Brain AT₁ Angiotensin Receptor Subtype Binding: Importance of Peptidase Inhibition for Identification of Angiotensin II as Its Endogenous Ligand

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Abstract

The existence and localization of brain angiotensin receptors is well established. However, questions regarding the endogenous ligand for brain angiotensin type 1 (AT₁) receptors necessitates re-examination of brain angiotensin receptor binding studies. To assess the ability of angiotensin II to bind to the brain AT₁ receptor, radioligand binding studies of rat brain AT₁ receptors were performed using both [¹²⁵I]-angiotensin II and [¹²⁵I]-sarcosine¹, isoleucine⁸ angiotensin II. Determination of binding kinetics and competition by an AT₁ receptor antagonist was carried out to reveal the identity of the membrane binding sites and to identify the bound [¹²⁵I]-labeled molecules. Initial analysis of [¹²⁵I]-angiotensin II binding to hypothalamic membranes using an established protocol revealed that a negligible amount of intact radioligand was bound to the membranes. In contrast, binding of [¹²⁵I]-sarcosine¹, isoleucine⁸ angiotensin II was saturable, of high affinity, and primarily as intact radioligand. Sequential addition of four peptidase inhibitors—o-phenanthroline, puromycin, phenylmethylsulfonyl fluoride, and glutamate phosphonate—to the assay buffer dramatically increased the binding of [¹²⁵I]-angiotensin II to rat brain membranes: more than 75% of the bound [¹²⁵I] was the intact radioligand, and the binding was of high affinity and saturable. Some, but not all, of the binding could be displaced by the AT₁-selective antagonist losartan. This demonstrates that angiotensin II can bind to brain AT₁ receptors and does not require conversion to [¹²⁵I]-angiotensin III to bind to brain AT₁ receptors.

After the discovery of the brain renin-angiotensin system (Ganten et al., 1971), angiotensin (Ang) II binding sites in rat (Glossmann et al., 1974) and bovine brains were observed (Bennett and Snyder, 1976). Angiotensin receptors occur in the brains of other species (Harding et al., 1981; Speth et al., 1985; Allen et al., 1987), including human (Allen et al., 1987). In vitro autoradiographic studies have localized angiotensin receptor binding sites to specific brain nuclei (Mendelsohn et al., 1984; Speth et al., 1985). Both AT₁ and AT₂ subtypes occur in the brain (Rowe et al., 1990). However, these studies faced a major pitfall—metabolic degradation of the radiolabeled angiotensin, which precludes accurate measurement of binding assays (Karamyan and Speth, 2007).

There were two major problems: 1) inability to reach steady-state conditions—the time course for binding of radioligand was bell-shaped; and 2) angiotensin fragments, along with intact radioligand, bound specifically (Harding et al., 1986; Abhold et al., 1987; Abhold and Harding, 1988). The reduction of [¹²⁵I]-Ang III binding when its degradation to [¹²⁵I]-des Asp¹,des Arg² Ang II ([¹²⁵I]-Ang IV) was inhibited contributed to the discovery of a novel binding site for Ang IV (Harding et al., 1992; de Gasparo et al., 2000) now known to be insulin-regulated aminopeptidase (Albiston et al., 2001).

Approaches used to overcome metabolic degradation of angiotensins during binding studies included 1) addition of protease inhibitors to the incubate, e.g., sulphydryl reagents (dithiothreitol and β-mercaptoethanol), chelating agents

ABBREVIATIONS: Ang, angiotensin; SI Ang II, sarcosine¹, isoleucine⁸ angiotensin II; AT₁, angiotensin receptor type 1; AT₂, angiotensin receptor type 2; PD123319, S-(+)-1-(4-(dimethylamino)-3-methylphenyl)[methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo(4,5-c)pyridine-6-carboxylic acid; GluP, glutamate phosphonate (4-amino-4-phosphonobutyric acid); JA-2, N-[1-(R,S)-carboxy-3-phenylpropyl]-Ala-Alb-Tyr-p-aminobenzoate; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride.
Angiotensin II Binds to Brain AT₁ Receptors

Materials and Methods

Adult Sprague-Dawley rats (Harlan, Indianapolis, IN), weighing 300 to 350 g, were used for this study. Rats were maintained under a 12-h light/dark cycle and fed ad libitum. The protocol for these experiments was approved by the University of Mississippi Institutional Animal Care and Use Committee.

