Angiotensin Binding Sites in Bovine and Human Retinal Blood Vessels

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Blood vessels isolated from bovine and human retinas have sites that specifically bind 3H-angiotensin II (3H-Ag II) with an apparent dissociation constant (Kd) of 14 nM and a capacity of binding (Bmax) of 0.82 pmol/g. The binding sites for 3H-Ag II appear to be influenced by guanine nucleotides (GTP) and cations (Mg2+ and Na+) in a way that resembles angiotensin II receptors in other tissues. The physiologic effect of blood-borne or locally-formed angiotensin II on retinal blood flow remains to be defined. Invest Ophthalmol Vis Sci 28:1747-1751, 1987

The octapeptide angiotensin II (Ag II) has well-defined actions on several tissues such as adrenal cortex, vascular smooth muscle, liver, kidney, heart, and both central and peripheral neurons. These actions of Ag II are mediated through specific receptors located in the plasma membranes of target cells in these tissues.1-4 The receptors bind the peptide angiotensin II, which is formed from the precursor angiotensin I by angiotensin-converting-enzyme located in certain parts of the vascular tree and in some target tissues.

As a circulating vasoconstrictor, angiotensin II seems to help maintain normal blood pressure. It is secreted in large amounts to prevent impending shock, for example from blood loss.5 It has a pathogenic role in several forms of experimental and clinical hypertension,6 and therefore converting-enzyme inhibitors can be used to control essential hypertension.7

Experimental angiotensin-induced elevation of blood pressure caused intraocular pressure to be more effective in blocking axonal transport at the optic nerve head. We suspected that the vasculature of the optic nerve head might undergo a vasoconstrictive response to the peptide.8 In general, central nervous system vessels do not respond directly to intravenous angiotensin, but constrict as a secondary autoregulatory response to the high blood pressure.9,10

In contrast to the lack of a direct response to intravascular angiotensin, the CNS vessels do have a direct contraction with administration to the external surface of the vessel,10,11 and this has been shown also in retinal vessels of cats.12

Because of the possible relevance of a direct vasocontractile response to the pathogenic mechanism of glaucomatous cupping,13 we used in vitro binding methods to confirm the presence of angiotensin binding sites that might serve as hormone receptors in both animal and human eyes. These in vitro techniques with highly specific radioactive ligands have previously been used to characterize Ag II receptors in several other vascular tissue preparations.3,14-16

Materials and Methods

Retinal vessels were prepared from retinas removed from enucleated bovine eyes obtained at a local abattoir, and human retinal vessels were prepared from eyes obtained 12 to 24 hours postmortem from the Florida Lions Eye Bank. From each human donor the two eyes were pooled. All the isolation procedures were performed as previously described.17

Binding Studies

Binding assays were performed basically as described by others.3,12 In brief, the particulate vascular fractions were separately diluted 1:10 in the “assay buffer” consisting of 50 mM Tris-HCl buffer, pH 7.2, 5 mM MgCl2, 0.25% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.0025% bacitracin. Aliquots of the freshly diluted particulate fractions (8-10 mg of wet tissue, approximately 300 μg of protein) were incubated with concentrations of 0.5 to 40.0 nM 3H-Angiotensin II (New England Nuclear, Boston, MA; 21.8 Ci/mmol) in 5 ml capacity polystyrene tubes in a final volume of 300 μl of “assay...
buffer”. After 45 min incubation at 25°C, the mixture was diluted 1:10 with ice-cold NaCl 0.9%. The bound and free radioactivity was separated by rapid filtration through glass-fiber filters prewetted with fresh “assay buffer” to avoid adsorption of the 3H-ligand to the glass. The tubes and the filters were rinsed thrice with 3 ml of ice-cold 0.9% NaCl, and the radioactivity retained was determined by liquid scintillation spectroscopy. Non-specific binding was determined by measuring filter-retained radioactivity from duplicate tubes incubated under the same conditions but in the presence of 1 nM unlabelled angiotensin II (Sigma Chemical Co., St. Louis, MO).

Competition curves with Ag II and Ag III were performed by incubating aliquots of particulate tissue with 20 nM 3H-Ag II to which was added one in a series of concentrations (0.1 nM-10 μM) of Ag II or Ag III (Sigma) as the competing ligands. The conditions for the binding were as described above.

