Differentiation Between Two Forms of Angiotonin by Means of Spirally Cut Strips of Rabbit Aorta

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ABSTRACT

HELMER, O. M. (Indianapolis Gen. Hosp., Indianapolis, Ind.) Differentiation between two forms of angiotonin by means of spirally cut strips of rabbit aorta. Am. J. Physiol. 188(3): 571-577. 1957. By means of a spirally cut strip of rabbit thoracic aorta, it has been shown that angiotonin exists in two forms. One form, angiotonin II, causes a contraction of the strip. The other, angiotonin I, is inactive. They are equally pressor when injected intravenously in animals. An enzyme in plasma converts the inactive form to the active form. The identical pressor activity can be explained by the excess of the converting enzyme in the plasma of the intact animal which rapidly converts angiotonin I to angiotonin II. Some patients with hypertension have a greater content of the converting enzyme in their plasma than is found in plasma of normotensive subjects. In addition to the converting enzyme, other factors in plasma enhance the ability of angiotonin and catechol amines to induce constriction of the strip of aorta. These factors may sensitize the mechanisms in the muscle which set up the process of contraction.

While investigating the effect of epinephrine and angiotonin on strips of rabbit aorta, it was noticed that whereas the amplitude of contractions produced by equal doses of epinephrine remained the same or increased, those produced by angiotonin became less with each succeeding addition of this agent to the muscle chamber; and in some instances disappeared entirely. The ability of angiotonin to cause contraction could be restored by the addition of human, dog, cat or rabbit plasma which by themselves had no apparent effect. The contraction produced by epinephrine could also be enhanced. This report is chiefly concerned with the nature of these plasma factors which enhance angiotonin activity and the two forms of angiotonin which can be distinguished by means of the aorta strip. One form causes a contraction of the strip; the other does not; they are equally pressor when injected intravenously in animals.

METHODS

Spirally cut strips of rabbit thoracic aorta as described by Furchgott and Bhadrakom (1) were used. The strips were mounted in a muscle chamber of 20 ml capacity with the aid of stainless steel hooks. The strip was bathed with Krebs-bicarbonate solution (2) containing 0.01 M glucose. Through the solutions in the reservoir, 95% O2-5% CO2 were passed to maintain the pH at 7.4. The isotonic lever was adjusted to give a ninefold amplification and counterweighted to exert 4 gm tension on the strip. The contraction was recorded on 'Waxer' paper (Gorrell and Gorrell) by means of a heat stylus attached to the lever. A small vibrating motor was clamped to the rod holding the lever to eliminate any friction of the stylus on the paper.

The strip was not used for comparative tests until maximum sensitivity to epinephrine or norepinephrine was attained. Test substances were washed out of the chamber within 1-2 minutes after maximum contraction occurred, or as soon as the strip showed signs of relaxing.

The angiotonin used was standardized by pressor assay in pithed cats against a lyophilized laboratory standard angiotonin powder, 30 mg of which is designated as 1 unit.

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RESULTS

In figure 1 is shown an example of the reaction of strips of rabbit aorta to epinephrine and angiotonin. The same dose of epinephrine administered over a period of 6 hours caused a like degree of contraction, whereas the contraction in response to a standard concentration of angiotonin decreased progressively. This figure also shows the different types of response to these substances. With epinephrine the shortening begins almost immediately and with moderate doses the contraction is complete in about 2-4 minutes before shortening begins. The period necessary for maximum contraction is from 10 to 20 minutes. With angiotonin, the relaxation after washing is much slower than with epinephrine; the complete cycle often requires about 60 minutes.

The degree of contraction caused by these two agents differs greatly from strip to strip. For instance, in one of two strips that show the same sensitivity to epinephrine, angiotonin will cause a strong contraction; in the other, angiotonin will cause only an insignificant shortening. This is similar to their behavior when given intravenously to animals in which their relative pressor activity varies greatly from animal to animal.

Activation of Angiotonin by Plasma. Figure 2 shows the effect of incubating angiotonin with heparinized plasma from a patient with hypertension. In this instance, plasma which itself had little effect on the muscle strip greatly enhanced the ability of angiotonin to cause contraction when it was added to angiotonin in the muscle chamber. This property is shared by plasma of all species so far tested (man, cat, rabbit, hog and dog). Untreated dog plasma, however, shows a low degree of activity.

