Angiotensin, the Renal Pressor Hormone

By F. Merlin Bumpus, Ph.D., Robert R. Smeby, Ph.D., and Irvine H. Page, M.D.

Since 1898 it has been known that an extract of kidneys called "renin" would cause an increase in blood pressure upon intravenous injection. Goldblatt et al. in 1934 demonstrated that permanent hypertension could be produced by partial constriction of the renal arteries. The groups of Page and Helmer and Braun-Menendez et al. demonstrated that this vasopressor action is mediated through a substance liberated by the action of renin upon a plasma protein (an \( \alpha_2 \)-globulin). This pressor substance was shown to be a polypeptide that was inactivated by most proteolytic enzymes called "angiotensinases" and by such chemical transformations as acetylation and benzoylation. This peptide originally named both "angiotonin" and "hypertensin" is now called "angiotensin."

Renin substrate has been purified approximately 1000-fold over its concentration in hog's blood but has not been obtained in pure form. From this, we prepared a partially purified angiotensin and showed it to have aspartic acid as the amino terminal and leucine or isoleucine as the carboxyl terminal amino acids.

Angiotensin was first isolated in pure form in 1956 from the reaction product of rabbit renin and beef blood. It was found to be a decapeptide (10 amino acids) and to have the amino acid sequence shown in figure 1. Skeggs and co-workers showed there were two forms of angiotensin, which were separated by countercurrent distribution. One of these, the direct product of renin, is the decapeptide and is converted by a plasma enzyme into an octapeptide, by splitting off the dipeptide histidyl-leucine from the carboxyl end. Both peptides increase blood pressure greatly, but it is thought that the decapeptide (angiotensin I) is active only because the plasma converts it to the octapeptide.

Early preparations of angiotensin possessed two biological effects. They caused an elevation of blood pressure as well as a contraction of uterine muscle. These two activities were partially separated by our group. It soon became obvious that the uterine contracting substance that we had partially separated was the same as angiotensin II. To prove this we carried out the chemical synthesis from the naturally occurring amino acids. The synthetic angiotensin proved to be identical in both physical and biological properties with the naturally occurring octapeptide, angiotensin II, and established beyond doubt the composition and structure of natural angiotensin.

By splitting renin substrate with trypsin, a peptide containing 14 amino acids was formed. Ten of this sequence are contained in angiotensin I. This tetradecapeptide, shown in figure 1 (\( R = OH \)), when incubated with renin gave rise to the biologically active decapeptide by hydrolyzing the leucyl-leucine bond.

It is not known how living organisms can translate activity from a particular peptide structure. At the present time it seems reasonable to think that biological activity is associated with the sequence of amino acids in the chain, as well as with the spatial configuration of the entire molecule.

To gain at least slight knowledge of how a peptide can possess an activity, a number of substitutions of amino acids in the chain of angiotensin II have been made. By comparing the biological activities of the substituted analogues a correlation may be made between peptide structure and biological activity. An inhibitor of angiotensin could possibly result from such studies. Some of the...
substituted compounds show complete loss of both pressor and oxytocic activities, while others have only a slight depression of these properties.

An improved synthesis of isoleucyl-5 angiotensin II has been carried out in our laboratory by methods that have been shown to cause little racemization. This was accomplished by starting with phenylalanine-p-nitrobenzyl ester and adding amino acids one at a time (with the exception of valyl-tyrosine, which was added by the azide method) until the protected octapeptide is obtained. All protecting groups were simultaneously removed by hydrogenolysis, and the product was finally purified by cellulose chromatography using butanol:acetic acid:water (4:1:5) as the solvent system. The main product from chromatography had 12,700 units per mg. as compared to that isolated by Skeggs and co-workers of 13,000 units per mg. A number of angiotensin II analogues have been synthesized in our laboratory by a similar method.

A comparison of the vasopressor activity of angiotensin II analogues has been made. The assays were carried out in different laboratories using different standard angiotensin preparations and different assay animals. We can make only very rough comparisons from these data. All comparisons in our laboratory are made using isoleucyl-5 angiotensin II as a reference.

