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REVIEW

Angiotensin II-Generating Enzymes

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Abstract—The renin–angiotensin system (RAS) is a system of enzymes and hormones that regulate blood pressure and electrolyte and fluid homeostasis in mammals. Angiotensin II (Ang-II) is one of the most important and well-known components of RAS. It is formed from the protein precursor angiotensinogen by the sequential actions of proteolytic enzymes. The classic pathway of Ang-II generation includes a reaction catalyzed by angiotensin-converting enzyme (ACE). However, there are alternative pathways for the generation of Ang-II. In this paper, possible routes of formation of Ang-II in the human body are reviewed. Various Ang-II-generating enzymes (tonin, cathepsin G, chymase, etc.) and their properties are considered. The classification of these enzymes is also considered.

Key words: angiotensin, angiotensin II-generating enzymes, serine proteinases, Ang-I-converting enzyme

Angiotensin II (Ang-II), an octapeptide with the structure Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, has potent vasoconstrictor and aldosterone secretion activities. It is formed *in vivo* from the larger parent protein precursor angiotensinogen, which circulates in blood plasma. Angiotensinogen (molecular weight 62-65 kD) is serum protein of the α-globulin fraction [1, 2]. Upon cleavage of angiotensinogen by the enzyme renin (EC 3.4.99.19), a decapeptide known as Ang-I and having the amino-acid sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu is released. This peptide has no significant biological activity and requires further conversion to Ang-II [1-4]. The conversion of Ang-I to Ang-II is catalyzed by angiotensin-converting enzyme (ACE), which removes the histidyl-leucine dipeptide from the carboxyl end of the Ang-I molecule [5-10]. The further splitting of Ang-II by angiotensinases produces Ang-III and Ang-IV. Angiotensins contribute to the pathogenesis of hypertension, arterial disease, cardiac hypertrophy, heart failure, and diabetic renal disease [1, 4]. Ang-II stimulates a variety of physiologic responses that support arterial blood pressure and electrolyte and fluid homeostasis; it is the best known and most powerful hypertensive substance [1, 4].

Abbreviations: Ang) angiotensin; ACE) angiotensin-converting enzyme; BPTI) basic pancreatic trypsin inhibitor; DFP) diisopropyl fluorophosphate; LBTI) lima bean trypsin inhibitor; PMSF) phenylmethylsulfonyl fluoride; RAS) renin–angiotensin system; SBTI) soybean trypsin inhibitor; t-PA) tissue plasminogen activator.

Renin, angiotensinogen, Ang-I, ACE, and Ang-II participating in the conversions form the blood and tissue renin–angiotensin system (RAS). Now the existence of two independent RAS systems, circulating and tissue (local) is known [4, 11-16]. The main components of the circulating RAS are secreted into the blood by the kidneys (renin) and liver (angiotensinogen) [4, 13]. Plasma-derived Ang-II is formed from angiotensinogen through the action of renin and ACE. However, enzymatic pathways independent from renin and ACE may also contribute to the generation of Ang-II. Several enzymes capable of generating Ang-I and/or Ang-II from angiotensinogen have been described [2, 3, 11, 14-18]. In addition, some of these enzymes are able to convert prorenin to active renin [4, 15] (see Fig. 1).

Thus, Ang-II may can be formed by different enzymes—ACE, tonin, chymase, etc. The properties and possible functions of these enzymes will be considered below.

ANGIOTENSIN-CONVERTING ENZYME

ACE (EC 3.4.15.1) is also called Zn-dependent peptidyl-dipeptidase [5-8, 19, 20]. ACE also inactivates bradykinin by liberating a C-terminal dipeptide [6, 7, 9]. The most useful synthetic ACE-substrates are Hip-His-Leu, Hip-Gly-Gly, Cbz-Phe-His-Leu, furanacyl-Phe-Gly-Gly, furanacyl-Phe-Phe-Arg, Abz-Gly-Phe(NO_2)-Pro, and dansyl-Gly-Phe(NO_2)Pro, Bz(NO_2)Gly-Trp-Gly [7].

