Angiotensin II Binding Receptors in Retinal and Optic Nerve Head Blood Vessels

An Autoradiographic Approach

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Angiotensin II (AII) binding sites were identified in cross-sections of the cat retinal and optic nerve vasculatures. The authors used $^3$H-AII and $^{125}$I-saralasin, an agonist and a high-affinity antagonist of AII receptors, respectively, to generate light microscopy autoradiograms in resin-embedded tissues. With both radioligands the presence of AII binding sites was confirmed in retinal arterioles but not in the veins or capillaries of the retina. Additionally the presence of such binding sites in the capillaries of the optic nerve head was shown. These results support the hypothesis that microvascular tone and perhaps autoregulatory responses of optic nerve capillaries might be influenced by vasoactive substances, such as AII, either leaking from the choroid or locally synthetized.

The presence of specific receptors for angiotensin II (AII) has been established in several circulatory beds in mammals where it is important in producing vascular tone. Of particular interest to us has been the potential effect of vasoactive substances, such as AII, on vascular tone in the optic nerve head by virtue of their diffusion from the adjacent choroid. Such externally induced tone might reduce autoregulatory dilation of optic nerve head vessels when blood flow is challenged by an elevated intraocular pressure (IOP). Variation in the autoregulatory capacity might explain the greater alteration in the level of IOP than an individual eye can tolerate.

The influence of AII specifically in the ocular vascular beds has been difficult to assess. In the retina, circulating AII has limited accessibility to its target, muscular coating, because of the blood-retina barrier formed by the endothelial tight junctions. Even where there may be bioaccessibility to the smooth muscle, compensatory reflex or autoregulatory responses in an intact animal might mask the direct vascular effects of the peptide. Conversely, the vasoconstriction observed after systemic administration of AII might be an indirect response (eg, retinal vascular constriction in response to elevated blood pressure), with the vessels not directly stimulated by it.

To understand the ocular vascular responses more precisely, we studied AII chemistry and physiology in retinal vessels in detail. We found that the retina possesses angiotensin-converting enzyme (ACE) and the biochemical machinery to produce AII locally. Additionally, the retinal vessels have specific binding for sites AII, and in fact, the arterioles show direct specific contraction when AII reaches the external vascular layers.

Although the retinal vessels were the easiest ocular vessels on which to initiate studies, our main interest was to determine if there were such mechanisms in the vessels of the optic nerve head. Because of the small size of the optic nerve head and its vessels, almost exclusively capillaries, quantitative binding studies and reliable observations of physiologic responses are difficult. We therefore decided to use autoradiography with specific radiolabeled ligands to determine the presence or absence of AII binding sites. We not only confirmed the presence of such binding sites in the larger retinal arterioles but determined that these sites are present in the capillaries of the optic nerve head.

Materials and Methods

All applicable federal guidelines and the ARVO Resolution on the Use of Animals in Research were followed. Four cats of either sex, 2–3 kg, 1–1½ years old, were killed with an overdose of nembutal (1
g/kg). Both eyes were enucleated, immediately placed in ice, and dissected under a dissecting microscope. After anterior structures and the vitreous were removed and discarded, five or more small squares of the ocular coat sclera (including choroid and retina) and pieces of the optic nerve head were obtained. Thin slices were made by hand from each tissue block and incubated under appropriate conditions with $^3$H-AII (four eyes from two animals) and $^{125}$I-saralasin (four eyes from two animals). From each of the four animals, one eye was used for total binding and one eye was used for nonspecific binding (in the presence of an excess of nonradioactive ligand). These procedures were done as four separate experiments, one animal used on each of four separate occasions.

$^3$H-AII Binding

Several pieces of ocular tissue were placed in 5 ml of 50 mM Tris HCl buffer, pH 7.2, containing 5 mM MgCl$_2$, 0.25% bovine serum albumin, 0.0025% bacitracin, and 10 nM $^3$H-AII (22 Ci/mmol; New England Nuclear, Boston, MA) for total binding. The same solution with the addition of 1 juM unlabeled All (four eyes from two animals). From each of the four animals, one eye was used for total binding and one eye was used for nonspecific binding (in the presence of an excess of nonradioactive ligand). These procedures were done as four separate experiments, one animal used on each of four separate occasions.

$^{125}$I-Saralasin Binding

The procedure was the same as described for $^3$H-AII binding. For total binding, tissue slices were incubated in 0.1 nM $^{125}$I-saralasin (2200 Ci/mmol; New England Nuclear) in a vehicle of 50 mM Tris plus 120 mM NaCl, 1 mM MgCl$_2$, and 0.25% bovine serum albumin.

Two groups were obtained to show nonspecific binding: one had 100 nM of unlabeled All added and the other, 100 nM unlabeled saralasin (Sigma). Incubation, washes, fixation, sectioning, and the rest of the method was as described for $^3$H-AII binding, except that autoradiograms were developed after 5–7 days.

Autoradiographic Microscopy and Quantitation

The sections were observed under phase-contrast illumination and the association of silver grains with blood vessels noted. For quantitation, selected regions were photographed; these were chosen because they were representative and were free of scratches, wrinkles, or other artifacts. Photographs were taken with the emulsion layer in focus to show the silver grains. Therefore the tissue beneath was not in optimum focus.

