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Preliminary biochemical characterization of the novel, non-AT1, non-AT2 angiotensin binding site from the rat brain

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Abstract

A novel binding site for angiotensins II and III was recently discovered in brain membranes in the presence of the sulfhydryl reactive angiotensinase inhibitor parachloromercuribenzoate. This binding site is distinctly different from the other known receptors for angiotensins: AT_1, AT_2, AT_4, and mas oncogene protein (Ang 1-7 receptor). Preliminary biochemical characterization studies have been done on this protein by crosslinking it with ^{125}I-labeled photoaffinity probes and solubilizing the radiolabeled binding site. Polyacrylamide gel electrophoresis studies and isoelectric focusing indicate that this membrane bound binding site is a protein with a molecular weight of 70–85 kDa and an isoelectric point of ~7. Cyanogen bromide hydrolysis of the protein yielded two radiolabeled fragments of 12.5 and 25 kDa. The protein does not appear to be N-glycosylated based upon the failure of PNGaseF to alter its migration rate on a 7.5% polyacrylamide gel. The binding of angiotensin II to this protein is not affected by GTPγS or Gpp(NH)p, suggesting that it is not a G protein-coupled receptor. Further characterization studies are directed to identify this protein either as a novel angiotensin receptor, an angiotensin scavenger (clearance receptor) or an angiotensinase.

Keywords

Angiotensin II binding; Brain; Parachloromercuribenzoic acid; ^{125}I-angiotensin II analogs; Photaffinity crosslinking; Protein purification; Gel electrophoresis

Introduction

The brain renin-angiotensin system (RAS) is most studied and known for its role in cardiovascular and hydromineral balance regulation [1–5]. While this function of the brain

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RAS is extensively studied, less is known about other possible roles of this system [6–8]. Nevertheless, it is a well-established fact that a number of central components of the brain RAS are abundantly localized not only in brain areas involved in the above-mentioned functions, but also in areas playing roles in cognition, analysis of new information, memory, reward, and addiction, etc. [9–18]. This simple fact suggests that our current understanding of the nature and function of the brain RAS is incomplete, and that future discoveries will expand our knowledge of the functions of the brain RAS.

Recently, we discovered a novel, high affinity angiotensin binding site in rat, mouse, and human brain membranes [19–21]. This novel binding site has nanomolar affinity for angiotensin (Ang) II and Ang III, and is different from classical type 1 (AT1) and type 2 (AT2) angiotensin receptors as it is insensitive to blockade by specific angiotensin receptor antagonists, and is present in mouse brains deficient in AT1 and AT2 receptors [19,20]. The novel angiotensin binding site is also abundantly distributed throughout the rat brain [22] and has been shown to be present in abundance in the testis as well as several other peripheral tissues [23] suggesting that it may have many yet 3 unrecognized function(s).

The identity of the novel angiotensin protein is still unknown and efforts are being made to identify this protein. Here, we report the results of our recent studies on isolation and preliminary biochemical characterization of this novel angiotensin binding site from the rat brain.

Results

Binding of radioiodinated angiotensin photoprobes SBpa-Ang II and azido-Ang II to the novel angiotensin binding site in the rat forebrain membranes was compared with that of ^{125}I-SI-Ang II binding (Fig. 1). Both photoprobes were found to have high affinity for the novel angiotensin binding site with maximal binding densities slightly less than that of ^{125}I-SI Ang II.

Figure 2 represents photolabeling of the novel angiotensin binding site with ^{125}I-azido-Ang II and approximation of its molecular weight using SDS-PAGE analysis. Optimal photolabeling conditions were worked out beforehand; it was found out that 5 min UV exposure (Rayonet Photochemical Reactor) of membrane suspensions (plus blockers and PCMB) and ^{125}I-azido-Ang II gave reproducible and un-dissociable crosslinking (data not shown).

Photocrosslinking and SDS-PAGE separation experiments were also carried out using radiolabeled photoprobe ^{125}I-SBpa-Ang II. As in the case of ^{125}I-azido-Ang II, ^{125}I-SBpa-Ang II also reproducibly and specifically (i.e., 10 μM Ang II replaceable) labeled a protein in rat forebrain membranes with 70–85 kDa apparent molecular mass (Figs. 3, 4). Based on these results our further experiments were carried out using ^{125}I-SBpa-Ang II.

