Is Renin a Factor in the Etiology of Essential Hypertension?

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SUMMARY The widespread clinical study of converting-enzyme inhibitors has shown that they are effective antihypertensive drugs even in patients who may manifest either normal or decreased plasma renin activity. This suggests either that renin in a site other than plasma may play a contributory role in essential hypertension or that the hypotensive effect is caused by increased concentrations of kinins and prostaglandins, both demonstrated consequences of converting-enzyme inhibitor administration. Specific renin inhibitors appropriate for studies in humans would aid in the resolution of this question. Four classes of compounds have been demonstrated to be renin inhibitors of high potency: specific antibody, general peptide inhibitors of acid proteases, analogs of angiotensinogens, and peptides that are related to the amino-terminal sequence of prorenin. With the purification of renin, specific polyclonal or monoclonal antibodies have become available. The former have already been used extensively in physiologic studies in intact animals. Pepstatin is an inhibitor of many acid proteases. Its in vivo application has been retarded by its relative insolubility, but recent chemical modifications, particularly the addition of charged amino acids at the carboxy terminus, have rendered it more useful. The minimal substrate for renin is an octapeptide segment of the protein substrate: His-Pro-His-Leu-Leu-Val-Tyr. Variants of this sequence have resulted in competitive inhibitors that are useful in vivo. Recently, remarkably active inhibitors have been synthesized by reducing the peptide bond that is cleaved by renin, producing what may be a transition state inhibitor. Several of these peptides have been shown to be effective as in vivo inhibitors of the hypertensive effect of the enzyme. The development of inhibitors based on prorenin sequences is awaited with interest. Substrate analog inhibitors have now been studied in dogs and monkeys, and, most recently, preliminary studies have been reported in humans. A hypotensive response has been demonstrated in sodium-replete, normal human subjects as well as in a low-renin hypertensive subject. The mechanism of this unexpected finding needs to be explained. (Hypertension 5 (supp V): V-8-V-15, 1983)

KEY WORDS • renin • converting enzyme • peptide inhibitors • pepstatin • essential hypertension

CARDIOVASCULAR disease remains the principal cause of death in developed countries, and hypertension is one of the major risk factors leading to its development.1 It is now well accepted that the treatment of hypertension results in reduced cardiovascular mortality and morbidity.2-9 More than 95% of all patients with high blood pressure are classified as having “essential hypertension,” a symptom complex without known cause. Thus, treatment, while effective, is directed at a manifestation of one or more disease processes rather than at the cause. When treatment is stopped, hypertension recurs in most instances. This leads to a curious paradox: patients must faithfully and regularly take a drug for the duration of their lives in order to control a disease process that is generally asymptomatic until the end of its course. Even though presently available drugs are often well tolerated, many patients have side effects that frequently are more uncomfortable than the symptoms of the disease. It is not at all surprising, then, that the major impediment to widespread and effective treatment of hypertension is patient compliance. The discovery of the etiology of essential hypertension must have a very high priority in medical research.

Physiologic control of the circulation is incompletely understood and sufficiently complex that, when modeled as a circuit diagram, its plan occupies many pages.10 Considerable progress has been made in understanding the relative roles of neural, endocrine, and renal control in the normal individual, but the distortion of that control in essential hypertension has eluded the investigator. A major impediment is the lack of an animal model that is a convincing analog of the human disease. Clues must be sought in the observation and study of patients.
One apparently important regulatory mechanism is the renin-angiotensin system. While initially believed to be of relevance only in certain uncommon forms of secondary hypertension, such as renovascular hypertension, a flurry of interest was created by the demonstration that some patients with essential hypertension had abnormal plasma renin levels, either elevated or depressed. This observation did not lead to further insight until drugs became available that blocked the renin-angiotensin system by inhibiting the activity of angiotensin-converting enzymes. It was expected that the drugs would be effective in lowering blood pressure in those patients who had elevated plasma renin levels, but investigators were soon surprised to discover that some patients with normal or even depressed renin levels showed a significant fall in blood pressure. Unfortunately, converting-enzyme inhibitors are relatively nonselective agents.