Ang II and SI Ang II were obtained from Phoenix Pharmaceuticals (Belmont, CA). Fragments of the angiotensins were purchased commercially or synthesized by Dr. Gerhard Munske (Washington State University, Pullman, WA). AT₂ receptor antagonist PD123319 was purchased from Tocris Bioscience (Ellisville, MO), and AT₁ receptor antagonist losartan was from Sigma-Aldrich (St. Louis, MO). Glutamate phosphate (4-amino-4-phenonybutyric acid; GluP) was synthesized in house or by Thesys Chemistry (Mentor, OH). In brief, the in-house procedure was as follows: the GluP precursor ester methyl-3-(ethoxyphosphono)-3-aminopropanoate was synthesized in three steps from readily available ethyl-4-chloro-4-oxobutyrate (Kowalik et al., 1981) and subsequently treated with 9 N HCl under reflux for 20 h. The product was precipitated with methanol/propanoic acid 1:1, isoleucine 1 analog (Marshall et al., 1970) may not fully reproduce agonist binding of natural sequences to angiotensin receptors. Sarcosine (N-methyl glycine) is devoid of a side chain in contrast to the bulky alkyl carboxylate moiety of aspartic acid. Thus, sarcosine 1 Ang II may better mimic Ang III than Ang II (Wright et al., 1989). This takes on great importance considering the ongoing debate regarding the importance considering the ongoing debate regarding the role of angiotensin II versus the heptapeptide Ang III (Harding and Felix, 1987; Zini et al., 1996; Kokje et al., 2007; Bodineau et al., 2008; Speth and Karamyan, 2008). Moreover, if only Ang III can activate brain AT₁ receptors (the angiotensin III receptor), then Ang II either does not bind to AT₁ receptors in the brain, or it binds to the brain AT₁ receptors without causing a response (Kokje et al., 2007; Speth and Karamyan, 2008).

Because receptor binding studies require that ligand and receptor metabolism does not occur (Bennett, 1978), binding assays using 125I-Ang II must document that specifically bound radioligand is unchanged. If the 125I-Ang II is metabolized to 125I-Ang III, this could support the hypothesis that Ang III is the active angiotensin in the brain (Abhold and Harding, 1988; Bodineau et al., 2008).

The purpose of this study was 2-fold: first, to establish experimental conditions using protease inhibitors to protect 125I-Ang II from metabolic degradation; and second, to determine whether Ang II must be converted to Ang III to bind to rat brain AT₁ receptors. Metabolic studies determined the effectiveness of the protease inhibitors in protecting 125I-Ang II from degradation during the binding assays. Dithiothreitol and other sulphydryl-reducing agents previously used to inhibit 125I-Ang II from degradation (Glossmann et al., 1974; Bennett and Snyder, 1976) were not used because they impair AT₁ receptor binding (Chiu et al., 1989; Speth et al., 1991).

Binding of radioiodinated Ang II to the brain AT₁ receptor was compared with that of sarcosine 1, isoleucine 1 Ang II (SI Ang II), considered to be the standard for angiotensin receptor binding studies. In addition, the binding of radioligands to the liver AT₁ receptor assessed possible differences in their affinities and possible negative effects of protease inhibitors.

(EDTA, EGTA, and o-phenanthroline), pure protease inhibitors (leupeptin, pepstatin, bacitracin, amastatin, bestatin, and phenylmethylsulfonyl fluoride), and unrelated peptides (glucagon, insulin, and bovine serum albumin) (Glossmann et al., 1974; Bennett and Snyder, 1976; Abhold et al., 1987; Abhold and Harding, 1988) 2) use of purified plasma membranes to avoid receptor-mediated endocytosis (Harding et al., 1986); and 3) development of peptidase-resistant antagonist analogs of angiotensins, e.g., sarcosine 1 substituted for aspartic acid 1 to provide metabolic stability against acid aminopeptidases (Regoli et al., 1974).

