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 fluid 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 apropinin-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)
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 Ficoll-Hypaque 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 W220F, Heat Systems-Ultrasonics, Inc., Plainview, New York) 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.

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 dimercaptopropanol, 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 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 (ala)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 active renin. The inhibitors employed were 1) EDTA (0.25 mM) plus captopril (10^-4 M); 2) sodium tetragenate (0.25 mM), N-ethylendiamine (1 mM) plus iodoacetat (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,
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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 subjected 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-activating activity were pooled, dialyzed with 0.1 M sodium acetate, pH 5.0, and applied to carboxymethyl-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 to purified cathepsin G was obtained from a goat as described. This antiserum yielded a single precipitin arc and a reaction of complete identity when diffused against 2 M NaCl extract of 2 x 10^6 human neutrophils and 7.5 μg of cathepsin G. The antiserum failed to yield precipitin arc when diffused against 10 μg of leukocyte elastase.

Antiserum to Cathepsin G
Antiserum to purified cathepsin G was obtained from a rabbit immunized with pure human renal renin. Antiserum R1723 has a ED50 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, elastase, renal kallikrein, or pepsin. This antiserum binds both renin and prorenin.

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), respectively. 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 neutrophil-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 proteases(s).

Neutrophil Release of Prorenin-Activating Enzyme
Since neutral proteases are constituents of neutrophil granules, human neutrophils were examined for release of the prorenin-activating enzyme. Duplicate samples containing 10^6 neutrophils in 1 ml reaction volume were exposed to 2.5 X 10^-6 M, 2.5 X 10^-8 M, and 2.5 X 10^-10 M f-met-leu-phe in the presence and absence of cytochalasin B, and 200 μl of release supernatant were retained and assessed for prorenin activation. In addition, β-glucuronidase and lactic dehydrogenase (LDH) activities were also determined. At all concentrations of f-met-leu-phe, LDH release was less than 5%. In the presence of cytochalasin B, f-met-leu-phe resulted in the release of prorenin-activating enzyme(s) into the supernatant (Table 1). Neither f-met-leu-phe nor cytochalasin B alone resulted in the release of the enzyme. The release of prorenin-activating enzyme(s) into the supernatants followed a dose-response relation to the concentration of f-met-leu-phe in the presence of cytochalasin B (Figure 3). The amount of prorenin-activating enzyme(s) in response to the above 3 concentrations of f-met-leu-phe represented 13, 23, and 61% of total cellular activity, respectively. In parallel, β-glucuronidase release was...
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 x 10⁻⁸ M fMLP + cyto B</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>PMN + 2.5 x 10⁻⁸ M fMLP + cyto B</td>
<td>34 ± 2</td>
</tr>
<tr>
<td>PMN + 2.5 x 10⁻⁶ M fMLP + cyto B</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>PMN + 2.5 x 10⁻⁸ M fMLP + buffer</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>PMN + 2.5 x 10⁻⁶ M fMLP + buffer</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>PMN + 2.5 x 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 x 10⁻⁸ M fMLP + buffer</td>
<td>0</td>
</tr>
<tr>
<td>Buffer + 2.5 x 10⁻⁶ M fMLP + buffer</td>
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</tr>
<tr>
<td>Buffer + 2.5 x 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 x 10⁻⁶ to 10⁻¹⁰ M final concentration); cyto B = cytochalasin B (5 µg/ml final concentration).

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

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

HBSS = Hank’s balanced salt solution.

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 x 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 assessed 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 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.

Figure 4. Elution profile of aprotinin-affinity chromatography. Note that the prorenin activating enzyme (Panel B) co-eluted 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 AI/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>0.5 M NaCl</td>
<td>57.8</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.
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were treated with antiserum specific for cathepsin G (Figure 5). Indeed, prorenin activation in both pools was greatly inhibited by anticathepsin 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 $10^{-4}$ 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, we performed experiments with various concentrations of 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. Moreover, with the nearly complete inhibition of the activity by anticathepsin 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 microenvironment. 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 $I_2$ and $E_2$.

**FIGURE 5.** A representative experiment on the effect of anticathepsin 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) $\times$ 100.
by blood vessel walls and the release of norepinephrine from peripheral nerve endings. 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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