Computer-assisted morphometrics were used to calculate the vessel areas. The outlines of the vessel walls (inner and outer perimeters) in the micrographs were traced using a Zeiss MOP Videoplan A (Kontron Electronik, West Germany). The correspondinc areas were obtained using the Standard Measuring Program $Y_1$/ $Y_2$. To obtain the silver-grain density of the “vascular area,” silver grains were counted only over the vascular wall areas previously traced with the computer.

Silver-grain density of the “surrounding area” was determined by tracing a large area of the micrograph, subtracting the area occupied by the vessel walls (total tissue area minus vascular area), and counting the silver grains over areas not occupied by vascular walls. This was done for total and nonspecific micrographs for the retina and optic nerve for both the agonist and antagonist bindings. Mean and standard deviation of silver-grain density (number of silver grains/100 $\mu$m$^2$) were obtained from several micrographs of every tissue for total and nonspecific binding. Analysis of variance and an unpaired student t-test were used to determine the statistical significance of differences.

Results

Retinal Vessels

A representative autoradiograph of retinal-vessel cross-sections is shown in Figure 1 for total (A) and nonspecific binding (B) of $^{125}$I-saralasin. About 90% of the vascular sections examined (range, 85–150 $\mu$m) showed silver grains on the major arterioles; in smaller arterioles (less than 100 $\mu$m in diameter), capillaries, veins, and venules, there was little, if any, evidence of the presence of All- or saralasin-specific

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binding sites (Fig. 1A). Nonspecific binding was slightly lower when $^{125}$I-saralasin (compared with $^3$H-AII) was used as a radioligand (not shown).

**Optic Nerve Capillaries**

About 80% of the capillaries observed in the optic nerve head at the level of the choroid showed silver grains clustered at the capillaries against an evenly distributed background of nonspecific labeling. By visual inspection the specificity of the binding was more evident with $^{125}$I-saralasin. However, the abundance and distribution of the specific binding sites were the same with $^3$H-AII and $^{125}$I-saralasin. A representative photograph is shown in Figure 2.

**Quantitation**

To document the equivalence of the two ligands, the silver grains in several tissue sections of each specimen were counted to determine the silver-grain densities in the optic nerve capillaries for total and nonspecific binding of $^3$H-AII and $^{125}$I-saralasin (Table 1). Analysis of variance did not reveal any statistical difference in the grain counts between the two ligands. Because of this and the approximate numeric
The equivalence of grain counts with the two ligands in each group, we combined the results with the two ligands for further analysis.

The total ranges of grain densities for total and nonspecific binding in the optic nerve capillaries overlapped by only one data point, confirming the impression from microscopic inspection of the autoradiographs that there was a clear association of grains with capillaries. After the data from each animal was reduced to an average specific and nonspecific grain count over capillaries for that animal, specific binding was shown as a statistically significant difference between total and nonspecific binding in the two groups of four eyes in each group (one-tailed unpaired t-test, \( P < 0.05 \)).

As a control, we examined the retinal arterioles (already known to have specific binding sites for \(^3\)H-AII\(^4\)). The pooled results for \(^3\)H-AII and \(^{125}\)I-saralasin showed a statistical difference between total and nonspecific binding only in the vascular areas (\( P < 0.05 \), Table 2), confirming our method. For the retina, we did not count a sufficient number of sections for each ligand to be able to analyze the results from each ligand separately.
Discussion

We showed (for the first time to our knowledge) the presence of specific binding sites for All in the capillaries of the optic nerve head. We validated the method by confirming the known presence of such sites in retinal arterioles. The relatively low amount found in autoradiograms agrees with the low quantity of binding sites shown in the retinal vessels with the use of binding studies. However, since All is known to be very potent, this density of receptors should be adequate to mediate vasoconstriction. Indeed, the retinal arterioles do show a specific direct physiologic response to very low concentrations of All.

The presence of binding sites, which may be receptors that mediate physiologic reactions to All, raises the question of how All may participate in the physiology and pathophysiology of these ocular tissues. Receptors for All and all other vasoactive agents may be ubiquitous in all vascular beds but play a role only where the agent reaches the receptors. For example, several vasoactive agents affect choroidal vascular resistance. The All binding sites are present in choroidal arteries (unpublished results), and choroidal vessels are leaky. It can therefore bathe both inner and outer cell layers of other blood vessels, and they can respond.

In the retinal vascular bed, the All sites were localized mainly in arterioles larger than 100 μm, suggesting that, as in other microvascular beds, the vasoconstrictive responses to this agent might be dominated by these high-resistance structures. In previous studies, All constricted relatively large arterioles when the external surface of the retinal vascular tree was exposed to exogenous All. Although these arterioles also constrict in response to circulating All, we suspect this is a reflex response to elevated blood pressure rather than a direct contraction induced by it (the endothelial tight junctions prevent it from reaching the muscular coating). There has not yet been a clear demonstration that retinal arterioles react directly to intraluminal All.

