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Enzymatic pathways of the brain renin-angiotensin system: unsolved problems and continuing challenges

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Review

Enzymatic pathways of the brain renin–angiotensin system: Unsolved problems and continuing challenges

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Abstract

The brain renin–angiotensin system continues to be enigmatic more than 40 years after the brain was first recognized to be a site of action of angiotensin II. This review focuses on the enzymatic pathways for the formation and degradation of the growing number of active angiotensins in the brain. A brief description and nomenclature of the peptidases involved in the processing of angiotensin peptides in the brain is given. Of primary interest is the array of enzymes that degrade radiolabeled angiotensins in receptor binding assays. This poses major challenges to studies of brain angiotensin receptors and it is debatable whether an accurate determination of brain angiotensin receptor binding kinetics has yet been made. The quandary facing the investigator of brain angiotensin receptors is the need to protect the radioligand from metabolic alteration while maintaining the characteristics of the receptors *in situ*. It is the tenet of this review that we have yet to fully understand the binding characteristics of brain angiotensin receptors and the extent of their distribution in the brain because of our inability to fully protect the angiotensins from metabolic alteration until equilibrium binding conditions can be attained.
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Keywords: Angiotensin receptors; Metabolism; Radioligand binding assays; Angiotensinases; Peptidases

Contents

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1. Introduction

The first definitive demonstration of an effect of angiotensin II (Ang II) in the brain was the cross perfusion study of Bickerton and Buckley [1]. However, this effect was produced by blood-borne Ang II later recognized to be mediated by the circumventricular organs of the brain which are outside of the blood–brain-barrier. A subsequent casual observation by Booth [2] revealed that Ang II acts within the brain which initiated an interest in the possible existence of a brain renin–angiotensin system (RAS). A few years later, Ganten et al. [3] reported the existence of renin-like activity in the brain and research on the brain RAS began in earnest.

1.1. Roles of the brain renin–angiotensin system

The influence of the brain RAS on hydromineral balance, arterial blood pressure, neurosecretory functions and overall body homeostasis is well documented. There are several excellent review articles covering early studies of the brain RAS [4–6].

While these 'traditional' functions of angiotensins in the brain have been established for some time, the recognition of the presence of multiple receptor subtypes for angiotensins in the brain as well as novel functions for metabolites of the octa- and heptapeptide angiotensins has greatly extended the range of activities attributed to the brain RAS. More recent reviews cover these aspects of the brain RAS [7–12].

1.2. Generation and degradation of angiotensin peptides in the brain

The rate of synthesis as well as the rate of degradation of neurotransmitters and neuromodulators is an important factor in initiating and terminating their biological effects. This principle applies to peptide hormones as well. They are regulated by proteases (otherwise called peptidases, proteinases or proteo-

lytic enzymes) that generate and metabolize them [13–15]. The human genome encodes several hundred proteases, of which the function of many has not yet been determined. Peptidases involved in processing of angiotensin peptides have been collectively termed "angiotensinases". While the term has largely been used to infer degradation, it is now know that these enzymes can generate active angiotensins. Angiotensinases are comprised of three groups of peptidases: amino-, endo- and carboxypeptidases. Aminopeptidases have traditionally been viewed as the most important group, accounting for 60–90% of angiotensinase activity in various tissues [16] however, it is now known that angiotensin peptides are processed by a broad variety of peptidases.

2. Angiotensin-forming enzymes

The formation of the primary active angiotensin, Ang II is considered to occur via a cascade of enzymatic reactions, starting with a large protein precursor. The fact that the enzyme renin was the first component of the RAS to be discovered has led to the naming of this hormonal system as the renin–angiotensin system. However, as will be seen, renin is certainly not the only angiotensin-forming enzyme. However, out of respect for the discovery of renin by Tigerstedt and Bergstrom [17], use of the term renin–angiotensin system likely will continue. In Section 2 the primary focus will be on formation of Ang II and Ang III from angiotensinogen and Ang I, as they are the angiotensins that act on the classical AT1 and AT2 receptors. The formation of the other active angiotensins from Ang I, Ang II and Ang III, which act on receptors other than AT1 and AT2, are pirmarily addressed in Section 3.

2.1. Renin

The only known substrate for renin is angiotensinogen, which is the only known precursor for the active octapeptide

angiotensin II (Ang II). In the brain, the primary source of angiotensinogen is astroglia which are reported to constitutively secrete it into the extracellular fluid of the brain [18]. By the classical pathway of the RAS it is cleaved by renin (EC 3.4.23.15) between Leu[10]–Leu[11] residues to yield angiotensin I (Ang I) (Fig. 1) [6,19,20]. Renin is an aspartyl peptidase of the A1 family. In the brain of rodents and humans renin is expressed in two forms: one is that secreted either as active renin from the secretory/storage granules, or is formed extracellularly from

secreted, inactive prorenin. The other form is a more recently described non-secreted, brain-specific intracellular renin, which in comparison with secreted renin is expressed in much higher amounts in the brain tissue [21–26].