Renin is a proteolytic enzyme synthesized by the kidney, but also found in the brain and other organs, which acts on a substrate, angiotensinogen, to release the hemodynamically inactive prohormone, angiotensin I. Converting enzyme, a dipeptidyl carboxypeptidase, converts the prohormone to angiotensin II, the effector of this system. Captopril and enalapril are drugs available for clinical study that block the activity of this enzyme and thereby decrease the concentration of circulating angiotensin II. Another role for converting enzyme, however, is the metabolism of bradykinin, a potent vasodilator, to inactive peptide fragments. Could some of the effects of converting-enzyme inhibitors be caused by the vasodilatory effects of bradykinin?

Another element of uncertainty in interpretation was added by the observation that the vasodilatory prostaglandin, PGE₂, was increased in concentration in the plasma of patients having a hypotensive response to captopril associated with an increased urinary kinin excretion. It was later shown that the inhibition of prostaglandin synthesis blunted the hypotensive response to converting-enzyme inhibition and diminished its hypotensive effect in sodium-depleted subjects.

It is clear that highly selective tools are needed to help define the role of the renin-angiotensin system in essential hypertension. A specific inhibitor of renin that could be used in clinical investigation would be the most desirable. The most promising inhibitors of renin include four classes of compounds: specific antibody, general peptide inhibitors of acid proteases, angiotensinogen analogs, and peptides that are fragments of the prorenin sequence. Each of these classes will be discussed, particularly with respect to potential for future development as practical tools in clinical investigation.

**Renin-Specific Antibodies**

Renin antibodies have been used as physiologic reagents for many years, yet their specificity was in doubt since we now know that the preparations then used as immunogens contained less than 1% of the enzyme. Dzau et al. purified canine renin some 600,000-fold in an eight-step process that yielded a product homogeneous by several criteria. Antibodies specific for purified canine renin raised in a goat inhibited the pressor action of the enzyme but did not modify the capacity of either angiotensin I or II to raise blood pressure. This antibody preparation did not have any effect on the hemodynamics of the unanesthetized, sodium-replete dog, while a significant hypotensive effect was noted in the sodium-depleted dog when the renin-specific antiserum was injected intravenously. Parallel to the fall in blood pressure, a decrease in both plasma renin activity and angiotensin II concentrations was observed, indicating that the antibody was exerting its effect by inhibiting the enzymatic action of renin on its substrate.

Intact antibody has a number of troublesome properties when used as a drug. When the source is a homologous species, it is an immunogen. After the first use, an immune response develops, which may result in anaphylaxis, serum sickness, or, at best, accelerated elimination. The hypotensive effect of unmodified antibody is very persistent. Antibody is initially eliminated by metabolism, with the half-life for endogenous immunoglobulins measured in days or weeks depending on the species and the immunoglobulin isotype. When anti-antibodies form, elimination is more rapid and occurs via phagocytosis by the reticuloendothelial system. At this point the antibodies are of very little use since they are no longer likely to bind renin. Immune complexes are also undesirable by virtue of their renal toxicity. In hemodynamic studies, vasoactive peptides by activation of complement may confuse physiologic measurement.

One solution to these difficulties lies in the cleavage of the native antibody molecule into smaller fragments by the enzyme, papain. The resultant Fabs bind 1 mole of antigen each, whereas the Fc fragment contains the complement binding site. Fab has a number of desirable properties when compared with the intact molecule. IgG: equilibrium distribution in extracellular fluid is achieved more rapidly; the volume of distribution is greater; and the fragment is eliminated with a far shorter half-life. In addition, when injected intravenously, Fab is less immunogenic than IgG. The immune complexes that may be formed are smaller than those that cause nephrotoxicity (comprising a single antigen molecule with several Fab attached), and complement cannot be fixed because the relevant binding sites on the Fc have been lost. Renin-specific Fab is an effective physiologic reagent. When compared with intact antibody, the initiation of hypotension is more rapid, and the duration of the effect very much shorter.

