ANGIOTENSIN-CONVERTING ENZYME INHIBITORS: BIOCHEMICAL PROPERTIES AND BIOLOGICAL ACTIONS

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I. INTRODUCTION

Careful scrutiny of scientific records can show that the connection between the kidney and hypertension had been discovered early in the 19th century, but undoubtedly it is the undisputed legacy of Harry Goldblatt to have clearly demonstrated in 1934 that chronic reduction of the blood supply to the kidney can lead to the development of hypertension in animals.[1,2] This observation marked the real beginning of the conceptualization of the renin-angiotensin system, since Goldblatt's experiments had provided clear evidence for the humoral nature of the connection between renal ischemia and experimental hypertension. Clarification of the molecular basis of this mechanism took approximately 30 years.[3] The various components of the renin-angiotensin system and their interrelations are depicted in Figure 1.

Two enzymes play a key role in this system: renin and angiotensin-converting enzyme (ACE). Renin is a highly specific endopeptidase that cleaves the *alpha*-globulin substrate angiotensinogen at a single peptide bond to release an N-terminal decapeptide, angiotensin I (AI). Even though there are other acidic proteases that can catalyze this specific hydrolytic step, there are no other known substrates for renin, except angiotensinogen or fragments incorporating an extensive portion of its N-terminal sequence. On the contrary, ACE, the peptidyldipeptide carboxyhydrolase (EC 3.4.15.1) that separates the C-terminal dipeptide from the decapeptide AI to give the octapeptide angiotensin II (AII), is a rather nonspecific peptidase that cleaves dipeptides from a wide variety of natural and synthetic peptides.[4]

AII is one of the most potent vasoconstrictor agents known, but it has several other important biological effects, including stimulation of the adrenal secretion of aldosterone, regulation of thirst mechanisms and water intake, control of renin release through a negative feedback effect, facilitation of adrenergic transmission, and inhibition of vagal activity.[5-8]

AII can also be generated in vivo directly from angiotensinogen through the action of nonspecific proteases such as tonin, a serine protease of the submaxillary gland, or cathepsin G, a neutral protease from neutrohiles.[10] Tonin is also capable of converting inactive renin to renin.[9] Since ACE inhibitors can block almost completely the formation of AII in vivo, it is doubtful that these two enzymes play any significant role in the economy of the renin-angiotensin system.

Aldosterone is not strictly a component of the renin-angiotensin system, since its production and secretion are not solely regulated by the end product AII, but also by such factors as blood levels of potassium ion and ACTH. Aldosterone induces sodium retention and enhances potassium excretion, and through these actions AII plays an important role in modulating electrolyte balance and blood volume. Removal of the N-terminal aspartic acid residue of AII leads to a heptapeptide with decreased vasoconstrictor activity but with intact aldosterone-stimulating activity.[5,11] However, it is not yet clear whether this enzymatic

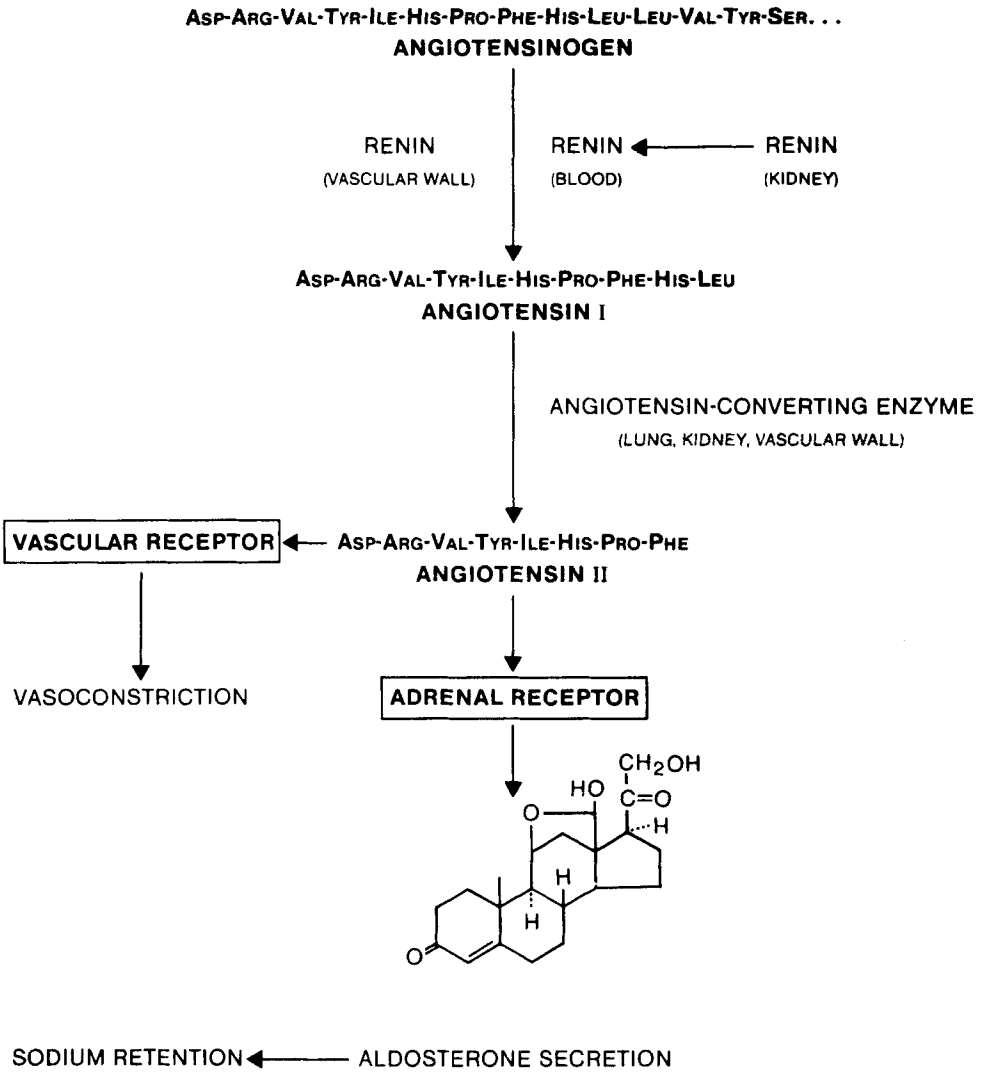


FIGURE 1. Renin-angiotensin system.

processing of AII to the so-called angiotensin III (Figure 2) has any physiological relevance. Further proteolysis of angiotensin III leads to the destruction of all the biological activities of this peptide.[12]

II. BLOCKADE OF THE RENIN-ANGIOTENSIN SYSTEM

As with many other humoral systems, the availability of agents capable of specifically blocking each individual step has contributed significantly to the better understanding of the interrelations among the different components of the renin-angiotensin system. Even though there has been considerable skepticism concerning the relevance of this system in any but the renovascular form of human hypertension, the therapeutic potential of an effective blockade of generation or action of AII has never been in doubt.

Examination of the sequential steps involved in the activation of the renin-angiotensin system (Figure 1) suggests a variety of alternate methods for blocking the final effects of the system:

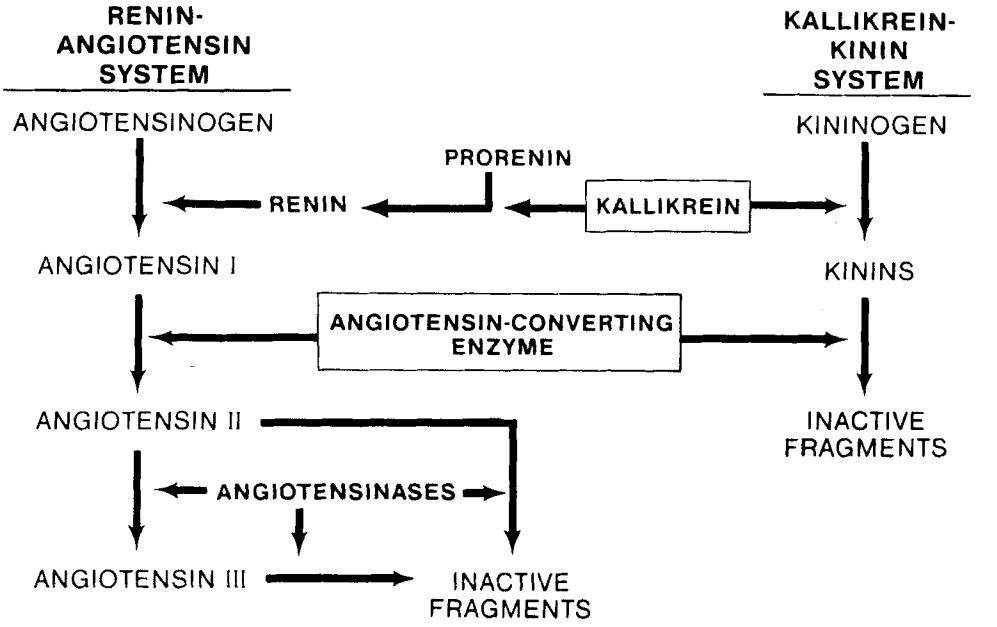


FIGURE 2. Interactions between renin-angiotensin and kallikrein-kinin systems.

1. Depletion of angiotensinogen
2. Inhibition of renin synthesis, activation, or release
3. Inhibition of the renin-angiotensinogen reaction
4. Inhibition of ACE synthesis or activation
5. Inhibition of ACE activity on AI
6. Blockade of the action of AII on its receptors

There is no evidence that ACE is synthesized as a proenzyme or that its synthesis is under specific feedback control; therefore, alternative four is not relevant at this time. Inhibition of ACE, alternative number five, is the main topic of this review. For the sake of brevity, we have used previous reviews as a point of departure and have only attempted to cover the most recent literature. The reader should consult the reviews cited in the text to obtain a more comprehensive view of the development of this field.