2.2. Renin-like enzymes

Several other peptidases, in addition to renin, are capable of forming Ang I from angiotensinogen. Among acid proteases in
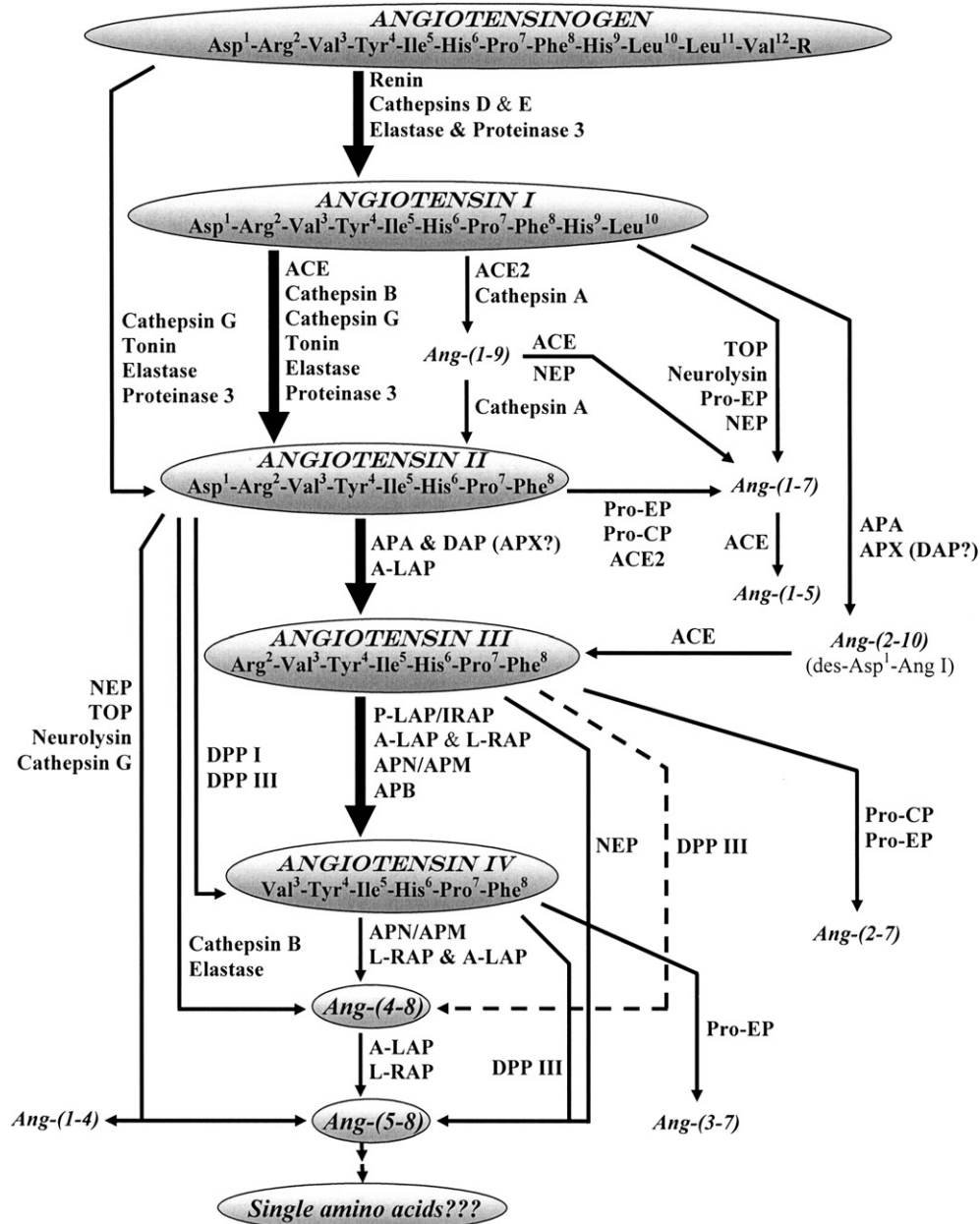
Fig. 1. Pathways of formation of angiotensin peptides in the brain. Abbreviations used: ACE — angiotensin converting enzyme; ACE2 — human homolog of angiotensin converting enzyme; APA — aminopeptidase A; A-LAP — adipocyte derived leucine-aminopeptidase; L-RAP — leukocyte-derived arginine aminopeptidase; NEP — neutral endopeptidase; TOP — thimet endopeptidase; Pro-EP — prolyl-endopeptidase; Pro-CP — prolyl-carboxypeptidase; APX — aminopeptidase X; DAP — aspartyl aminopeptidase; P-LAP/IRAP — placental leucine-aminopeptidase/insulin-regulated aminopeptidase; APN/APM — aminopeptidase N/M; APB — aminopeptidase B; DPP I — dipeptidyl peptidase I; DPP III — dipeptidyl peptidase III. Numbering of amino acid residues in all fragments is based on the numbering in angiotensinogen. Larger sized arrows indicate the "classical" metabolic pathways for angiotensin peptides.

the brain cathepsins E (EC 3.4.23.34) and D (EC 3.4.23.5), aspartic proteinases belonging to the A1 family, can form Ang I from angiotensinogen substrate [27–30] (Fig. 1). Cathepsin D is a typical lysosomal enzyme, whereas cathepsin E has been found in the endoplasmic reticulum, in endosomes and other cell compartments. Both enzymes are involved in the processing of various peptide precursors [29].

Two neutral peptidases are also capable of generating Ang I from angiotensinogen in the brain: elastase and proteinase 3 [7,31,32] (Fig. 1). Furthermore, two other neutral peptidases — cathepsin G (EC 3.4.21.20) and tonin, together with elastase and proteinase 3, can form Ang II directly from angiotensinogen, without Ang I production as an intermediate, by cleaving the peptide bond between the Phe^8–His^9 residues [32–37] (Fig. 1). Cathepsin G, tonin, elastase and proteinase 3 are neutral serine proteinases (S1 family). They are localized mainly to endosomes and lysosomes, and are widely expressed in different mammalian tissues including the brain [31,34–36]. They are an essential part of the cell proteolytic machinery with a still growing list of known physiological substrates, and have a role in many biological processes.

