Converting Enzyme Activity in Human Plasma
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ABSTRACT
Human plasma converting enzyme activity was investigated by comparing biological with immunological activity of added or generated angiotensin after plasma incubation. Sodium citrate (2 X 10^{-2} M), 2:2'-dipyridyl (1 X 10^{-4} and 1 X 10^{-3} M), chromotropic acid (3 X 10^{-2} and 3 X 10^{-1} M) and desferrioxamine (10 and 100 mg/100 ml) did not interfere with converting enzyme. By contrast, EDTA (3 X 10^{-2} M and 3 X 10^{-3} M), EGTA (3 X 10^{-2} M), 8-quinolinol (1 X 10^{-3} M), and oxalic acid (3 X 10^{-3} M) were shown to interfere with plasma converting enzyme activity. The EDTA inhibition of converting enzyme activity could be partially reversed by molar excess amounts of calcium, zinc and cobalt. The ability of these ions to restore converting enzyme activity to the plasma was quantitatively variable. Inhibition of converting enzyme activity by EDTA could not be reversed by the addition of iron, copper or manganese to the samples.

KEY WORDS angiotensin I chelating agents angiotensin II calcium renin activity cobalt hypertension

Various aspects of the renin-angiotensin system have been under investigation for many years. It has been established that the renal enzyme, renin, acts on renin substrate, a glycoprotein. This reaction results in the formation of the decapeptide angiotensin I, generally believed to be biologically inactive. To achieve biological activity, angiotensin I must be cleaved by a "converting enzyme," as yet unidentified. This reaction produces the biologically active octapeptide angiotensin II (1). Except for the work of Skeggs et al. (2), other investigators (3, 4), and especially Ng and Vane (5), who indicated that most angiotensin conversion occurs in the lungs, little is known about human converting enzyme. In particular, little is known about plasma converting enzyme. Skeggs et al. reported that EDTA was capable of inhibiting plasma converting enzyme, and more recently Ryan (6) showed that use of Dimercaprol in plasma also inhibits the conversion of angiotensin I to angiotensin II. Since the use of EDTA to inhibit angiotensinase activity is an integral part of most renin activity methods, it seemed imperative to obtain more information about its influence on converting enzyme, if the angiotensin II radioimmunoassay was to be used to measure plasma renin activity. The availability of radioimmunoassay for angiotensin II (7), and more recently angiotensin I (8), offered a unique and specific approach to an evaluation of converting enzyme activity in plasma.

Materials and Methods
Synthetic valine-5-angiotensin II amide (Hyptensin, Ciba) was used as the standard for both the angiotensin II radioimmunoassay and the rat blood pressure bioassay. Valine-5-angiotensin I amide was used as the standard for the angiotensin I radioimmunoassay and occasionally for biological assay in special circumstances.

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Native human angiotensin I was also prepared in our laboratories by incubation of excess human renin (approximately 0.05 Haas-Goldblatt units/mg protein) with human substrate. Its identity was confirmed by angiotensin I radioimmunoassay. Human plasma was used in all experiments. The human renal renin used in these experiments was prepared in our laboratories, using Method A described by Haas et al. (9). The ethylene-diaminetetraacetic acid (EGTA) (10), chromotropic acid, 2,2'-dipyridyl, oxalic acid, 8-quinolinol and sodium citrate were all general laboratory grade reagents. EDTA and EGTA were used as 0.3M solutions adjusted to pH 7.3 with NaOH. Chromotropic acid and 2,2'-dipyridyl were also used at pH 7.3. Oxalic acid was made up as a 0.5M solution, and 8-quinolinol was made up as a 1.0M solution in ethanol. Sodium chloride, calcium chloride, magnesium chloride, manganese chloride, copper sulfate and ferrous sulfate were all analytical grade reagents. The cobaltous acetate, ferric chloride and zinc acetate were general laboratory reagents. Visking dialyzers were general laboratory grade reagents. Visking dialysis cassettes 8/32 were used for all dialysis procedures.

Biological Assays.—The standard rat blood pressure bracket bioassay was used (11). When extracts were to be compared with angiotensin I by radioimmunoassay, the biological assay was performed using the valine-S-angiotensin I amide standard.

Radioimmunoassay.—The techniques used for extraction, elution, and assay were those developed in this laboratory for the specific radioimmunoassays of angiotensin II (7) and angiotensin I (8).

