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ACTIVATION OF INACTIVE RENIN DURING THE SELECTIVE DESTRUCTION OF PROTEINASE INHIBITORS IN HUMAN PLASMA BY A METALLOPROTEINASE IN *BITIS ARIETANS* VENOM

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Summary

Puff adder venom, which has been pretreated with phenylmethylsulphonyl fluoride and extensively dialysed, is capable of destroying selectively proteinase inhibitory activity in human plasma by an action of an EDTA-sensitive venom proteinase. We found that incubation of 1/5 vol. of such venom with human plasma at 25°C leads to a concomitant increase in renin to 4.4 times control by 5 h. The activation of inactive renin was abolished by 10 mM EDTA and the rate of activation was reduced by 50% in the presence of 5 mM phenylmethylsulphonyl fluoride and by 90% when 0.32 mg/ml soybean trypsin inhibitor and 5 mM *N*-ethylmaleimide were added as well. The venom proteinase thus appears to activate inactive renin via an activation of endogenous plasma proteinases. This may be accomplished either by activation of proteinase precursors or action on proteinase inhibitor-proteinase complexes. By destroying proteinase inhibitors at the same time as it activates endogenous proteinases, *Bitis arietans* metalloproteinase would appear to be particularly useful for studies of the activation of inactive renin in human plasma, since endogenous proteinases are then free to activate inactive renin without subsequent inhibition by endogenous proteinase inhibitors.

Introduction

Renin (EC 3.4.99.19) is a highly specific carboxyl proteinase which cleaves the Leu^{10}-Leu^{11} bond of angiotensinogen in plasma to give angiotensin I. Angiotensin I is converted to angiotensin II which has important roles in the regulation of sodium and fluid homeostasis and blood pressure [1]. Under

normal circumstances 60—90% of renin in human plasma and amniotic fluid is in an inactive form [2—4]. This may be a prorenin or a noncompetitively inhibited form and is also found in kidney [1]. Inactive renin can be activated by the serine proteinases trypsin [5,6], kallikrein [7,8] and fibrinolysin [9,10] or by the carboxyl proteinases pepsin [5,11,12] and cathepsin D [11]. However, the possibility of activation occurring in plasma in vivo is lessened by the fact that endogenous proteinases are present as inactive protein-bound or precursor forms. The presence of endogenous proteinase inhibitors thus limits studies of potential endogenous enzymatic activation of inactive renin.

A potent proteolytic activity, identified recently in snake venoms of the Crotalid, Viperid and Colubrid families, preferentially attacks the proteinase inhibitors in human serum and has only a limited activity towards other protein substrates [13]. Incubation with venom results in a gradual loss of all detectable trypsin and chymotrypsin inhibitory activity in human serum [14]. Two proteinases with this activity have been purified from *Crotalus adamanteus* venom; both require Zn^{2+} or Ca^{2+}, do not require thiol compounds for activation, and are not inhibited by diisopropylphosphorofluoridate [13] or phenylmethylsulphonyl fluoride [14]. Of the venoms tested, that of the puff addder, *Bitis arietans*, has the most powerful inactivating activity [14] and the present study examined its ability to activate inactive renin.

Materials and Methods

Materials. Citrated plasma from individual subjects was obtained from the Sydney Blood Bank within an hour of collection and stored at −20°C before use. Lyophilised *Bitis arietans* venom was from Sigma (Lot No. 53C-2080), as were phenylmethylsulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide.

Incubation with venom. A 6 mg/ml solution of venom (12 A_{280} units/ml, see Ref. 14) in 50 mM Tris buffer, pH 7.4 or 8.0, containing 2 mM $CaCl_2$, was incubated with 5 mM phenylmethylsulphonyl fluoride at 25°C for 15 min to inhibit its serine proteinase activity, and then dialysed overnight in 50 vol. Tris-HCl buffer, with three changes to remove excess, unbound phenylmethylsulphonyl fluoride [14]. Portions of 0.05 ml were then incubated with 0.2 ml portions of human plasma at 25°C for 0—5 h [14]. The reaction was stopped by the addition of 0.01 ml each of solutions of EDTA, phenylmethylsulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide added to give 10 mM, 5 mM, 0.32 mg/ml and 5 mM, respectively. In different experiments, these inhibitors were added alone or in combination before addition of plasma to observe their effect on the rate of activation of inactive renin by the phenylmethylsulphonyl fluoride-treated, dialysed venom.