These approaches partly protected angiotensin ligands from degradation; however, none of the approaches alone or in combination has been completely successful (Regoli et al., 1974; Abhold et al., 1987; Karamyan and Speth, 2007). Moreover, studies of the identity of brain membrane-bound 125I-Ang II derived from 125I-Ang II predated the discovery of multiple Ang II receptor subtypes (Chiu et al., 1989; Whitebread et al., 2008; Speth and Karamyan, 2008). Moreover, if only Ang III is important considering the ongoing debate regarding the effectiveness of the protease inhibitors in protecting 125I-Ang II from degradation; and second, to determine whether Ang II must be converted to Ang III to bind to rat brain AT₁ receptors. Metabolic studies determined the effectiveness of the protease inhibitors in protecting 125I-Ang II from degradation during the binding assays. Dithiothreitol and other sulphydryl-reducing agents previously used to inhibit 125I-Ang II from degradation (Glossmann et al., 1974; Bennett and Snyder, 1976) were not used because they impair AT₁ receptor binding (Chiu et al., 1989; Speth et al., 1991).

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Thus, it is not known whether the intact 125I-Ang II bound to the AT₁ receptor, the AT₂ receptor, or both. An additional concern is that the sarcosine 1 analog or the isoleucine 1 analog (Marshall et al., 1970) may not fully reproduce agonist binding of natural sequences to angiotensin receptors. Sarcosine (N-methyl glycine) is devoid of a side chain in contrast to the bulky alkyl carboxylate moiety of aspartic acid. Thus, sarcosine 1 Ang II may better mimic Ang III than Ang II (Wright et al., 1989). This takes on great importance considering the ongoing debate regarding the role of angiotensin II versus the heptapeptide Ang III (Harding and Felix, 1987; Zini et al., 1996; Kokje et al., 2007; Bodineau et al., 2008; Speth and Karamyan, 2008). Moreover, if only Ang III can activate brain AT₁ receptors (the angiotensin III receptor), then Ang II either does not bind to AT₁ receptors in the brain, or it binds to the brain AT₁ receptors without causing a response (Kokje et al., 2007; Speth and Karamyan, 2008).

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Saturation binding studies were carried out by incubation of 50 μl of membrane preparation with six concentrations of 125I-Ang II (0.3–6 nM) and 125I-SI Ang II (0.25–3 nM) in 100 μl of total assay volume for 1 or 2 h at 24°C, respectively. The incubation times are based on time course analyses indicating maximal specific binding at 60 min for 125I-Ang II and near-equilibrium binding at 2 h for 125I-SI Ang II in hypothalamic membranes (data not shown). Nonspecific binding was estimated in the presence of 3 μM SI Ang II for 125I-Ang II or 3 μM Ang II for 125I-SI Ang II. Values shown represent specific (total minus nonspecific) binding.
hypothalamic or liver membrane suspensions (equal to 12.5 and 5 mg initial wet weight, respectively) in 500 μl of assay buffer were incubated with ~1 nM ^125^I-Ang II or ^125^I-SI Ang II at 24°C for 1 or 2 h, respectively. Incubation was stopped by centrifugation at ~13,000 g for 3 min, and the supernatant was discarded. The pellet was resuspended in HPLC mobile phase: 21% acetonitrile/79% triethylamine phosphate (83 mM phosphate, pH 3.0), periodically vortexed during a 20-min extraction period, and recentrifuged. The supernatant was filtered through a 0.22-μm filter, applied to Sep-Pak (Waters, Milford, MA) C18 column, and eluted with 21% acetonitrile/79% triethylamine phosphate (83 mM phosphate, pH 3.0). The eluate (~99% recovered radioactivity) was evaluated by HPLC using a reverse-phase (C18) column with a mobile phase of either 13% acetonitrile/87% triethylamine phosphate (83 mM phosphate, pH 3.0), to resolve 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, SI Ang II, and fragments were identified based on the elution times of radioiodinated angiotensin fragment standards as monitored using a flow-through radioiodine detector (model 170; Beckman Coulter, Fullerton, CA) under the same HPLC conditions. Fifteen-second fractions of the column eluates were collected and counted in a γ-counter to calculate the ratio of peaks corresponding to angiotensins and fragments relative to the total amount of radioactivity applied for HPLC analysis. Values reported are mean percent ± S.E.M.