Even though blockade of the renin-angiotensin system through interference with the renin-angiotensinogen reaction or the AII receptor step is not the purpose of this review, we will briefly summarize the present status of these areas, since they are of great potential value for the development of novel approaches to antihypertensive therapy. For an in-depth review of this area, see References 4 and 4a.

A. Renin-Angiotensinogen Reaction

Angiotensinogen is a plasma glycoprotein having a molecular weight of approximately 60,000 that serves as a substrate for the enzyme renin.[13] The renin-catalyzed cleavage of the peptide bond between leukyl residues 10 and 11 of angiotensinogen (Figure 1) releases the N-terminal decapeptide AI. The amino acid sequence of the N-terminal portion of human angiotensinogen in the vicinity of the scissile peptide bond is slightly different from that of other species (Figure 3).

Tewksbury et al. have hypothesized that these differences might be responsible for the species specificity of the renin-angiotensinogen reaction.[14] Enzymatic studies with human and dog renin and synthetic trideca- and tetradecapeptides with variable sequences between

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
HUMAN	ASP	-ARG	-VAL	-TYR	-ILE	-HIS	-PRO	- PHE	-HIS	-LEU	-VAL	-ILE	-HIS	-ASN-
RAT	ASP	-ARG	-VAL	-TYR	-ILE	-HIS	-PRO	- PHE	-HIS	-LEU	-LEU	-TYR	-TYR	-SER-
HORSE	ASP	-ARG	-VAL	-TYR	-ILE	-HIS	-PRO	- PHE	-HIS	-LEU	-LEU	-VAL	-TYR	-SER-

FIGURE 3. Amino terminal amino acid sequence of angiotensinogen from different species.

residues 10 and 14 indicate that the histidyl residue in position 13 is responsible for the resistance of human angiotensinogen to cleavage by nonprimate renins.[15] However, other mammalian angiotensinogens, with the possible exception of that of rat,[15a] are cleaved by human renin.

In human serum there is also a high molecular weight form of angiotensinogen which appears to increase disproportionately in high estrogen states, such as pregnancy.[16] Alterations of angiotensinogen levels under various conditions have been reviewed by Reid et al.[17]

Renin is a very specific peptidase for which the only known substrates so far found are angiotensinogen or partial sequences of its N-terminal portion. The minimal sequence that can serve as substrate is the octapeptide His-Pro-Phe-His-Leu-Leu-Val-Tyr.[3] The kidney is the most abundant source of this enzyme, which is concentrated in granules within the cells of the juxtaglomerular apparatus.[18] Renin has also been found in the submaxillary glands (but only in white male mice), brain, vascular walls, pituitary, and amniotic fluids. The role of the renin present in vascular tissue may be of critical importance for understanding the relevance of the renin-angiotensin system in human hypertension.[19,20] Renin release from juxtaglomerular cells into the blood stream is influenced by a variety of factors, e.g., tubular Na^+, K^+, or Cl^- concentration, fluid pressure, sympathetic activity, etc. At this time it does not seem possible to block effectively the renin-angiotensin system through inhibition of renin release. The pharmacological alteration of renin release has been extensively reviewed by Keeton and Campbell.[21]

The most well-characterized form of renin is that occurring in the submaxillary gland of white male mice. The complete amino acid sequence has been established both by classical protein sequencing procedures[23,23] and also by a combination of protein and gene sequencing techniques.[24] Mouse submaxillary gland renin (36,000 mol wt) is composed of light and heavy polypeptide chains joined by a single disulfide bridge; in addition, the heavy chain has an intrachain disulfide bridge and two free cysteine residues.[23] No carbohydrate moieties are present in this molecule, but renins from other tissues are glycosylated.

Evidence from several laboratories has clearly established that an inactive form of renin, called prorenin, can be found in kidneys and plasma.[25] The studies on gene sequencing of mouse submaxillary gland renin[24] have shown that prorenin and preprorenin forms are also encoded, but the proenzyme has not yet been directly isolated from this tissue.

Activation of prorenin by conversion to renin through one or more specific proteolytic cleavage steps may be an important mechanism regulating the overall activity of the renin-angiotensin system. Kallikrein, the kinin-generating enzyme, has been implicated in the conversion of prorenin to renin.[26,27] This would indicate that there is a very close relationship between these two humoral systems (Figure 2). If kallikrein or any other specific enzyme is conclusively shown to be involved in this activation step, a new avenue to the blockade of the renin-angiotensin system might be opened. However, it is presently unclear whether inactive renin is converted to an active form in vivo, although many proteases can activate it in vitro.

Studies of the active site of purified rat submaxillary gland renin have shown that the residues involved in catalysis are the same as those present in acidic proteases, namely two

aspartic acid residues and a tyrosine residue; also, the heavy and light chains show more than 40% homology with N- and C-terminal sequences, respectively, of porcine pepsin.[28] These similarities are reinforced by the fact that renin is also inhibited by the pepsin inhibitor pepstatin, albeit with a considerably higher K_i value.[29] However, the optimal pH of renin is in the neutral range, while acidic proteases derive their generic name from their acid pH optima.

The search for inhibitors of the renin-angiotensinogen reaction has been undertaken in several laboratories. Pepstatin and pepstatin derivatives, peptide substrate analogs, and renin antibiodies have shown significant inhibition in vitro and in vivo, but lack of oral activity has so far precluded their therapeutic application. (For a review, see Reference 4.) Recent novel entries in this area are the potent and specific substrate analog inhibitors with a reduced carbonyl at the scissile peptide bond (–CONH– → –CH_2NH–).[30]

B. Angiotensin II Antagonists

Antagonists of the effect of AII on its receptors were among the first useful blockers of the renin-angiotensin system to be developed. (For reviews, see References 31 and 32.) The AII antagonists available so far are analogs of AII with amino acid substitutions at positions 1 and 8 (Figure 4).

Replacement of the phenylalanine in position 8 of AII with an aliphatic amino acid is critical for the transformation of this agonist into antagonist. Unfortunately, the agonistic activity is not completely eliminated by such a substitution, and, to different degrees, all the antagonists available so far retain agonistic activity.[33] The most widely used substituent at the N-terminal of AII antagonists is sarcosine, which apparently increases potency and duration of action by increasing resistance to enzymatic degradation and by increasing receptor binding. In spite of the shortcomings of residual agonistic activity and lack of oral activity, angiotensin antagonists have provided solid evidence of the contribution of the renin-angiotensin system in animal models of experimental hypertension, and of the relevance of this system in clinical hypertension. The most widely used AII antagonist is the octapeptide saralasin.[34]

III. ANGIOTENSIN-CONVERTING ENZYME

A. Distribution and Assay

ACE, the exopeptidase that converts AI to AII by removal of the C-terminal dipeptide, is widely distributed in mammalian tissues, and a similar enzyme has been found in prokaryotes.[35] The highest concentrations have been found in lung, and male reproductive tissues, but it also occurs in plasma, kidney, brain, intestine, adrenal cortex, and occular tissue,[4] and it has recently been isolated from skin.[36] Cultured vascular endothelial cells and human monocytes also have high levels of ACE.[35,37,38] Histological studies indicate that this enzyme is localized on endothelial cells facing the vascular lumen and on epithelial cells bordering kidney tubular or intestinal lumina.[35,39]

Homogeneous ACE has been obtained from lung, kidney, plasma, and reproductive tissues of several species.[4,35] The most common methods of assay utilized for the determination of ACE activity involve measuring the extent of hydrolysis of synthetic tripeptide substrates, such as benzoyl-Gly-His-Leu, furanacryloyl-Phe-Gly-Gly, or analogs, by radiochemical, spectrophotometric, or spectrofluorometric procedures.[4]

Since ACE was first isolated and characterized as a consequence of studies on AI and bradykinin metabolism, it is not surprising that these two peptides are often considered to be its "natural substrates". However, the wide distribution of this enzyme and the lack of stringent specificity requirements for its substrates would seem to indicate that there might be other, yet undiscovered, functions for this peptidase.

	1	2	3	4	5	6	7	8
ANGIOTENSIN II	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe
SARALASIN	Sar	Arg	Val	Tyr	Val	His	Pro	Ala
[Sar^1,Ile^8]-AII	Sar	Arg	Val	Tyr	Ile	His	Pro	Ile
[Sar^1,Thr^8]-AII	Sar	Arg	Val	Tyr	Ile	His	Pro	Thr

FIGURE 4. Structures of angiotensin II and antagonist.

B. Physicochemical Properties

ACE is a glycoprotein with a carbohydrate content of 8 to 32% and a molecular weight of 130,000 to 160,000, both depending on the tissue of origin. Due to the high carbohydrate content, certain methods often used to estimate molecular weights of proteins, particularly dextran gel permeation chromatography, have been misleading, and values from 100,000 to 300,000 have been reported for ACE from some sources.[4,35] The ACE from rabbit testis, however, although immunologically and catalytically similar to ACE from other sources, has a lower molecular weight of 100,000 whether obtained from the tissue or synthesized in a cell-free system using mRNA from testis.[40,40a]

Whatever the source from which it was isolated, ACE has proved to be an acidic protein (pI approximately 4.5), and to depend on a metal ion for full enzymatic activity. Rabbit and dog lung and horse plasma ACE have all been shown to contain one gram-atom of zinc per mole. Amino acid compositions have only been reported in a few cases, and only a preliminary report on the amino acid sequence of the amino and carboxyl terminal portions of enzymes from rabbit lung and testis[41] and from human kidney[42] has appeared.

