Angiotensin II–Induced Constrictions Are Masked by Bovine Retinal Vessels

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PURPOSE. To unmask the vasoconstricting effect of angiotensin II (Ang II) on retinal smooth muscle by studying its interaction with endothelium-derived paracrine substances. This study focused specifically on determining the changes in vascular diameter and the release of endothelial-derived vasodilators, nitric oxide (NO) and prostaglandin (PG) I₂, from isolated retinal microvessels.

METHODS. Bovine retinal central artery and vein were cannulated, and arterioles and venules were perfused with oxygenated/heparinized physiological salt solution at 37°C. This ex vivo perfused retinal microcirculation model was used to observe the contractile effects of Ang II on arterioles and venules of different diameters. The NO and PGI₂ synthase inhibitors, L-NOARG and flurbiprofen, respectively, were used to unmask Ang II vasoconstriction; the changes in vascular diameters were then measured. Enzyme immunoassays were used to measure the release of cGMP (an index of NO release) and 6-keto-PGF₁α (a stable metabolite of PGI₂) from isolated bovine retinal vessels.

RESULTS. Topically applied Ang II (10⁻¹⁰ M to 10⁻⁴ M) caused significant (P < 0.05) arteriolar and venular constrictions in a dose-dependent manner, with the smallest retinal arterioles (7 ± 0.2 μm luminal diameter) and venules (12 ± 2 μm luminal diameter) significantly more sensitive than larger vessels. After the inhibition of endogenous NO and PGI₂ synthesis by L-NOARG and flurbiprofen, respectively, the vasoconstriction effects of Ang II became more pronounced. Again, the smallest vessels tested were significantly more sensitive, and synthesis of endothelial-derived relaxing factor (EDRF), therefore, may be most important in these vessels. Vasoactive doses of Ang II (10⁻¹⁰ M to 10⁻⁴ M) caused a dose-dependent increase in the release of NO and PGI₂ from isolated bovine retinal vessels, indicating that the increase in EDRF may nullify direct Ang II–induced vasoconstriction. Interestingly, intraluminal administration of Ang II caused only vasodilation.

CONCLUSIONS. This study demonstrates that the retinal vascular endothelium acts as a buffer against the vasoconstricting agent Ang II via release of vasodilators NO and PGI₂, and the vasoconstriction effects due to Ang II are most prominent in the smallest diameter vessels. (Invest Ophthalmol Vis Sci. 1999;40:721-726)
II. In fact, the A1 and A2 rat cremaster arterioles, which did not show a contractile response to Ang II alone, constricted in response to the topical application of Ang II after PG synthesis was inhibited by cyclooxygenase inhibitors such as indomethacin or mefenamic acid.

In most microcirculatory beds, blood flow is regulated via vasoactive substances (such as NO, PGs, and endothelin) and adrenergic and cholinergic autonomic innervations. However, an interesting point is that retinal microcirculation is not innervated by adrenergic nerves, despite the presence of α- and β-adrenergic receptors. Thus, the endothelial-derived relaxing factors play a crucial role in retinal microcirculation regulation.

The primary goal of this study was to further investigate the endothelium-dependent response of the retinal microvasculature to various concentrations of Ang II. Based on our previous knowledge of Ang II effects on other vascular beds, discussed previously, we formulated the hypothesis that Ang II probably stimulates the release of NO and PGs into the retinal vascular bed and counterbalances the vasoconstriction effect. Second, with the blockage of the release of these vasocontractor factors, the vasoconstrictor effect of Ang II should be more pronounced in smaller arterioles than in larger ones, because the smaller the diameter, the greater the percentage of constriction will be. And, finally, we wanted to study the correlation between the concentration of Ang II applied and the amount of NO and PGs released.

We searched for the answers to these questions via two routes. An extro perfused bovine retinal microcirculation model was used to observe the contractile effects of Ang II on retinal arterioles and venules of different diameters. Second, effects of Ang II on release of NO and PGs (PGI2 in this case) from isolated bovine retinal vessels were determined via enzyme immunoassays.