**Results**

The identity of the ^125^I-labeled material bound to hypothalamic and liver membranes with incubation of ^125^I-Ang II and ^125^I-SI Ang II in the initial assay buffer, with no additional protease inhibitors, after 1- and 2-h incubation at 24°C, respectively, is summarized in Table 1. In the hypothalamic membranes, more than 80% of the bound ^125^I-derived from ^125^I-Ang II was ([125^I]tyrosine). Conversely, ^125^I-SI Ang II remained as substantially (56.5%) intact bound radioligand in hypothalamic membranes. Binding characteristics (K_d and B_max values) of ^125^I-Ang II and ^125^I-SI Ang II to AT_1 receptors under the same conditions are summarized in Table 2. The profile of the membrane-bound fragments and the specific binding of ^125^I-Ang II is shown in Fig. 1, a and a’). The calculated B_max and K_d values for ^125^I-Ang II (represented by the dotted line in Fig. 1a’) were not statistically significantly different from zero and are not reported.

The binding and metabolism of ^125^I-Ang II and ^125^I-SI Ang II in the liver in the initial assay buffer were profoundly different from the hypothalamic membrane preparation. Both ^125^I-Ang II and ^125^I-SI Ang II binding were predominantly represented by the intact radioligand with minimal (^125^I-SI Ang II) or moderate (^125^I-Ang II) metabolism to (^125^I)tyrosine (Table 1). Both of these radioligands exhibited abundant, high-affinity, specific binding to liver membrane preparations (Table 2). It is noteworthy that the K_d value of ^125^I-SI Ang II was significantly less than that of ^125^I-Ang II, indicating that ^125^I-SI Ang II binds to liver AT_1 receptors with higher affinity than endogenous Ang II when it is radiolabeled.

The addition of 1 mM o-phenanthroline and 3 mM puromycin dramatically improved the specific binding of ^125^I-Ang II (Fig. 1b’) to the hypothalamic membrane preparation. The metabolic profile was also markedly changed (Fig. 1b). The binding was now predominantly made up of ^125^I-Ang III (26.3 ± 1.5%, the des Asp^3^ des Phe^8^ hexapeptide [Ang (2-7)] fragment (31.1 ± 0.6%), and intact ^125^I-Ang II (19.7 ± 3.0%). The B_max value was substantially increased, whereas the K_d value (1.9 nM) revealed a high-affinity specific binding (Fig. 1b’).

The effects of puromycin and o-phenanthroline on ^125^I-Ang II metabolism were not determined individually in this study. However, a previous study (Saylor et al., 1993) reported that the addition of o-phenanthroline plus bovine serum albumin to assay buffer protected unbound ^125^I-SI Ang II from metabolic degradation and substantially increased ^125^I-SI Ang II binding to brain AT_1 receptors on slide-mounted sections of rat brain. Moreover, the superiority of EDTA plus o-phenanthroline against either of the inhibitors alone in protecting ^125^I-Ang IV and increasing its binding affinity also has been convincingly demonstrated (Laeremans et al., 2005).

The addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) to the hypothalamic membrane preparation along with puromycin and o-phenanthroline further increased the specific binding of ^125^I-Ang II (Fig. 1c’). It also blocked the formation of the des Asp^4^, des Phe^8^ hexapeptide [Ang (2-7)] metabolite of ^125^I-Ang II. In the presence of these three added peptidase inhibitors, ^125^I-Ang III made up 57.7 ± 2.7% of the binding to hypothalamic membranes, whereas ^125^I-Ang II made up 27.5 ± 3.5% (Fig. 1c).