Catalytically essential functional groups at the active site of ACE include the amino acids arginine, tyrosine, lysine, and glutamic acid, and the complexed zinc ion.[43-47] A small peptide containing the active-site glutamic acid residue has been isolated, but not yet sequenced.[45] There is no information yet as to what amino acid residues serve as ligands for the zinc ion of ACE, or as to whether it is present in a pentacoordinate state as it has been recently demonstrated for carboxypeptidase A.[48] As with carboxypeptidase A, the zinc ion of ACE does not play any role in the binding of the substrate,[47] but it is apparently involved in the cleavage of the scissile amide bond by polarization of the amide carbonyl and facilitation of the nucleophilic attack of the glutamic carboxylate. The arginine and lysine residues present at the active site of ACE are probably required for the binding of the substrate (Figure 5).

The arginine is most likely required for ionic interaction with the C-terminal carboxyl group of the substrate, as has been clearly shown for carboxypeptidase A in crystallographic studies.[49] The lysine might be involved in the mechanism of chloride ion activation.[43,46,51] Chloride or other monovalent anions increase the activity of ACE to an extent that varies in accordance with the structure of the substrate[50] and pH.[51] At all pH and with most substrates, chloride activation is exerted through a lowering of K_m, while increase of k_{cat} is seen only at some pH with some substrates.

C. Substrate Specificity

Although ACE was discovered because of its specific cleavage of a dipeptide residue from the C-terminal sequence of AI, it soon became clear that a large variety of peptides with different C-terminal amino acid sequences could serve as substrates. The only apparently

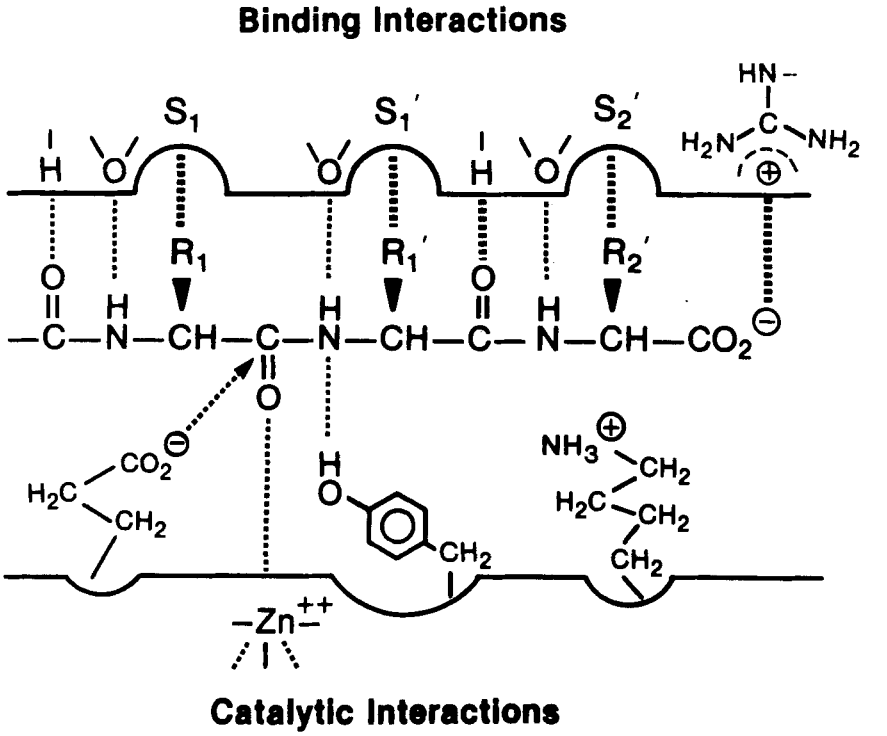


FIGURE 5. Hypothetical model of substrate binding at the active site of angiotensin-converting enzyme.

absolute requisites for an ACE substrate are (1) an amino acid with a free carboxylic acid function at the C-terminal end, and (2) an amino acid other than proline on the penultimate position.[35,51a] Two recent reports have questioned these requirements. Synthetic peptides with a C-terminal *p*-nitrobenzyl amide moiety are cleaved by homogeneous human ACE to release glycine-*p*-nitrobenzylamide and the next C-terminal dipeptide sequence.[52] Highly purified preparations of hog ACE can release C-terminal tripeptides from substrates having a proline residue in the penultimate position.[53] In both cases the hydrolytic activity is enhanced by chloride ion, and inhibited by specific ACE inhibitors. Although the catalytic efficiency of ACE for these reactions is considerably lower than that observed in the classical peptidyldipeptide carboxyhydrolase activity, it is significant enough to warrant further investigation.

Peptides containing acidic amino acids are poor substrates for ACE, while peptides with aromatic amino acids are usually very good substrates.[4,54] Only after the isolation of peptide inhibitors from snake venoms[55,56] was it realized that peptides with C-terminal proline can bind exceptionally well to ACE and can, therefore, provide good substrates or inhibitors depending on other features of the sequence.

ACE, like many other peptidases, can also cleave ester bonds, albeit at a lower rate.[57] This behavior had been predicted on the basis of the structure-activity relationships observed with modified analogs of the peptidic inhibitors found in *Bothrops jararaca* venom.[58]

IV. ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

A. Peptide Inhibitors

The first well-characterized inhibitors of ACE were the peptides isolated from the venom of the South American pit viper *B. jararaca*. The amino acid sequences of these peptides

bear very little resemblance to those of AI or bradykinin. However, the venom peptides are very potent competitive inhibitors of ACE with K_i values in the range of 10^{-6} or 10^{-7} *M* (Table 1).

The structure-activity relationships in this area have been reviewed.[59,64] The most potent inhibitor of cell-free ACE in vitro is the pentapeptide 1.3 (BPP5a or SQ 20,475), but the most potent in vivo is the biologically stable nonapeptide 1.4 (BPP9a, SQ 20,881, or teprotide).[58] The study of many analogs has clearly indicated that the C-terminal tripeptide sequence Trp-Ala-Pro or the related Phe-Ala-Pro leads to the most potent inhibitors.[64] As will be seen, this observation has played a key role in further development of ACE inhibitors.

More recently, ACE inhibitory activity has also been found in peptide fragments of bradykinin[60] (2.1 to 2.3, Table 2), casein[61] (2.4, Table 2), fibrinogen[62] (2.5, Table 2), and ACTH[63] (2.6 to 2.8, Table 2).

The pentapeptide Ala-Arg-Pro-Ala-Lys was shown to block the hypertensive activity of AI and potentiate the hypotensive activity of bradykinin but with a very short duration of action.[62]

No significant amount of effort seems to have been devoted to further modifications of teprotide, since they were reviewed last,[64] but analogs containing 3,4-dehydro-L-proline, instead of L-proline, have been reported;[65] although more active in vitro than teprotide, they do not show any significant improvement over this nonapeptide in their antihypertensive activity.[66] The metabolic fate of intravenously administered teprotide labeled with tritium in the pyroglutamic acid residue has been reported. The main metabolites found are the fragments <Glu-Trp, <Glu-Trp-Pro-Arg-Pro-Gln, and <Glu-Trp-Pro-Arg-Pro-Gln-Ile, and 25 to 80% of the radioactivity was excreted in the urine within 2 hr after injection.[67]

A systematic study of the inhibitory potency of dipeptides composed of L amino acids shows a range of four orders of magnitude from Val-Trp (I_{50} = 1.6 μ*M*) to Pro-Gly (I_{50} = 17 m*M*).[50] In spite of its high intrinsic activity, L-Val-L-Trp does not inhibit the contractile activity of AI in the guinea pig ileum, or the hypertensive activity of this decapeptide.

Dipeptides of cysteinyl-L-proline (3.1 and 3.2, Table 3) show considerably higher inhibitory activity vs. isolated ACE than Val-Trp, and the D-L analog is ten times more potent than the L-L analog. These results can only be interpreted by assuming an interaction of the sulfhydryl-bearing side chain of the cysteine residue with the zinc atom of the enzyme, as it occurs with mercaptopropanoyl amino acid inhibitors (see below).[68,69] Therefore, these dipeptides are to be considered analogs of captopril [S-1-(3-mercapto-2-methylpropanoyl)-L-proline], rather than true dipeptides. If the sulfhydryl group of Cys-Pro is alkylated or acylated there is still a substantial amount of inhibitory activity remaining. However, these modified inhibitors are now noncompetitive in action.

B. Peptide Analog Inhibitors

1. Introduction

Studies with peptidic inhibitors of ACE had reached a dead end in the early 1970s. The smaller peptides with the optimal side chains for interaction with the active site of ACE had very short lived inhibitory activities in vivo, and all attempts to increase duration by replacing peptide bonds with proteolytically stable linkages were unsuccessful.[58] Longer peptides, such as teprotide, had shown substantial in vivo activity but only when administered parenterally. The molecular size and the peptidic structure of these inhibitors made it unlikely that an orally active form could be derived from them.