2.3. Angiotensin-converting enzyme (ACE)

In the classical RAS cascade, the decapeptide Ang I is converted to Ang II by angiotensin-converting enzyme (ACE, kininase II, EC 3.4.15.1). ACE is one of the most extensively studied mammalian peptidases. It exists in soluble and membrane-bound forms, and is a member of the M2 family of zinc metallopeptidases. ACE possesses dipeptidyl carboxypeptidase activity and can selectively convert Ang I to Ang II, des-Asp-angiotensin I to Ang III, Ang 1–7 to Ang 1–5, and bradykinin to its inactive metabolite, in addition to metabolizing several other peptides [15,38–42]. However, ACE does not metabolize Ang II, presumably due to its inability to metabolize the His^6–Pro^7 bond [43]. The soluble form of ACE is distributed extracellularly while the membrane-bound form is on the external face of the plasma membrane. Thus the formation of Ang II from Ang I by ACE is thought to occur extracellularly only [44].

2.4. Other carboxypeptidases

Two other peptidases — cathepsin A (carboxypeptidase A, lysosomal protective protein, deamidase, EC 3.4.16.5) and human homologue of ACE (ACE2, ACEH) can remove His^{10} from Ang I, generating Ang-(1–9) [7,43,45,46]. Ang-(1–9) can be further metabolized to Ang II by cathepsin A [7,45] (Fig. 1). Cathepsin A is a multifunctional lysosomal, acidic, serine carboxypeptidase (S10 family) that also functions as a protective and an activator protein for neuraminidase and beta-galactosidase [47–49]. Cathepsin A also acts as an esterase/C-terminal deamidase at neutral pH and both carboxypeptidase and esterase/C-terminal deamidase functions are fully separated from its protective function [50]. ACE2, like ACE, is a member of the M2 family of zinc metallopeptidases, existing in a membrane-bound form and

widely expressed in a variety of mammalian tissues including the brain [15,41,51]. In contrast to ACE, ACE2 cleaves only a single C-terminal residue from its peptide substrates including Ang I and Ang II, but not Ang-(1–9) and Ang-(1–7) [43,46]. Another differentiating feature of ACE2 from ACE is that it is unable to cleave bradykinin and Hip–His–Leu, and is insensitive to ACE inhibitors, e.g., lisinopril and captopril [15,41]. In addition to its role as a carboxypeptidase, ACE2 is also the receptor for severe acute respiratory syndrome (SARS) coronavirus [52,53].

Elastase, proteinase 3, tonin, cathepsin B and G can also cleave the carboxy terminal His–Leu from Ang I to form Ang II [32,36,54] (Fig. 1) in a manner similar to the well-characterized formation of Ang II by ACE. Cathepsin B is a member of cysteine cathepsins (C1 family). It is a ubiquitous mammalian lysosomal peptidase, expressed in brain, that functions to convert several peptide precursors into active peptides [54,55].

Chymase is another enzyme capable of forming Ang II from Ang I [56]. This enzyme is largely associated with mast cells, and aside from the pineal gland and the pituitary, there is little chymase activity in the brain [57].

2.5. Aminopeptidases

Aminopeptidases have traditionally been thought to be inactivators of Ang II, with the exception of aminopeptidase A-mediated formation of the generally short-lived heptapeptide, Ang III [58–60]. The des–Asp^1, des–Arg^2 Ang II hexapeptide (Ang IV) has little activity at the classical Ang II receptor mediating the pressor, dipsogenic, salt appetite-inducing and hormone-releasing effects of Ang II and thus was considered to be a weak agonist at best. However, the discovery of high affinity binding sites for Ang IV in the brain [61] and the reported memory enhancing effects of Ang IV [62], yet another active angiotensin was discovered. Thus aminopeptidases are newly cast as angiotensin-synthesizing enzymes in addition to their continuing role as angiotensin inactivators. Indeed, it has been suggested that aminopeptidase A activity is essential for formation of an active angiotensin peptide in the brain [63]. Although recent studies from our laboratory have challenged that hypothesis [64,65].

2.5.1. Aminopeptidase A (APA)

One of the most known and accepted peptidases responsible for further processing of Ang II is APA (glutamyl aminopeptidase, EC 3.4.11.7), which cleaves Asp^1 from Ang II, generating the heptapeptide Ang III [58–60]. Ang III can also be generated from Ang I without Ang II production as an intermediate through formation of des–Asp^1–Ang I (Ang 2–10) by APA and aminopeptidase X (see also Section 2.5.3), and subsequent carboxypeptidase cleavage of Leu–His by ACE [60,66,67] (Fig. 1). APA is a widely expressed mammalian membrane-bound aminopeptidase, a member of the M1 family of zinc metallopeptidases [14,68]. It is abundantly expressed in the brain [69]. The catalytic domain of the peptidase is in the ectodomain of the protein thus it is

positioned to metabolize peptides that are in the extracellular milieu. It specifically cleaves acidic residues (aspartic acid or glutamic acid) from the N-terminus of peptide substrates, predominantly from Ang I, Ang II and cholecystokinin-8 [14,60,68,70].