Addition of the aminopeptidase A inhibitor glutamate phosphonate (Lejczak et al., 1993; Vazeux et al., 1997) to the hypothalamic membrane preparation along with puromycin, o-phenanthroline, and PMSF did not increase specific binding of ^125^I-Ang II or change its K_d value (Fig. 1d’). However, it substantially increased the proportion of intact ^125^I-Ang II bound to the hypothalamic membranes to 75.6 ± 3.2%, while simultaneously reducing the proportion of ^125^I-Ang III bound to the hypothalamic membranes to 10.8 ± 1.3% (Table 3; Fig. 1d). Addition of the four peptidase inhibitors to liver membrane preparations improved the protection of ^125^I-Ang II and ^125^I-SI Ang II from metabolic degradation to more than 95% (Table 3).

**TABLE 1**

<table>
<thead>
<tr>
<th>Metabolism of ^125^I-Ang II and ^125^I-SI Ang II bound to rat hypothalamic and liver membrane preparations in assay buffer after 1- and 2-h incubation at 24°C, respectively</th>
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</thead>
<tbody>
<tr>
<td>Data are mean ± S.E.M., n = 3.</td>
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<tr>
<td><strong>Tyre</strong></td>
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<tr>
<td>Hypothalamic membranes</td>
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<tr>
<td>^125^I-SI Ang II</td>
</tr>
<tr>
<td>Liver membranes</td>
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<tr>
<td>^125^I-SI Ang II</td>
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</table>
TABLE 2

Binding characteristics of 125I-Ang II and 125I-SI Ang II to rat hypothalamic and liver AT$_1$ receptors in the presence and absence of additional protease inhibitors after 1-and 2-h incubation at 24°C, respectively.

Data are mean ± S.E.M., n = 3 to 6.

<table>
<thead>
<tr>
<th></th>
<th>Hypothalamic Membranes</th>
<th>Hypothalamic Membranes + Inhibitors</th>
<th>Liver Membranes</th>
<th>Liver Membranes + Inhibitors</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ ($nM$)</td>
<td>$B_{max}$ ($fmol/mg$ wet wt)</td>
<td>$K_d$ ($nM$)</td>
<td>$B_{max}$ ($fmol/mg$ wet wt)</td>
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<tr>
<td>125I-Ang II</td>
<td>—</td>
<td>—</td>
<td>3.17 ± 0.47</td>
<td>0.34 ± 0.45</td>
</tr>
<tr>
<td>125I-SI Ang II</td>
<td>0.55 ± 0.13</td>
<td>0.54 ± 0.09</td>
<td>0.93 ± 0.37*</td>
<td>0.37 ± 0.13</td>
</tr>
</tbody>
</table>

* Significantly different from 125I-Ang II ($p < 0.05$).

** In some groups of animals $B_{max}$ values of 125I-Ang II and 125I-SI Ang II binding in liver membranes were substantially higher (15-21 fmol/mg wet wt) compared with the values reported in Table 2.

Specific binding is not significantly different from zero.

Comparison of 125I-Ang II and 125I-SI Ang II binding to hypothalomic membranes in the presence of the four additional protease inhibitors and PD123319 (specific AT$_2$ receptor antagonist) revealed that the $B_{max}$ for AT$_1$ receptors in the hypothalamus was not significantly different between the radioligands (Table 2; Fig. 2). Under the same conditions, the $K_d$ value for 125I-SI Ang II was significantly less than that of 125I-Ang II, indicating that 125I-SI Ang II binds to hypothalomic AT$_1$ receptors with a higher affinity than the radiodinated endogenous ligand.

The $B_{max}$ values for binding of 125I-Ang II and 125I-SI Ang II to the liver membrane preparation did not differ. The $K_d$ value of 125I-SI Ang II for liver membranes was again significantly less than the $K_d$ value of 125I-Ang II (Table 2; Fig. 2).