The breakthrough was made in the mid 1970s with the development of the carboxyalkanoyl and mercaptoalkanoyl amino acid inhibitors.[70] These derivatives were as potent or more potent than the most active venom peptide inhibitors, while having lower molecular weights and no true peptide bonds. Both of the latter factors improve the probability for oral absorption; indeed, the oral absorption of the mercaptoalkanoyl amino acid inhibitors is quite

Table 1
BINDING CONSTANTS FOR PEPTIDE SUBSTRATES AND INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME[59]

No.	Compounds	K_i (μM)	K_m (μM)
Substrates			
1.1	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu		30—80
1.2	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg		0.9
Inhibitors			
1.3	<Glu-Lys-Trp-Ala-Pro	0.09	
1.4	<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	0.8	

Table 2
PEPTIDE INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

No.	Compounds	K_i (μM)
2.1	Arg-Pro	16
2.2	Arg-Pro-Pro	5.9
2.3	Phe-Ser-Pro	5.2
2.4	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	77[a,b]
2.5	Ala-Arg-Pro-Ala-Lys	19[b]
2.6	ACTH 1-39	1.5[a,b]
2.7	ACTH 7-38	0.75[a,b]
2.8	ACTH 4-11	24[a,b]

[a] Noncompetitive inhibition.
[b] I_{50}.

Table 3
CYSTEINYL DIPEPTIDES AS ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

No.	Compounds	K_i (μM)
3.1	L-Cys-L-Pro	0.055
3.2	D-Cys-L-Pro	0.0057
3.3	Captopril	0.0017
3.4	N-Z-S-Z-D-Cys-L-Pro	2.5[a]
3.5	S-Bzl-L-Cys-L-Pro	5.2[a]

[a] Noncompetitive inhibitors.

significant.[71] The development of these inhibitors has already been reviewed.[59,64] Here it is only important to reemphasize the concept that supported this development, namely the postulation of a hypothetical model of the active site of ACE and the stepwise maximization of the interactions between inhibitors and the postulated binding sites. Since this hypothetical model has guided the subsequent design of all new types of ACE inhibitors, it is worthwhile to describe it in some detail (Figure 5).

In the binding of any peptidic substrate or inhibitor to the active site of peptidases or proteases, there are four basic types of interactions: (1) peptide backbone interactions; (2)

terminal amino and carboxyl group interactions; (3) side chain interactions; and (4) interactions involved in the hydrolytic mechanism. With the exception of type four, the same types of interactions are involved in the binding of peptides to peptide receptors.

The most likely interactions between the peptide backbone and the enzyme are those between hydrogen bond donors and acceptors. It is possible that electrophilic metal cations might also play a role in the binding of carbonyl residues from peptide bonds, but no specific case has yet been documented. C-terminal carboxyl group interactions are most likely to be ionic in character; the cationic center on the enzyme active site could be provided by an arginine residue, as in the case of carboxypeptidase A or B, or by the positive end of a dipole formed by a suitably positioned *alpha*-helical sequence.[72] N-terminal amino group interactions can be ionic or through hydrogen bonding.[73] Coordination to an enzyme metal ion and ionic interaction with an enzyme carboxylate group has been recently postulated for the N-terminal amino group of the dipeptide Gly-Tyr and carboxypeptidase A based on X-ray crystallographic analysis.[74]

Side chain interactions involve hydrophobic, dispersion, hydrogen bond interactions, or a mixture of all three, depending on the nature of the side chains.[75] These interactions constitute the enthalpic component ($\Delta H°$) of the free energy of binding ($\Delta G°$).

$$\Delta G° = \Delta H° - T\Delta S°$$

The entropic component, $\Delta S°$, can also be manipulated to decrease $\Delta G°$ (to make it more negative). When enzyme and inhibitor bind together there is an overall loss of translational, rotational, and vibrational freedom, with the corresponding decrease in entropy. If the rotational freedom of the inhibitor in its solution form is reduced, the loss of rotational entropy upon binding is proportionally decreased ($\Delta S°$, less negative; $\Delta G°$, more negative), and overall binding is increased.

The degrees of rotational freedom of a peptide chain (A, Figure 6) can be restricted in at least three ways:

1. Introduction of a substituent on the *alpha*-carbon increases the energy barrier for rotation around the C_α-CO bond, and decreases substantially the number of allowed conformations for dipeptides (B, Figure 6). The introduction of a single methyl group is sufficient to achieve this effect; any further increase in the size of the substituent does not alter substantially this restriction.[76]
2. Formation of a ring between the *alpha*-carbon and the nitrogen of the same residue eliminates all possibilities for rotation around the N–C_α bond, and restricts significantly not only the allowed conformations for the carboxyl or carbonyl group of the same residue, but also those of the preceding residue (C, Figure 6). These effects are responsible for the special conformational properties of proline-containing peptides.[76,77]
3. Formation of a ring between the nitrogen and the *alpha*-carbon of the preceding residue can also introduce rotational restrictions (D, Figure 6).[78]

How important are these interactions to the overall binding interactions of an inhibitor? If the height of the rotational barriers of a conformationally mobile molecule are small, the relative proportion of conformers will not influence binding significantly.[79] However, if the heights of these barriers are significant, and the most abundant conformers can retain the efficient enthalpic contributions of the most flexible analog, the more conformationally restricted analogs should show significantly increased binding.

The interactions between peptide substrates and catalytic residues at the active site required for peptide bond hydrolysis can be of great value in inhibitor design, if they are clearly understood. In the case of ACE, the presence of a zinc ion at the catalytic center explains

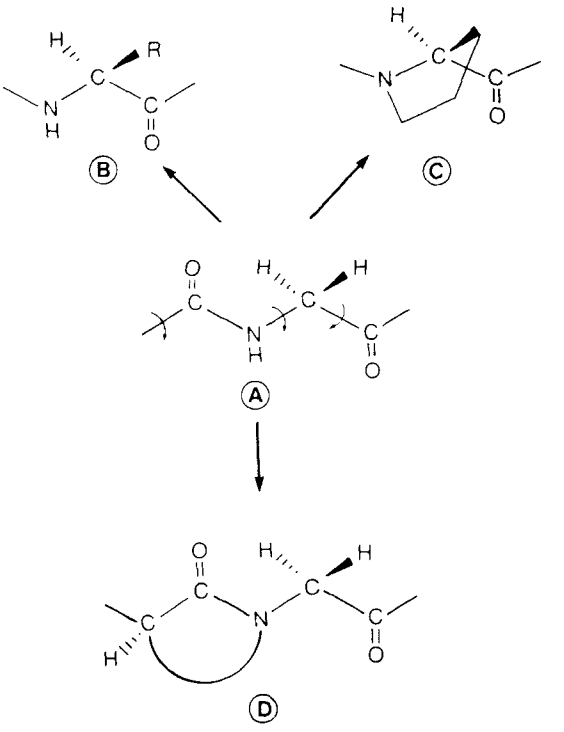


FIGURE 6. Restrictions on rotational freedom of peptide backbone after substitution.

without difficulty the significant binding of those inhibitors which carry a carboxyl group in the appropriate position (Figure 7). The assumption that this ionic interaction was of paramount importance in the binding of carboxyalanoyl amino acid inhibitors to ACE led to the development of mercaptoalkanoyl amino acid inhibitors such as captopril, in which this interaction was maximized. The "hard" nucleophile oxygen was replaced by the "softer" nucleophile sulfur, a better ligand for Zn^{+2}, which is a "soft" or, at best, "medium hard" electrophile. It was clearly demonstrated by comprehensive structure-activity relationships that this interaction between ligand and zinc ion alone is not sufficient to produce efficient inhibitors, but in concert with other interactions, as shown in Figure 7 for captopril, high inhibitory activity can be achieved.[80] It should also be noted that since the inhibitor molecule is providing only one ligand to the zinc ion, it is not functioning as a chelating agent, and, therefore, its inhibitory activity is not reversed by the addition of zinc.[68] Direct evidence for the proposed interaction between the sulfur and the metal ions at the active site of metallopeptidases has been provided by the spectroscopic studies of Holmquist and Vallee[81] and the crystallographic studies of Mozingo and Matthews with a sulfhydryl-containing inhibitor of thermolysin.[82]

2. Dipeptide Analogs

If we consider the enzyme-binding interactions of carboxyalkanoyl and mercaptoalkanoyl inhibitors beyond those involved with the zinc atom of ACE (Figure 7), it is clear that they are virtually the same as those expected for dipeptide inhibitors or for C-terminal dipeptide residues of peptide substrates (Figure 5). Thus, these inhibitors could properly be considered to be dipeptide analogs with an added zinc-binding function. In view of the structure-activity relationships discussed in the section on peptide inhibitors, it is not surprising that all dipeptide analog inhibitors have been modeled after the favorable C-terminal sequence alanyl-

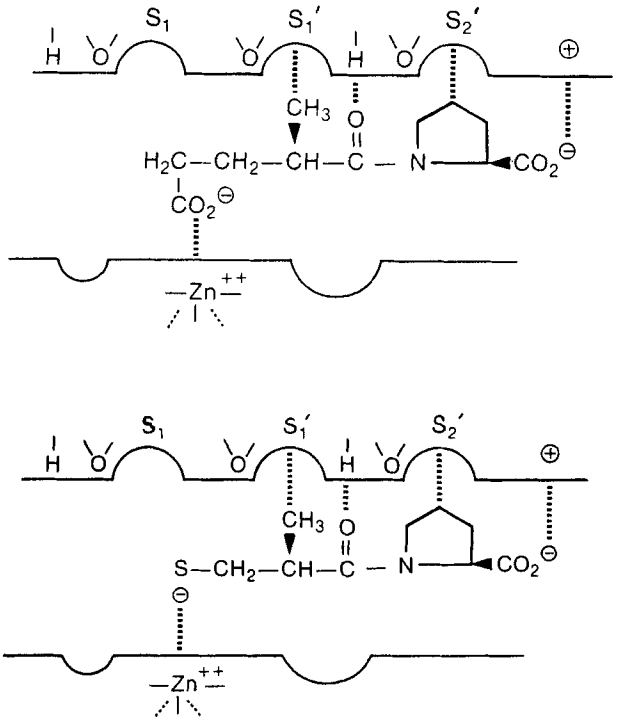


FIGURE 7. Proposed binding of carboxyalkanoyl and mercaptoalkanoyl-L-proline at the active site of angiotensin-converting enzyme.