Effect of Dialysis on Plasma Activity. When plasma was dialyzed against several changes of isotonic sodium chloride solution at 3°C for 1-3 days or against running tap water overnight and made isotonic with sodium chloride, there was no change in its ability to activate angiotonin. When human plasma was dialyzed against running tap water and adjusted to pH 5.3 and centrifuged to remove the bulky precipitate, there was no change in its activity. In contrast, the removal of this precipitate greatly increased the ability of dog plasma to enhance the contraction induced by angiotonin. To a much lesser extent, the potencies of rabbit and cat plasmas were also increased.

The greater activity of dog plasma after dialysis and removal of the pH 5.3 precipitate probably may be explained by the fact that this procedure will remove plasmin. The plasminogen of dog plasma is spontaneously converted to plasmin when withdrawn from the body. Human plasma shows little spontaneous proteolytic activity (3).
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Nature of Plasma Factors. Dialyzed plasma as well as untreated plasma added to the muscle chamber will also enhance the contraction caused by epinephrine. Figure 3 shows the increased constriction of the rabbit aorta produced by epinephrine and angiotonin in the presence of dialyzed plasma.

Plasma could potentiate the action of angiotonin and epinephrine on muscle strips in several ways. It could, especially with epinephrine, bind trace amounts of heavy metals which might catalyze the auto-oxidation of epinephrine in alkaline solution in the presence of oxygen; it could sensitize the strip to constrictor agents, or change the properties of the effector substances by an enzymatic process.

Furchgott (4) has shown that ethylenediamine tetra-acetic acid (EDTA) will prevent the oxidation by metal ions of epinephrine in oxygenated Krebs solution. Therefore, the contraction produced on the strip by epinephrine was tested with and without the addition of EDTA. The addition of EDTA not only potentiated the degree of contraction induced by epinephrine but also extended the duration. The addition of plasma to the same strip in the presence of an optimal amount of EDTA caused a greater shortening of the strip, indicating that dialyzed plasma potentiates epinephrine in some manner other than preventing oxidation. In the absence of EDTA, part of the enhancement of epinephrine by plasma in all probability is due to metal binding.

When epinephrine is incubated with plasma at pH 7.4 in Krebs solution and the reaction stopped by boiling at pH 5.0 and this reaction mixture added to the muscle chamber, no increase in contraction occurred over that produced by epinephrine itself. In most instances, a decreased response occurred. Consequently, it appears that plasma sensitizes the strip to the constrictor action of epinephrine rather than producing a direct effect on the epinephrine molecule.

In contrast to the results obtained with epinephrine, EDTA does not potentiate the contraction of the aortic strip induced by angiotonin. The enhancing action of plasma then appears to be due to some other process than that of preventing the oxidation of angiotonin by metal ions. Plasma could potentiate angiotonin by sensitizing the strip to the action of this agent, by changing the structure of the polypeptide, or possibly by both mechanisms.

In order to determine which of these mechanisms might be concerned, a comparison was made of the relative ability of the same amount of dialyzed human plasma to activate angiotonin by incubation with angiotonin outside the muscle chamber and in the chamber. With a mixture of angiotonin incubated at 37°C for 30 minutes at pH 7.4 and boiled at pH 5.2 to stop the reaction, an amount of the mixture equivalent to 0.2 angiotonin units and 0.125 ml of plasma caused a contraction recorded as 21.5 mm. When 0.15 ml of the same plasma was incubated with 0.2 U of angiotonin in the muscle chamber, only a 1.5-mm response occurred, a shortening which was the same as that produced by 0.2 U of untreated angiotonin. Apparently an amount of plasma unable to activate angiotonin in the muscle chamber can potentiate the activity of angiotonin when the incubation is performed before the mixture is added to the bath. However, when dialyzed plasma (0.5 ml) is added to fully activated angiotonin in the muscle chamber, a stronger contraction occurs. Even if the plasma is added at the height of the contraction produced by angiotonin, a further shortening of the aortic strip occurs.

It appears, therefore, that plasma potentiates the effect of angiotonin on the aortic strip in two ways: one, by sensitizing the muscle and two, by an action on angiotonin itself. The nature of the first effect, which is similar to the potentiation of epinephrine by plasma, is obscure. The activation of angiotonin by incubation with plasma appears to be enzymatic. The evidence for the enzymatic nature of this process will be presented in the following sections.