These studies have shown that the following requirements are necessary for pressor activity:

Position 8: Phenylalanine as the C-terminal amino acid was shown to be important several years ago by its removal with carboxypeptidase. The complete lack of activity of the alanyl-8 angiotensin shows the importance of the phenyl residue in this position. That the free carboxyl group was necessary was first suggested by the fact that the decapeptide is almost completely inactive on uterine muscle. This suggestion was confirmed by the very slight activity of the octapeptide phenylalanine ester and amide.

Position 7: Proline is necessary as shown by the inactivity of alanyl-7 octapeptide.

Position 6: No amino acid substitutions have been made in this position.

Position 5: Valyl-5 and isoleucyl-5 angiotensin were shown by Gross and Turrian to have identical vasopressor activity. This might be expected, since these amino acids both have branching on the beta-carbon atom. The hypertensive activity is lowered to a significant extent when leucine is substituted in this position.

Position 4: Replacement of tyrosine in the hexapeptide with either phenylalanine or alanine results in complete loss of activity. Likewise, substitution of p-fluorophenylalanine for tyrosine results in a completely inactive hexapeptide. One must conclude that the phenolic group is necessary in this position.

Position 3: Replacement of valine with leucine had no effect upon biological activity.

Position 2: Since the hexapeptide (octapeptide minus amino acids 1 and 2) is slightly pressor, it appears that position 2 is of little importance. Substantiating this is the fact that nitroarginyl-2 and ornithyl-2 peptides retain considerable pressor activity. The strongly basic guanidine group of arginine seems to have very little significance.

Position 1: The heptapeptide without aspartic acid retains about one-third of the pressor activity of angiotensin II. The acidity of the beta-carboxyl group of aspartic acid in angiotensin II has no function as indicated
by the equivalence of activity of the asparaginyl analogue to the natural compound. The ratio of oxytocic to pressor activity of the arginine derivative is almost four times that of the natural isoleucyl-octapeptide. N-p-nitrobenzoyl-asparaginyl-1 angiotensin II retains pressor activity indicating that the amino group is not essential.

The structural requirements of angiotensin II for biological activity are summarized as follows:
1. A free C-terminal carboxyl group
2. Phenyl group as side group of amino acid number eight
3. Phenolic group on amino acid number four
4. Proline in position seven (may be for configuration only)
5. A definite degree of spatial order may be required
6. At least six amino acids from the C-terminus

Urea and arginine in high concentrations inhibit the oxytocic action of angiotensin almost 50 per cent (fig. 2). These reagents, which are known to inhibit hydrogen bonding, probably change the spatial configuration of angiotensin. Oxytocin, which has a stable configuration owing to its disulfide bridge, is not affected by these reagents. It may be that the amino acid sequence is of significance only in that certain side groups of amino acids must be in particular positions relative to one another that they may interact with another molecule. It has seemed that polypeptides have less specificity than the hormones of smaller molecular size because a portion of the amino acids in the chain serve only to produce the spatial configuration necessary for activity. Some of these amino acids can be substituted without loss of activity while others of the amino acids that are probably involved in a binding between the peptide and the "receptor site" are necessary for activity and specificity.

**Biological Activity of Angiotensin Decapeptide**

We have shown that the "angiotensin pressor principle," which is now known to be the decapeptide, has very little effect on uterine muscle. More recent comparisons show it to have only 2 to 5 per cent of the oxytocic activity and 64 per cent of the pressor activity of the octapeptide. Skeggss, Kahn, and Shumway showed on an isolated rat's kidney perfused with physiological salt solution that angiotensin II was a powerful vasoconstrictor substance, while angiotensin I was almost completely inactive. Helmer obtained similar results when comparing the octa- and decapeptides on an isolated rabbit aortic strip. The work on these isolated preparations.