The presence of receptors for this vasoactive hormone in the optic nerve head is a necessary condition for the hypothesis that circulating agents might diffuse into the optic disc from the choroid and affect vascular tone, change the autoregulatory responsiveness, and alter susceptibility of the optic disc to different levels of IOP. Although our findings would permit All to be a physiologic factor in the genesis of glaucomatous optic nerve damage, further work is needed to show that it, or any other vasoconstrictor, actually contributes to the process.

Of particular general interest is that, in the optic nerve, the blood vessels are mainly capillaries without smooth muscle. These capillaries consist of pericytes and nonfenestrated endothelial cells. Presumably there is some contractile capacity in these capillaries. That optic disc capillaries constrict and relax is demonstrated by the fact that optic nerve blood flow is autoregulated. These capillaries are presumably responsible, perhaps responding to Po2 and Pco2. There is evidence that pericytes contract. It is also possible that endothelial cells contract in response to All. In this study we could not determine if the All binding sites were located in endothelial cells, in pericytes, or in both. Further studies at the electron-microscope level might answer this question.

Whatever role All receptors might play in these capillaries, the source of the agonist must be considered. The All might diffuse to the receptors from the bloodstream by leaking out of the choroidal vessels and affect both the vessels of the choroid and the capillary bed in the optic nerve head. However, the distance of diffusion may be limited by rapid degradation caused by serum angiotensinases. If inactiva-

Table 1. Number of silver grains/100 μm² area in optic nerve capillaries (mean ± SD)

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<th>Total</th>
<th>Nonspecific</th>
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<tr>
<td>³H-Angiotensin II</td>
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<tr>
<td>Surrounding area</td>
<td>4.53 ± 2.24</td>
<td>4.28 ± 2.17</td>
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<tr>
<td>(n = 3)</td>
<td>(n = 4)</td>
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<tr>
<td>Vascular area</td>
<td>27.67 ± 12.69</td>
<td>6.38 ± 5.3</td>
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<td>(n = 3)</td>
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| ¹²⁵I-Saralasin        |                     |                     |
| Surrounding area     | 3.75 ± 2.14         | 2.93 ± 2.3          |
| (n = 5)              | (n = 5)             |                     |
| Vascular area        | 27.60 ± 15.84       | 3.0 ± 3.69          |
| (n = 5)              | (n = 5)             |                     |

* Total tissue area minus vascular area.
† n is the number of tissue regions counted. The total of 17 sections came from eight eyes of four cats. From each cat one eye was used for total binding and the other for non-specific binding. For two animals the ligand was angiotensin and for the other two the ligand was saralasin. The indicated number of sections (n) came from one block for each eye.
‡ From two blocks from two cats, one eye from each animal.
§ From two blocks from two cats, the companion eyes of those used for total binding.

Table 2. Number of silver grains/100 μm² area in retinal arterioles (mean ± SD)

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<tr>
<td>³H-Angiotensin II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surrounding area</td>
<td>3.05 ± 1.55</td>
<td>0.88 ± 0.13</td>
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<tr>
<td>(n = 4)</td>
<td>(n = 4)</td>
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<tr>
<td>Vascular area</td>
<td>15.25 ± 8.76</td>
<td>0.93 ± 0.47</td>
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<td>(n = 4)</td>
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* Total tissue area minus vascular area.
† Number of sections examined from four eyes of four cats in each group.

The question of how All may participate in the physiology and pathophysiology of these ocular tissues raises that, as in other microvascular beds, the vasoconstrictive responses to this agent might be dominated by these high-resistance structures. In previous studies, All constricted relatively large arterioles where the agent reaches the receptors. For example, several vasoactive agents affect choroidal vascular resistance. The All binding sites are present in choroidal arteries (unpublished results), and choroidal vessels are leaky. It can therefore bathe both inner and outer cell layers of other blood vessels, and they can respond.

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tion is rapid, vasoconstriction deep in the optic nerve head might still occur if precursors diffuse from the bloodstream and are locally converted by the ACE present in this area. We also note that All can be synthesized by endothelial cells and that the ACE activity in these cells can be enhanced by substances that increase cyclic adenosine monophosphate. Since All-like activity has also been found in smooth muscles of peripheral blood vessels, it would not be surprising if endothelial-synthesized All diffused into smooth muscles and influenced vascular tone. So it is possible that the All locally synthesized in endothelial cells might act on these cells themselves or on the pericytes as part of the normal physiology that determines tone and vascular responsiveness, with additional pathologic influence from the precursor (AI) or All that diffuses into the optic nerve from the choriocapillaris.

In conclusion, binding sites for All are present in the retinal arterioles and optic nerve capillaries. Further study is needed to show: (1) on which layer of the vessels these sites reside, (2) if they are receptors that mediate physiologic responses, (3) if these vessels synthesize their own peptide, (4) what role All might play in normal physiologic control of circulation, and (5) if the All that reaches the optic nerve from distant sites plays a role in the pathophysiology of diseases such as glaucomatous cupping.

Key words: retinal vessels, optic nerve, capillaries, angiotensin II receptors, autoradiography

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References