**MATERIALS AND METHODS**

**Perfused Bovine Retinal Microcirculation Model**

Bovine eyes were obtained through a local slaughterhouse and on removal were immediately placed in a flask containing 150 ml of ice-cold heparinized physiological salt solution (PSS) and transferred to the laboratory. The PSS contained heparin (1%; 100 U/ml) and the following chemical compounds (in millimoles): NaCl, 130; NaHCO3, 14.9; KCl, 4.7; KH2PO4, 1.17; CaCl2, 1.6; MgSO4, 0.7; dextrose, 5.5; and EDTA, 0.03. The globe was dissected free of any muscle and placed in a Petri dish containing 50 ml ice-cold PSS that was heparinized and oxygenated with O2 (95%) and CO2 (5%). An incision on the bovine eye was made in the sclera just posterior to the limbus, and the scleral portion of the eye was then completely excised. The eye was then lifted up by the optic nerve, and the anterior portion was pulled off the eye with slight pressure. The vitreous body was carefully removed using forceps. After the vitreous body was removed, oxygenated PSS was added to the resultant cup to prevent the retina from drying out. The retina was then carefully teased free of the choroid with a cotton-tipped applicator. The anterior edge of the retina was first completely separated from the choroid before working the rest of the way down to the optic nerve. The detached retina was then placed in the muscle chamber containing PSS bubbled with 95% O2 and 5% CO2. The retina was then stretched across the muscle chamber, and small pieces of clay were used to delicately tack the retina down, exposing the central artery and vein.

Once the retina had been completely tacked down, an incision on the central retinal artery and one on the retinal vein were made using microscissors. Close to the optic nerve, the retinal artery and retinal vein were cannulated using toothless forceps. The free end of the arterial-side cannula was connected to a minipulse pump, and the free end of the venular cannula was placed in a measuring cylinder. Then, whole retinal circulation was perfused at 20 μl/min with oxygenated/heparinized PSS. At 20 μl/min perfusion rate, the perfusion pressure was 80 mm Hg (Kulkarni et al. and Kulkarni and Payne).

The perfused retinal vasculature preparation in heparinized and oxygenated PSS was observed using closed-circuit video microscopy as follows. The preparation was positioned on the portable stage of a triocular microscope, and light was passed through the optical port in the bath through the tissue and into the microscope. The image of the microvessels was transferred via a video camera to a video monitor and simultaneously stored on videotape for analysis at a later time. Images (magnification, ×1000) of A1 and V1 (just above the first branch), A2 and V2 (just above the second branch), and A3 and V3 (subsequent branches) were recorded on the television screen and used for analysis. Before and after drug administration, the vascular diameters (at the same site for each vessel) were measured by a caliper of the selected vessels at the same site to determine changes in diameters in each branch.

Rat retinal arterioles were identified on the basis of their branching pattern. The primary arteriole supplying the retina (i.e., central retinal artery) was designated the first-order arteriole (A1). Arterioles branching from A1 were defined as A2, and subsequent branches were termed A3 arterioles. Venules branching from the primary retinal vein (V1) were designated as V2 and subsequent branches as V3. In observations from 24 bovine retinal preparations, luminal diameters of A1, A2, and A3 arterioles ranged from 45 ± 2 μm, 32 ± 2 μm, and 7 ± 0.2 μm, respectively. Diameters of V1, V2, and V3 were 65 ± 2 μm, 45 ± 1 μm, and 12 ± 1 μm, respectively (n = 24).

**Retinal Vascular Responses to Vasoactive Compounds in the Presence and Absence of Metabolic Inhibitors**

Angiotensin II, saralasin (Ang receptor antagonist), flurbiprofen (a cyclooxygenase inhibitor), and l-NOARG (a NO-synthase inhibitor; Sigma Chemical; St. Louis, MO) were used. Retinal arteriolar preparations are equilibrated in heparinized and oxygenated PSS at 37°C for 1 hour before cumulative dose responses to extraluminal or intraluminal applications of Ang II. After each dose, a minimum 10-minute recording period was allowed to attain the maximum response before the next dose was applied. After completion of each dose response, the preparation was washed with fresh heparinized and oxygenated PSS at 15-minute intervals for 1 hour to allow arteriolar diameters to return to baseline. Then, either saralasin (10⁻⁷ M), or flurbiprofen (10⁻⁶ M), or l-NOARG (10⁻⁷ M), or both flurbiprofen and l-NOARG were added to the bathing medium at least 15 minutes before obtaining a second dose-response curve to Ang II.
**Effects of Ang II on Release of NO and PGI₂ from Isolated Bovine Retinal Vessels**

Bovine retinal vessels were isolated according to the procedure described previously by Kulkarni and Payne. Angiotensin II and 6-keto-PGF₁α were used as index for NO and PGI₂ release, respectively. Concentrations of Ang II used in this study were concentrations that induced vasoconstrictions of bovine retinal vessels in ex vivo preparations. Isolated bovine retinal vessels were incubated with different concentrations of Ang II for 30 minutes in 1 ml cell culture medium (Cellgro: Dulbecco’s modified of Eagle’s medium IX; Fisher Scientific; Pittsburgh, PA) at 37°C, and the reactions for PG and NO synthesis were stopped by acidifying samples. Measurements of cGMP (and index for NO) and 6-keto-PG-F₁α (a stable metabolite of PGI₂) in tissues and media samples were performed as described in their specific enzyme immunoassay kits (Amersham Life Science, Arlington Heights, IL). Protein content of the isolated vessels was determined by Bio-Rad assay kits (Richmond, CA).