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*Figure 2 reproduced from Bumpus et al.: Biochem. et biophys. acta 46: 42, 1961. By permission of Elsevier Publishing Co., Amsterdam.*

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**Figure 3**

Effect of tissue extracts on the oxytocic activity of angiotensin decapeptide.
ANGIOTENSIN, THE RENAL PRESSOR HORMONE

Table 1

<table>
<thead>
<tr>
<th>Tissue extract</th>
<th>B Homogenates (total protein)</th>
<th>C Oxytocic to pressor ratio Dose = 0.2</th>
<th>Ratio C/B</th>
<th>No. of incubations</th>
<th>Inhibition of DFP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta (emulsion)</td>
<td>0.213 mg.</td>
<td>5.42</td>
<td>25.44</td>
<td>6</td>
<td>(+)</td>
</tr>
<tr>
<td>Aorta (suspension of pieces)</td>
<td>—</td>
<td>1.96</td>
<td>—</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Heart muscle</td>
<td></td>
<td>0.522 mg.</td>
<td>3.98</td>
<td>7.62</td>
<td>3</td>
</tr>
<tr>
<td>Heart muscle</td>
<td></td>
<td>(outer layer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart muscle</td>
<td></td>
<td>(inner layer)</td>
<td>—</td>
<td>0.104</td>
<td>1</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.44</td>
<td>0.89</td>
<td>8.55</td>
<td>5</td>
<td>(—)</td>
</tr>
<tr>
<td>Liver</td>
<td>0.216</td>
<td>4.10</td>
<td>18.63</td>
<td>3</td>
<td>(—)</td>
</tr>
<tr>
<td>Spleen</td>
<td>1.05</td>
<td>1.52</td>
<td>1.44</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>0.621</td>
<td>0.67</td>
<td>1.07</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Uterus</td>
<td>—</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ileum</td>
<td>—</td>
<td>1.51</td>
<td>—</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Red blood cells</td>
<td>—</td>
<td>1.45</td>
<td>—</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>0.45</td>
<td>3.8</td>
<td>7.26</td>
<td>13</td>
<td>(—)</td>
</tr>
</tbody>
</table>

*DFP = diisopropylfluorophosphate.

(without plasma) indicates that the decapptide possesses only slight activity as compared with the octapeptide.

From comparisons made on toad hindquarter vessels and isolated guinea-pig ileum, Carlini, Picarelli, and Prado10 showed some similarity of activity between the two peptides. However, after prolonged washing of the vessels of the rat hindquarter with a perfusion medium containing no plasma, a large decrease in response to the decapptide was observed, indicating possibly that an enzyme adsorbed on the inner surface of the vascular system was responsible for the conversion of the decapptide to the octapeptide. Washing had no effect on the response of the other two assay preparations to the decapptide.

Halvorsen et al.26 concluded that angiotensin I need not be converted to angiotensin II to produce constriction of blood vessels. This view is based on observations of similar physiological activity between these two peptides on several preparations. They report that angiotensin I and II produce the same vasoconstrictor effect in vascular preparations of toad perfused without blood and give the same rise in pressure when injected into the artery leading to a perfused (blood) leg or kidney. They believe the decapptide is pressor as such, that conversion to the octapeptide is unnecessary.

We have incubated homogenates of heart, liver, aorta, uterus, and ileum with angiotensin I to determine whether the converting enzyme is present in these tissues. The results are shown in table 1. The effect of these homogenates on the oxytocic activity of isoleucyl-5 angiotensin I is illustrated in figure 3. The oxytocic activity initially increases as angiotensin II is formed and then decreases as further hydrolysis occurs and the octapeptide is destroyed.

The degree of conversion to angiotensin II was measured by the increase in oxytocic activity at the point of maximum activity. As shown previously, angiotensin II and the heptapeptide, arginyl-valyl-tyrosyl-isoleucyl-histidyl-prolylphenylalanine, are the only peptides which could arise from angiotensin I that show significant oxytocic activity. Since both peptides are formed by removal of histidine and leucine from the C-terminus of angiotensin I that show significant oxytocic activity. Since both peptides are formed by removal of histidine and leucine from the C-terminus of angiotensin I, the measurement of the increase in oxytocic activity is a direct measurement of conversion. All these tissue homogenates, except that of uterine muscle, have

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been shown to contain an enzyme or enzymes that rapidly carry out this conversion. Since only uterine muscle homogenates do not have the ability to hydrolyze angiotensin I to II and since the decapeptide is almost completely inactive on this muscle, it appears likely that the octapeptide is the active form of the angiotensins. The fact that the other homogenates tested can form the active octapeptide from the decapeptide suggests that the variable results reported above are due to a partial conversion of the inactive decapeptide to the active form during assay.