Semipurified, radio-photolabeled angiotensin binding site was obtained by large-scale separations of SDS-solubilized ^{125}I-SBpa-Ang II photolabeled membranes on 7.5% SDS gels, followed by identification of segments of lanes (~0.3 cm) corresponding to specific binding (10 μM Ang II displaceable) of the ligand and extraction of proteins from the gel segments.

Incubation of the semipurified, radio-photolabeled angiotensin binding site with PNGaseF did not alter its molecular weight as revealed by SDS-PAGE analysis followed by Coomassie blue staining and monitoring of ^{125}iodine migration through PNGaseF-treated and non-treated lanes of the gels (Figs. 3, 4). In contrast, the control protein, fetuin, which is

known to be N-glycosylated [24,25] showed a reduction in molecular mass by SDS-PAGE analysis following incubation with PNGaseF.

CNBr digestion of the semipurified, radio-photolabeled binding site generated two radiolabeled bands with ~25 and 12.5 kDa molecular mass (Fig. 4). 2D gel electrophoresis of the semipurified, radio-photolabeled protein (n = 3) indicated that the isoelectric point of the binding site was approximately 7.

Incubation of the cerebral cortical membranes with either of the two stable GTP analogs did not alter the ability of Ang II to compete for ^{125}I-SI Ang II binding to the novel angiotensin binding site (Fig. 5). In contrast, the ability of Ang II to compete for ^{125}I-SI Ang II binding in the liver was decreased in the presence of the stable GTP analogs (Fig. 5).

Discussion

The primary purpose of this study was to develop an approach for isolation and purification of the protein representing the novel, non-AT1, non-AT2 angiotensin binding site. Biochemical characterization, including sequencing, of this unidentified protein could give further clues on the function of the novel angiotensin binding site and define its role among other, well-studied members of the RAS. For this purpose we applied photoprobe angiotensin analogs ^{125}I-azido-Ang II and ^{125}I-SBpa-Ang II previously used in AT1 and AT2 angiotensin receptor studies [26–30]. As presented in Fig. 1, both radioiodinated photoprobes showed high affinity binding to the novel angiotensin binding site which was comparable to the binding of ^{125}I-SI-Ang II.

In order to track and isolate the novel binding site, in the next step we worked out photolabeling (UV crosslinking) conditions for both photoprobes. UV crosslinking experiments using the radioiodinated angiotensin photoprobes successfully radiolabeled the novel angiotensin binding site allowing tracking of the protein by polyacrylamide gel electrophoresis without dissociation of the photoprobe from the binding site (Figs. 2, 3). Notably, to avoid tracking of non-specifically photolabeled protein(s) all experiments were carried out with parallel use and comparison of total and non-specific samples. Presence of 10 μM Ang II in non-specific samples insured minimal or no binding and labeling/crosslinking of radioiodinated photoprobes to the novel angiotensin binding site. Thus, specifically photolabeled angiotensin binding site was identified by tracking of ^{125}iodine migration in parallel SDS-PAGE lanes of “Non-specific” and “Total” samples (Fig. 2). As presented in Figs. 2 and 3 both photoprobes gave similar results specifically labeling a protein with an apparent molecular size of 70–85 kDa. Importantly, with our limited information about the nature of the novel angiotensin binding site, we cannot be sure that both photoprobes labeled the same protein in our experiments. On the other hand, both photoprobes reproducibly labeled a protein with the same apparent molecular mass, and that binding was Ang II displaceable. Based on these results, all our further experiments were carried out with ^{125}I-SBpa-Ang II photoprobe.

The relatively high molecular mass of the novel angiotensin binding site compared with reported masses of numerous G protein-coupled receptors and receptor kinases made us test the glycosylation status of the novel angiotensin binding site. For this purpose we semipurified the radio-photolabeled angiotensin binding site using conventional gel electrophoresis methodology. To determine the N-glycosylation status of the binding protein the semipurified binding site was subjected to N-Glycosidase F (PNGaseF), which specifically cleaves the N-glycan chains from glycopeptides and glycoproteins [31]. The lack of change in the migration of radio-photolabeled binding protein (migration of ^{125}iodine) confirmed the absence of N-glycosylation of the binding protein. Notably, this

observation is unlike the AT1 and AT2 angiotensin receptors, which are known to undergo post-translational N-glycosylation [30,32,33].