To further confirm that the estimated specific binding of 125I-Ang II and 125I-SI Ang II in hypothalomic membranes was to the AT$_1$ receptor, the observed binding (in the presence of four additional protease inhibitors and PD123319) was compared with specific binding of radioligands under the same experimental conditions plus 10 μM losartan (specific AT$_1$ receptor antagonist). This comparison revealed that only a part of the specific binding of 125I-Ang II (~38% of total specific binding; $B_{max}$ = 0.19 ± 0.03 fmol/mg wet weight; $K_d$ = 2.0 ± 0.31 nM) and 125I-SI Ang II (~33% of total specific binding; $B_{max}$ = 0.25 ± 0.01 fmol/mg wet weight; $K_d$ = 0.3 ± 0.08 nM) in hypothalomic membranes was losartan displaceable, i.e., AT$_1$ receptor binding (Fig. 3, a and b).

In the liver, in the presence of all protease inhibitors, virtually all specific binding of 125I-Ang II and 125I-SI Ang II was losartan displaceable (Fig. 3, c and d). Several other peptidase inhibitors and amino acids were tested for their ability to protect 125I-Ang II from metabolic degradation (Table 4). None of these inhibitors improved the binding of 125I-Ang II to hypothalomic membranes beyond that seen with the initial assay buffer at the concentrations used.

**Discussion**

Binding and metabolism experiments were carried out with 125I-Ang II and 125I-SI Ang II in rat brain hypothalomic membranes, with a goal of developing an assay protocol ensuring protection of the radioligands with no impairment of binding to the AT$_1$ receptor. Initial and final (in the presence of additional protease inhibitors) assay conditions also were used to estimate metabolism and binding of the radioligands in liver membranes.

Under initial experimental conditions (assay buffer with no additional protease inhibitors) virtually all of the bound radioactivity derived from 125I-Ang II in hypothalomic membranes consisted of fragments of the peptides, primarily free 125I-Tyr (Table 1). Notably, under these assay conditions, there was no significant saturable binding of 125I-Ang II in hypothalomic membranes. In contrast, more than half of the bound radioligand arising from 125I-SI Ang II was intact 125I-SI Ang II, and the binding displayed a high affinity and saturability as seen in previous studies (Rowe et al., 1992; Speth, 2003). Also noteworthy was the stability of 125I-Ang II in the liver membrane preparation in which the majority of the bound 125I was intact 125I-Ang II, and the binding displayed high affinity and saturability equivalent to that seen for 125I-SI Ang II.

Thus, it is clear that the hypothalomic membrane preparation contains peptidases that effectively metabolize 125I-Ang II. As noted previously (Bennett, 1978), determination of binding kinetics requires that the radioligand remain intact throughout the binding assay. If this criterion cannot be met, the kinetics of the binding cannot be reliably determined, nor can the identity of the bound radioligand be established. As the extent of degradation of the radioligand increases, the error also will increase.

Addition of four peptidase inhibitors—puromycin, o-phenanthroline, PMSF, and glutamate phosphonate—dramatically reduced the metabolic degradation of 125I-Ang II such that more than 75% of the 125I bound to the hypothalomic membranes was intact radioligand. Associated with this improved preservation of the radioligand was the ability to demonstrate high-affinity binding of 125I-Ang II to the hypothalomic membranes in amounts that were not significantly different from those determined using 125I-SI Ang II (Table 3). These four peptidase inhibitors also increased the proportion of intact radioligands bound to liver membranes (Table 3).

Losartan displacement studies revealed that only a part of 125I-Ang II-specific binding to hypothalomic membranes was accounted for by the AT$_1$ receptor, i.e., was losartan-displaceable ($B_{max}$ = 0.19 ± 0.03 fmol/mg wet weight; $K_d$ = 2.0 ± 0.31 nM; n = 3) (Fig. 3a). This phenomenon was true for 125I-SI Ang II as well (Fig. 3b), which bound to a similar number of AT$_1$ receptors ($B_{max}$ = 0.25 ± 0.01 fmol/mg wet weight) but, as was expected, with higher affinity ($K_d$ = 0.3 ± 0.08 nM). Notably, a portion of specific binding of both 125I-Ang II and 125I-SI Ang II in hypothalomic membranes was losartannondisplaceable (Fig. 3, a and b). The observed losartannondisplaceable binding probably arose from degradation products of radioligands carrying 125I, which apparently did...
not bind to the AT$_1$ receptor. Consistent with this assumption in the presence of additional protease inhibitors only 76% $^{125}$I-Ang II and 79% $^{125}$I-SI Ang II are intact after incubation with hypothalamic membranes (Table 3). In contrast, virtually all specific binding of $^{125}$I-Ang II and $^{125}$I-SI Ang II in the liver was to the AT$_1$ receptor, i.e., losartan displacable (Fig. 3, c and d), where more than 95% of both ligands are protected from proteolytic degradation (Table 3).