2.5.2. Oxytocinase subfamily

Another aminopeptidase that can cleave Asp[1] from Ang II is known as adipocyte-derived leucine aminopeptidase (A-LAP, puromycin insensitive leucyl-specific aminopeptidase, PIL-SAP) [71,72]. Moreover, it can sequentially cleave N-terminal amino acids from generated angiotensins up to His–Pro–Phe [72,73] (Fig. 1). A-LAP is a member of the oxytocinase subfamily of M1 aminopeptidases, found in soluble and membrane-bound forms. It is extensively expressed in the brain and many other tissues [71,72]. Together with other members of this subfamily: placental leucine aminopeptidase (P-LAP, cysteinyl aminopeptidase, EC 3.4.11.3, EP 11.3), also known as insulin-regulated aminopeptidase (IRAP) and the Ang IV receptor [74], and leukocyte-derived arginine aminopeptidase (L-RAP), it plays an important role in the maintenance of homeostasis during pregnancy, memory retention, blood pressure regulation and antigen presentation [71,72].

2.5.3. Aspartyl aminopeptidase (DAP)/aminopeptidase X (APX)

Aminopeptidase X (amastatin-, bestatin- and EDTA-insensitive aminopeptidase) activity described by Sim and coworkers [66,75] can cleave Asp[1] from Ang I and Ang II forming the 2–10 nonapeptide fragment of Ang I and Ang III, respectively. This enzyme is likely the same as aspartyl aminopeptidase (DAP, EC 3.4.11.21) first described by Kelly et al. [76] and later characterized by Wilk et al. [67]. It is a member of the M18 family of metalloproteinases. This DTT and o-phenanthroline sensitive, but amastatin, bestatin, relatively EDTA, insensitive enzyme was capable of efficiently metabolizing Ang II to Ang III [67]. As noted above, des–Asp[1]–Ang I can be processed to Ang III by ACE [38,60] (Fig. 1).

3. Angiotensin degradation

As noted above, the line between angiotensin synthesis and degradation has blurred due to the discovery of physiological actions and receptors for angiotensin peptides previously considered to be inactive metabolites. However, when considering the primary actions of the RAS, in particular, effects at the AT1 receptor subtype which mediates nearly all of the physiological and pathophysiological effects of Ang II, anything less than the carboxy terminal heptapeptide is considered to be an inactive metabolite.

3.1. Aminopeptidases

Historically aminopeptidases have been viewed as the primary metabolic enzymes for degradation of Ang II [16]. However, it is recognized now that the effects of peptidases are more complex.

3.1.1. Aminopeptidase A (APA) and aspartyl aminopeptidase (DAP)/aminopeptidase X (APX)

APA and DAP have a strong preference for acidic amino acids and are considered to be the primary enzymes responsible for the degradation of Ang II to Ang III. However, since Ang III is thought to be a fully active agonist at AT1 and AT2 receptors, this metabolic step can hardly be considered to be a degradation. On the other hand, Ang III appears to be highly labile and short-lived [77,78] so the process of formation of Ang III could be considered to be a prelude to inactivation of the peptide, at least as an AT1/AT2 agonist. Of interest is the observation that Ang IV binds to a soluble form of human APA, but is very slowly metabolized by it, thereby acting as a competitive inhibitor [79]. These investigators also reported a small amount of Ang III metabolism to Ang IV by this soluble APA. In addition to its ability to form Ang III from Ang II, the reported ability of APX (likely DAP) to form des Asp[1] Ang I from Ang I has been suggested to result in the formation of a physiological antagonist of the AT1 angiotensin receptor subtype [80]. An interesting inference of this observation is that ACE inhibitors could then be prolonging the lifespan of this endogenous AT1 receptor antagonist.

3.1.2. Oxytocinase subfamily

A-LAP (described in Section 2.5.2) is capable of cleaving N-terminal amino acids starting from Ang II up to tripeptide His–Pro–Phe [72,73]. Thus it is capable of metabolizing angiotensins to completely inactive peptides (Fig. 1). Interestingly, the closely related P-LAP/IRAP and L-RAP are not able to metabolize Ang II [72,74,81,82]. P-LAP/IRAP is reported to metabolize Ang III to IV, whereas L-RAP has ability to metabolize Ang III down to the tripeptide His–Pro–Phe by sequential elimination of N-terminal amino acids [72,82].

3.1.3. Aminopeptidase N (APN) and aminopeptidase B (APB)

APN (E.C. 3.4.11.2, aminopeptidase M, alanyl aminopeptidase, CD13) and APB (E.C. 3.4.11.6, arginyl aminopeptidase) have also generally been considered to be inactivating enzymes because they form an angiotensin fragment that has negligible affinity for the classical Ang II receptors. In cleaving Arg[1] from Ang III they form des–Asp[1], des–Arg[2] Ang II (Ang IV) [14,83,84], which is predominantly involved in learning and memory functions mediated mainly by Ang IV receptors [62]. In addition to its ability to cleave Arg[1] from Ang III to form Ang IV, APN can also cleave Val[1] from Ang IV to form Ang-(4–8) (Fig. 1), a putatively inactive angiotensin fragment [85]. APN and APB are members of the M1 family of Zn-metallopeptidases. APN is predominantly characterized as a membrane-bound peptidase with its catalytic site in its extracellular domain [14,84]. APB is both a secreted and membrane-bound protease [86]. Both APN and APB are ubiquitously distributed in different tissues of mammalian organisms, including brain. APN metabolizes regulatory peptides by removing the N-terminal amino acid with a preference towards, neutral residues. APB demonstrates strict specificity for Arg and/or Lys residues at the N terminus of various peptides. This suggests a dominant role of APB, rather than APN, in the processing of Ang III [14,84,86,87]. However it