To gain further insights into biochemical properties and structure of the novel angiotensin binding site, the semipurified, radio-photolabeled protein was applied for 2D electrophoresis. The isoelectric point of the binding site was approximately 7 (data not shown), which again is unlike the AT1 and AT2 angiotensin receptors [30,32,33]. The next step in determining the biochemical properties of the novel angiotensin binding site was cyanogen bromide (CNBr) hydrolysis of the semipurified, radio-photolabeled protein. This yielded 25 and 12.5 kDa radiolabeled fragments, suggesting that there are at least 2 methionine residues in this protein. Furthermore, no labeling occurs on any of these methionine residues due to the lack of CNBr-dependant ligand release [26,34]. Importantly, these fragmentation patterns also indicate that the novel angiotensin binding site differs from classical AT1 and AT2 angiotensin receptors as the obtained hydrolysates have a larger molecular mass than the CNBr hydrolysates of AT1 and AT2 angiotensin receptors [30,32,33].

The apparent molecular mass of the novel angiotensin binding site is within the range of reported masses of membrane receptors. To test whether this angiotensin binding protein could be a G protein-coupled receptor we used one of classical approaches in receptor pharmacology by adding a stable GTP analog to the homogenate and looking for a shift (decrease) of binding affinity of the protein for a putative agonist [35]. As illustrated in Fig. 5, neither of the two stable GTP analogs (GTPγS or GppNHP at 50 μM final assay concentration) altered binding affinity of Ang II for the novel angiotensin binding site. Conversely, and as was expected, the affinity of Ang II for the AT1 angiotensin receptor was decreased in the presence of both GTP analogs. These results suggest that the novel angiotensin binding site is most likely not a G protein-coupled receptor. However, it is possible that the novel angiotensin binding site might still function through a G protein. Agonist binding to at least two other G protein-coupled receptors; AT2 angiotensin, and SST1 somatostatin receptors, has previously been shown to be insensitive to GTP analogs [36–41]. Moreover, it is also possible that PCMB interferes with G protein coupling to G protein-coupled receptors or with GTP binding to G proteins [42,43].

In summary, we successfully developed an approach for photolabeling and purification of the novel non-AT1, non-AT2 angiotensin binding site from rat brain. Preliminary biochemical characterization of the purified protein provides additional evidence that the novel angiotensin binding site is different from classical AT1 and AT2 angiotensin receptors, is not N-glycosylated, and does not seem to be coupled to a G protein. Obviously, the most important question that is un-answered at present is the identity of this novel binding protein. While our efforts on further purification and sequencing of the binding site are continued, we took advantage of an available database; the Mouse Genome Informatics (MGI) database; (www.informatics.jax.org) on protein expression patterns in the mouse to theoretically estimate and gain some information on possible candidate proteins for the novel angiotensin binding site [23]. For this purpose we used some of the information obtained during this study: protein size (70–85 kDa) and absence of glycosylation sites, as well as results of our previous studies [20], cellular localization (membrane protein fraction), and tissue distribution (highest expression in testis and brain) in adult mice [23]. These characteristics served as criteria for a global search of tissue expression patterns using the MGI database. Eight candidate proteins were identified: Cldn 17, Emp1, Fbox2, Maged1, Mmp24, Rxfp2, Septin 3, and St8sia3. Three of the eight proteins, Maged1, Mmp24, and Rxfp2, were ranked highest with respect to agreement with characteristics of the novel angiotensin binding protein. Further analysis of tissue distribution patterns and functional properties of these candidates narrowed down the list to one protein:

metalloproteinase Mmp24 (MT5-MMP). It most closely parallels the reported biochemical characteristics of the novel angiotensin binding site described herein and previously. While there are still differences between some features of MT5-MMP and the novel angiotensin binding site, the concordance of this protein with the novel angiotensin binding site is not entirely surprising considering the ability of PCMB to inhibit other metallopeptidases EC 3.4.24.15, (thimet oligopeptidase) and EC 3.4.24.16, (neurolysin) [44,45]. Moreover, the role of a number of other metalloproteinases (ACE, ACE2, insulin-regulated aminopeptidase, aminopeptidase A, neutral endopeptidase, etc.) in regulation of the renin-angiotensin system is well established, see reviews [46,47].