Evidence for Enzymatic Nature of the Activity of Angiotonin. The substance in plasma which activates angiotonin has the properties of a protein. It is nondialyzable through cellophane membrane; it is inactivated by boiling at pH 5.3; it is precipitated with ammonium sulfate. In fact, the fraction of hog plasma precipitated between 1.5 and 2.1 saturation which contains the renin substrate (hypertensinogen) is a good source of the factor.

The factor also shows many properties of
an enzyme. Activity is influenced by varying the hydrogen ion concentration. The optimum pH is between 7.0 and 8. At pH 5.0, it is inactive. There is insignificant activity at pH 6.0; above pH 8.0, activity begins to fall off. Renin substrate, in 0.9% sodium chloride, prepared from hog renin was used as a source of the enzyme. The pH was adjusted with hydrochloric acid or sodium hydroxide. The velocity of the reaction is greater with increasing plasma concentration. With an excess of plasma the reaction is very rapid, being completed in less than 5 minutes, the shortest period of incubation tested.

In this experiment, dialyzed human plasma was used. The plasma was incubated for 20 minutes at pH 3.9 to destroy angiotonase and adjusted to pH 7.2. To 8 ml of this plasma, 2 ml of an angiotonin preparation containing 2 U of angiotonin/ml were added and the mixture incubated for 5 minutes at 37°C. The reaction was stopped by adding hydrochloric acid to pH 5.2, and boiling the mixture in a water bath for 10 minutes. After centrifuging to remove coagulated protein, the clear supernatant fluid was added to the muscle chamber amounts equivalent to 0.5 U of angiotonin. As controls, similar mixtures were prepared with boiled plasma and unboiled plasma in which the incubation time was 60 minutes. The results of this experiment are shown in figure 4.

The rapid activation of angiotonin with an excess of plasma, as will be discussed later, may account for the identical pressor responses to the two forms of angiotonin when injected intravenously in animals.

Another property of an enzyme is that it is not used up in the reaction. The following experiment demonstrates that the plasma factor fulfills this criterion.

As shown in figure 5, a mixture of 12 ml of dialyzed human plasma, treated to remove angiotonase, was incubated with 8 ml of angiotonin (2 U/ml) at pH 7.2 for 30 minutes at 37°C. To a 5 ml aliquot of the incubated mixture, 5 ml of a 0.9% sodium chloride solution was added, the mixture heated to boiling for 10 minutes after adjustment to pH 5.3. The coagulum was removed by centrifugation, and the clear supernatant (fraction A) was stored in a frozen condition until tested on muscle strip.

The remainder of the mixture was dialyzed overnight in cellophane membranes against running tap water. To a 5 ml portion, 5 ml of 0.9% sodium chloride was added and the resulting solution incubated 30 minutes at 37°C at pH 7.2. At the end of the incubation this was treated in the same way as the previous fraction. The supernatant was labeled fraction B.

To 5 ml of the remaining dialyzed mixture, 2 ml of angiotonin (2 U/ml) and 3 ml of 0.9% sodium chloride were added. The mixture was incubated and treated under the same conditions as before (fraction C).

When each of the three fractions was tested on an aortic strip in quantities equivalent to 0.15 ml of original plasma, fraction A gave a 22.5 mm response, fraction B an insignificant response of 1 mm, and fraction C a shortening recorded as 22.5 mm.

Skegg's, Marsh, Kahn and Shumway (5) have found by counter-current distribution studies that there are two forms of angiotonin (hypertensin I and hypertensin II) and that a nondialyzable protein fraction of plasma can convert hypertensin I to hypertensin II. This enzyme is activated by chloride ion. Consequently, the effect of chloride on the plasma factor which enhances the ability of angiotonin to contract the rabbit aortic strip was investigated.

Angiotonin was prepared from renin and renin substrate that had been dialyzed against several changes of distilled water. This angio-
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Angiotonin was incubated with dialyzed plasma with and without sodium chloride. Very little, if any, activation was noted when the incubation occurred in the absence of chloride. In the presence of chloride, full activation of the angiotonin was obtained.