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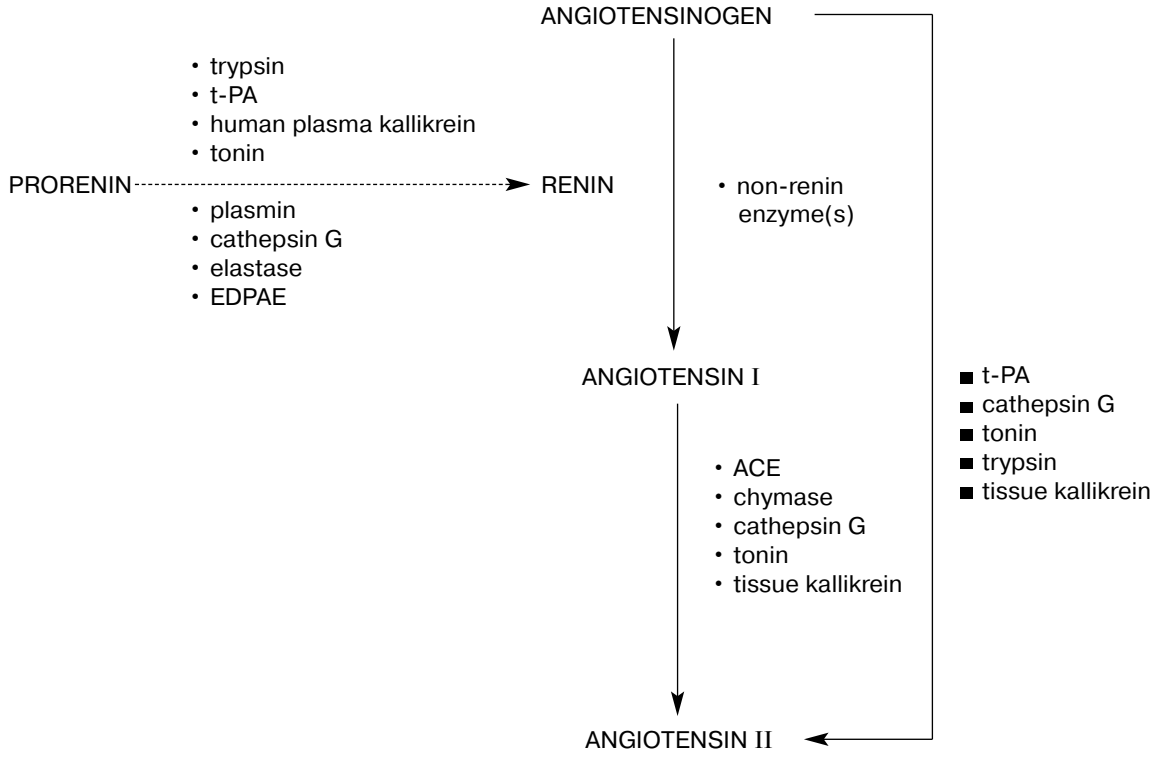

Fig. 1. Pathways of Ang-II production. Designations: ACE, angiotensin-converting enzyme; EDPAE, endothelium-derived prorenin-activating enzyme; t-PA, tissue plasminogen activator.

There are several forms of ACE [5, 21, 22]; all forms are heavily glycosylated. Somatic ACE (130-180 kD) is mainly located in endothelial, epithelial, and neuronal cells [6, 9, 21-24]. This enzyme is a classic type of integral enzyme consisting of a single polypeptide chain with two-domain structure (N- and C-domains). Structural analysis showed that ACE is mainly a membrane-bound protein. Each domain contains a Zn-dependent catalytically active site [5, 21, 22]. The active sites of the enzyme are situated extracellularly [5, 23].

It is argued that ACE could act on lumenally delivered Ang-I and produce Ang-II outside the cell. ACE functions in the body mainly in the membrane (m-) form, though it may be present in soluble (s-) form (in plasma, lymph, cerebrospinal, and other biological fluids). The s-form is generated from the m-form by posttranslational hydrolysis of a hydrophobic peptide anchor [5, 25].

The molecule of somatic ACE is characterized by high internal homology between two large catalytically active domains. However, the domains are not identical; they are distinguished by their catalytic properties and the profile of activation by Cl^- [5]. It has been suggested that the ACE domains may have different functions in the human body [5, 22].

It was found that man and other mammals have natural one-domain ACE forms. The testicular isoenzyme (90-110 kD) synthesized by the testis is identical to the C-domain of somatic ACE except for a small N-terminal region [5, 22]. The soluble form of ACE, identical to the N-domain, was found in patient's "intestinal fluid" after surgery and in human urine [5].

ACE is a metalloproteinase. It is inhibited by EDTA and *o*-phenanthroline but not by diisopropyl fluorophosphate (DFP) or β-mercaptoethylamine [9, 25]. The ACE inhibitor captopril is widely used in science and medicine. Captopril (D-3-mercapto-2-methyl-propanoyl-L-proline; SQ 14225) is a competitive inhibitor of ACE with K_i value of $1.3 \cdot 10^{-9}$ M [5, 21, 26]. The pH optimum for ACE is 7.2-7.6 [9, 19].