GENERAL EXPERIMENTAL PROTOCOL

Unless otherwise specifically indicated, all experiments were conducted according to one protocol. After various experimental plasma treatments, angiotensin I and II were generated by incubation renal renin (0.0062 Haas-Goldblatt units) with plasma for 70 minutes at 37°C. The experiments were designed so that a heparinized plasma aliquot was treated only with renin, to serve as an internal control. Samples were then extracted immediately on 100 mg fuller’s earth (Hopkins & Williams, MFC) using a batch technique with continuous mixing. After subsequent washes with water and methanol, the fuller’s earth was eluted with 40% NH₄ (0.88) in methanol (v/v). Eluates were then dried in an air stream at 40°C. The amount of angiotensinogen present in each extract was then determined by bioassay. The remainder of the same sample was then subjected to radioimmunoassay for angiotensin II. The biological activity was taken as representing the sum of angiotensin I and angiotensin II in the sample. This value, expressed as ng of valine-S-angiotensin II amide/ml of extract, was compared with the value obtained by radioimmunoassay for angiotensin II. When only angiotensin II was present in the extract, the values were, of course, identical (Fig. 1). In early studies the low values in the angiotensin II radioimmunoassay compared with the biological assay were taken as indirect evidence that the material generated by the action of renin on renin substrate (plasma) in the presence of chelating agents was angiotensin I. More recently, the availability of a specific radioimmunoassay for angiotensin I has allowed this assumption to be confirmed, and the identity of the material as angiotensin I to be established (Fig. 2).

Effect of pH on Formation of Angiotensin.—Citrate blood bank plasma was dialyzed for 96 hours at 4°C against 1% NaCl, previously adjusted to pH 5.5 or pH 7.0. The saline was changed daily. Following dialysis the plasma was centrifuged, pH was confirmed, renin was added, and the experiment was completed according to the protocol above.

Effect of pH on Conversion of Angiotensin I to II.—Heparinized, fresh human plasma was also dialyzed at 4°C against 0.03M barbital sodium acetate buffer at pH 4.5, 5.5, 6.5, and 7.5. An additional sample was dialyzed against 0.1M acetate buffer pH 5.5 for 18 hours. All plasma samples were made 0.15M with respect to NaCl. In these experiments, renin was not added, but native human angiotensin I, final concentration 750 ng/ml, was added to each sample. They were then incubated for 30 minutes at 37°C and were

Circulation Research, Vol. XXVIII, February 1971
Comparison of biological activity with radioimmunoassay of angiotensin II. $x =$ Standard radioimmunoassay curve, valine-5-angiotensin II amide. $\bullet =$ Pressor material obtained when heparinized plasma from a nephrectomized patient was incubated with renin for 70 minutes at 37°C. Points on the abscissa were located by bioassay. This material is 80% as active as valine-5-angiotensin II on immunoassay. $\triangle =$ Pressor material obtained when the same plasma was treated with $3 \times 10^{-5}$M EDTA prior to incubation with renin; since no binding occurred this material presumably contains no angiotensin II. $\circ =$ Pressor material obtained when the same EDTA-treated plasma was also treated with $3.5 \times 10^{-5}$M cobalt acetate for 30 minutes prior to incubation with renin. When points on abscissa are located by biological activity, 40% of the material binds as angiotensin II.

Effect of EDTA: Comparison of biological activity with radioimmunoassay of angiotensin I and angiotensin II. $x =$ Angiotensin standard curve. On the left this is the standard synthetic val-5-angiotensin II amide; On the right it is standard val-5-angiotensin I. $\circ =$ Pressor material resulting from incubation of renin with $3 \times 10^{-5}$M EDTA-treated fresh normal human plasma. No binding occurs with angiotensin II antibody, but binding with angiotensin I antibody is nearly complete.
CONVERTING ENZYME ACTIVITY IN HUMAN PLASMA

extracted, eluted, and assayed as described previously.

Effect of Time on the Formation and Conversion of Renin-Generated Angiotensin I to Angiotensin II.—Fresh heparinized human plasma was treated with renin and samples were incubated for 15 minutes, 30 minutes, 45 minutes, and 60 minutes at 37°C. The generated angiotensin was then extracted, eluted, and assayed by bioassay and angiotensin II radioimmunoassay.

Effect of Chelating Agents on Converting Enzyme.—Fresh, human heparinized plasma from normal subjects or from nephrectomized patients was used for all experiments in which chelating agents were added directly to the plasma. In these experiments various chelating agents were added to plasma samples, followed 30 minutes later by addition of renin. For comparison, each experiment included a separate plasma aliquot designated "heparin control," which was incubated with renin but no chelating agent. In other respects the experiment was conducted according to protocol.

In some experiments, either fresh or frozen human heparinized plasma was dialyzed against the chelating agent (in 1% NaCl solution) for 24 hours, followed by a 24-hour dialysis against only 1% NaCl solution. After dialysis the sample was then centrifuged and the supernatant fraction was treated with renin. The remainder of the experiment was conducted according to protocol.