Future studies, directed at sequence elucidation and identification of the novel angiotensin binding site will provide valuable information for characterization of the physiological functions and possible pathophysiological processes in which this protein is involved.

Materials and methods

Adult Sprague-Dawley rats (Harlan) 300–350 g maintained under a 12 h light/dark cycle, fed ad libitum were used for the study. The protocol for this study was approved by the University of Mississippi IACUC.

Ang II and Sar^{1}-Ile^{8}-angiotensin II (SI-Ang II) were purchased from Phoenix Pharmaceuticals. Photoprobe analog of Ang II Sarcosine^{1}-*p*-benzoyl-$_{L}$-phenylalanine^{8}-Ang II (SBpa-Ang II) was prepared at the Department of Pharmacology, Université de Sherbrooke according to a previously established procedure [48]. The second photoprobe analog of Ang II, [Sar^{1},(4-N_3)Phe^{8}]-Ang II (azido-Ang II) was a gift of Dr. Kevin J. Catt (NICHD, NIH, Bethesda, MD). ^{125}I-SI Ang II, ^{125}I-azido-Ang II, and ^{125}I-SBpa-Ang II were prepared in house by the chloramine T procedure and purified by reverse-phase HPLC as described previously [49]. Losartan was a gift of Dr. Ron Smith of Dupont Merck. PD123319 was purchased from Tocris, *p*-chloromercuribenzoic acid (PCMB) sodium salt was obtained from MP Biomedicals. All reagents for SDS-PAGE analysis were purchased from BioRad. All other reagents were purchased from major commercial suppliers.

Saturation binding experiments using ^{125}I-azido-Ang II, ^{125}I-SI-Ang II and ^{125}I-SBpa-Ang II were performed in membrane preparations of rat forebrain using established procedures [19,20].

Photolabeling of the non-AT1, non-AT2 angiotensin binding site in membrane preparations of rat forebrains was carried out using ^{125}I-SBpa-Ang II or ^{125}I-azido-Ang II. Membrane preparations were prepared from frozen Sprague-Dawley rat forebrains in a similar way as for saturation binding experiments. The final pellet was resuspended by homogenization in the assay buffer (150 mM NaCl, 5 mM EDTA, 0.1 mM bacitracin, 50 mM Na_2HPO_4, pH 7.1–7.2) to obtain 50 mg/ml initial wet tissue weight suspension. Losartan, PD123319 (final concentration of 10 μM) and PCMB (0.3 mM final concentration) were added into the membrane homogenate. Homogenate was incubated with ~1 nM ^{125}I-SBpa-Ang II or ^{125}I-azido-Ang II for 60 min at room temperature with and without 10 μM Ang II (for estimation of the non-specific binding). Incubation was followed by UV exposure for 5 min using a Rayonet Photochemical Reactor. The homogenate was then centrifuged at ~13,000×*g* for 3 min. The pellet was resuspended in ice-cold assay buffer and recentrifuged to wash away the non-specifically bound radioligands. This step was repeated twice. The pellets from the “Non-specific” and “Total” binding treatments were then dissolved in SDS sample buffer (62 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue), heated for 5 min at 100°C, and separated on a 7.5 or 12% SDS-Tris ready-gel (BioRad). The lanes with “Non-specific” and “Total” binding samples were sliced into 3–5

mm sections and counted in a gamma counter to track the migration of ^{125}iodine. For partial purification of the novel angiotensin binding site the gel sections of the "Total" binding samples showing substantially higher radioactivity compared with gel sections of the "Non-specific" binding samples from the same migration level (corresponding to 70-85 kDa) were combined and the radioactivity was extracted into Tris-glycine SDS-PAGE running buffer at 4°C for 3 days (>90% recovery of the ^{125}iodine). The extracted samples were concentrated using a Centriplus centrifugal filter (Millipore). An aliquot of concentrated sample was resolved on a 7.5% SDS-Tris ready-gel to check the presence of the band (Coomassie blue staining) that was not observed in the "Non-specific" sample of the membrane homogenate.