proline. The superiority of the C-terminal proline in dipeptide analog inhibitors was clearly established in an extensive comparison of various mercaptopropanoyl amino acids.[83] The overall activity of ACE inhibitors in isolated tissues or in vivo is governed by factors beyond simple in vitro potency. For example, tryptophan derivatives are equal to or more potent than proline derivatives in vitro, but are less potent in isolated smooth muscle tests and in vivo.[50,117]

Since the discovery of captopril, a considerable effort has been put into the development of analogs in which the proline moiety has been replaced with other amino acids. Imino acids have always shown the highest inhibitory activity, particularly when incorporated into cyclic or bicyclic structures, which emphasizes the importance of a conformationally restricted amino acid residue on the C-terminal position of ACE inhibitors. The most interesting examples have been collected in Table 4.[84-90] A large number of other analogs can also be found in the patent literature, but, since in many of those cases no significant activity data are included, they will not be reviewed here.

Some examples included in Table 4 illustrate further the importance of conformational effects on the interactions of the C-terminal amino acid with ACE. If bulky substituents are introduced next to the nitrogen in the thiazolidine ring, 2-alkyl substitution on the mercaptoacyl side chain can actually decrease inhibitory potency (compare 4.4 and 4.6) or produce only a very minor increase in activity (compare 4.7 and 4.8). The most likely reason for this anomaly is that the ring substituent will now occupy the space required for the *alpha*-substituent on the acyl side chain. When the ring substituent is oriented towards the rear (S configuration, 4.5) it will occupy the same position as that of the unfavorable R-2-methyl substituent in the side chain of captopril[83] and, as expected, decreased inhibitory activity will be obtained. Conversely, 2-substituted thiazolidines with the R configuration at that center have enhanced inhibitory activity[91] (4.4 vs. 4.3). In the case of derivatives 4.13 and

Table 4
MERCAPTOALKANOYL AMINO ACID INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

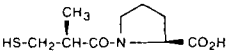
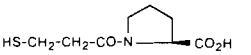
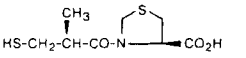
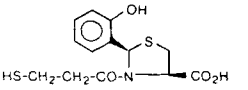
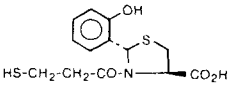
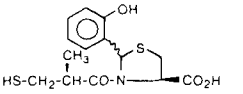
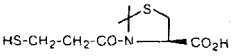
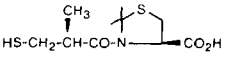
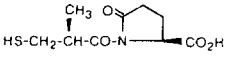
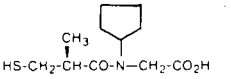
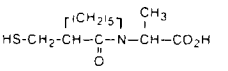
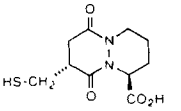
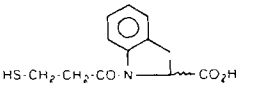
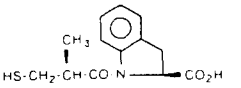
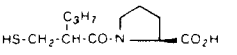
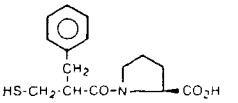
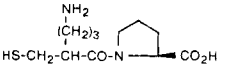
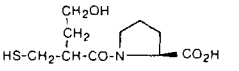
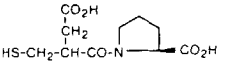
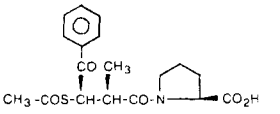
No.	Compounds	I_{50} (n*M*)[a]	Ref.
4.1		23	87
4.2		240	87
4.3		260	84
		9	93
4.4		7	92
4.5		>10000	91
4.6		210	84
4.7		4000[b]	
4.8		2500[b]	
4.9		4	85
4.10		—[c]	
4.11		53	78
4.12		38	96
4.13		140	89
4.14		4	89
4.15		82[b]	
4.16		61[b]	

Table 4
MERCAPTOALKANOYL AMINO ACID INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

No.	Compounds	I_{50} (n*M*)[a]	Ref.
4.17		69[b]	
4.18		251[b]	
4.19		>10000[b]	
4.20		55	90

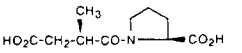
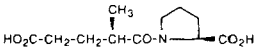
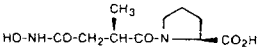
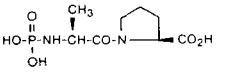
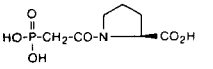
[a] Concentration of inhibitor in nmol/ℓ needed for 50% inhibition of isolated angiotensin-converting enzyme.
[b] Cushman, D. W. and Ondetti, M. A., unpublished results.
[c] Reported at 17 n*M* in Suh, J. T., Skiles, J. W., Wlliams, B. E., and Schwab, A., U.S. Patent 4,256,761. No comparative data given.

4.14, the phenyl ring of the indoline moiety bisects the positions of the R- and S-*alpha* substituents of the mercaptoacyl side chain, and allows the increase in binding provided by this substitution to be observed. These conformational restrictions do not apply to the N-cyclopentyl analog 4.10, since there is more flexibility in this molecule. Analogs 4.3 (YS-980), 4.4 (SA-446), and 4.10 (RHC-3659) have been extensively studied as ACE inhibitors in vivo.[88,92,93]

Analog 4.9 appears to be slightly more potent than captopril.[85] This result could be interpreted as being due to an increase in the population of the *trans* form of the amide bond in solution, which might be the form in which these inhibitors bind to the enzyme.[86] This proposal has been confirmed by the studies with conformationally restricted analogs of captopril (4.11 and 4.12, Table 4).[78,96] Nuclear magnetic resonance spectra (^{13}C) of aqueous solutions of captopril[94] or other mercaptoalkanoyl amino acids[86] indicate the presence of significant amounts of the *cis* form of the amide bond, depending on the pH.[94] Only one conformer is seen in the NMR spectra of 4.9 in aqueous solutions.[95]

Table 4 also includes dipeptide analog inhibitors with modifications on the mercaptoacyl moiety that correspond to variations on the N-terminal amino acid of the analogous dipeptide (4.15 to 4.19). It is readily apparent that no significant increase in binding can be obtained by further substitution of the mercaptoalkanoyl side chain after the introduction of the *alpha*-methyl group with the appropriate configuration. This would indicate that the major contribution in the increased binding observed in going from 3-mercaptopropanoyl-L-proline (4.2) to 3-mercapto-2-S-methylpropanoyl-L-proline (4.1) is mainly due to a conformational effect, namely, a restriction in the number of allowed conformations, one of which is optimal for interaction with the enzyme active site (see above). Analog 4.20 shows a pattern of substitution on the mercaptoacyl side chain that does not correlate with that of natural dipeptides; the additional substituent does not appear to contribute significantly to enzyme binding, but a quantitative comparison is not possible, since the active form of this type of inhibitor, the free sulfhydryl form, is unstable.[90]

Table 5
DIPEPTIDE ANALOG INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME WITH DIFFERENT ZINC-BINDING MOIETIES

No.	Compounds	I_{50} (n*M*)[a]	Ref.
5.1		22000	87
5.2		4900	87
5.3		600	98
5.4		1[b]	101
5.5		8400	102

[a] Concentration of inhibitor in nmol/ℓ needed for 50% inhibition of isolated angiotensin-converting enzyme.

[b] K_i (n*M*).

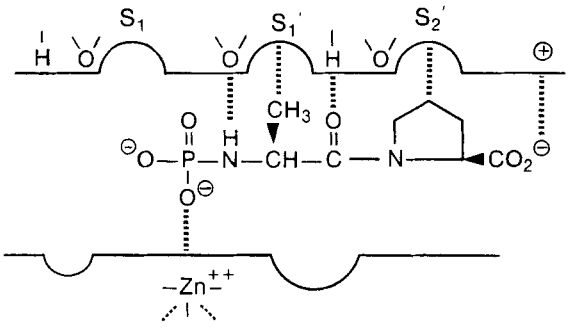


FIGURE 8. Proposed binding of N-phosphoryl-L-Ala-L-Pro at the active site of angiotensin-converting enzyme.

The bicyclic analog (4.12) is an interesting example because its conformation has been correlated with that of captopril based on X-ray and NMR data, aided by computer graphics. The conformation of 4.12 was found to fall within the "space" proposed for the conformation of captopril bound to the active site of ACE. Since these bicyclic analogs are aimed at restricting rotations around the –CO–N–, and the $-C(CH_3)-CO$ bonds, which are probably already restricted to a significant extent in the pyroglutamyl derivative 4.9, the activity of the optimal analog in this series, whenever it is synthesized, might not be higher than that of 4.9.[96]

As important as the structure of the dipeptide moiety is in these dipeptide analog inhibitors, the nature and localization of the zinc-binding function are essential. The studies that led to the development of captopril had identified three types of zinc-binding functions: carboxylic acid, hydroxamic acid, and sulfhydryl.[97,98] A more recent development has been the introduction of phosphorous-containing zinc-binding functions. Table 5 lists the most characteristic examples in each of these classes.