An antiserum is a mixture of several hundred antibodies, all capable of binding to the immunizing antigen. These antibodies vary considerably both with respect to affinity for the antigen and specificity of recognition. The mixture of antibodies contained within an antiserum is different among even inbred (geneti-
cally identical) individual animals as it is in the serum of the animal collected at varying times after immunization. Supplies of antisera are thus necessarily limited. Köhler and his colleagues showed that antibodies might be produced by the technique of somatic cell fusion. Normal lymphocytes and cells from a malignant plasmacytoma are fused to produce a hybrid that incorporates both properties of antibody production (from the normal lymphocyte) and growth in vitro (from the malignant plasmacytoma) into the product (colloquially named "hybridoma"). Cells that grow in culture may be cloned so that all the progeny are daughters of a single precursor cell. A homogeneous culture of this type produces a single antibody that is uniform in structure and antigen-binding properties. Since the cultures may be stored indefinitely at low temperatures, the same antibody may always be recovered. Production in industrial quantities is now possible utilizing large-scale fermenters.

Because the amount of renin-specific antibody available from conventional sera was severely limiting, we set out to make monoclonal, renin-specific antibodies by the somatic cell-fusion method. These antibodies vary greatly in their affinity for renin, in species specificity, and in their capacity to inhibit the catalytic activity of the enzyme. Since monoclonal antibodies bind to a single epitope on the surface of a protein, only a fraction of these antibodies is likely to affect the catalytic site. Thus, one must search diligently among many fusions to find a clone producing a single antibody that is uniform in structure and antigen-binding properties. Since all the acid proteases studied are inhibited to varying degrees, how is a physiologic change to be interpreted as reflecting renin inhibition? May not some other acid proteases be involved in the regulation of the circulation? The desire to obtain an agent suitable for clinical investigation is certainly not satisfied by a general protease inhibitor, regardless of its efficacy.

Mouse monoclonal antibodies have now been used in the therapy of lymphoid malignancies and kidney transplant rejection. While initially effective in both of these conditions, anti-antibodies soon formed, blunting their effectiveness. In both of these studies, intact xenon antibodies were used so that the formation of an immune response is not at all surprising. As indicated above, Fab, which is one-third the molecular size of antibody, is less immunogenic than the intact molecule. The fragment of minimal size that has thus far been shown to have unimpaired antigen binding is Fv, one-half the molecular size of Fab. Careful examination of crystal structures of antibodies suggests that this is still not the minimal-size fragment that can bind antigen. Novotny et al. have proposed that a stable, 12,000-dalton fragment could be constructed comprising the binding site and supporting structures only.

This fragment would have to be assembled by genetic engineering methods from several cDNA or synthetic polynucleotide fragments. Once optimal size had been determined, an amino acid sequence of minimal immunogenicity could be specified. Presumably one would start with a human antibody structure. By combining the techniques of in vitro immunization and human myeloma cell-lymphocyte fusion, the biosynthesis of a human antibody to human renal renin is a realistic goal.

**Pepstatin and Its Congeners**

Renin is an enzyme that, together with such enzymes as pepsin and cathepsin D, is considered an aspartyl protease because of the presence of two aspartic acid residues at the active site that are intimately involved in catalysis. In 1971, Aoyagi and colleagues discovered that the bacterial peptide they had been studying as an inhibitor of pepsin was also an effective blocker of a number of acid proteases. Shortly thereafter, Gross and his colleagues demonstrated that in vivo inhibition of the renin-angiotensin system could be effected with pepstatin. This work was difficult to reproduce, probably because of the insolubility of the peptide, and did not lead to the widespread adoption of pepstatin in physiologic experiments. Improvements in solubility, first by acetylation of pepstatin and then by the substitution of hydrophilic amino acids at the carboxy terminus of the molecule, yielded more convincing results. Although pepstatin derivatives may eventually prove to be adequate in vivo renin inhibitors, their use as investigative agents is limited conceptually by their lack of specificity for renin. Since all the acid proteases studied are inhibited to varying degrees, how is a physiologic change to be interpreted as reflecting renin inhibition? May not some other acid proteases be involved in the regulation of the circulation? The desire to obtain an agent suitable for clinical investigation is certainly not satisfied by a general protease inhibitor, regardless of its efficacy.