N-glycosylation of the novel binding site was tested by incubation of semipurified, radio-photolabeled binding site with N-Glycosidase F (PNGaseF, 100,000 U/ml, 2 h at 37°C according to manufacturer's instructions; New England, BioLabs) followed by separation by 7.5% SDS-PAGE and Coomassie blue staining. Fetuin was used as a positive control in these deglycosylation reactions. Additionally, the lanes with semipurified, radio-photolabeled binding site ± N-Glycosidase F were sliced into 3–5 mm sections and counted in a gamma counter to track the migration of ^{125}iodine.

For further purification of the novel angiotensin binding site the semipurified sample of the radio-photolabeled protein was precipitated by trichloroacetic acid (TCA), resuspended in rehydration buffer (7 M Urea, 2 M Thio-Urea, 5% CHAPS (w/v), 0.8% Ampholine (w/v), 0.2% Tergitol NP7, 100 mM DTT, with a trace of bromophenol blue). Resuspended proteins were isofocused on IPGphore strips (pH 3–10; Pharmacia Plus One) using an Ettan IPGphorII apparatus from Amersham Bioscience. Strips were incubated in a reducing buffer for 10 min (50 mM Tris HCl, pH 6.8, 6 M Urea, Glycerol 30% (v/v), SDS 2% (w/v), 65 mM DTT, traces of bromophenol blue). Strips were then incubated in an alkylation buffer for 15 min (50 mM Tris HCl, pH 8.8, 6 M Urea, Glycerol 30%, SDS 2% (w/v), 81 mM Iodoacetamide, traces of bromophenol blue). Treated strips were then loaded on 10% SDS-PAGE gels, covered with agarose and migrated 5–6 h at 200 V (BioRad). Gels were stained with silver nitrate and exposed overnight on a Kodak autoradiography film at −80°C.

The solubilized, radio-photolabeled binding site (up to 5000 cpm), with or without deglycosylation (±PNGaseF) was diluted 3:5 in a mixture of 30% TFA and 50 mg/ml cyanogen bromide (CNBr) (w/v; dissolved in 100% acetonitrile) to prepare a CNBr digest. Samples (100 μl) were incubated at room temperature in the dark for 18–24 h. Water (1 ml) was added to terminate the reaction. The CNBr and PNGaseF-treated samples were lyophilized, dissolved in 1× Laemmli buffer, and resolved on a 16.5% Tris-tricine-SDS gel (Bio-Rad).

Following electrophoretic separation of the samples, the gel was fixed in a 10% glycerol (v/v), 40% methanol (v/v), and 7.5% acetic acid (v/v) aqueous solution and dried on a Bio-Rad model 583 gel drier at 65°C for 3 h. Dried gels were exposed overnight on a Kodak autoradiography film at −80°C.

Coupling of the novel angiotensin binding site with a G protein was studied using two different stable GTP analogs; GTPγS and GppNHP, and compared with the effect of GTP analogs on the AT1 angiotensin receptor in liver. Briefly, brain cortical and liver membranes were obtained according to procedures mentioned above. Final resuspension of the pellet (50 mg/ml initial wet tissue weight for cerebral cortex, and 20 mg/ml for liver) was performed in a modified assay buffer consisting of 150 mM NaCl, 5 mM EGTA, 10 mM $MgCl_2$, 0.1 mM bacitracin, pH 7.2. Competition binding studies were conducted by incubation of 50 μl of membrane preparation with ~0.25 nM ^{125}I-SI-Ang II in the presence of 1 nM–1 μM concentrations of Ang II, and in the presence or absence of 50 μM GTPγS or Gpp(NH)p in

100 μl total assay volume for 45 min at 24°C. Non-specific binding was estimated in the presence of 3 μM Ang II. In cortical membranes 10 μM losartan, 10 μM PD123319, and 0.3 mM PCMB were also present. Incubation was stopped by running the reaction medium through a glass fiber filter (Whatman, S&S, #32 glass), pre-wetted with 1 mg/ml bovine serum albumin, using a cell harvester (Model M24R, Brandel). The bound radioactivity was counted in a Beckman Gamma 5500 gamma counter. Determination of IC_{50} values was carried out using a one-site competition binding model of Prism software (Graphpad Software).