It is interesting to note the increase in activity in going from succinyl to glutaryl derivatives (5.1 and 5.2). This result was not expected, since the perfect isosteric replacement of an

acyldipeptide had already been achieved with a succinyl amino acid. However, this observation was confirmed with the development of the carboxyalkyldipeptide inhibitors which will be described later.

N-phosphoryl dipeptides such as 5.4 (Figure 8) are variations on a theme encountered with the naturally occurring metallopeptidase inhibitor phosphoramidon. This inhibitor of the zinc-containing endopeptidase thermolysin was isolated from cultures of streptomyces.[99] The X-ray picture of phosphoramidon bound to the active site of thermolysin[100] clearly shows that the phosphoryl anion is bound as a ligand for the zinc ion. *N*-phosphoryl dipeptides are very potent inhibitors of ACE, but their inherent instability has hindered their development as therapeutic agents.[80,101] Phosphonic acid derivatives (5.5) are considerably more stable but also considerably less potent.[102] Phosphinic acids and phosphonamidates, two other zinc-binding phosphorous-containing inhibitor classes, will be discussed under tripeptide analogs, since by allowing the extension of the chain beyond the zinc-binding moiety, they permit the introduction of side chains that can interact with binding sites beyond those accessible to dipeptides or dipeptide analogs.

3. Tripeptide Analogs

In this class are included those inhibitors which contain moieties that are likely to undergo interaction with enzyme-binding sites specifically involved in the binding of tripeptides.

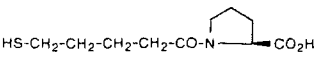
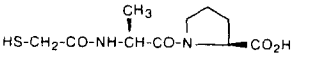
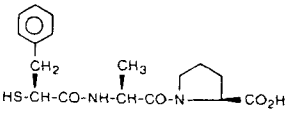
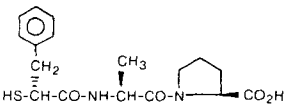
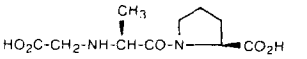
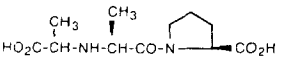
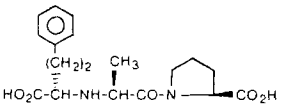
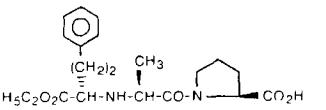
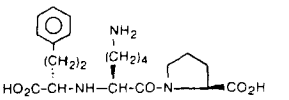
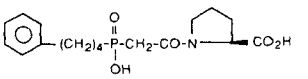
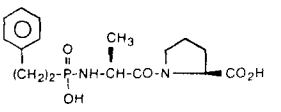
The first generation of inhibitors, the carboxyalkanoyl and mercaptoalkanoyl amino acids, did not incorporate substituents that could interact with binding sites for the antepenultimate amino acid of a substrate. However, it was later found that mercaptoalkanoyl dipeptides like 6.2 (Table 6) show a surprisingly high degree of inhibitory activity if one compares them with mercaptoalkanoyl amino acids of comparable length (6.1 and 6.2). It is possible that the hydrogen-bonding possibilities of the second amide bond and its conformational rigidity are responsible for this increased activity. Further substitution of the mercaptoacyl moiety in the S or R configuration (6.3 and 6.4) does not appear to elicit any further binding interactions.[79]

The most successful attempt to incorporate such added binding interactions into a molecule containing a carboxyl as a zinc-binding moiety was achieved with the design of the carboxyalkyl dipeptides by Patchett and co-workers.[103] The most active compounds in this series are 6.7 (MK-422) and 6.9. Compound 6.9 and the monoethyl ester of compound 6.7 (6.8) have been advanced to clinical studies as antihypertensive agents under the code numbers MK-521 and MK-421 (enalapril), respectively. The most obvious interpretation of the binding of these inhibitors to the active site of ACE must invoke interactions similar to those advanced in the design of their predecessors, the carboxyalkanoyl amino acid inhibitors (Figures 8 and 9). Besides those interactions, the more potent carboxyalkyldipeptides may also bind to the enzyme via added interactions of the imino nitrogen and phenethyl side chain. However, since the introduction of the imino function does not increase significantly the inhibitory activity of the lower and more flexible analog 6.5 (compare with 5.2), it is not clear whether this group functions as a hydrogen bond donor or acceptor, or if it interacts with the enzyme at all. The striking increase in potency obtained by introduction of a single methyl group next to the imino group (compare 6.5 and 6.6) indicates once again the occurrence of preferred conformations with high rotational barriers for the segment $-CH(CH_3)-NH-CH(CH_3)-$. Larger substitutents, such as phenethyl, are expected to undergo the added hydrophobic interaction postulated for the antepenultimate aromatic amino acid of peptide substrates.

Analogs of enalapril in which proline has been substituted with 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid and perhydroindole-2-carboxylic acid have recently been described.[104,105]

In the phosphinic acid inhibitor 6.10 and the phosphoramidate 6.11, the phenylalkyl side chains most likely interact with the enzyme active site in a manner similar to that of

Table 6
TRIPEPTIDE ANALOG INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

No.	Compounds	I_{50} (n*M*)[a]	Ref.
6.1		83000	80
6.2		60	80
6.3		50	80
6.4		60	80
6.5		2400	103
6.6		90	103
6.7		1.2	103
6.8		1200	103
6.9		1.2	103
6.10		180	102
6.11		7	106

[a] Concentration of inhibitor in nmol/ℓ needed for 50% inhibition of isolated angiotensin-converting enzyme.

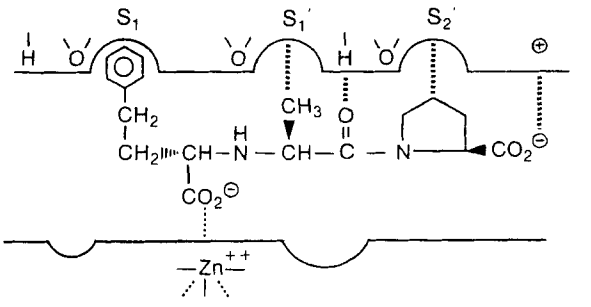
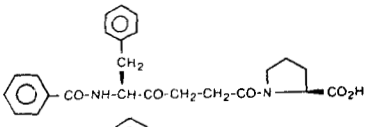
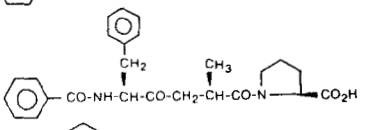
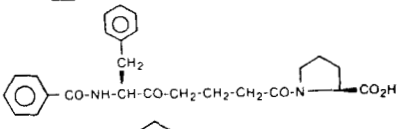
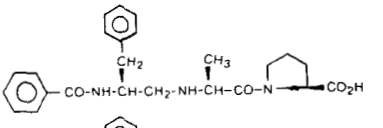
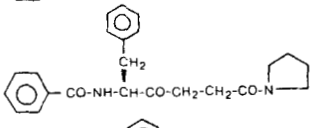
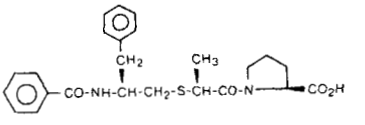


FIGURE 9. Proposed binding of the carboxyalkyl dipeptide inhibitor MK-422 at the active site of angiotensin-converting enzyme.

Table 7
TRIPEPTIDE ANALOG INHIBITORS OF ANGIOTENSIN-CONVERTING ENZYME

No.	Compounds	I_{50} (n*M*)[a]	Ref.
7.1		3	108
7.2		1	111
7.3		42	109
7.4		>10000	107
7.5		"Inactive"	109
7.6		>10000	107

[a] Concentration of inhibitor in nmol/ℓ needed for 50% inhibition of isolated angiotensin-converting enzyme.

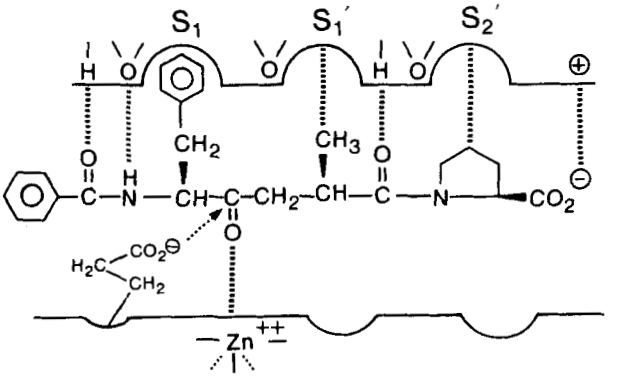


FIGURE 10. Proposed binding of the ketomethylene inhibitor 7.2 at the active site of angiotensin-converting enzyme.

carboxylalkyl dipeptides. However, the structural requirement for optimal enzyme binding of phosphinic acids is somewhat different from that observed with phosphoramidates.[102] Structure-activity relationships in the latter series have been described by Thorsett et al.[106]

The tripeptide analog inhibitors shown in Table 7 are quite different from those discussed above in that they contain no strong zinc-binding moiety, although one could postulate a weak interaction between the ketone and the zinc ion, particularly if we postulate the nucleophilic assistance by the carboxylate group (Figure 10). The importance of the ketone