ACE was purified from various human and animal tissues (lung, heart, liver, brain, blood plasma, etc.). None of these preparations are distinguished by molecular weight, p*I*, pH optimum, and inhibitor constants for known ACE inhibitors. However, their immunological and catalytic properties are different [8, 10]. For example, ACE from human heart hydrolyzed atriopeptin 2 (Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gly-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg) and its C-terminal analog Bz-Gly-Ser-Phe-Arg [8]. These data confirm the assumption [27] that atriopeptin 1 (atrium natriuretic peptide, modulating natriuresis and diuresis) is formed in the myocardium from atriopeptin 2, and

Table 1. Natural ACE substrates [5-7]

Substrate	C-Terminal dipeptidase	C-Terminal tripeptidase	N-Terminal tripeptidase
Ang-I Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	+	—	—
Bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	+	—	—
des-Arg^9-bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	—	+	—
Hemopoietic peptide AcSer-Asp-Lys-Pro	+	—	—
Substance P Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-MetNH_2	+	+	—
Encephalins Tyr-Gly-Gly-Phe-Met/Leu	+	—	—
Neurotensin <Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	+	—	—
Luteinizing hormone releasing hormone <Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2	—	+	+

ACE is involved in this process. The functional difference between the cardiac and pulmonary isozymes with respect to atriopeptin 2 provides additional experimental support for organ specificity of ACE. The existence of a large spectrum of physiologic ACE substrates also is a consequence of organ specificity.

ACE can cleave substrates other than Ang-I or bradykinin by peptidyl-dipeptidase action. Among these peptides are substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2), opioid brain peptides (encephalins and their analogs, precursors of encephalins, neurotensin), and hemopoietic peptide [5-8]. ACE can also act as peptidyl-tripeptidase when it hydrolyzes substance P. In addition, after removal of the C-terminal Arg of bradykinin by carboxypeptidase N, ACE can cleave the remaining octapeptide to release the tripeptide Ser-Pro-Phe [5-7] (see Table 1).

SERINE Ang-II-GENERATING ENZYMES

At the beginning of 1970s it was found that in addition to the classic pathway of Ang-II generation with catalysis by renin and ACE, there is alternative pathway where a serine proteinase acts in place of ACE [4, 15, 28-30] (see Fig. 1). Multiple lines of evidence suggest that heart, lung, large arteries, and kidney contain in addition to ACE other alternative Ang-II-forming enzymes [3, 4, 14].

Arakawa [31] divided Ang-II-forming serine proteinases into 2 groups—aprotinin-sensitive or kallikrein-like enzymes (trypsin and kallikrein) and chymostatin-sensitive or chymase-like enzymes (chymase) (see Fig. 2). The classification of Arakawa [31], especially division on aprotinin- and chymostatin-sensitive, is not complete because cathepsin G, which is also an Ang-II-forming enzyme, is inhibited by both aprotinin and chymostatin. We suggested a fuller scheme for Ang-II-generating serine proteinases because tonin, cathepsin G, and other enzymes (in addition to those listed by Arakawa [31]) are also Ang-II-forming enzymes. Our classification is based on the nature of the active site of the enzyme: trypsin-like proteinases (trypsin, kallikrein, tonin, etc.) and chymotrypsin-like proteinases (cathepsin G and chymases) [32]. The main physicochemical characteristics of Ang-II-forming enzymes are given in Table 2.

Trypsin-like Ang-II-generating enzymes. Trypsin (EC 3.4.21.20) is a pancreatic serine proteinase that is secreted into the intestinal tract and digests food proteins. Trypsin hydrolyzes protein bonds X–Y containing a basic amino acid such as Lys or Arg at the X position. The optimum pH for trypsin varies from 7.0 to 8.0 depending on the substrate. The presence of Ca^{2+} in the medium is needed for its activation and stabilization [20, 33]. Trypsin can generate *in vitro* bradykinin from kininogens; hence, it is a kinin-forming enzyme. It is known that trypsin can also activate a prorenin and generate Ang-II from angiotensinogen [34].

Arakawa et al. [34] showed that trypsin is able to generate Ang-II from angiotensinogen. A vasopressor substance formed by trypsin from renin-free human plasma protein was isolated, purified to homogeneity, and identified as Ang-II [34]. The identity of this substance with Ang-II was supported by: 1) a similar R_f value (on thin-layer chromatography and electrophoresis) as those of synthetic [Ile5]-Ang-II; 2) a similar amino acid composition to that of human Ang-II, namely, Asp, Arg, Val, Tyr, Ile, His, Pro, and Phe all in equimolar ratio [34].

The proteolytic enzymes called kallikreins (EC 3.4.21.34-35) are widely distributed in tissues and biolog-