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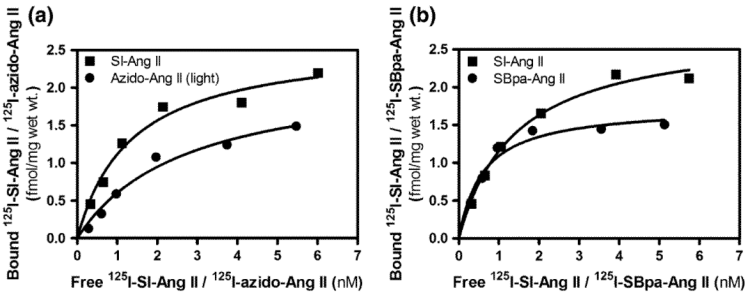

Fig. 1.

High affinity binding of ^{125}I-azido-Ang II, ^{125}I-SBpa-Ang II, and ^{125}I-SI-Ang II to the novel angiotensin binding site in rat brain membranes. **a** Representative saturation analyses of ^{125}I-SI-Ang II (B_{max} = 2.6 ± 0.2 fmol/mg wet wt, K_d = 1.35 ± 0.3 nM) and ^{125}I-azido-Ang II (B_{max} = 2.24 ± 0.3 fmol/mg wet wt, K_d = 2.7 ± 0.78 nM) binding in rat cerebral cortical membranes in the presence of 10 μM PD123319, 10 μM losartan, and 0.3 mM PCMB (1 h incubation at 24°C). **b** Representative saturation analyses of ^{125}I-SI-Ang II (B_{max} = 2.77 ± 0.15 fmol/mg wet wt, K_d = 1.4 ± 0.2 nM) and ^{125}I-SBpa-Ang II (B_{max} = 1.75 ± 0.1 fmol/mg wet wt, K_d = 0.6 ± 0.1 nM) binding in rat forebrain membranes in the presence of 10 μM PD123319, 10 μM losartan, and 0.3 mM PCMB (1 h incubation at 24°C); n = 3 for each assay

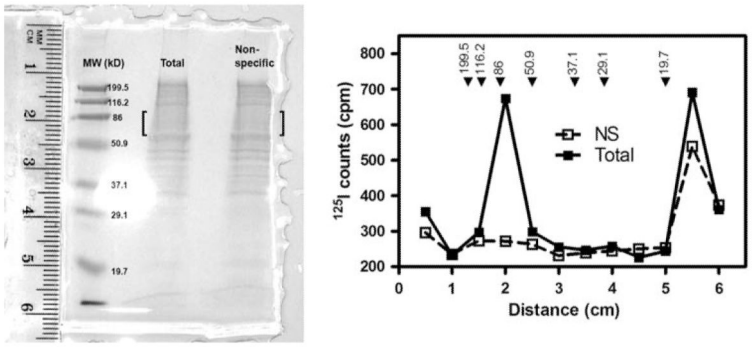

Fig. 2.

Radiolabeling of the novel angiotensin binding site with photoaffinity probe ^{125}I-azido-Ang II in rat forebrain membranes (representative results from four independent experiments). *Left* SDS-PAGE (12%) analysis of radio-photolabeled rat forebrain membranes followed by Coomassie blue staining; *bracketed areas* represent a segment of the lanes (between molecular masses 86 and 50 kDa) corresponding to the highest specific binding (10 μM Ang II displaceable) of the radio-photoligand ("Total"—binding of ^{125}I-azido-Ang II to the membranes in the presence of 10 μM losartan, 10 μM PD123319, and 0.3 mM PCMB; "Non-specific"—binding of ^{125}I-azido-Ang II under the same experimental conditions plus 10 μM Ang II). *Right* migration of ^{125}iodine in the "Total" and "Non-specific" lanes of the same SDS gel (0.5 cm sections)