Nature of Reaction Product. The reaction product obtained by the incubation of angiotonin with the plasma factor retains the properties of a polypeptide. It is inactivated by proteolytic enzymes such as trypsin and by red cell and kidney angiotonase. The kidney angiotonase is the most active.

Although the inactivated and activated forms of angiotonin behave so differently on the aortic strip, that is, at a given dose the inactivated form does not produce contraction of the strip while the activated form causes a strong contraction, both give the same pressor response when injected intravenously in a pithed cat.

The two forms of angiotonin described by Skeggs and his colleagues are equally pressor. In order to determine whether the two forms of angiotonin described in this paper were related to those described by Skeggs, angiotonin which had been activated by incubation with dialyzed human plasma was subjected to countercurrent distribution.

The angiotonin used for this experiment was prepared by Dr. Carl Kuether. He found that it corresponded to the form described as hypertensin I by Skeggs. One thousand units of this angiotonin was incubated for 1 hour at 37°C with 350 ml of dialyzed angiotonase free human plasma at pH 7.4. It was then adjusted to pH 5.2 with hydrochloric acid and dialyzed in Visking casings against 2.0 liters of distilled water at 0°C for 24 hours. It was dialyzed two more times. The dialysates were concentrated and dried in a frozen state. Dr. Kuether subjected this preparation to countercurrent distribution in a 10 tube Craig apparatus, using the same n-butanol-0.1 N sodium bicarbonate system as was used by Skeggs. The upper and lower fractions of each tube were assayed for pressor activity in a pithed cat. The results of the pressor assay are shown in figure 6 along with graphs of hypertensin I and II as published by Skeggs. It appears that the activation in our experiment was not complete. However, it was possible to select samples which according to Skeggs' data should contain hypertensin I or hypertensin II. These fractions were tested on the strip for their ability to cause contraction. Amounts that caused the same elevation of arterial pressure in cats were added to the muscle chamber.

Fraction 5 U, which, according to Skeggs' data should contain mostly hypertensin I, did not cause a contraction of the strip. Fraction 2 U gave a 10-mm contraction. When 5 U was incubated with human plasma, a 12-mm response occurred. The ability of fraction 2 U to cause a shortening of the strip was not enhanced by incubation with plasma. So it appears that by means of the aortic strip it is possible to differentiate biologically the two forms of angiotonin that behave similarly when injected intravenously in animals but show different characteristics when subjected to countercurrent distribution.

At this stage of the work, I wrote to Dr. Leonard Skeggs, telling him of our findings. He kindly sent me highly purified samples of hypertensin I, hypertensin II and converting enzyme. The hypertensin I contained 7150 u/mg of nitrogen, hypertensin II 13,700 u/mg of nitrogen. His unit is approximately six times stronger than the one used in our laboratory.

As shown in figure 7, when hypertensin II
FIG. 7. Response of strip to 0.02 U of Skeggs' hypertensin I and II before and after incubation with human plasma in the muscle chamber. 1 and 3—hypertensin II; 2—hypertensin I; 4—0.5 ml human plasma; 5—hypertensin I added without washing; 6—0.5 ml human plasma; 7—hypertensin II added without washing.

FIG. 8. Mean blood pressure tracing of pithed nephrectomized cat. 1 and 3—i.v. injection of 0.08 U of hypertensin II; 2 and 4—0.08 U of hypertensin I. Ordinates mm of Hg. Time marker—1 min.

FIG. 9. Contraction produced when incubation mixtures of angiotonin and plasma were added to muscle chamber. At 1, 3 and 6, normal plasma incubates; at 2, 4 and 5, hypertensive plasma incubates.

was added to the muscle chamber in quantities equivalent to 0.02 Skeggs units, contractions of 18 and 24 mm were recorded. The same amount of hypertensin I caused no contraction. This figure also shows that when each of these forms of angiotonin was incubated with human plasma in the muscle chamber, a response of the same order occurred.

In addition to converting hypertensin I to II by enzymatic action, plasma increases the contraction produced by the fully activated polypeptide by a non-specific action on the muscle strip. It is also interesting to note the differences in the responses of hypertensin I and II treated with plasma in the muscle chamber. The curve representing the fully activated hypertensin II has a much steeper slope. The slower rate of contraction produced by hypertensin I is due to its activation during the contractile process.