**Statistical Analysis**

Changes of vascular luminal diameters are expressed as a percentage of basal luminal diameter for each branch, whereas cGMP and 6-keto-PGF₁α content are expressed as per milligram tissue protein. Results are given as mean ± SEM with the number of preparations (control and experimental after inhibition of PG, NO, or both). Vascular diameters were compared in the same preparations. For the statistical analysis the level of significance (P < 0.05) is accepted using paired Student’s t-test.

**RESULTS**

**Effects of Topical Ang II on Bovine Retinal Microarterioles and Venules**

Angiotensin II (0.001 μM to 10 μM) applied topically has a small contractile effect on A1, A2, and A3 arterioles, with A3 vessels being the most responsive (P < 0.05). Also in these preparations, V1, V2, and V3 responded (Table 1) in smaller but significant (P < 0.05) vasoconstrictions to Ang II (0.001 μM to 10 μM). Angiotensin II (10 μM)-induced vasoconstrictions in arteriolar and venular preparations were completely antagonized by the Ang II receptor antagonist saralasin (0.1 μM; Table 1).

**Vasoconstricting Effects of Topical Ang II on Bovine Retinal Arterioles and Venules after Cyclooxygenase Inhibition**

Figure 1 shows bovine retinal arteriolar (A1, A2, A3) and venular (V1, V2, V3) dose-response curves of topical Ang II (10⁻² M to 10⁻⁴ M) in the presence and absence of flurbiprofen (10⁻⁶ M; a cyclooxygenase inhibitor). Angiotensin II-induced vasoconstriction curves were obtained before the addition of flurbiprofen.

This figure shows that there was a significant (P < 0.05) and dose-dependent vasoconstriction of all arterioles and venules to Ang II (10⁻¹⁰ M to 10⁻⁴ M). When PG synthesis was inhibited by flurbiprofen, the vasoconstriction to topical Ang II was slightly enhanced in A1, A2, V1, and V2 vessels. However, in the smaller vessels (A3, V3), Ang II-induced constrictions were significantly increased after flurbiprofen treatment (n = 6, P < 0.05) and were dose dependent in the 10⁻¹⁰ M to 10⁻⁷ M range.

**Vasoconstrictor Effects of Topical Ang II on Bovine Retinal Arterioles and Venules after NO Synthase Inhibition**

Figure 2 demonstrates the dose-response curves of bovine retinal arterioles (A1, A2, A3) and venules (V1, V2, V3) to Ang II. All arterioles and venules (n = 6) responded with significant dose-dependent vasoconstrictions to topical Ang II (10⁻¹⁰ M to 10⁻⁴ M). Inhibition of NO synthase by I-NOARG significantly enhanced Ang II-induced vasoconstrictions in all vessels (n = 6, P < 0.05). When I-NOARG was added to the bathing medium before Ang II, it caused significant vasoconstriction of all vessels. A3 and V3 vessels were significantly more responsive to this than were the larger vessels. Angiotensin II-induced constrictions were dose dependent over the range of 10⁻¹⁰ M to 10⁻⁷ M in these vessels.

**Table 1. Effects of Ang II on Bovine Retinal Arterioles (A1, A2, A3) and Venules (V1, V2, V3)**

<table>
<thead>
<tr>
<th>Ang II Concentration (μM)</th>
<th>% Change in Basal Luminal Diameter</th>
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<tbody>
<tr>
<td></td>
<td>A1</td>
</tr>
<tr>
<td>0.000</td>
<td>100 ± 0</td>
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<tr>
<td>0.001</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>0.01</td>
<td>97 ± 0.8*</td>
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<tr>
<td>0.1</td>
<td>96 ± 0.03*</td>
</tr>
<tr>
<td>1</td>
<td>95 ± 0.2*</td>
</tr>
<tr>
<td>10</td>
<td>93 ± 0.3*</td>
</tr>
<tr>
<td>10 + 0.1 saralasin</td>
<td>100 ± 0*</td>
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Numbers in column 1 are micromolar concentrations of Ang II and saralasin, a specific Ang II receptor antagonist, added to tissue media in a cumulative fashion; numbers in other columns represent percent change in basal luminal diameters ± SEM of six vessels. Luminal diameters of A1, A2, and A3 arterioles were 52 ± 1 μm, 27 ± 1 μm, and 7 ± 0.2 μm, respectively, whereas basal luminal diameters of V1, V2, and V3 were 65 ± 1 μm, 41 ± 1 μm, and 14 ± 0.5 μm, respectively. Percent inside arteriolar and venular basal diameters ± SEM were calculated as percent of luminal diameters prior to cumulative additions of Ang II to muscle bath.