Table 1
Affinities of some aminopeptidases for angiotensin peptides

	Ang II	Ang III	Ang I
APA	Ki=15 μM, Km=0.13 mM [171];Ki=24 μM [172]; Km=35.3 μM [59]	No complete inhibition [171]	No complete inhibition [171]
APM/N	No complete inhibition [171]; no degradation [173]	Ki=3 μM, Km=0.24 mM [171];Ki=0.34 μM, Km=2 μM [173]	No complete inhibition [171]
DPP III	Ki=0.34 μM [90]	Ki[a]=0.3 μM [90]	

[a] Analogs of Ang III.

has been suggested that APN is the predominant enzyme responsible for brain Ang III metabolism in the mouse [88] and rat [89] brain. Besides having peptidase activity, APN has also been shown to be a receptor of coronaviruses, a human herpesvirus and Bacillus thuringiensis CryIA(c) toxin in their target tissues [14,84].

3.1.4. Diaminopeptidases

In addition to APA, DAP and A-LAP, dipeptidyl peptidase I (DPP I, EC 3.4.14.1, previously called cathepsin C or J) and dipeptidyl peptidase III (DPP III, EC 3.4.14.4) can also be responsible for N-terminal amino acid cleavage of Ang II. These aminopeptidases are responsible for processing of various proteins and peptides, including angiotensins [90,91] They remove the Asp^1–Arg^2 dipeptide from Ang II forming Ang IV [45,90–92] (Fig. 1).

Diaminopeptidases are also reported to cleave Val–Tyr from Ang IV to form the putatively inactive Ang-(5–8) fragment [85,90]. DPP I and DPP III were first characterized as acidic lysosomal and basic cytosolic cysteine peptidases respectively, however, their membrane-associated forms were also described [91]. As shown in Table 1, DPP III possesses substantially higher affinity for Ang II than does APA [90]. The affinity of DPP III for Ang III analogs is similar to the affinity of APN for Ang III leading to the suggestion that DPP III also metabolizes Ang III to the inactive Ang-(4–8) fragment [90] (Table 1).

3.2. Endopeptidases

Several endopeptidases metabolize Ang II in the brain. Neprilysin, thimet oligopeptidase, and neutral endopeptidase cleave the Tyr^4–Ile^5 bond forming two tetrapeptides [93–95] (Fig. 1). Prolyl endopeptidase can also metabolize Ang II by cleaving the C-terminal Phe^8 from Ang II to form Ang 1–7 [7,96,97] (Fig. 1). Moreover, all 4 of these endopeptidases are able to cleave Ang I at the Pro^7–Phe^8 bond to form Ang-(1–7) [45,95,98–100] (Fig. 1). Up until the observation was made that Ang-(1–7) could stimulate vasopressin release from hypothalamic explants [101] the removal of phe^8 from Ang II was thought to be a metabolic inactivation of the peptide. However, Ang-(1–7) is widely recognized as having activity on its own right [102], with its own receptor [103]

which may play a role in hippocampal plasticity [104]. Thus the formation of Ang-(1–7) by prolyl carboxypeptidase must be viewed as an enzymatic step leading to the formation of an active angiotensin.

3.2.1. Neprilysin

Neutral endopeptidase (EC 3.4.24.11, EP 24.11, neprilysin, NEP) is a member of the membrane-bound, M13 family of zinc-dependent metalloproteases, that cleaves peptide bonds on the amino acid side of hydrophobic amino acid residues [105,106]. It is constitutively expressed in several tissues including the brain and kidney, but is developmentally regulated in other cell types (e.g. lymphocytes). It terminates the activity of peptides involved in cardiovascular regulation, inflammatory phenomena, and is critical for synaptic neuropeptide metabolism. Neutral endopeptidase has been called the "cholinesterase" of peptidergic synapses by some authors [105,106]. Of note neutral endopeptidase is reported to cleave Ang-(1–9) to Ang-(1–7), Ang III to Ang-(5–8), and Ang-(1–7) to Ang-(1–4) [43,107,108].

3.2.2. Thimet oligopeptidase and Neurolysin

Thimet oligopeptidase (EC 3.4.24.15, EP 24.15, thimet endopeptidase, Pz-peptidase, endo-oligopeptidase A) and neurolysin (EC 3.4.24.16, EP 24.16, "neurotensin-degrading enzyme") are members of the M3 family of Zn-dependent metalloendopeptidases ubiquitously distributed in the central nervous system and in peripheral organs of mammals [109,110]. Initially, both proteases were considered to be soluble enzymes, of which thimet oligopeptidase predominantly nuclear, while neurolysin was cytosolic. Later it was demonstrated that in rat brain 20–30% of thimet oligopeptidase activity is associated with membranes, including plasma membranes, endosomes and synaptic vesicles [94,111–114]. Moreover, Shivakumar et al. [95] recently showed that thimet oligopeptidase is associated with AT1 and B2 receptors in kidney cells, both at the plasma membrane and after receptor internalization, suggesting a possible mechanism for endosomal disposition of ligand that could facilitate receptor recycling. In the case of neurolysin, it has now been shown that the enzyme is mainly cytosolic in astrocytes, but is largely membrane-associated in neurons [115].