Addition of Metals after Chelation of Plasma.—In most of these experiments, EDTA, a slight molar excess of metal salt, and renin were all added to plasma sequentially and incubated in the usual way. In all experiments an unchelated sample was kept as a "heparin control." In addition, a second control consisted of chelated plasma to which none of the experimental ions had been added. In this way an estimate of the metal ion under investigation. To insure the absence of preformed angiotensin, heparinized plasma for these experiments was obtained from nephrectomized patients maintained on peritoneal dialysis. In dialysis experiments, citrated plasma from normal subjects was dialyzed against EDTA for 24 hours; this was followed by dialysis against distilled water for 24 hours. All plasma samples were made 0.15M with respect to NaCl before a molar excess of metal salt, based on the original concentration of EDTA, and renin were added.

In two experiments EGTA was used as the chelating agent. In other respects regarding addition of metal salts and renin, the experiments were conducted as indicated.

Effect of Duration of EDTA Treatment.—In this experiment 3 X 10^-4M EDTA was added at 4°C to heparinized plasma (obtained from a nephrectomized patient) 84 hours, 7/2 hours, 4 hours, and 30 minutes prior to addition of cobalt acetate 3.5 x 10^-4M. After a further 30 minutes at 4°C, renin was added. The samples were then incubated at 37° for 70 minutes prior to the usual extraction, elution, and assay.

Results

Identity of Pressor Material Formed by Incubation of Renin with EDTA-Treated Plasma.—Pressor material generated by renin in three separate plasmas first treated by EDTA and containing no angiotensin II on radioimmunoassay, were analyzed by angiotensin I immunoassay after biological activity in terms of valine-5-angiotensin I amide had been determined. In these three samples 95%, 85%, and 70% of the pressor activity was accounted for by angiotensin I.

Factors Influencing Conversion of Generated Angiotensin I to Angiotensin II.—Time: In these experiments, angiotensin measured by bioassay was generated by added human renin in normal heparinized plasma at the rate of 0.2 to 0.8 ng/ml/minute. At this rate, after 15 minutes incubation, 50% of the pressor material present was angiotensin II, whereas after 60 minutes of incubation, 90% of the generated material was angiotensin II. (2) pH: When citrated blood bank plasma was dialyzed against 1% saline at pH 5.5 and 7.0 and then treated with renin for 60 minutes, 65% of the pressor material at both pH levels was angiotensin II. An undialyzed aliquot of this plasma, similarly treated, yielded 70% angiotensin II. When fresh heparinized plasma was dialyzed overnight against the barbital acetate buffers, no conversion of angiotensin I (added to plasma) occurred at pH 4.5, but with increasing pH, conversion progressively improved so that at pH 7.5, 85% of the angiotensin I had been converted to angiotensin II (Fig. 3). When plasma was dialyzed to pH 5.4 against acetate buffer no conversion of added angiotensin I occurred.

Chelating Agents (Table 1)—Sodium cit-
rate, 2,2'-dipyridyl, chromotropic acid, and desferrioxamine\(^2\) added to plasma in the concentrations shown did not significantly interfere with converting enzyme. EDTA (Figs. 1 and 2), EGTA, and 8-quinolinol all interfered with converting enzyme activity when added to citrated or heparinized plasma. EDTA also was shown to interfere with conversion when it was used in place of heparin as an anticoagulant. Conversion of generated angiotensin was also inhibited in citrated plasma dialyzed against \(3 \times 10^{-3}\) M EDTA. This inhibition of converting enzyme activity was unaltered by subjecting the samples to a second dialysis against distilled water and also occurred after citrated samples had been dialyzed against \(3 \times 10^{-3}\) M EDTA in 1% NaCl at either pH 7.0 or pH 5.5. When \(3 \times 10^{-4}\) M oxalic acid was added to plasma 1 hour before renin was added, only about 40% of the pressor material was angiotensin II. When \(3 \times 10^{-4}\) M oxalic acid was added 18 hours before incubation with renin, the inhibition of conversion was complete and no angiotensin II resulted from the action of renin on the plasma.