Table 8
ACTIVITY OF ANGIOTENSIN-CONVERTING ENZYME INHIBITORS ON ISOLATED ENZYME AND GUINEA-PIG ILEUM PREPARATIONS

No.	Compounds	I_{50}[a] ACE (nM)	EC_{50}[b] AI (nM)	AC_{50}[c] Bk (nM)
1.4	<Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	560[59]	60[120]	1.5[120]
1.3	<Glu-Lys-Trp-Ala-Pro	60[59]	>2000[120]	20[120]
4.1	$HSCH_2CH(CH_3)CO$-Pro	23[70]	23[70]	3.2[70]
8.1	$HSCH_2CH(CH_3)CO$-Trp	35[d]	1700[d]	12[d]
4.3	YS-980	260[118]	160[118]	3.5[118]
4.4	SA-446	71[84]	28[84]	0.7[84]
6.7	MK-422	1.2[119]		0.08[119]
7.3	Ketone tripeptide analog inhibitor	5[108]	230[108] 900[120]	

[a] See Table 4.
[b] Concentration of inhibitor in nmol/ℓ needed for 50% inhibition of the angiotensin I response.
[c] Concentration of inhibitor in nmol/ℓ needed for 50% augmentation of the bradykinin response.
[d] In this study[117] the EC_{50} for captopril was 210.

group is quite clear, since tripeptide analogs without it (7.6) are practically inactive.[107] Introduction of an extra methylene between the carbonyl and the amide function (7.3 vs. 7.1) can be achieved with substantial retention of activity, a result which is not altogether surprising, since in carboxyalkanoyl amino acids this modification enhances activity. Further structure-activity relationships have been reported.[102,108-111]

Though kinetic studies on the inhibition of ACE by 7.1[108-112] have not given a pure competitive behavior for this inhibitor, the facts that it protects the enzyme against inactivation by acetylimidazole and that removal of the C-terminal carboxyl (7.5) eliminates activity support the interpretation that these inhibitors bind at the active site in a manner similar to that of substrates or other competitive inhibitors (Figure 10). In spite of their high inhibitory activity in vitro, these inhibitors are poorly active in vivo, particularly when administered by the oral route.[109]

4. Inhibitor Classification

The concept of a biproduct analog played a key role in the development of the first generation of nonpeptidic ACE inhibitors.[59,64] As conceived by Byers and Wolfenden, biproduct analog inhibitors are those compounds which incorporate in one molecule certain modes of enzyme binding of each of the two products of the enzymatic reaction.[113] The first specific nonpeptidic ACE inhibitors, the succinyl and glutaryl derivatives of proline, were designed according to this general biproduct analog concept. As shown in Figure 11 for 2-D-methylglutaryl-L-proline, the carboxyl group of the carboxyacyl moiety is considered to satisfy some important enzyme-binding interaction normally involving the terminal carboxylate of one of the products of enzymatic hydrolysis, while the remainder of the molecule mimics the mode of binding of the other product, in this case the dipeptide residue Ala-Pro.

Although a rationale is suggested for optimizing the binding of the moiety representing the "amino product" (analogous to a dipeptide), the biproduct analog concept does not

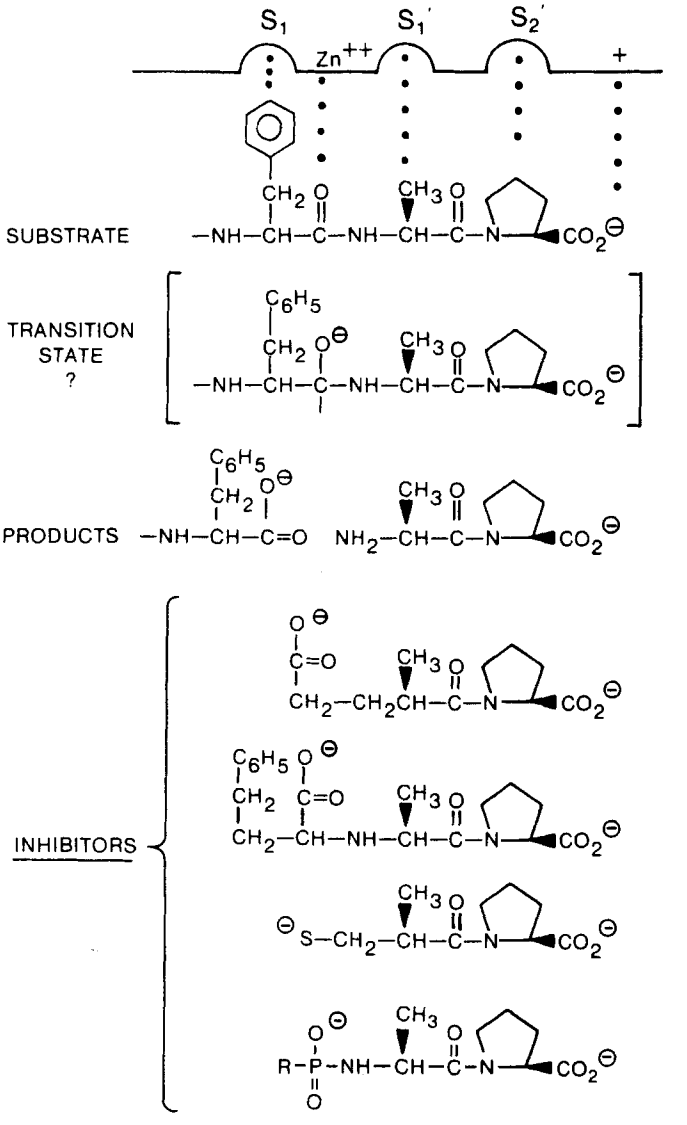


FIGURE 11. Comparison of binding modes of substrate, transition state, products, and competitive inhibitors of angiotensin-converting enzyme.

suggest any further modification to improve the potency of this type of inhibitors. In order to do that it was necessary to "postulate" more specifically that the role of the carboxyl group of the succinyl moiety was to become a ligand to the active-site zinc. This, in turn, led to the search for more effective ligands (see above). The mercaptoalkanoyl amino acid inhibitors derived from this specific approach cannot properly be called biproduct analogs, and the authors have purposefully refrained from creating a special nomenclature for them. In connection with the biproduct analog concept, it is interesting to note that the methyleneamino tripeptide analog 7.4 (Table 7) can also be considered a biproduct analog, since it contains all the binding interactions of the "amino" product Gly-Pro, and some of those belonging to the "carboxyl" product benzoyl-Phe. However, it has a very low level of ACE inhibitory activity, most likely because it lacks a strong zinc-binding moiety, which is undoubtedly one of the key features of the original biproduct analogs.

The recently introduced carboxyalkyl dipeptide inhibitors have been broadly classified as transition-state inhibitors.[103] A simple comparison of these inhibitors with the substrate and with the carboxyalkanoyl amino acid inhibitors (Figure 11) makes it obvious that they could, as well, be considered biproduct analogs. A tetrahedral carbon next to the imino group of a dipeptide moiety is not sufficient to assure potent inhibitory activity; such a requirement would also be satisfied by the poorly active inhibitor 7.4 (Table 7).

The most important guiding principle in the design of potent and specific enzyme inhibitors is the identification of the potential binding interactions that can occur at the active site, irrespective of whether the structure of the enzyme active site is known or merely postulated. The putative inhibitor molecule should contain functional groups that can maximize these binding interactions, even if those functional groups are of a chemical nature quite different from that of those present in the substrate.

Since the increased affinity expected in the transition state[114] could only be explained by the generation of increased binding interactions beyond those present in the ground state, identification of the nature of those new binding capabilities could be utilized with advantage in the design of inhibitors. If the zinc-binding interaction is conceived as being an interaction only present in the transition state, then all ACE inhibitors with a zinc-binding function are transition-state analogs whether or not the contain a "magic" tetrahedral atom. It is therefore obvious that these denominations are arbitrary and not of much use in inhibitor design.

V. BIOLOGY OF ANGIOTENSIN-CONVERTING ENZYME INHIBITORS

A. In Vitro Evaluation

In the discussion of structure-activity relationships among ACE inhibitors we have only used their activity as inhibitors of the isolated ACE, mostly that of rabbit lung. Although the I_{50} value obtained is the primary tool for evaluation of an ACE inhibitor, it can be misleading if one wishes to draw conclusions about the ultimate use of such inhibitors in treating hypertension. The experience accumulated in the evaluation of different types of ACE inhibitors indicates that testing in a variety of biological systems of increasing complexity is the best procedure for thorough evaluation of new types of inhibitors.