Renin assay. Renin was measured as its initial velocity of formation of angiotensin I during incubation at 37°C, pH 7.4, with $40 \times K_m$ angiotensinogen, added as 4 vol. plasma from nephrectomized sheep, and angiotensin I was quantified by radioimmunoassay [4,11]. Results were expressed as mean ± S.E.

Results

Addition of 1/5 vol. of phenylmethylsulphonyl fluoride-treated, dialysed venom to pooled samples of human plasma in 15 experiments, resulted in a

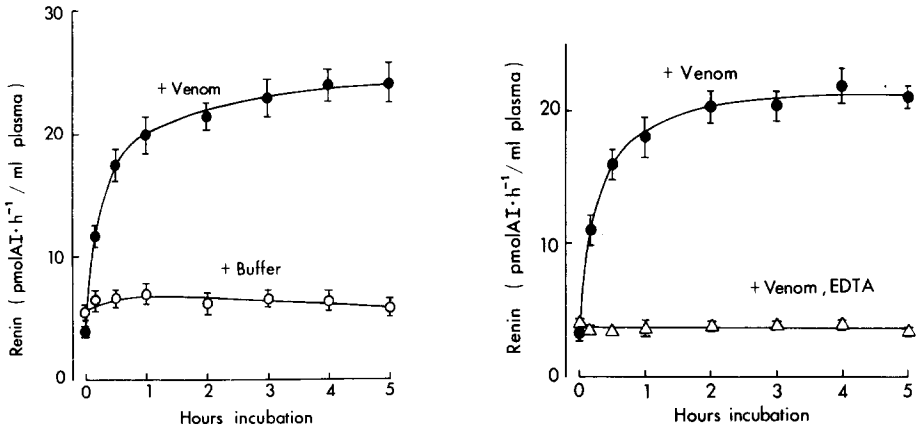

Fig. 1. Activation of inactive renin during 25°C incubation of phenylmethylsulphonyl fluoride-treated *Bitis arietans* venom with human plasma in $n = 15$ experiments. The plasma was used as a different pool (made from 8—12 subjects) in each experiment. As a control, Tris-HCl buffer was added instead of venom. AI, angiotensin I.

Fig. 2. Complete inhibition of venom-mediated activation of inactive renin in the presence of 10 mM EDTA ($n = 7$). AI, angiotensin I.

rapid increase in renin (Fig. 1). The initial rate of increase in renin was 1 pmol angiotensin I/h per ml per min and the average time needed for 50% activation (half-time) was 20 min. The activation of inactive renin was essentially complete by 3 h. At this time the inhibitory activity of α_1-proteinase inhibitor, a major inhibitor of endogenous serine proteinases, has been shown to be eliminated completely by the concentration of venom used [14]. Renin did not increase in control plasma incubated in the absence of venom. Other controls indicated negligible formation of angiotensin I (less than 1 pmol/ml) during the 25°C incubation of plasma with venom, thus ruling out the possibility that the results may have been due to renin-like activity in the venom hydrolysing endogenous plasma angiotensinogen. Furthermore, the similarity in zero time points with and without venom in Fig. 1 shows that any renin activity in the venom did not hydrolyse angiotensin I from exogenous sheep angiotensinogen during the renin assay. Preparations of venom having an amorphous appearance were not able to activate inactive renin and, interestingly, Kurecki et al. [13] have reported that such venoms lack α_1-proteinase inhibitor inactivating activity.

The degree of activation using venom was similar to that achieved by the most commonly used method of activation, viz. dialysis to pH 3.3 for 24 h at 4°C before dialysis to and assay at pH 7.4 [2].