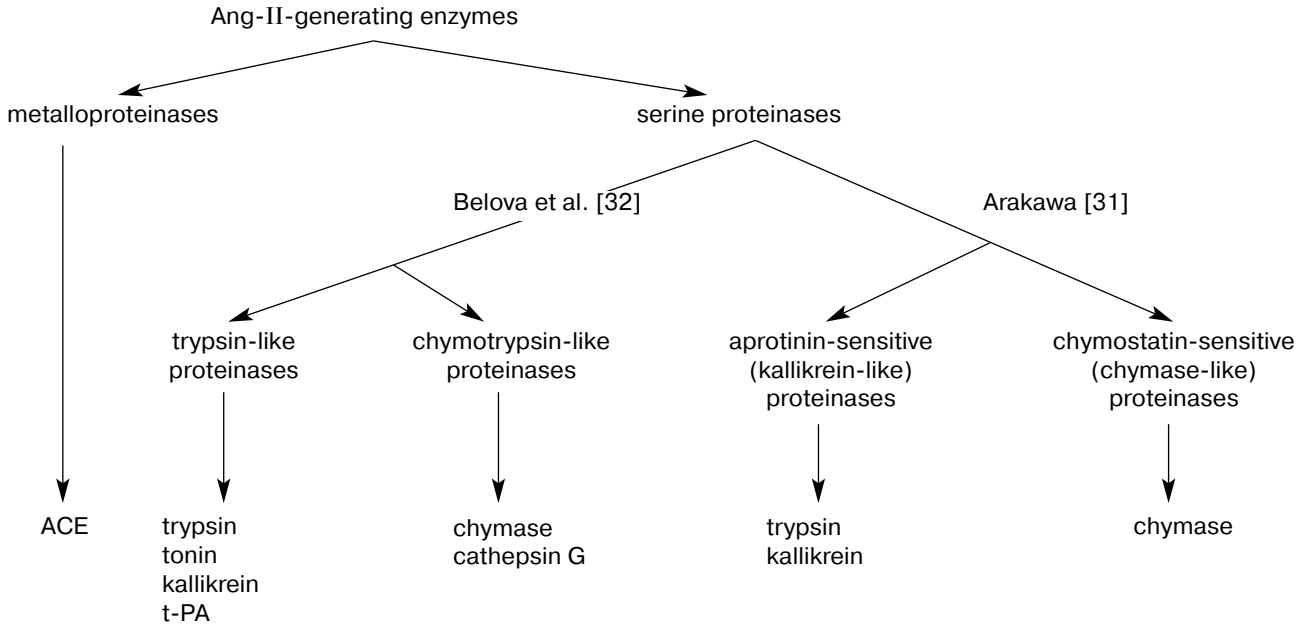

Fig. 2. Classification of Ang-II-generating enzymes.

ical fluids including blood [20, 35, 36]. Kallikreins are similar to trypsin in some properties [20, 35]. For example, they can hydrolyze low-molecular-weight arginine esters (BzArgOEt, BzArgOMe, TosArgOMe). However, kallikreins do not hydrolyze similar amide substrates.

DFP, PMSF, and soybean trypsin inhibitor (SBTI) are inhibitors of trypsin and kallikrein. Mammalian tissues (lung, pancreas, and parotid and salivary glands) have a number of trypsin and kallikrein inhibitors, the most known from which is the basic pancreatic trypsin inhibitor (BPTI) from bovine organs (commercial names are trasylol, gordox, aprotinin, contrykal, etc.) [20, 35]. In contrast to trypsin, kallikrein is practically not inhibited by ovomucoids and lima bean trypsin inhibitor (LBTI) [20].

Plasma kallikrein (EC 3.4.21.34) (molecular weight 97 kD) is produced by liver as an inactive precursor—prekallikrein [20, 35]. Secretions of many glandular organs (pancreatic juice, saliva, sweat, tears, urine) have tissue kallikreins (EC 3.4.21.35) in active forms. The molecular weights of kallikreins from urine, pancreas, and submaxillary gland are 32, 33, and 36 kD [35]. Plasma and tissue kallikreins differ in immunological and physicochemical properties [35, 36]. Plasma kallikrein liberates bradykinin from kininogen, but pancreatic kallikrein and kallikreins of other glands produce the decapeptide kallidin, which is converted to bradykinin in plasma by an aminopeptidase. The biological effect of kinins is opposite to Ang-II because they are vasodilatators, and thus they can decrease blood pressure [35, 36].

As has been shown, pancreatic kallikrein can generate Ang-II from angiotensinogen at pH 4.0-5.0 [37, 38].

We assume that the generation of Ang-II by pancreatic kallikrein and trypsin has no physiologic significance because the substrate and enzyme are separated in space. But it is possible that *in vivo* other trypsin-like enzymes (including other, not pancreatic, kallikreins) can generate Ang-II. Tissue kallikrein purified from the rat submandibular gland and human urine can also convert Ang-I to Ang-II [39].

A kallikrein-like enzyme that is able to generate low-molecular-weight kinins from kininogen and Ang-II from Ang-I was isolated from dog heart [39]. This enzyme is different from cathepsin D, cathepsin G, and chymase. The kinin-forming enzyme is a glycoprotein with an apparent relative molecular weight of 65 kD by SDS-PAGE. The pH optimum is 8.0 for kininogenase activity and is 6.5 for Ang-I-converting activity [39].

Nerve growth factor γ, which belongs to the kallikrein family of proteins and also exhibits trypsin-like activity, specifically cleaves the Phe-His bond of the synthetic renin substrate Ang-(1-14) (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) [40].