Diisopropylfluorophosphate (DFP) does not inhibit the plasma converting enzyme but almost completely inhibits that in homogenates of aorta. Liver homogenates have a very high concentration of converting enzymes and these are inhibited only slightly by DFP. This suggests the possibility that plasma converting enzyme is synthesized in the liver. The kidney enzymes responsible for the destruction of angiotensin II are inhibited by DFP as illustrated in figure 3. Thus, this destruction is not carried out by the converting enzyme.

Summary

We have shown the side groups of angiotensin that are necessary for biological activity. Additional evidence is given that angiotensin decapeptide has little or no biological activity and exerts its effect only through the octapeptide. Even though none of the analogues of angiotensin have inhibitory action on the parent compound, the information obtained from studying these compounds could lead to the synthesis of inhibitors.

References

Discussion

Dr. Sjoerdsma: Is it known, or would you care to guess, whether the peptides are excreted in the urine after their intravenous administration?

Dr. Bumpus: To my knowledge, no one has found angiotensin in the urine. This is understandable because a large number of known proteolytic enzymes, except the converting enzyme and renin, are angiotensinases inasmuch as they destroy angiotensin. Peptidases and proteinases, for example, are present in urine and for that reason angiotensin could not be expected to exist there as such. The study of angiotensin half-life "in vivo" is difficult because of the numerous enzymes (angiotensinases) that hydrolyze it. If, for example, the tyrosine of angiotensin is tagged with radioactive iodine, much care must be taken to measure the half-life of angiotensin rather than that of tyrosine.

Dr. Laragh: Have you tested adrenal tissue for angiotensinase?

Dr. Bumpus: No, we have not.

Dr. Wakerlin: Have you prepared human angiotensin from human renin and human renin substrate? It seems to me that it would be very valuable to know the amino acids of human angiotensin I and II. Also, do you have any information from these excellent biochemical studies as to why hog renin is unable to split off angiotensin from human renin substrate?

Dr. Bumpus: Regarding the second question, renin is a very specific enzyme. We have made peptides up to 5-amino acids in length around the leucyl-leucine bond (L-histidyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine) and none of these is split by renin. Dr. Skeggs states that the smallest peptide hydrolyzed by hog renin is an octapeptide containing the four amino acids on either side of the leucyl-leucine bond of their peptide renin substrate. This indicates the great specificity of renin. The fact that human plasma is not a substrate for other mammalian renin suggests a slight variation in its structure.

We have not attempted to prepare human angiotensin. It would be very helpful to know its structure. The work of Walaszek and Huggins may shed some light on this subject. They have prepared a pressor material called "substance A" from an incubation mixture of alpha-amylase and Cohn's fraction IV of human plasma. According to Dr. Walaszek "substance A" is pharmacologically identical with isoleucyl-5 angiotensin II. If the structure of purified "substance A" is shown to be the same as isoleucyl-5 angiotensin II, then one might suggest that "substance A" is human angiotensin. The suggestion has been made that "substance A" is formed not by alpha-amylase but by a contaminating proteolytic enzyme.

Dr. Helmer: Since Dr. Judson and I are working with something we think is like renin in the peripheral blood of hypertensive patients, we are interested in that problem. By incubation at proper pH we can now obtain a stable substance that behaves in every way like angiotensin. Could the urea technique you mentioned help us to decide whether this substance is angiotensin?

Dr. Bumpus: This technique may not help you much. The inhibitory action of urea has not been shown to be specific for angiotensin. We have determined that it does not affect oxytocin, bradykinin, and some of the pressor amines, but strict specificity has not been proven.
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