3.2.3. Prolyl endopeptidase

Despite being an endopeptidase, prolyl endopeptidase (3.4.21.26, prolyl oligopeptidase, post-proline cleaving enzyme), a member of the prolyl peptidase subfamily of serine proteases, can cleave Pro–Xaa peptide bonds (where Xaa is any amino acid) even when the Xaa amino acid is the omega amino acid of the peptide [116,117]. As such, it also has the ability to form Ang-(1–7) from both Ang II and Ang I, Ang-(2–7) from Ang III, and Ang-(3–7) from Ang IV [7,45,96,97,118,119]. Prolyl endopeptidase is an intracellular enzyme responsible for degradation of several peptide hormones and neuropeptides, which is highly conserved in mammals and is one of the most abundantly expressed brain peptidases [116,117,120,121].
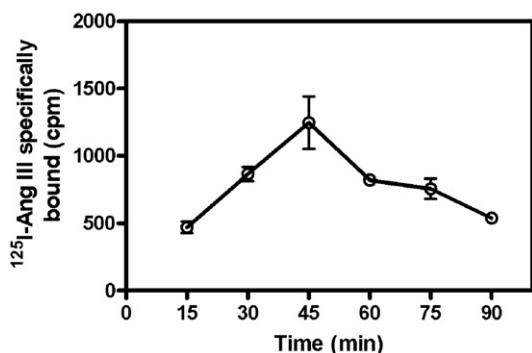
Fig. 2. Time course of specific binding of ^{125}I–Ang III to a rat brain membrane preparation. Rat brain membranes (50 mg initial wet weight/ml) were prepared as described previously [169]. The incubation medium contained standard assay buffer 150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, and 50 mM NaPO$_4$, pH 7.1–2, plus the following peptidase inhibitors: o-phenanthroline (1 mM), puromycin (3 mM) phenylmethylsulfonyl fluoride (1 mM). A total of 2.5 mg initial wet weight of brain membranes was present in 100 μl for this assay which was carried out at 21–24 °C. Nonspecific binding was determined in the presence of 3 μM Ang II and subtracted from total binding to derive specific binding.

3.2.4. Other endopeptidases

Cathepsin B and elastase (described above for their ability to form Ang I from angiotensinogen) have also been shown to be able to cleave the Val^3–Tyr^4 bond of Ang II, whereas cathepsin G cleaves the Tyr^4–Ile^5 bond [32,54] (Fig. 1). The various fragments formed by these endopeptidases are virtually devoid of biological activity.

3.3. Carboxypeptidases

3.3.1. ACE2 and ACE

As noted above, ACE2 can cleave Leu^{10} from Ang I, and Phe^8 from Ang II, to make Ang-(1−9) and Ang-(1−7) respectively, and thus is viewed primarily as an angiotensin-forming enzyme [43,46]. Interestingly the latter two peptides seem to not be metabolized by ACE2. Of note, Ang-(1−7) is a substrate for ACE, APN and DPPIII [85,90], whereas Ang-(1−9) is metabolized to Ang II by cathepsin A (described above in Section 2.4.) [7,45] (Fig. 1).

3.3.2. Prolyl carboxypeptidase

Prolyl carboxypeptidase (EC 3.4.16.2, angiotensinase C, peptidyl prolylamino acid hydrolase) is another carboxypeptidase capable of cleaving Ang II and Ang III at the Pro–Phe bond, to yield Ang-(l−7) and Ang-(2−7), respectively [45,120,122−125] (Fig. 1). It exists as membrane-associated and extracellular forms, that remove the omega amino acid from peptides when the penultimate amino acid is a proline [116,117]. It has an acidic pH optimum (pH = 5.0) when hydrolyzing short synthetic peptide substrates, but retains significant activity in the neutral range with longer, naturally occurring peptides (e.g. Ang II, Ang III, des–Arg^9–bradykinin) [122,124,126].

4. Protection of radiolabeled angiotensins in binding assays

After the discovery of the brain RAS, binding sites for angiotensin peptides in the brain were extensively studied using receptor binding techniques. The presence of angiotensin binding sites in the brain has been demonstrated in a variety of species [127–136] and subsequent in vitro autoradiographic studies have directly localized these binding sites to specific brain nuclei [137–141].

However, from the beginning all these studies faced a major pitfall — severe metabolic degradation of angiotensin peptides, which could and did negatively influence correct generation and interpretation of binding experiments. There were two major problems: 1) impossibility to reach steady-state conditions — the time course for binding of radiolabeled angiotensins has a bell shape (Fig. 2), and, 2) identification of different angiotensin fragments, along with intact ligands, bound specifically [133,134,142,143]. There have been three types of attempts to solve these problems: addition of protease inhibitors to the incubation medium, use of purified plasma membranes and development of peptidase-resistant analogs of angiotensins.

4.1. Peptidase inhibitors

Attempts to overcome the problem of metabolic degradation of angiotensin ligands during receptor binding studies have invariably included a variety of protease inhibitors. The most common of which include: sulfhydryl reagents (dithiothreitol, β-mercaptoethanol), chelating agents (EDTA, EGTA, o-phenan-throline), pure protease inhibitors (leupeptin, pepstatin, bacitra-cin, amastatin, bestatin, PMSF), unrelated peptides (glucagon, insulin, bovine serum albumin) [128,136,143,144]. Of note, addition of peptidase inhibitors does not always help to protect the ligand from degradation. One of the commonly used peptidase inhibitors in angiotensin receptor binding assays, bacitracin, is reported to activate aspartyl aminopeptidase (DAP) [67]. This would promote its conversion to Ang III, thus distorting the observations of binding kinetics. Moreover, dithiothreitol (DTT) and other disulfide reducing agents are capable of activating thimet oligopeptidase and DPPIII [90,145–147].