Addition of EDTA to the venom-plasma incubates abolished activation (Fig. 2). EDTA directly inhibits the specific proteinase in venom that is responsible for inactivation of proteinase inhibitory proteins [13,14]. The result also indicates that the phenylmethylsulphonyl fluoride-treated venom cannot activate inactive renin itself via an action of its own serine proteinases, these presumably having been inhibited during pretreatment of venom with phenylmethylsulphonyl fluoride. Phenylmethylsulphonyl fluoride, when added to the venom-plasma incubates, reduced the initial rate of activation 50% and

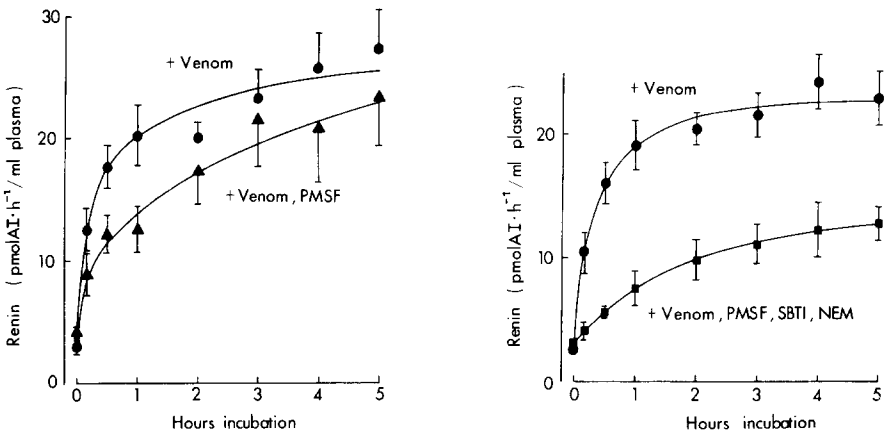

Fig. 3. Partial inhibition of the rate of venom-mediated activation of inactive renin by 5 mM phenylmethylsulphonyl fluoride (PMSF) (n = 5). AI, angiotensin I.

Fig. 4. Inhibition of the rate of venom-mediated activation of inactive renin by a mixture of 5 mM phenylmethylsulphonyl fluoride (PMSF), 0.3 mg/ml soybean trypsin inhibitor (SBTI) and 5 mM *N*-ethylmaleimide (NEM) (n = 6). AI, angiotensin I.

caused a 4-fold prolongation of half-time (Fig. 3). The plateau of renin was approached by 5 h, indicating that residual proteinase activity may be capable of eliciting complete activation of inactive renin. Results for 5 and 10 mM phenylmethylsulphonyl fluoride were similar. 5 and 10 mM *N*-ethylmaleimide doubled the half-time and 0.3 mg/ml soybean trypsin inhibitor had little effect by itself. The most potent inhibition of activation was achieved when a combination of phenylmethylsulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide was used (Fig. 4). This mixture caused a 90% reduction in the initial rate of activation and prolonged the half-time 20-fold.

Discussion

The results show that puff adder venom can activate inactive renin in human plasma and that an EDTA-sensitive venom proteinase is involved. A venom metalloproteinase has been shown to inactivate human plasma proteinase inhibitors under the incubation conditions employed [13—15]. With the exception of α_2-macroglobulin, inactivation does not involve complex formation and thus differs from the usual interaction of proteinase inhibitors with proteinases [14,15]. Since phenylmethylsulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide could inhibit the rate of activation 90%, it would appear that venom proteinase activates endogenous plasma serine and thiol proteinases and these, in turn, activate inactive renin. Moreover, a substantial increase in caseinolytic activity has been observed by us in preliminary experiments.