Degradation of 3H-Ag II during the binding assay was assessed by thin layer chromatography. Membrane fractions incubated with 1 nM 3H-Ag II during 45 min at 25°C, were centrifuged at 15,000 rpm for 15 min at 4°C. The supernatant was saved on ice. The pellet was resuspended in 0.03 M acetic acid and left on ice for 30 min. After recentrifugation, the acid eluate (neutralized with NaOH) was chromatographed on Avicel plates in a mixture of n-butanol:glacial acetic acid:water (25:4:10) together with the supernatant, pure 3H-Ag II, and unlabelled angiotensin II. Dried chromatograms were scanned for radioactivity by liquid scintillation spectroscopy. Chemical purity was also compared with the pure unlabelled angiotensin II band, determined with 0.4% ninhydrin. The effects of GTP (Sigma) and Na+ were tested in individual, but simultaneous, sets of experiments performed with and without the agents tested at a concentration of 3H-angiotensin II that assured saturation. Specific binding of 3H-Ag II was calculated by subtracting the values of non-specific binding from the total binding. The apparent dissociation constant (Kd) and the capacity of binding (Bmax) were calculated from Scatchard plots by linear regression analysis.

Results

The relative purity of the vascular fraction in our preparation was previously illustrated.17

Since adsorption to glass may sometimes produce inaccuracy in binding assays with peptides, we tested for any adsorption of radioactive angiotensin II to the glass fiber filters we used before performing the saturation studies. The amount (counts per minute) of radioactive angiotensin present on glass fiber filters prewetted with “assay buffer” (see Materials and Methods) on which 10 nM 3H-Ag II in “assay buffer” was poured through and rinsed three times was similar to the counts present in prewetted filters not exposed to the radioligand, which demonstrates the absence of 3H-Ag II adsorption or binding to the filters.

Even though phenylmethylsulfonylfluoride and bacitracin (inhibitors of proteolytic enzymes) were present in the routine incubation medium (“assay buffer”), we also tested for the possibility of some degradation of the 3H-Ag II after exposure to the tissue. As shown in Figure 1, both the unbound 3H-Ag II remaining in the supernatant (effluent) and the bound 3H-Ag II eluted from the tissue were unchanged after incubation at 25°C for 45 min, both co-chromatographed with authentic 3H-Ag II and with unlabelled Ag II, which shows that little if any catabolism of the peptide took place.

The characteristic of the Ag II binding sites were determined by incubating the bovine retinal vessel membrane preparation and the retinal neuronal membranes with 3H-Ag II in a concentration range of 0.5 to 40 nM (Fig. 2A). In the vascular preparation, non-specific binding (defined with 1 μM unlabelled Ag II) was always between 35-50% of the total binding at concentrations up to 20 nM, but markedly increased at higher concentrations. As shown in Figure 2B, the Scatchard plot indicates that the specific
binding sites have a Kd of 14 nM and a Bmax of 0.82 pmol/g of vascular tissue. When data from binding of 3H-Ag II at concentrations higher than 40 nM were examined it appeared as if more than one type of binding site or affinity state might be present, but we did not perform any of the detailed analyses required to document the presence of multiple sites.

In the retinal fraction homogenates, unlike the vascular preparation, the non-specific binding and the total binding were not statistically different at concentrations up to 100 nM, which indicates an absence of specific binding sites in this neuronal tissue, at least in that range of concentrations used.

Since guanine nucleotides and cations influence binding of radiolabelled Ag II to its receptor sites in a variety of tissues, we tested the effects of Na+ and GTP, without the addition of a regenerating system, in the presence and absence of Mg²⁺, which is known to increase the binding of Ag II to its receptors. The addition of 20 mM Na⁺ induced a 30% increase in the binding of 3H-Ag II to the vascular preparation when compared to controls without the monovalent cation (P < 0.01) (Fig. 3). On the other hand, GTP at a concentration of 1 mM, without Mg²⁺, decreased binding of Ag II by 26% of control (P < 0.05). Lower concentrations of GTP did not have any effect on the binding. The addition of 5 mM Mg²⁺ to the incubation medium inhibited the attenuation of binding induced by the nucleotide.