4.2. Subcellular fractionation

Virtually all brain angiotensin receptor binding assays have used a membrane fraction. In most cases this involves mechanical and osmotic disruption of the cells, centrifugal precipitation of the membrane fraction, with the soluble/cytosolic enzymes as well as the microsomal membrane fractions being discarded in the supernatant. On occasion, synaptosomal preparations have been used for receptor binding assays [142,148] to focus on receptors expressed on the extracellular side of the plasma membrane. However, additional steps must be taken to ensure that receptor–ligand internalization (receptor-mediated endocytosis) does not alter the binding kinetics and lead to subsequent degradation of receptor-associated angiotensin peptides [149].

4.3. Peptidase-resistant analogs

Initial studies of brain angiotensin receptors used ^{125}I–Ang II. However, subsequent studies using angiotensin analogs modified in the 1 and 8 position gave much better results

[140,143,144,150–152]. The most common amino terminal substitution is sarcosine (*N*-methyl glycine) for aspartic acid. Because sarcosine is a secondary amine, it resists degradation by aminopeptidases. The lack of a side-chain on the alpha carbon also contributes to its metabolic stability since it is a poor substrate for the acid aminopeptidases (APA, DAP) primarily responsible for removal of Asp[1] of Ang II [58,59,67]. The most common substitution to the carboxy terminal is the introduction of an alkyl side chain-containing amino acid, with isoleucine being the most often used amino acid. The substitution of the aromatic phenylalanine with an aliphatic amino acid converts the peptide to an antagonist [153,154], although there are reports suggesting that some agonistic properties of the peptide are retained [155].

The advantage of using antagonistic angiotensin peptides is that the primary angiotensin receptor subtypes: AT1 and AT2 are G protein-coupled receptors, which show different agonist binding affinity states depending on the presence of GTP or GDP [156]. Of note, while the characteristic agonist affinity shift for AT1 receptors has been demonstrated [157], the AT2 receptor does not show this shift [158], except in the presence of dithiothreitol [159]. The AT2 receptor also appears not to internalize [159,160]. However, it should be considered, that unnatural sequences in angiotensin peptide analogs may not fully represent agonist binding to angiotensin receptors.

4.4. Shortcomings of angiotensin protection in receptor binding assays

Although all the above-mentioned approaches have had a positive impact in preserving angiotensin ligands from degradation, none of them has been completely successful and metabolic degradation still continued [143,148,161] (Fig. 3). Moreover, both Grover et al. [148] and Abhold et al. [143] suggested that binding assays using angiotensin peptides should include a measurement of ligand metabolism, an identification of specifically bound radioactivity; and a correction of specific binding based on the amount of authentic radioligand bound.

However, it should be considered that, metabolism might occur post-binding, in which case radiolabeled metabolites may represent a portion of the specific binding of the radioligand. On the other hand, the suggested correction of the specific binding based on observed metabolism and identification of the specifically bound radioactivity is a technically challenging and cumbersome procedure. This might explain the paucity of reports that have addressed this problem, i.e. correction of the specific binding based on observed metabolism and identification of specifically bound radioactivity to fully determine the binding characteristics of angiotensin ligands in brain tissue.

It should also be noted that metabolic degradation can also affect the ability of non-radiolabeled angiotensin peptides (usually termed *cold*), used for estimation of the non-specific binding of a redioligand, to fully compete for specific binding sites. Determination of the affinity (expressed as IC_{50} or K_I values) of these non-radiolabeled angiotensin analogs for binding to brain angiotensin receptors can also be differentially affected by their susceptibility to metabolism by brain peptidases.

The most disappointing outcome of the practice of preserving angiotensin ligands from metabolic degradation in receptor binding assays is the case of sulfhydryl reducing agents, in particular DTT. Pioneering studies of Glossmann et al. [127], and Bennett and Snyder [128] indicated that the addition of DTT was required for protection of the angiotensin radioligands from metabolic degradation. Later it was shown that DTT had the ability to increase the binding affinity for Ang II in brain with no change in the density of the binding sites [132,136]. At that period nearly all angiotensin receptor binding studies conducted in brain were carried out in the presence of up to 5 mM DTT. Printz et al. [136] in their paper mention: "...*In studies in our laboratories there is no question but that thiols are essential for optimum binding of Ang II by membranes isolated from brain and adrenal medulla...*"

However, again starting from early studies, it was observed that Ang II binding sites in non-neuronal tissues such as vasculature [162], liver [163], and anterior pituitary [136] showed reduced Ang II binding in the presence of DTT. Consistent with this observation is the report by Ellis and Nuenke [145] that β-mercaptoethanol enhances the activity of pituitary DPP III, which converts Ang II to Ang IV. Moreover, activation of thimet oligopeptidase by DTT is also well documented [146,147].