The activation of endogenous proteinases could occur in two ways. Firstly, as well as inactivating proteinase inhibitors, the venom metalloproteinase may also act on proteinase inhibitor-proteinase complexes to give fully active proteinase either in the free form (such as occurs after treatment of the α_1-proteinase inhibitor-trypsin complex with strong nucleophilic agents [16] or

still attached to the disrupted inhibitor chain (such as occurs after treatment of α_2-macroglobulin-serum proteinase complexes with sodium dodecyl sulphate [17]). However, in the case of α_1-proteinase inhibitor (M_r 53 000), which accounts for 90% of the trypsin inhibitory capacity of plasma [18], inactivation by an analogous proteinase activity in *Crotalus adamanteus* venom involves enzymatic cleavage of an X-Met bond 8 residues N-terminally from the usual site of proteolytic attack that leads to removal of a M_r 7000 N-terminal fragment and formation of inhibitor-enzyme complex [19]. Thus, *C. adamanteus* venom proteinase may not act on α_1-proteinase inhibitor in its complex because the X-Met bond would no longer be present. Furthermore, in preliminary experiments we have found that treatment of the α_1-proteinase inhibitor-trypsin complex with *B. arietans* venom proteinase does not release caseinolytic activity. The site of hydrolysis of other proteinase inhibitor-proteinase complexes by venom proteinase has not been investigated. Fibrinolysin, which can activate inactive renin [9,10] is bound primarily with α_2-plasmin inhibitor [20,21]. Selective hydrolysis of α_2-thiol proteinase inhibitor [22] may activate proteinase inhibitable by *N*-ethylmaleimide and an action on α_2-macroglobulin may activate a variety of proteinases, including kallikrein, since it binds serine, carboxyl, thiol and metalloproteinases [23].

The second way in which an EDTA-sensitive venom proteinase in *B. arietans* venom could increase endogenous proteolytic activity is by activation of proteinase precursors.

In either case, the concomitant inactivation of proteinase inhibitors by venom proteinase eliminates the potential for inactivation of proteinases once they have been activated by venom proteinase. This is essential if valid studies are to be performed to identify the endogenous enzymes or enzymatic pathways involved in the activation of inactive renin.

Finally, we wish to consider the possibility of our results being due to direct release of renin from a hypothetical proteinase inhibitor-renin complex. It has been shown that mouse renin is not bound by proteinase inhibitors in human or mouse plasma during incubation in vitro [24] and hog renin is not inhibited by α_2-macroglobulin, soybean trypsin inhibitor and Cl inactivator [25]. This may be a consequence of renin's lack of general proteinase activity [26], since proteolysis of proteinase inhibitor seems to be required to trigger binding of proteinases. Curiously, partial unfolding of mouse renin in 5 M guanidine and refolding (which inactivates it) bestows on renin the ability to bind to α_2-macroglobulin, inter-α-trypsin inhibitor, α_2-antithrombin, α_1- and β-lipoprotein, albumin and an unidentified protein in human and mouse plasma [24]. Moreover, a M_r 80 000 form of renin in mouse plasma that can be converted to M_r 40 000 native renin by acidification or limited proteolysis is thought to be an α_2-macroglobulin-renin complex [24]. However, there is no evidence for such a complex in human plasma, although a competitive inhibitory interaction of α_1-proteinase inhibitor in human plasma with hog renin has been reported [25]. If inactive renin was a proteinase inhibitor-renin complex and if activation was due solely to digestion of proteinase inhibitor by venom proteinase, then we should have been unable to inhibit activation using phenylmethylsulphonyl fluoride, soybean trypsin inhibitor and *N*-ethylmaleimide. The fact that inhibitors of serine and thiol proteinases were able to inhibit

activation of inactive renin mitigates against this proposal. The alternatives of secondary reaction of these activated proteinases with renin-protein complexes or with putative renin precursor remain open to investigation as possible mechanisms of activation of inactive renin. Nevertheless, the present results do not exclude a minor role of venom proteinase itself in such actions.

The present work suggests that endogenous neutral proteinases may be capable of causing maximal proteolytic activation of inactive renin. A partial role had been demonstrated previously in studies using cold or pH 3.3 to dissociate the proteinase inhibitors: with cold, activation is incomplete and with pH 3.3 pepsin has a substantial role as well [4,27]. It should be noted that EDTA does not affect activation with cold or acid. Metalloproteinase in *B. arietans* venom appears to unmask neutral proteinase activity in plasma at physiological pH and room temperature. The accompanying increase in renin suggests a link between the activation of endogenous neutral proteinases and the activation of inactive renin.

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