The Ang-II-generating enzyme tonin (EC 3.4.21.35) was isolated from rat submaxillary glands [28, 29, 36, 40]. Tonin belongs to the same family of serine proteinases as glandular kallikrein and the γ-subunit of nerve growth factor [2, 28, 36, 41]. Tonin liberates Ang-II from angiotensinogen, Ang-(1-14) and Ang-I, but in contrast to ACE does not inactivate bradykinin [28, 29, 41]. Tonin has a trypsin-like activity since it hydrolyzes the majority of substrates cleaved by trypsin. In general tonin shows more esterolytic than amidolytic activity. The optimum pH of tonin for Tos-Arg-OMe is 8.5, 9.0 for Bz-Arg-OEt, 9.0-9.5 for Bz-Arg-OMe, and above 10.0 for Bz-Arg-

Table 2. Physicochemical and kinetic characteristics of Ang-II-generating enzymes

Enzyme (source)	Molecular mass, kD	pH_{opt}	k_{cat}/K_m*, $\mu M \cdot min^{-1}$	Inhibitor	References
ACE (human lung)	150	7.2-7.4	125	EDTA, captopril	[5, 21]
Trypsin (bovine pancreas)	23	6.0	—	DFP, PMSF, SBTI, LBTI, BPTI, ovomucoid	[20, 38]
Kallikrein (porcine pancreas)	33	4-5	—	DFP, PMSF, SBTI, BPTI	[35, 37]
Kallikrein-like enzyme (dog heart)	65	6.5	—	BPTI	[39]
Tonin (submaxillary gland of rat)	28.7	6.8	68	SBTI, BPTI	[28, 41]
t-PA	72	<7	—	DFP, PMSF	[17]
Cathepsin G (human neutrophils)	23-30	7.0-7.5		DFP, PMSF, SBTI, BPTI, chymostatin	[3, 29]
Chymase (human skin and heart mast cells)	30-35	8.5	198	DFP, PMSF, SBTI, BPTI, chymostatin	[18, 58]

* For conversion of Ang-I to Ang-II [67].

pNA. Among these substrates, Bz-Arg-OEt is the best (on the base of the k_{cat} value) [41]. Substrates containing tyrosine or phenylalanine residues, which are easily hydrolyzed by chymotrypsin, are not appreciably hydrolyzed by tonin [36, 41, 42]. Although tonin displays tryptic activity but no activity on synthetic chymotrypsin substrates, it exerts exclusively a chymotrypsin-like activity on the Phe-His bond of Ang-I and (des-Asp1)-Ang-I [28, 29, 41]. The optimum pH of tonin with Ang-I or Ang-(1-14) is 6.8 [28]. Tonin is inhibited by BPTI and SBTI. However, the serine proteinase inhibitors DFP and PMSF, which inhibited trypsin and chymotrypsin almost completely at a molar inhibitor/enzyme ratio higher than 100, inhibited tonin only by 40% when present at a molar ratio exceeding 10,000 [41]. Tonin is not inhibited by pepstatin, EDTA, SQ 20881, or SQ 14225 [28, 41].

Thibault and Genest [41] suggested that tonin is identical to salivain (molecular weight 30 kD, p*I* about 6.0), an alkaline protease purified from the submaxillary glands of rats. It possesses high activity with proteins and synthetic substrates (BzArgOEt and BzArgOMe) at pH 9.0-9.3 [20, 43]. This enzyme was also inhibited by DFP and BPTI but not by LBTI or ovomucoid [20, 43].

A number of authors have suggested that kallikrein-like Ang-II-generating enzymes (including tonin) play an important role in regulation of the brain RAS [40, 44].

Arakawa et al. [38, 39] proposed the term "kinin–tensin system" for serine proteinases (tonin, trypsin, and tissue kallikrein) that form Ang-II from angiotensinogen and kinins from kininogen. Thus two opposing activities—depressor and pressor—are built into one enzyme system, and the direction of the reaction depends on pH. These enzymes act as kininogenase generating kinins at pH 8.0-9.0, and they are Ang-II-generating enzymes at pH 4.0-6.5 [37-39].

t-PA (EC 3.4.21.68) is also able to convert Ang-I to Ang-II, and this may have physiological value [17]. t-PA catalyzes the conversion of the inactive proenzyme plasminogen into the active proteinase plasmin, and it is an important component of the fibrinolytic system. t-PA is synthesized by the endothelium and is localized at the blood vessel wall [45]. Dzau et al. [15, 17] demonstrated that t-PA can cleave Ang-II from either Ang-(1-14) or purified human angiotensinogen. t-PA is synthesized *in vivo* as a single-chain polypeptide (molecular weight 72 kD) that is proteolytically cleaved into a double-chain form by a variety of endogenous proteinases, including plasmin, tissue kallikrein, and activated factor X. The double-chain form is more potent than the single-chain precursor [45]. The pH optimum for conversion of angiotensinogen to Ang-II is acidic [15, 17]. Thus, t-PA as an Ang-II-generating enzyme may act intracellularly

or at site of vascular injury and necrosis where the pH is 4.0-6.5.