This mystery continued until the late 80's–early 90's. During this time, sulfhydryl reducing agents continued to be widely used in angiotensin receptor binding assays in neuronal but not
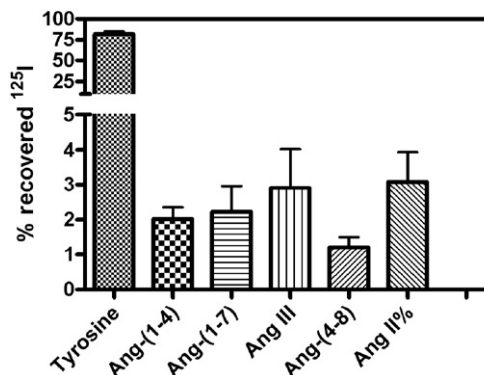

Fig. 3. Metabolic fate of ^{125}I–Ang II bound to a rat brain membrane preparation. Rat brain membranes were prepared as described previously [169]. The incubation medium contained 150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, and 50 mM NaPO$_4$, pH 7.1–2. A total of 12.5 mg initial wet weight of brain membranes was present in 500 μl for this assay, which was carried out for 60 min at 21–24 °C. After one hour incubation the membrane suspension was centrifuged and the supernatant discarded. The pellet was resuspended in HPLC mobile phase: 21% acetonitrile: 79% triethylamine phosphate (83 mM phosphate, pH 3.0), periodically vortexed during 20 min and recentrifuged. The supernatant was fitered through a 0.22 μm filter, applied to Sep-Pak® C18 (Waters Inc.) column and eluted with 21% acetonitrile: 79% triethylamine phosphate (83 mM phosphate, pH 3.0). The eluate was run on a reverse-phase (C$_{18}$) column with a mobile phase of either 13% acetonitrile: 87% triethylamine phosphate (83 mM phosphate, pH 3.0), to allow better resolution of smaller fragments, or 21% acetonitrile: 79% triethylamine phosphate (83 mM phosphate, pH 3.0) at a flow rate of 1.2 ml/min. Radiolabeled Ang II and fragments were identified based on the elution times of radioiodinated standards of Ang II and its fragments under the same HPLC conditions. 15 s fractions of the column eluate were collected and counted in a gamma counter.

peripheral tissues. The discovery of angiotensin receptor subtypes led to the revelation that sulfhydryl agents severely impair binding at AT1, but not at AT2 receptors [164–167]. Recognizing the fact that sulfhydryl reducing agents impaired binding of angiotensin ligands to AT1 receptors, Speth and co-workers [166] in a paper showing sulfhydryl agent-sensitive and non-sensitive (AT1 and AT2 containing) nuclei distribution in the rat brain noted: "… *An inescapable conclusion from these studies, however, is that the vast majority of Ang II receptor binding studies in brain homogenates, and a large number of in vitro receptor autoradiographic studies were carried out under conditions that should have effectively inhibited binding to the AIIα subtype* (today known as AT1). *Thus only a portion of receptors were characterized for responsivity to various conditions…*"

5. Future perspectives

It is clear that the metabolic pathways for angiotensins are complex and that we still have much to learn about the metabolic fate of the various angiotensin peptides in the brain. The relative importance of many of the pathways is unknown due to uncertainties related to their cellular localization, affinity for angiotensin peptides, their catalytic capacity, and their expression levels in the brain. Other factors that can affect the metabolic fate of angiotensins include developmental differences in enzyme expression, the presence of activators and inhibitors of these enzymes, and the redox state of the brain.

It is also apparent that claims of specificity for various enzyme inhibitors must be considered in the light of the expanse of enzymes capable of metabolizing angiotensin peptides. Defining an enzyme inhibitor as a specific inhibitor when only 2 or 3 enzyme preparations are tested is inappropriate because it ignores the possibility that other, untested enzymes could be affected. For example, enzyme inhibitors that interact with sulfhydryl groups are likely to have widespread effects on a large number of proteins, and not just enzymes, as has been noted with the AT1 receptor.

Another interesting development has been the observation that different angiotensin peptides can have opposing actions, e.g., the Ang II counteracting effects of Ang-(1–7) [12,102] and Ang-(2–10) [80], or complementary actions, e.g., the Ang II protecting effects of Ang IV by virtue of its ability to inhibit aminopeptidases [79]. It may be that other angiotensin fragments are also capable of indirectly interacting with the metabolic processes of angiotensin peptides.

Despite more than 30 years of research on brain angiotensin receptors, the problem of metabolic degradation of angiotensin ligands in brain Ang II receptor binding studies remains unsolved. None of the reported binding studies for angiotensin receptors are immune to the challenge that; 1) the radioligand did not remain intact throughout the incubation period, 2) the bound radioligand may not be representative of the binding of the initial radioligand or 3) the conditions under which the angiotensin peptide was protected from metabolism compromised the ability of the angiotensin receptors to bind angiotensins.

Correct characterization of the receptor binding kinetics of angiotensin peptides can be carried out only in conditions where peptide ligands as well as the receptors are preserved from metabolic degradation, at least until steady state conditions can be attained. However, at the same time, one needs to insure that the ability of angiotensin receptors to interact with ligands will not be impaired by procedures designed to protect ligand integrity.

Continuing studies of brain angiotensin receptors in our laboratory have focused on resolving this problem and are beginning to show some success[168]. However they have also provided some unanticipated results: in the presence of p-chloromurcuribenzoic acid (PCMB), a novel, non-AT1, non-AT2 binding site for angiotensin peptides can be observed in the brain[169]. However, PCMB, like DTT and β-mercaptoethanol inhibits Ang II binding to AT1 receptors, presumably by altering critical cysteine residues in the receptor. Thus it is necessary to continue to be vigilant to alterations in angiotensin receptor behavior while developing effective inhibitors of the peptidases that metabolize angiotensins.

Acknowledgments

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