Since it has been postulated that Ag III (inactivated angiotensin II) has a higher affinity than Ag II at Ag II receptors in rat portal vein, we compared the ability of both peptides to compete for 3H-Ag II binding presented in a Scatchard plot. The data points are the means of six separate experiments, each performed in duplicate. Apparent dissociation constant, Kd = 14 nM, and capacity of binding at saturation, Bmax = 27.6 pM (0.82 pmol/g vascular tissue).

Results from binding to human retinal vessels are summarized in Table 1. We did not perform saturation curves as we did with the bovine tissues, but simply measured the amount of specific binding sites in the vessels obtained from each donor with 20 nM of 3H-Ag II, which should occupy approximately 60% of the binding sites. In some of the samples the binding of 3H-Ag II was very low, but did represent measurable specific binding with the non-specific binding always between 30–50% of the total binding. The results were very different among the individuals tested, revealing the biological variability in humans. We cannot say whether or not this variability is due to pathologic or drug therapy conditions, because complete medical information on the donors was not available. Comparable variability might also have occurred among cattle eyes, but would have been masked by the pooling of retinas to obtain a sufficient quantity for analysis by saturation curves. This individual variability must be considered when seeking physiologic or pathophysiologic correlations among individuals of different ages, or between health and disease states.
Discussion

The data show that binding sites for Ag II are present on bovine and human retinal vascular trees.

The presence of 5 mM Mg\(^{2+}\) in the incubation medium increased the binding in our retinal vessel preparation, as has been reported for other tissues.\(^{3,14,18}\) An observed increase in binding induced by Na\(^{+}\) has been previously reported for the mesenteric artery Ag II receptor\(^{18}\) and in bovine adrenal cortex.\(^{19}\) However, in tissues such as rabbit ventricles,\(^{14}\) myocardial sarcolemmal membranes,\(^{20}\) and rabbit smooth muscle,\(^{21}\) Na\(^{+}\) has no effect on or decreases Ag II binding to the tissue. The mechanism by which Na\(^{+}\) affects the binding of Ag II is not clear, but the different responses reported in the literature show the heterogeneity of the Ag II receptors in different tissues and species. Our data suggest that Ag II binding sites in bovine retinal vessels may be of the type associated with a membrane monovalent cation site.

GTP at a concentration of 1 mM produced a decrease in the binding of Ag II, but in the presence of Mg\(^{2+}\) the attenuation was not observed (perhaps because the Mg\(^{2+}\) stimulates GTPases, and no GTP regenerating system was present). However, in other tissues, cations modulate the effect of guanine nucleotides when interacting with the receptor-associated nucleotide-sensitive site, and GTP effects on angiotensin binding to its receptors require Mg\(^{2+}\).\(^{14}\) Thus, cations and guanine nucleotides seem to interact in a complex fashion to regulate the affinity of the Ag II site. The biochemical nature of these interactions, however, may vary from tissue to tissue and even from vessel to vessel.

From these data alone we cannot specify the physiological role of the specific binding sites, but we believe that these are the receptors responsible for the observed vasoconstrictive response to retinal vessels to angiotensin.\(^{12}\) Ag II is normally a vasoconstrictor,
although it may induce dilation under certain circumstances.\textsuperscript{22}

The concentration of circulating Ag II is remarkably low, but it is a very efficacious and potent agonist. In fact, a maximum contraction was obtained in the rat portal vein with only 20\% occupation of the receptor site,\textsuperscript{15} and cat retinal vessels are able to contract with concentrations of Ag II as low as 100 pM.\textsuperscript{12}

An increase in vascular permeability might be another physiologic response with great importance in the physiology of tissues that bear a blood-organ barrier and lack endothelial fenestrations, such as the retinal vessels. Intraventricular Ag II, for example, produces a transient increase in brain capillary permeability by unknown mechanisms.\textsuperscript{23} Angiotensin also induces endothelial cell contraction in the aorta, consequently increasing the permeability of the vascular wall.\textsuperscript{24}

In conclusion, the retinal vascular bed possesses Ag II binding sites, and it is known that retinal vessels respond to exogenous Ag II. These findings indicate that either circulating or locally-formed peptide may participate in physiology and pathophysiology of retinal and other CNS vessels.

**Key words:** angiotensin binding sites, Angiotensin II, bovine retinal blood vessels, human retinal blood vessels

**References**