In vivo, t-PA release can occur both after mechanic injury of tissue and during hypoxia connected with restriction of normal blood flow to tissues due to thrombi [20]. Hence, t-PA (as an Ang-II-generating enzyme) released in a site of vessel injury or occlusion may locally regulate tonus and vasospasm.

Chymotrypsin-like Ang-II-forming enzymes. The cleavage site between Ang-I and Ang-II is a phenylalanyl–histidyl peptide bond, which should be susceptible to enzymes of the chymotrypsin family. In addition, chymotrypsin and chymotrypsin-like enzymes easily hydrolyze the Pro7–Phe8 bond [2, 15].

Though chymotrypsin (EC 3.4.21.2) hydrolyzes in proteins peptide bonds X–Y containing aromatic amino acids (Trp, Tyr, or Phe) or Leu at the X position (pH 7.5-8.0), it does not generate Ang-II from Ang-I or angiotensinogen [20, 33, 46].

The two proteinases cathepsin G and chymase are chymotrypsin-like Ang-II-forming enzymes. Comparison of the amino-acid sequences of chymase and cathepsin G showed high homology (47%) between them. Their activity toward low-molecular-weight ester substrates is similar; however, they hydrolyze different peptide and protein substrates [36, 47]. The positive charge on the chymase and cathepsin G molecules is favorable for their connection with heparin or heparin-sulfate, which enhance their stability and protect from the action of other proteinases and serum inhibitors of serine proteinases [48].

In the early 1980s, a serine proteinase that could convert circulating angiotensinogen to Ang-II was found in mammalian neutrophils [3, 29, 49]. Tonnesen et al. [30] identified the angiotensin-II-generating proteinase of human neutrophils as cathepsin G (also having fibrino(geno)lytic activity) on the basis of subcellular localization (in lysosomes), substrate specificity, physicochemical properties, and antigenic characteristics.

Cathepsin G (EC 3.4.21.20) is a chymotrypsin-like proteinase of neutrophils and spleen. The molecular weight of cathepsin G is 23-30 kD [29, 30, 49-53]. Cathepsin G, located in azurophile granules, has microbicidal activity and the capacity to degrade a number of proteins including complement components, immunoglobulins, and fibronectin [2, 42, 50-53]. Cathepsin G is a cell proteinase that gets into blood either via stimulation or after secretor granule degradation [52]. Cathepsin G can influence vascular tone, enhancing endothelium permeability due to its cationic nature [53]. Cathepsin G is a chymotrypsin-like proteinase and can cleave Ang-I at two sites: bonds Tyr4–Ile5 and Phe8–His9. It was shown [2] that *in vitro* cathepsin G preferentially cleaves Ang-I at the Phe8–His9 bond, and the pH optimum is 7.0-7.5 [29]. In addition, cathepsin G can generate Ang-II from angiotensinogen [30].

Suc-Val-Pro-Phe-NA and Suc-Phe-Pro-Phe-NA are the best substrates for cathepsin G (the pH optimum is 7.2) [2, 42]. In contrast to other serine proteinases (trypsin, chymotrypsin, and blood proteinases), which are stabilized by $CaCl_2$, cathepsin G is inhibited by Ca^{2+} [42]. Cathepsin G requires high ionic strength or detergents (the buffer must contain 1 M NaCl or 0.5 M NaCl with Brij-35) for its enzymatic activity [42, 51]. Its activity is not significantly affected by dimethylsulfoxide (2.5-18% v/v) [42], and the addition of polyanions (heparin or heparin-sulfate) at concentration 30 μg/ml to cathepsin G enhanced the enzymatic activity by 25% (with BzTyrOEt) [53].

Owen and Campbell [54] found that human neutrophils express inducible, catalytically active cathepsin G on their cell surface. Membrane-bound cathepsin G also has Ang-II-generating activity and can convert both angiotensinogen and Ang-I to Ang-II. In contrast to soluble cathepsin G, the membrane-bound enzyme is substantially resistant to inhibition by plasma proteinase inhibitors and converts Ang-I to Ang-II even in undiluted plasma.

Cathepsin G activity is inactivated by low-molecular-weight inhibitors of serine proteinases—PMSF and DFP. Protein inhibitors of cathepsin G include soybean and lima bean trypsin inhibitors, α-1-anti-chymotrypsin, α-1-proteinase inhibitor, and α-2-macroglobulin [30, 42, 51]. The most active synthetic inhibitor of cathepsin G is Z-Gly-Leu-Phe-CH_2Cl, with $k_{app}/[I] = 51\ M^{-1}\cdot sec^{-1}$ [51]. Captopril (SQ 14225) and teprotyd (SQ 20881) do not inhibit the angiotensin-converting activity of cathepsin G [29].