**Effect of Metals on EDTA Inhibition** (Table 2, Fig. 4). The EDTA-induced inhibition of converting enzyme was not altered by the addition of a slight molar excess of ferric chloride, ferrous sulfate, or copper sulfate. Metals which were further investigated are listed in Table 2. Plasmas made \(3 \times 10^{-3}\) M with EDTA were especially resistant to the effect of excess metal addition, since little return of converting enzyme activity could be obtained with any metal. The maximum return in these experiments was only 7% (with zinc—column 1, Table 2). A greater, though variable degree of returned converting enzyme activity could be obtained when a lower concentration of EDTA was present (column 2, Table 2). A typical experiment is shown in Figure 4. Complete return of converting enzyme activity could rarely be achieved with any metal, although calcium, zinc, and especially

\(^2\)Supplied by Ciba Laboratories, Ltd.
cobalt, all reverse EDTA inhibition to some extent. Cobalt and zinc are especially consistent in their ability to restore some converting enzyme in plasmas which had been treated by the addition of EDTA. Two experiments were performed in which metal ions were added back to a plasma which had been dialyzed against EDTA (column 3, Table 2). When EDTA had been removed by a second dialysis against 1% NaCl, the readdition of very small amounts of cobalt and zinc resulted in a modest return of converting enzyme activity, although similar amounts of calcium, magnesium, and manganese were without effect. In a second experiment a higher concentration of cobalt (3.5 X 10^{-3}M) was added to the EDTA-dialyzed plasma, and 100% of the pressor material formed was angiotensin II.

At higher molarity, EGTA (3 X 10^{-3}M) also inhibited converting enzyme. This inhibition could be partially reversed by addition of magnesium to the EGTA-treated sample, but the addition of similar amounts of calcium did not result in angiotensin II formation.

In the experiment in which the effect of duration of the previous EDTA treatment was studied, addition of cobalt resulted in approximately the same amount of angiotensin II.
Effect of metals on conversion of generated angiotensin I to angiotensin II in EDTA-treated plasma. x = Valine-S-angiotensin II amid standard curve. • = Heparinized plasma from nephrectomized patient incubated with renin for 70 minutes at 37°C. ● = The same plasma as above, made 3 x 10^-6 M with EDTA prior to incubation with renin. ○ = Heparinized, EDTA-treated plasma treated with 3.5 x 10^-6 M cobalt acetate for 30 minutes prior to incubation with renin. □ = Heparinized, EDTA-treated plasma treated with 3.5 x 10^-6 M zinc acetate for 30 minutes prior to incubation with renin. ◇ = Heparinized, EDTA-treated plasma treated with 3.5 x 10^-6 M magnesium chloride prior to incubation with renin.

Discussion

It is apparent from these experiments that the enzyme, or enzymes, in human plasma responsible for the conversion of angiotensin I to angiotensin II are metal-dependent, as was first suggested by Skoggs (1). The nature of this metal dependence is not clear, however. The experiments with the chelating agents were designed to cover a wide range of metals, and they give strong indication that neither iron nor copper is involved, since chelating agents specific for these metals did not inhibit conversion. This is confirmed by the inability of either of these two ions to reverse the EDTA inhibition of converting enzymes. It is also unlikely that either manganese or nickel are important in its activity, since the addition of neither metal reversed EDTA inhibition to any great extent. Of all the ions tested, cobalt was most consistent in its ability to cause the return of some converting enzyme activity after EDTA inhibition, in that it was found to be active over the widest range of experimental conditions. Zinc, calcium and, rarely, magnesium were also found to share this capacity, however, so it cannot be said with certainty which of these ions is of prime importance.

Thevariation noted in the ability of a given metal ion to return plasma converting enzyme in various chelated plasmas may be related to subtle differences between plasmas. This is especially likely since some of the plasma samples were obtained from nephrectomized patients. A similar variation could also occur if the capacity to convert angiotensin I to angiotensin II is shared by two or more
CONVERTING ENZYME ACTIVITY IN HUMAN PLASMA

plasma enzymes activated by different metals. It seems obvious that the metal involved is tightly bound to the enzyme, or enzymes, since potent chelating agents are required to produce inhibition and simple dialysis against saline does not interfere with conversion.

No effort was made to inhibit angiotensinase activity in the plasma since the major agents used in angiotensinase inhibition (chelating agents) also inhibit converting enzyme activity, as shown in this study. Converting enzyme activity in the plasma may partially be related to endopeptidase or other angiotensinase activity. This study therefore does not rule out the possibility that human plasma converting enzyme activity may in part be due to enzymes other than a specific "converting enzyme." Nevertheless, it does describe the metal dependence of the plasma enzyme or enzymes responsible for the production of angiotensin II from the interaction of plasma and angiotensin I, when further angiotensinase activity was prevented by the extraction on fuller's earth.

It was our hope that converting enzyme activity and angiotensinase activity could be easily separated by the use of selective chelating agents in combination with selective return of metal ions, so that the angiotensin II radioimmunooassay could be used for renin activity determinations. We were unable to induce return of converting enzyme activity with enough quantitative consistency to make this approach feasible, however. This suggests that renin activity in plasma should be measured by the rate of formation of angiotensin I, and this has been achieved by the development of an immunoassay for angiotensin I (8).

References

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