Renin is a fastidious protease. It requires a specific amino acid sequence in its substrate to cleave the bond that releases angiotensin I. If pepstatin derivatives could be rendered more specific by incorporating the recognition sequence of angiotensinogen, useful inhibitors might be obtained. Pepstatin inhibits pepsin with a K<sub>i</sub> < 10<sup>-13</sup> M. If a renin inhibitor could be constructed of similar potency, based on pepstatin's essential structures (such as the amino acid statine), one would have both a powerful and selective pharmacologic agent.

**Angiotensinogen Analogs**

Skeggs et al. defined the minimal sequence from natural protein substrate that interacts strongly with renin. The octapeptide sequence extending from histidine-6 through tyrosine-13 (table I) has kinetic parameters essentially the same as those of the full tetradecapeptide renin substrate. Kokubu et al. synthesized a number of analogs of the tetrapeptide found
between residues 10 and 13 (table 1) in the hope of creating an effective inhibitor. While inhibition could be shown, inhibitory constants were only in the millimolar range.

To produce more effective inhibitors Burton and colleagues synthesized analogs of a larger segment of renin substrate. Peptides were tested as renin inhibitors using a radioimmunoassay to measure decreases in the generation of angiotensin I from either natural protein substrate or the tetradecapeptide. Addition of the octapeptide analogs to the standard assay mixture decreased the rate of formation of angiotensin I. Data from these tests fit the standard Michaelis-Menten equation with a high degree of precision. All of the synthetic peptides tested were competitive inhibitors.

The native octapeptide sequence is both a competitive inhibitor and a substrate for renin. Edman degradation of the reaction product shows the enzyme quantitatively cleaves the leucyl-leucine bond in the octapeptide. The first modifications made in the octapeptide sequence were aimed at producing peptides that would bind but not be cleaved by renin. Replacement of either leucyl residue (10 and 11 in table 1) with the D-enantiomorph yields inhibitors that are not cleaved by renin. In addition, the (D-Leu)octapeptide binds renin one order of magnitude (3 μm) more tightly than the parent octapeptide (39 μm).

K, of the various inhibitors can be related to the lipophilicity of amino acid residues at the cleavage site. Replacement of the leucyl residues with phenylalanine yields an analog that binds about 40 times as well as Pro-octapeptide (K, 1 μm vs 39 μm). Chlorophenylalanine provides an even more hydrophobic residue at the cleavage site and yields a compound that is yet a more potent inhibitor. Two chlorophenylalanines at the site yield an insoluble peptide.

Parikh and Cuatrecasas synthesized inhibitor peptides of greater length, but there was no apparent advantage with respect to enhanced inhibition (table 1).

Solubility of peptides is a requirement for a physiologically applicable inhibitor since both a high concentration and tight binding to renin are required to compete with natural substrate. The effectiveness of an inhibitor is best judged by the ratio between solubility and K,. Addition of a single prolyl residue to the (phe)octapeptide doubled solubility and decreased K, so that this ratio increased from 6 to 100. A further improvement in the ratio was obtained by attaching a lysyl residue to the C-terminus of the (Pro), (PhePhe)octapeptide (RIP) (table 1). Solubility of this peptide was increased eightfold with only a doubling of K, yielding a solubility/K, ratio of 420:1. The pattern of solubility as a function of pH is also changed, significantly enhancing solubility at neutral pH as, of course, would be desirable in an inhibitor of physiologic utility.

Another approach to the construction of competitive inhibitors has been undertaken by Szelke and his collaborators. Instead of substituting amino acids for those normally at the cleavage site of a substrate analog, the peptide bond (-CO-NH-) has been entirely replaced with a reduced bond (-CH₂-NH-). It is likely that the reduced peptide bond acts as a transition state analog. One of these compounds (table 1) has been shown to be an effective renin inhibitor in the dog. Another is highly potent with respect to inhibiting human renin (table 1).