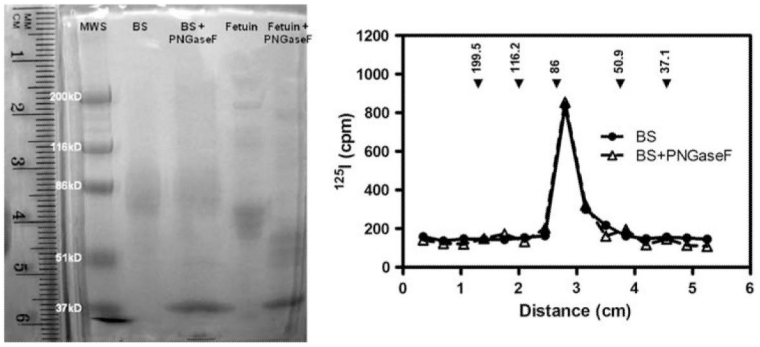

Fig. 3.

Analysis of N-glycosylation status of the novel angiotensin binding site (representative results from three independent experiments). *Left* SDS-PAGE (7.5%) analysis and Coomassie blue staining of radio-photolabeled (^{125}I-SBpa-AngII), semipurified novel angiotensin binding site without (BS) and with (BS + PNGaseF) PNGaseF treatment. Glycoprotein fetuin was used as a positive control in this experiment. *Right* migration of ^{125}iodine in the “BS” and “BS + PNGase” lanes of the same SDS gel (~0.3 cm sections)

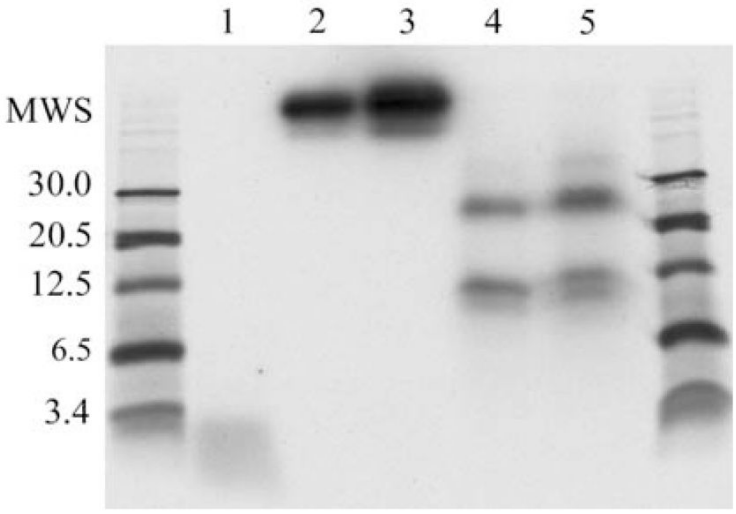

Fig. 4.

Representative autoradiogram of Tris-tricine-SDS-PAGE (16.5%) analysis of CNBr and PNGaseF digests of radio-photolabeled (^{125}I-SBpa-AngII) and semipurified novel angiotensin binding site. *Lane 1* contains free/unbound 125I-SBpa-AngII, *lane 2* contains untreated binding site, *lane 3* contains PNGaseF-treated binding site, *lane 4* contains CNBr-treated binding site, *lane 5* contains CNBr and PNGaseF-treated binding site

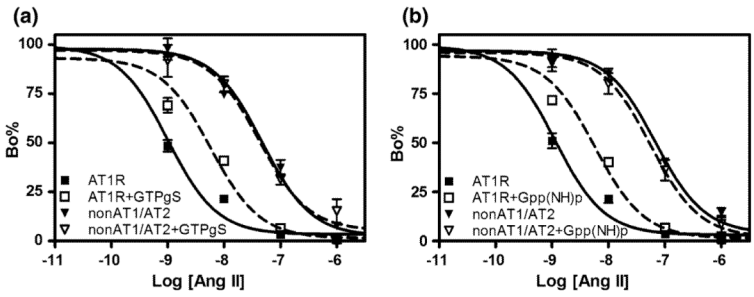

Fig. 5.

Competition by Ang II for ^{125}I-SI Ang II (~0.25 nM) binding to the AT1 angiotensin receptor (AT1R; in liver membranes) or the novel angiotensin binding site (non-AT1/AT2; in cerebral cortical membranes) in the presence and absence of 50 μM GTPγS (**a**) or 50 μM Gpp(NH)p (**b**); $n = 3$