Dzau et al. [55] examined whether neutrophils can influence angiotensin formation by activating human renin. When incubated with partially purified plasma and amniotic prorenin, sonicates from 10^5-10^6 neutrophils resulted in 120 ± 30 and 1240 ± 300% increase in renin activity, respectively. This activity is inhibited by serine proteinase inhibitors. The pH optimum of the neutrophil prorenin-activating enzyme is 6.5-7.0, and the enzyme is cathepsin G [55]. Thus, cathepsin G may be the main participant of the granulocyte–angiotensin system, taking part in the conversion of its components at various stages.

There has been much speculation as to the role of cathepsin G in physiological processes. It is possible that it takes part in the degradation of immune complexes, in the activation of the complement cascade, and in fibrin clots [2, 42, 51]. Cathepsin G, as one of the main neutrophil proteinases, may facilitate neutrophil egress from venules and their movement into extravascular tissues, or it may provide a mechanism for local control of blood flow during the inflammatory response [2, 15, 29, 30]. Cathepsin G (in its form in neutrophils) may represent a mobile system that allows local Ang-II formation and thus modulate local blood flow or cell infiltration at sites of inflammation [2, 15, 29].

Another chymotrypsin-like Ang-II-forming enzyme is chymase. Chymases (EC 3.4.21.39) mainly exist in

secretory granules of mast cells and in extracellular interstitium [48, 56]. Mast cells are present in various tissues (heart, lung, skin) [2, 18, 57]. Mammalian chymases can be divided into two groups, α- and β-chymases. α-Chymases include human chymase, canine chymase, murine chymase-5, rat chymase-3, and gerbil chymase-2. α-Chymases are specific enzymes that efficiently convert Ang-I to Ang-II by splitting the Phe8–His9 bond in the decapeptide Ang-I. β-Chymases include rat chymase-1 and -2, murine chymase-1, -2, -4, and -L, and gerbil chymase-1. β-Chymases have broad substrate specificity that is analogical to chymotrypsin, but they are not Ang-II-generating enzymes [58-62]. Only one α-type chymase gene has been identified in the human body [60].

Urata et al. [63] have shown widespread tissue distribution of human chymases. High levels of chymase-like enzymatic activity (on generation Ang-II from Ang-I) were detected in skin, esophagus, stomach, and uterus; moderate levels were found in both cardiac ventricles, lung, colon, tonsil, adenoid, and renal cortex; and low levels were found in the cardiac atria, coronary artery, aorta, spleen, renal medulla, and liver [57, 58, 63]. Chymase was also found in human heart in cardiomyocytes and endothelial cells [60, 64]. Enzyme isolated from human aortic tissues and designated as chymostatin-sensitive angiotensin II generating enzyme [65, 66] appears to be identical to chymase [58]. In vessel wall, chymase was distributed predominantly in the adventitia, while ACE was localized mainly in the endothelium [66]. The molecular weight of chymases from different organs is about 30-35 kD [18, 48, 58]. The optimum pH of purified chymase is 8.5 (between 6.5 and 10.5) [18, 58, 65].

Chymase from human heart and vascular tissues is a more specific enzyme than other Ang-II-generating enzymes and ACE. It does not hydrolyze peptide hormones including bradykinin, vasoactive intestinal peptide, substance P, and somatostatin and does not convert angiotensinogen to Ang-II [48, 58, 67]. Human heart chymase cleaves the Phe8–His9 bond with a 750-fold higher catalytic efficiency (k_{cat}/K_m) than the Tyr4–Ile5 bond in Ang-I [61]. Mast cell chymase from human lung also failed to hydrolyze the Tyr4–Ile5 bond of Ang-II, which is cleaved by chymotrypsin-like enzymes [18]. Thus, the generated Ang-II is not further degraded because the Tyr4–Ile5 bond in Ang-II is resistant to cleavage by human chymase. Rat chymase-1, in contrast, is an angotensinase because it readily splits the Tyr4–Ile5 and Phe8–His9 bonds in angotensins [14, 62]. Powers et al. [68] investigated chymase specificity by using *p*-nitroanilide substrates that contained a portion of the Ang-II sequence: Suc-His-Pro-Phe-NA, Suc-Ile-His-Pro-Phe-NA, and Suc-Val-Pro-Phe-NA. Human skin chymase hydrolyzed the latter substrate with the highest velocity [68].

Like all serine proteinases, chymases are inactivated by DFP and PMSF; in addition, they are inhibited by SBTI and chymostatin, but not by captopril, EDTA, and pepstatin [18, 48, 58, 67]. In contrast to cathepsin G, chymases are not inhibited by BPTI and are less susceptible to the action of LBTI and SBTI [18, 58]. Human heart chymase is partially inhibited by *p*-tosyl-L-Phe chloromethyl ketone, but is not inhibited by *p*-tosyl-L-Lys chloromethyl ketone [48, 67].