We have examined in vivo inhibition of renin by Pro-(PhePhe)octapeptidyl-lysine (RIP) (table 1) in the monkey, M. fascicularis. When we infused it into normotensive, sodium-replete monkeys, we observed no significant change in blood pressure.

### Table 1. Selected Substrates and Inhibitors of Renin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amino acid sequence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine substrate</td>
<td>Asp·Arg·Val·Tyr·Ile·His·Pro·Phe·His·Leu·Val·Tyr·Ser·</td>
<td>42</td>
</tr>
<tr>
<td>Human substrate</td>
<td>Asp·Arg·Val·Tyr·Ile·His·Pro·Phe·His·Leu·Val·Ile·His·</td>
<td>44</td>
</tr>
<tr>
<td>Minimal substrate</td>
<td>His·Pro·Phe·His·Leu·Val·Tyr</td>
<td>42</td>
</tr>
<tr>
<td>Early inhibitors</td>
<td>D</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>His·Pro·Phe·His·Leu·Val·Tyr</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>Asp·Arg·Val·Tyr·Ile·His·Pro·Phe·His·Leu·Val·Tyr·Ser·</td>
<td>45</td>
</tr>
<tr>
<td>In vivo inhibitors</td>
<td>Pro·His·Pro·Phe·His·Phe·Phe·Val·Tyr·Lys·</td>
<td>49</td>
</tr>
<tr>
<td>RIP</td>
<td>R</td>
<td>48</td>
</tr>
<tr>
<td>Potent inhibitor</td>
<td>R</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Pro·His·Pro·Phe·His·Leu·Val·Ile·His·Lys</td>
<td>54</td>
</tr>
</tbody>
</table>

D above an amino acid indicates the D stereoisomer; R above a bond indicates a reduced peptide bond. (Modified from ref. 43.)
Mean arterial pressure (MAP) reduction occurred in five studies of the normotensive sodium-depleted state following a bolus injection (2 mg/kg) of renin-inhibitory peptide (RIP) (p < 0.004). This was associated with significant cardiac acceleration (p < 0.003). The MAP reduction in response to the 1 mg of converting-enzyme inhibitor (CEI) per kg (p < 0.006) was not significantly different compared to the renin inhibitor peptide response. (Reprinted by permission of the National Academy of Sciences, from ref. 49.)

The effect of purified human renin was inhibited by an infusion of the peptide by the effects of angiotensin I and angiotensin II were not. We then tested the peptide in normal sodium-depleted animals. An intravenous bolus dose resulted in a prompt reduction in blood pressure; within 15 minutes the pressure had returned to normal (fig. 1). An intravenous dose of the converting-enzyme inhibitor, teprotide, resulted in an identical fall in pressure.

In sodium-depleted, renin-dependent hypertensive monkeys, the intravenous injection of RIP resulted in a prompt fall in blood pressure to normal levels. After hypertension was reestablished, teprotide caused an identical fall in pressure (fig. 2).

These experiments closely duplicate the observations previously detailed utilizing renin-specific antibody. The substrate inhibitor peptide acts specifically on renin and does not inhibit the pressor action of either angiotensin I or II. It appears to be well tolerated without any hemodynamic consequences in the normotensive, sodium-replete animal.

It is apparent that these compounds have immediate potential for exploring the role of renin in normal human cardiovascular homeostasis and in certain forms of renovascular and renal hypertension. As indicated earlier, the more exciting opportunity is the definition of the role of this enzyme in the genesis and maintenance of essential hypertension in humans.