The results of this study clearly indicate that $^{125}$I-Ang II binds to the brain AT$_1$ receptor. This binding is equivalent to the binding of $^{125}$I-SI Ang II, a synthetic peptide ligand (antagonist), considered as a standard for studies of angiotensin receptors. This observation has relevance to questions that have arisen regarding the identity of the active angiotensin in the brain (Harding and Felix, 1987; Zini et al., 1996). These authors postulated that Ang II must be converted to Ang III in the brain to activate brain Ang II receptors, now known to be the AT$_1$ receptor subtype. Also see recent reviews by Bodineau et al. (2008) and Speth and Karamyan (2008). If this supposition is correct, it would suggest two unlikely scenarios governing the interaction of Ang II with the brain AT$_1$ receptor: 1) Ang II does not bind to the brain AT$_1$ receptor; or 2) Ang II is an antagonist of the brain AT$_1$ receptor, or is at least a partial antagonist with respect to the pressor actions mediated by brain AT$_1$ receptors. The results of this study indicate that Ang II can bind to

Fig. 1. Metabolic fate ($n = 3$) and representative binding of $^{125}$I-Ang II to AT$_1$ receptors in hypothalamic membranes (1-h incubation at 24°C): a and a’, in the initial assay buffer with no additional protease inhibitors ($B_{max}$ and $K_d$ values were not significantly different from zero); b and b’, in the initial assay buffer with 1 mM o-phenanthroline (OPh) and 3 mM puromycin (PR); c and c’, in the initial assay buffer with 1 mM OPh, 3 mM PR, and 1 mM PMSF; and d and d’, in the initial assay buffer with 1 mM OPh, 3 mM PR, 1 mM PMSF, and 3 mM GluP.
TABLE 3
Metabolism of $^{125}$I-Ang II and $^{125}$I-SI Ang II bound to rat hypothalamic and liver membrane preparations in assay buffer with additional protease inhibitors (see Materials and Methods)
Data are mean ± S.E.M., n = 3.

<table>
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<tr>
<th></th>
<th>Tyr</th>
<th>Ang (1-4) or S-Ang (1-4)</th>
<th>Ang (4-7)</th>
<th>Ang (1-7)</th>
<th>Ang (2-7)</th>
<th>Ang (4-8)</th>
<th>Ang III</th>
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<td>Hypothalamic membranes</td>
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<tr>
<td>$^{125}$I-Ang II</td>
<td>1.37 ± 0.19</td>
<td>2.8 ± 0.75</td>
<td>4.1 ± 1.6</td>
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<td>10.8 ± 1.3</td>
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<td>78.8 ± 1.1</td>
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<td>Liver membranes</td>
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<td>$^{125}$I-Ang II</td>
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Fig. 2. Representative saturation binding analyses of $^{125}$I-Ang II and $^{125}$I-SI Ang II to AT$_1$ receptors in hypothalamic (a) and liver (b) membranes in the assay buffer with 1 mM o-phenanthroline, 3 mM puromycin, 1 mM phenylmethylsulfonyl fluoride, and 3 mM glutamate phosphonate (60- and 120-min incubation at 24°C, respectively). In hypothalamic membranes, $B_{max} = 2.53 ± 0.02$ fmol/mg wet weight, $K_d = 2.6 ± 0.58$ nM for $^{125}$I-Ang II; $B_{max} = 0.26 ± 0.03$ fmol/mg wet weight, $K_d = 0.78 ± 0.25$ nM for $^{125}$I-SI Ang II. In liver membranes, $B_{max} = 8.1 ± 0.17$ fmol/mg wet weight, $K_d = 1.6 ± 0.06$ nM for $^{125}$I-Ang II; $B_{max} = 7.1 ± 0.11$ fmol/mg wet weight, $K_d = 0.17 ± 0.01$ nM for $^{125}$I-SI Ang II.

the brain AT$_1$ receptor with high affinity, which refutes the first of these two unlikely scenarios.