After obtaining an I_{50} value vs. the isolated enzyme, testing in isolated smooth muscle preparations is the next logical step in the sequential evaluation of potent ACE inhibitors. Using such a system it is possible to obtain information on the activity of the compound per se on smooth muscle preparations, and whether the compound is an antagonist or a potentiator of various agonists, such as AI and AII, bradykinin, norepinephrine, acetylcholine, serotonin, etc. A specific ACE inhibitor should block the effect of AI and augment the effect of bradykinin in any tissue containing significant levels of ACE. Also, it should not influence the effect of any other agonist, or have any direct effect on smooth muscle tone. In the smooth muscle preparations that have been used for such evaluations,[115,116] AI in moderate concentrations has no effect on the tissue unless it is converted to AII by ACE, and the effect of bradykinin is limited by its degradation by ACE. In such tissues, the dual action described above, and the lack of any other effects on the tissue preparations, are the clearest indications of specificity for a putative ACE inhibitor. Also, among peptidic inhibitors, a low level of activity on isolated smooth muscle preparations usually correlates with poor in vivo activity. Among the mercaptoalkanoyl amino acid inhibitors, tryptophan derivatives are less potent antagonists of AI on smooth muscle preparations than are proline derivatives, and they are also less active in vivo. However, as inhibitors of the isolated enzyme, tryptophan derivatives are often more potent than proline derivatives.[50] Two other mercaptoacyl ACE inhibitors that have been fairly extensively studied in vitro and in vivo, YS-980 (4.3) and SA-446 (4.4), have in vivo activities that closely parallel their antagonistic activity on AI in smooth muscle. There is a close parallelism between inhibition of the

isolated enzyme, antagonism of AI activity in smooth muscle preparations, and in vivo activity by carboxyalkyl dipeptide ACE inhibitors. Another example of the predictive value of the smooth muscle test can be seen with the ketomethylene tripeptide analog inhibitors. Compound 7.1 (Table 7) is as potent as captopril in inhibiting the isolated ACE, but it is significantly less active in antagonizing AI on guinea-pig ileum.[108] Its in vivo activity is also considerably less than that of captopril.[110]

B. In Vivo Evaluation

1. Vasopressor Response to Angiotensin I

Since the hypertensive activity of AI depends on its conversion to AII, the most direct way to test ACE activity in vivo is to demonstrate blockade of the hypertensive effect of AI. After administration of the compound by the parenteral or oral route, the animal is challenged at suitable intervals with AI and the vasopressor response is recorded, usually as a percentage of the control response or as a percent of the inhibition of the control response. The animals most commonly used for these studies are normotensive conscious rats and dogs, although cats and monkeys have also been used. This approach was utilized extensively to study the parenteral activity of the peptide ACE inhibitors, such as BPP_{5a}[120a] and teprotide[120b] and the parenteral and oral activity of peptide analog inhibitors, such as captopril. (For reviews, see References 59, 64, and 121.) Since then, this procedure has been routinely used to demonstrate ACE inhibitory activity in all new ACE inhibitors, e.g., new captopril analogs — YS-980[93] and SA-446,[92] carboxyalkyldipeptides,[119] and ketone tripeptide analogs.[109] The fairly good quantitation of responses that can be achieved with this procedure permits a rapid evaluation of the ratio between equipotent oral (p.o.) and intravenous (i.v.) activity, which is usually a good indication of the extent of oral absorption. For example, the p.o./i.v. dose ratio of 366 obtained with the carboxyalkyl dipeptide 6.7 (Table 6) is comparatively high, while its monoethyl ester (6.8, MK-421) has a significantly lower p.o./i.v. dose ratio, which indicates that esterification has considerably increased the oral absorption.[119] The p.o./i.v. dose ratio observed with the mercaptoalkanoyl amino acids like captopril is low, as would be expected in view of its good oral absorption.[71] A very similar procedure could be used to demonstrate ACE inhibitory activity in vivo by demonstrating a potentiating effect on the hypotensive activity of bradykinin.[115,119] Actually this approach predates that of studying AI hypertension, since ACE inhibitors were actually discovered in the venom of *B. jararaca* through their bradykinin potentiating effect.[122]

2. Animal Models of Hypertension

The need for parenteral administration of peptidic ACE inhibitors precluded their extensive evaluation in various animal models of hypertension. However, they were clearly shown to be efficacious in the 2-kidney/1-clip renal hypertensive rats, thereby confirming the dependency of this model on the activation of the renin-angiotensin system.[122a,123] With the development of orally active inhibitors like captopril, it became possible to explore the effect of this approach to renin-angiotensin system blockade in all models of hypertension. The effects obtained with captopril in these studies have been reviewed in detail,[124,125] and can be briefly summarized as follows. Captopril is effective in 2-kidney/1-clip renal hypertensive rats and in aortic coarctated rats, both models in which the involvement of the renin-angiotensin system has been clearly implicated; it is also effective in 1-kidney/1-clip renal hypertensive rats but only after repeated administration. At somewhat higher doses than those used in the previous models, captopril is also effective in spontaneously hypertensive rats and, after repeated administration, it can produce a complete normalization of blood pressure. The antihypertensive activity of captopril in this model is significantly enhanced by the simultaneous administration of a diuretic.[126] Development of hypertension can be delayed by treatment of weanling spontaneously hypertensive rats with captopril. More

recently similar effects were obtained with two other ACE inhibitors, the captopril analog YS-980[127] and the carboxyalkyldipeptide inhibitor MK-421.[128] Captopril was also found to lower blood pressure and the incidence of cerebral stroke in stroke-prone spontaneously hypertensive rats.[129] Two-kidney perinephritic hypertensive dogs are also susceptible to the blood pressure-lowering effects of captopril. A hypertensive animal model in which the renin-angiotensin system is clearly suppressed, the DOCA hypertensive rat, is resistant to the effect of captopril, unless it is administered in conjunction with diuretics.[130] Captopril analogs and carboxyalkyl dipeptide inhibitors have also been studied in various models of animal hypertension.[92,131,132]

C. Clinical Studies

1. Hypertension

Since the introduction of teprotide to the clinics in 1973,[133] it has become customary to initiate clinical studies with ACE inhibitors by measuring inhibition of the AI hypertensive response in normal volunteers. Studies of this type have now been carried out with captopril,[134] the captopril analog RHC-3659[135] (4.10), and the carboxyalkyl dipeptides MK-421 (6.8), MK-422 (6.7), and MK-521 (6.9).[136] As in the case of the corresponding animal studies, monitoring of the AI response at different time intervals after the administration of the inhibitor permits the determination of potency and duration of action in a fairly quantitative manner.

The early studies of Laragh and collaborators[137] have clearly shown that teprotide, the nonapeptide ACE inhibitor, was capable of lowering blood pressure in hypertensive patients with elevated or normal renin levels, with the more pronounced effect being obtained in renovascular hypertensive patients with elevated plasma renin levels.

The clinical studies with captopril, the first orally active ACE inhibitor available for such studies, covered a wide variety of hypertensive patients, and they have been reviewed on several occasions.[138-142] In the early stages of clinical development, captopril was extensively studied in severe resistant hypertension and found to be equivalent to the "standard triple therapy" (diuretic + *beta*-blocker + vasodilator). In many of the patients studied, captopril alone did not achieve complete control of blood pressure unless a diuretic and sometimes a *beta*-blocker was added. Captopril is particularly effective in renovascular hypertension where small doses can produce a pronounced lowering of blood pressure. In mild-to-moderate hypertension (essential hypertension) captopril has efficacy similar to that of diuretics, and, in combination with diuretics, it can normalize blood pressure in 90% of the hypertensive population. Recent studies[143] have shown that this efficacious response in mild-to-moderate hypertension can be maintained even with low doses given twice daily.[144] Preliminary clinical studies with carboxyalkyl dipeptide ACE inhibitors have shown efficacious results in mild-to-moderate hypertension alone and in combination with diuretics.[145-147]

2. Congestive Heart Failure

The concept that lowering of peripheral vascular resistance could be beneficial for improving performance of the compromised heart prompted the evaluation of teprotide in congestive heart failure[148] where it was clearly shown that beneficial acute hemodynamic changes could be achieved. Acute and chronic therapy with captopril have demonstrated sustained improvement in the clinical status of patients with congestive heart failure.[149]

D. Mechanism of the Antihypertensive Action of Angiotensin-Converting Enzyme Inhibitors

ACE inhibitors were designed to inhibit specifically the generation of the potent hypertensive agent AII through the action of ACE, the only mechanism of AII formation of biological relevance. One would, therefore, have expected that their antihypertensive activity

would have been easily understood. However, this is not the case, mainly because there is not always a correlation between inhibition of the pressor effect of exogenous AI and lowering of blood pressure in hypertensive animals. When this discrepancy was first observed with captopril it was common to hypothesize, with no particular experimental support, that captopril might exert biological actions that were not dependent on its specific ACE inhibitory activity. However, practically all of the antihypertensive effects demonstrated with captopril have been shown also with the so-called nonsulfhydryl ACE inhibitors.[150,151]

Inhibition of the two well-demonstrated effects of AII, namely, vasoconstriction and release of aldosterone, is clearly involved in the antihypertensive activity of ACE inhibitors. However, ACE is also the key enzyme in the inactivation of the hypotensive nonapeptide bradykinin, and, therefore, ACE inhibitors could owe at least part of their antihypertensive activity to the potentiation of endogenous bradykinin. This point has not been resolved to everyone's satisfaction,[121,124,125,152-155] but certainly clear evidence for the involvement of the kinin system is lacking. Increased levels of kinins after ACE inhibition can activate the arachidonic acid cascade and lead to the formation of vasodepressor agents like PGI_2 and PGE_2. This possibility has been investigated extensively without conclusive results. For review, see Reference 156.

One consequence of the availability of potent and specific blockers of the renin-angiotensin system has been the confirmation that this system is involved in the regulation of other biological effects besides direct vasoconstriction and sodium balance. It is now apparent that the permissive effect of the renin-angiotensin system on adrenergic transmission plays a role in blood pressure regulation, and that the blockade of this system by ACE inhibitors may also play a role in the antihypertensive activity of these agents.[6,157,158] Also, the observation that ACE inhibitors produce vasodilation without increasing heart rate may be accounted for by blockade of the effect of the renin-angiotensin system on baroreceptor reflexes[159] or on vagal efferent nerve activity.[7,8] Studies in hypertensive patients indicate no alteration of baroreceptor or sympathetic reflexes, but increased parasympathetic (vagal) tone.[160]

In conclusion, the evidence accumulated so far in a considerable number of studies in animal models and in human hypertension indicates that the antihypertensive activity of ACE inhibitors is almost exclusively due to the blockade of AII formation. Thus, these agents continue to have great experimental and diagnostic value, as well as being significant tools for the clinical management of high blood pressure.

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