PHYSIOLOGICAL ROLE OF SERINE Ang-II-GENERATING PROTEINASES

There are conflicting results on the physiological importance of serine Ang-II-generating proteinases [3, 56, 69]. Human heart chymase is the most effective and specific Ang-II-generating enzyme [2, 14, 62, 70]. But ACE inhibitor therapy has proven to be highly effective in the treatment of hypertension and heart failure [26, 71].

However, the effect of ACE inhibitors can be explained by their action independent of conversion of Ang-I to Ang-II. Since ACE is a kininase (an enzyme that degrades the potent vasoconstrictor bradykinin), the possibility exists that kinins are involved in the cardiovascular effect of ACE inhibitors [4, 26, 39]. Sulfhydryl-containing ACE-inhibitors also have vasodilating properties independent of Ang-II (through the lipoxygenase vasoconstrictive pathway) [16, 26, 72]. Finally, arachidonic acid metabolites may be involved, because a direct stimulatory effect of captopril on prostacyclin synthesis has been shown [16, 73].

In biochemical studies using human heart tissues, it was shown that ACE inhibitors could block only 10 to 20% of Ang-I conversion [14, 56, 60, 67, 70, 74]. The remaining activity was blocked by PMSF and SBTI, pointing to serine proteinases as Ang-II-generating enzymes [71]. ACE inhibitors only partially reduced vessel wall Ang-II production, but the addition of chymostatin resulted in the complete blockade of the conversion of Ang-I to Ang-II in human arteries [65].

Wolny et al. [56] studied the effects of proteinase inhibitors on the Ang-I-induced (1 μM) contraction of human coronary arteries after long-term therapy with ACE inhibitors. Human hearts were obtained from heart-failure patients undergoing heart transplantation. The ACE inhibitor captopril did not block contraction, but chymostatin blocked it by 78%; the combination of ACE inhibitor and chymostatin, by 98%; and Ang-II receptor blocker, by 100%.

In contrast with these results, it was recently found that ACE inhibitors could reduce Ang-I conversion *in vitro* by 90% [75]. *In vitro* Ang-II forming capacity of ACE and chymase (extracted from cardiac tissue) depends on detergent concentration used to process the tissue [62]. The high detergent concentration used for chymase solubilization and following dialysis lead to loss of enzyme activity [56, 62].

In vivo, the physiological role of serine Ang-II-generating enzymes was examined in the studies described below. Ang-II increase in dog coronary sinus after coronary ligation was not inhibited by ACE inhibitors, but it was by the serine proteinase inhibitors [76]. BPTI, nafamostat (a serine proteinase inhibitor), and chymostatin, but not captopril, suppressed intracardiac Ang-II formation during myocardial ischemia in bilaterally nephrectomized dogs [39]. However, reduction in myocardial infarct size was observed only in captopril-treated dogs in which immunoreactive kinin levels in the left interventricular vein had increased significantly [39].

Exercise-induced increase in plasma Ang-II could not be prevented by inhibition of ACE, but it was reduced by a serine proteinase inhibitor [3, 77]. In healthy volunteers, both renin inhibitors and Ang-II antagonists induced renal vasodilator response ~50% larger than the maximal renal hemodynamic response to ACE inhibitors, so it is proposed that Ang-II generation occurs via a renin-dependent, but ACE-independent, pathway [3]. In patients with peripheral vascular disease, serine proteinase inhibitors significantly increased maximal walking distance and improved subjective symptoms [78]. In addition, long-term ACE inhibition in humans did not decrease tissue and plasma Ang-II levels [79].

Perhaps ACE is responsible principally for Ang-II formation on the luminal side, while chymase is important for Ang-II generation in the adventitial-outer medial area and modulates sympathetic neuro-effector functions and affects smooth muscle contractility [15, 18, 62, 65]. Arakawa [31] hypothesized that aprotinin-sensitive Ang-II-forming enzymes may make some contribution to the regulation of tissue perfusion, and chymostatin-sensitive ones may participate in structural remodeling of the heart and vessels.

Thus, the human body contains several Ang-II-forming enzymes that can be divided into 2 groups: 1) ACE, and 2) serine Ang-II-generating enzymes (see Fig. 2). ACE and serine Ang-II-forming enzymes are often localized in the same organs. And ACE or serine proteinase may become more active when one of the pathways is blocked [79]. The circulating RAS as the system of rapid reaction provides short-term cardio–renal homeostasis. The RAS in tissues (where ACE converts only 10-20% of Ang-I to Ang-II) are the systems of long-term regulation, and they influence the tonic control of vascular resistance and local tissue function [16, 23, 74].

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