Previous work from this laboratory (Kokje et al., 2007) using aminopeptidase-resistant analogs of Ang II included an experiment in which a $^{125}$I-labeled aminopeptidase-resistant Ang II analog, N-methyl l-Asp$^1$ Ang II, was administered intracerebroventricularly to rats. This peptide caused short-latency pressor and dipsogenic responses at a time when there was no discernible formation of $^{125}$I-$^{127}$I-Ang III. Other aminopeptidase-resistant analogs of Ang II also showed agonistic properties with potencies equal to or greater than Ang II, with latencies equal to or shorter than Ang II (Kokje et al., 2007). This argues strongly against the second unlikely scenario, that Ang II could be an antagonist of the brain AT$_1$ receptor.

It is noteworthy that $^{125}$I-Ang III was the major component of the bound radioligand when $^{125}$I-Ang II was incubated with hypothalamic membranes in the presence of puromycin, o-phenanthroline and PMSF but was a minor component of the bound radioligand with the further addition of glutamate phosphonate. This suggests that there is abundant aminopeptidase A activity in hypothalamic membrane preparations that escapes inhibition by the other peptidase inhibitors present in the assay buffer but which is susceptible to glutamate phosphonate at the concentration used. It is also possible that the effect of glutamate phosphonate could be due to inhibition of other acid aminopeptidases that have been reported to exist in neuronal tissues (Kelly et al., 1983; Speth and Karamyan, 2008) in view of the failure of the aminopeptidase inhibitors amastatin and bestatin to improve $^{125}$I-Ang II binding to these hypothalamic membrane preparations. The high proportion of $^{125}$I-Ang III binding that occurred with the addition of puromycin, o-phenanthroline, and PMSF suggests that one or more of these inhibitors was effective in blocking carboxypeptidase- and endopeptidase-mediated metabolism of $^{125}$I-Ang II and $^{125}$I-Ang III as well as aminopeptidase-mediated metabolism of $^{125}$I-Ang III.

The question of whether the rat brain AT$_1$ receptor differs from that in the periphery (for these studies, the liver was used as a representative peripheral AT$_1$ receptor) was not fully resolved by these studies. The $K_d$ values of both $^{125}$I-Ang II and $^{125}$I-SI Ang II tend to be lower in liver compared with brain; however, there is a possibility that the higher rate of degradation of these radioligands in the brain could explain this discrepancy. Another interesting possibility is that Ang II and Ang III might have different profiles of action, e.g., biased agonism (Violin and Lefkowitz, 2007) upon binding to the brain AT$_1$ receptor, which could explain discrepancies in the reported actions of angiotensins II and III in the brain.

In summary, the results of this study provide an abundance of novel and important information about the brain AT$_1$ receptors of the rat: as might be anticipated, rat brain AT$_1$ receptors bind $^{125}$I-Ang II as well as $^{125}$I-SI Ang II with high affinity in equivalent amounts. It is necessary to use an array of peptidase inhibitors to protect $^{125}$I-Ang II and to a lesser extent, $^{125}$I-SI Ang II from metabolic degradation by rat brain peptidases during radioligand binding assays.
the first time in brain angiotensin receptor binding studies, AT₁ receptor-specific binding of ¹²⁵I-Ang II was assessed as AT₁ receptor antagonist-displaceable specific binding. To date, this provides the most accurate estimation of AT₁ receptor density in rat hypothalamic membranes by virtue of establishing conditions whereby the majority of radioligand is protected from proteolytic degradation.

Overall, these results indicate that Ang II is an agonist for the rat brain AT₁ receptor akin to its characterization as an agonist for the AT₁ receptor in peripheral tissues and does not require prior conversion to Ang III to interact with the AT₁ receptor.

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References


Angiotensin II Binds to Brain AT1, Receptors


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