Zusman et al. have infused RIP intravenously into normal, salt-depleted human subjects and into low-renin, hypertensive patients, who were studied supine and after 1 minute of upright tilting to 70° following a 10-minute infusion. In the supine position and at an RIP dose of 1 mg/kg/min, blood pressure fell from 107 ± 3/65 ± 2 to 63 ± 13/23 ± 12 mm Hg (p < 0.05);
the heart rate was unchanged. On upright tilting, no audible blood pressure was obtainable. In comparison, converting-enzyme inhibition with captopril (up to 325 mg p.o. over 3 hours) reduced supine blood pressure from 108 ± 2/68 ± 2 to 89 ± 6/48 ± 5 mm Hg (p < 0.05); on upright tilting, blood pressure fell from 107 ± 2/76 ± 2 to 74 ± 5/41 ± 14 mm Hg (p < 0.05). Captopril also had no effect on heart rate. Of particular interest, when RIP was infused in a previously studied subject now on a 200 mEq sodium diet, a dose of 1 mg/kg/min reduced supine blood pressure from 117/69 to 88/50. Upright tilting resulted in a fall of blood pressure from 117/73 to an inaudible blood pressure, and the patient’s heart rate fell from 80 to 40 bpm. Captopril had no effect on blood pressure or heart rate under these conditions. In a low-renin hypertensive on a 10 mEq diet, RIP (1 mg/kg/min) reduced blood pressure on upright tilting from 135/113 to an inaudible blood pressure and heart rate from 116 to 60 bpm (fig. 3 left). In figure 3 right, the upright blood pressure levels following captopril administration show a gradual fall rather than the dramatic orthostatic changes observed with RIP.

These observations are highly provocative. In the nonhuman primate, observations with RIP were what might have been quite predictably expected with renin inhibition. There were no hemodynamic effects in the salt-replete animal; sodium-depleted animals became hypotensive with renin inhibition as did animals with renovascular hypertension. The effects of RIP could not be differentiated from those of captopril. On the other hand, at the higher doses of RIP utilized in humans, hypotension was noted in a salt-replete normal subject; under these conditions, captopril had no effect. Hypotension also was observed in a low-renin hypertensive patient. Bradycardia was noted in subjects who had very low blood pressure.

Is RIP acting at another site of renin action other than plasma? Does renin have a role in the neural regulation of blood pressure, heart rate, and peripheral resistance, or has an element of nonspecificity been uncovered at higher doses of RIP, which was not apparent in the nonhuman primate studies? These observations need to be extended before firm conclusions can be drawn.

Prorenin Peptides as Renin Inhibitors
Corvol et al. recently reported that a fragment of the prorenin sequence is a renin inhibitor. From x-ray crystallographic models of other acid proteases, it is apparent that the pro-sequence covers the catalytic site in the zymogen. Logic dictates that this amino acid sequence may be a key to the construction of inhibitors. We eagerly await further developments in this area.

Will Renin Inhibitory Peptides Evolve into Drugs
While the value of all these compounds as investigational tools is now certain, a central question remains: can any of these concepts be utilized in the creation of a drug useful in the treatment of hypertension? Oral administration is an obvious requirement for a drug in widespread use. The stringent specificity requirements of renin may make it difficult to reduce the size of the compound, yet considerable progress has already been made in creating analogs of rather large peptides (somatostatin) that survive proteolytic digestion, are absorbed by the gastrointestinal tract, and manifest full biologic activity.

![Figure 3. Effects of a renin inhibitory peptide (left) and captopril (right) on the blood pressure of a patient with low-renin, essential hypertension. Blood pressures were measured in the upright position.](http://hyper.ahajournals.org/)

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References


2. Veterans Administration Cooperative Study Group on Antihypertensive Agents: Effects of treatment on morbidity in hypertension: results in patients with diastolic blood pressures averaging 115 through 129 mm Hg. JAMA 202: 1028, 1967

3. Veterans Administration Cooperative Study Group on Antihypertensive Agents: Effects of treatment on morbidity in hypertension: II. Results in patients with diastolic blood pressures averaging 90 through 114 mm Hg. JAMA 213: 1143, 1970


43. Poulsen K, Burton J, Haber E: Competitive inhibitors of renin: Inhibitors effective at physiologic pH. Biochemistry 14: 1356, 1975


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