In figure 8, it can be seen that hypertensin I and II, when injected intravenously in a pithed nephrectomized cat, gave arterial pressure of 48–52 mm of mercury, hypertensin II 52–54 mm.

When hypertensin I was incubated at either pH 6.0 or 7.4 with purified converting enzyme, it caused the same contraction as hypertensin II. So it appears that these highly purified preparations from a different laboratory behave in the same way as the angiotonin prepared in this laboratory.

Content of Plasma Factor in Patients With Hypertension. Early in this study, it was found that the plasma of some patients with hypertension had a higher content of the activating enzyme than normal subjects. In these experiments, the angiotonin was activated by addition of the plasma to the muscle chamber.

Later the angiotonin was incubated with plasma at pH 7.4 for 15 minutes at 37°C before being added to the strip. The incubation was conducted for the short period to cut down on possible destruction of angiotonin by angiotonase in the plasma. Nonhemolyzed, heparinized plasma was used. The reaction was stopped by boiling at pH 5.2. The coagulated protein was removed by centrifugation. The clear supernatant fluid was added to the muscle chamber. Figure 9 illustrates the use of such an assay.

Eight of twenty plasmas from patients with hypertension had a definitely higher content of the enzyme which converts angiotonin to the active form than was found in plasmas of normotensive subjects. Many of these patients were undergoing some type of therapy. The effect of therapy on this reaction is unknown. These data must be regarded as preliminary in nature until a greater number of plasmas from untreated patients are assayed.

DISCUSSION

The experiments described demonstrate that by means of a spirally cut strip of rabbit thoracic aorta the difference in two forms of angiotonin can be detected. Both forms cause the same elevation of arterial pressure when injected intravenously in a cat. In equal pressor doses, one form causes a contraction of the strip, the other does not.

The two types of angiotonin found by means of countercurrent distribution by Skeggs, Marsh, Kahn and Shumway (5) when tested...
on the strip, could easily be differentiated. The type these investigators call hypertensin I was inactive; the other form, hypertensin II, caused a strong contraction. When hypertensin I was incubated with the plasma enzyme (called by them ‘converting enzyme’), it caused a contraction of the strip of equal magnitude to that produced by hypertensin II.

In 1940 Page and Helmer (6) found that the first injection of angiotonin into a rabbit’s ear perfused with Locke-Ringer’s solution caused vasoconstriction. Second and third injections were ineffective. The addition of normal plasma or plasma fractions restored the ability of angiotonin to induce constriction. At that time these authors concluded from their data that angiotonin, the reaction product of renin and its substrate, was vasoinactive and a factor from blood was necessary to convert angiotonin into a pressor substance. The enzymatic nature of the conversion of the inactive form of angiotonin to the active form was not proved at that time, nor was a differentiation made between other factors in blood or plasma which increase the contraction of isolated tissue preparations.

The data presented in this paper, along with that of Skeggs, Kahn and Shumway (7), definitely prove that angiotonin (hypertensin) occurs in two forms, and that the inactive form can be converted to the active form by an enzyme in plasma.

As shown in this paper, an excess of the plasma factor rapidly converts the inactive form to an active one, so that when both forms are injected intravenously they have equal pressor activity.

The preparation of this paper forcibly brought out the difficulty of discussing agents which have a double nomenclature. The simultaneous announcement (within 24 hr.) of this active pressor agent by the Buenos Aires group (8, 9) and the Indianapolis group (10, 11) was responsible for this confusion. The former group called it ‘hypertensin,’ the latter group ‘angiotonin.’

Stimulated by whatever phenomenon or set of circumstances which cause men separated by thousands of miles to think alike, our group at one time before a name was chosen, also considered calling this agent ‘hypertensin.’ However, because that name implied that this agent was the effector substance in essential hypertension, a nonspecific name was finally agreed upon. The term ‘angiotonin’ was suggested by Dr. A. C. Corcoran. On the basis of these considerations and Skeggs’ use of the figures I and II, the renin-angiotonin system can be outlined as follows:

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\text{renin + renin substrate} \rightarrow \text{angiotonin I (vasoinactive)}
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\text{angiotonin I + converting enzyme} \rightarrow \text{angiotonin II (vasopressor)}
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REFERENCES