinin-affinity chromatography. Note that the prorenin activating enzyme (Panel B) coeluted with cathepsin G activity, i.e., BTEE hydrolysis activity (Panel A) and conversion of AI to All (Panel C). Percent control (in ordinate) represents ratio of final activity divided by the basal renin activity in the fluid prior to incubation.

Table 2. CM-Cellulose Chromatography of Human Neutrophil Prorenin Activating Enzyme

<table>
<thead>
<tr>
<th>Activation of prorenin (pmol Al/mg)</th>
<th>Cathepsin G (units/mg)</th>
<th>Elastase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting material (after aprotinin affinity chromatography)</td>
<td>31.8</td>
<td>330.7</td>
</tr>
<tr>
<td>CM effluent</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CM eluate —</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>57.8</td>
<td>32.4</td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>107.2</td>
<td>&gt;800*</td>
</tr>
</tbody>
</table>

*Cathepsin G activity was beyond sensitivity of the assay, i.e., >479 nmol AI; hence, the specific activity was >800 nmol AI/mg.

Figure 3. Dose-response relation of neutrophil-release supernatants to the concentration of f-met-leu-phe in the presence of cytochalasin B (n = 4). Ordinate is percent activation that represents the increase in renin activity of amniotic fluid as a result of incubation with the supernatants divided by the basal renin activity in the fluid prior to incubation.
were treated with antiserum specific for cathepsin G (Figure 5). Indeed, prorenin activation in both pools was greatly inhibited by anticotathepsin G antiserum. This experiment was repeated twice with almost identical results. To confirm further that cathepsin G is responsible at least in part for neutrophil-dependent prorenin activation, amniotic fluid was incubated with various amounts of purified cathepsin G that contained no detectable elastase activity. Cathepsin G 10^-8 to 10^-6 M activated amniotic fluid prorenin in a dose-response fashion (Figure 6).

However, the presence of elastase, as well as cathepsin G activity in the 0.5 M NaCl pool, indicates that we cannot exclude elastase's contribution to prorenin activation in this pool. To examine whether elastase will activate human amniotic fluid inactive renin, in separate experiments, amniotic fluid was incubated with 4 x 10^-4 M to 4 x 10^-7 M purified elastase. Our results demonstrated that elastase also activated human prorenin (Figure 6). On the basis of these preliminary experiments, cathepsin G appears to be more effective than elastase in activating human prorenin. Unfortunately, we do not have antielastase antibody to examine its inhibitory effect on the prorenin-activating activity in the CM-cellulose column eluates. However, with the nearly complete inhibition of the activity by anticotathepsin G antibody and the higher relative specific activity of cathepsin G in the column eluates, one would expect that most of the enzymes present in these eluates are cathepsin G.

Discussion

The bulk of renin in plasma is prorenin. The mechanism and location of in vivo activation of this circulating molecule are unknown. Since recent evidence indicates that tissue localized angiotensin II formation may occur, potential pathways that may initiate or amplify angiotensin formation at a local tissue site are of interest. Renin proenzyme and its substrate, angiotensinogen, are of 65,000 MW or less and are therefore of appropriate size to pass from the intravascular to the extravascular environment. For this reason, extravascular formation of angiotensin II in the vicinity of its smooth muscle target is possible. The data in this study suggest that releasable granules of human neutrophils contain proteases capable of activating prorenin in human plasma and amniotic fluid. These proteases have many of the same properties as cathepsin G and elastase. Since previous data demonstrated that cathepsin G could cleave angiotensin II directly from angiotensinogen or angiotensin I, it is possible that a neutrophil dependent-angiotensin II forming pathway would provide a mechanism for local activation of prorenin and amplification of angiotensin II formation in the microcirculation by acting simultaneously at multiple sites of the renin-angiotensin cascade (Figure 7).

Local angiotensin II formation at inflammatory sites may play a role in mediating or modulating local vascular processes such as edema formation. Administration of captopril, an inhibitor of angiotensin-converting enzyme, reduced the rat cutaneous vascular permeability and edema formation in response to histamine, bradykinin, and serotonin, suggesting that angiotensin II may also facilitate vascular permeability responses to other vasoactive mediators. In addition, angiotensin may influence local vascular tone via the stimulation of the synthesis of prostaglandins I2 and E2.

Figure 5. A representative experiment on the effect of anticotathepsin G antiserum (anti-CG) on the neutrophil prorenin-activating enzyme eluted from CM-cellulose column. CM-1 is eluted by 0.5 M NaCl and CM-2 by 1 M NaCl. Note incubation of these fractions with amniotic fluid (AF) resulted in substantial increases in total renin activity. Prorenin activation was inhibited by anti-CG antiserum. This experiment was reproduced twice.

Figure 6. Activation of amniotic fluid inactive renin by purified prorenin by various concentrations of purified cathepsin G and elastase. The results are expressed as percent activation of amniotic fluid renin activity (RA): (total RA after activation) – (control RA)/(control renin RA) x 100.
by blood vessel walls and the release of norepinephrine from peripheral nerve endings. Angiotensin may be released by blood vessel walls and the release of norepinephrine.

Angiotensin may also play a role in mediating cellular response in granulomatous reactions. Indeed, in vivo experiments have demonstrated that the granulomatous inflammatory responses to *schistosoma mansoni* eggs and to bacille calmette-Guerin in mice were diminished by captopril treatment. Because neutrophil-dependent angiotensin generation is not inhibited by captopril, it is unlikely to account for these latter effects. On the other hand, experimental evidence demonstrates that the enhanced vascular permeability and increased blood flow associated with acute, cutaneous, zymosan-induced inflammation is neutrophil-dependent. The neutrophil-angiotensin pathway may represent a mobile system by which the neutrophil initiates or amplifies local generation of angiotensin II so as to modify local blood flow, vascular permeability, and cellular infiltration.

Whether the neutrophil-renin interaction occurs in vivo remains to be determined. Indeed, the physiologic role of the neutrophil-angiotensin pathway has not been determined. Our in vitro data preclude an accurate quantitation of the kinetics of activation or the capacity of the system in situ. However, extrapolation can be made from the in vitro experiments. In our experiments, 200 µl of released supernatants from 10⁶ neutrophils readily activated plasma or amniotic fluid prorenin whose concentrations in the test tube were approximately 20 ng Al/ml/hr. The concentrations of neutrophils and prorenin approximate their physiologic concentrations in the circulation. It is possible that the concentrations of neutrophils, prorenin, and angiotensinogen might be substantially higher at local tissue sites, and under these conditions neutrophil-influenced angiotensin II formation might proceed.

References


**KEY WORDS** • protein activation • cathepsin G • elastase
neutrophil-angiotensin pathway • tissue renin angiotensin
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