* P < 0.05.
Vasoconstricting Effects of Topical Ang II on Bovine Retinal Arterioles and Venules after Cyclooxygenase and NO Synthase Inhibition

Figure 3 shows that topical Ang II (10^{-10} M to 10^{-4} M) caused significant vasoconstriction of arterioles (A1, A2, A3) and venules (V1, V2, V3) in a dose-dependent manner (P < 0.05, n = 6). Flurbiprofen and 1-NOARG combined significantly enhanced the vasoconstriction of all vessels to Ang II doses (n = 6, P < 0.05). Flurbiprofen and 1-NOARG added together caused marked vasoconstriction of all vessels before Ang II treatment. Interestingly, from Figures 2 and 3 it appears that these two combined drug treatments resulted in significantly greater vasoconstriction of only A3-level arterioles, whereas Ang II-induced responses were dose dependent over the concentration range of 10^{-10} M to 10^{-7} M in A3 and V3 vessels.

Intraluminal Administration of Ang II

Figure 4 illustrates contractile effects of Ang II on bovine retinal microcirculation when perfused through retinal central artery. Ang II (10^{-10} M to 10^{-4} M) perfusion resulted in a
FIGURE 2. Dose-response curves of bovine retinal arterioles (A1, A2, A3) and venules (V1, V2, V3) to topical Ang II (10^{-10} M to 10^{-4} M) in the absence and presence of 10^{-7} M 1-NOARG. The basal inside diameters of A1, A2, A3 and V1, V2, and V3 were 45 ± 1 μm, 30 ± 5 μm, and 8 ± 2 μm and 68 ± 1 μm, 43 ± 2 μm, and 11 ± 1 μm, respectively. Percent inside vascular diameters were calculated as percent of basal inside diameters before cumulative Ang II additions to the muscle bath. Points represent mean of six vascular preparations in the absence and presence of 1-NOARG. Error bars, ±SEM. It should be noted that 1-NOARG applied topically before Ang II treatment caused vasoconstriction of all arterioles and venules.

Inhibition of Ang II-Induced Vasoconstriction by Saralasin

In six preparations, topical Ang II (10^{-10} M to 10^{-4} M) in the presence of flurbiprofen caused significant vasoconstriction responses, especially of A3 and V3 retinal vessels, which were completely antagonized by topical administration of the Ang II antagonist saralasin (10^{-7} M; data not shown).
**Effects of Ang II on Release of NO and PGI₂**

Angiotensin II (10⁻⁸ M to 10⁻⁴ M) increased the release of cGMP, used as a marker for NO, and 6-keto-PGF₁₀, a stable metabolite of PGI₂, from isolated bovine retinal vessels in a dose-dependent manner (Table 2).

**DISCUSSION**

This is the first study to demonstrate that Ang II causes a small but significant vasoconstriction of bovine retinal arterioles and venules via Ang II receptors in an ex vivo perfused bovine retinal microcirculation preparation. Interestingly, it has been...
reported in the past that topically applied Ang II does not constrict bovine retinal arteriolar rings; however, it constricts posterior ciliary arteriolar ring in vitro.12 Also, it has been previously demonstrated that intra-arterial injection of Ang II in cats does not affect retinal blood flow.11 In both of these studies11,12 vascular endothelial cells were intact. It has been reported that Ang II stimulates the release of potent vasodilators NO,1,2,5 PGI2, and PGE2,9,15 from vascular endothelium. Bovine retinal vascular endothelium also synthesizes NO,19,20 PGI2,17 and PGE2,19.

In 1987, Ang II was shown to stimulate the release of PGs from rat mesenteric vascular beds.3 This study indicated that Ang II does not constrict mesenteric arterioles; however, after inhibition of PG synthesis by indomethacin, constriction of these arterioles does occur due to Ang II. The study, thus, suggested that vasodilating PGs (I2 and E2) mask direct vasoconstrictor effects of Ang II in the rat mesenteric vascular bed. Now it is well known that PGE2, PGI2, and NO are potent vasodilators.5 Therefore, we examined our hypothesis that little or no effect of Ang II on bovine retinal vessels is due to the stimulation of vasodilating NO and PGs release from retinal vessels.

Although use of saralasin (a specific Ang II receptor antagonist) indicated no change in basal luminal diameter, the present study demonstrated that Ang II alone caused a small vasoconstriction of bovine retinal arterioles in a dose-dependent manner. After inhibition of the release of PGs by flurbiprofen, there was enhancement in Ang II-induced vasoconstrictions, especially in smaller vessels. After the inhibition of NO release, an even greater potentiation of these responses was observed in all retinal vessels. Interestingly, a significant and marked potentiation of Ang II was observed when NO and PG release was inhibited in smaller rather than larger vessels, suggesting that PGs and NO as vasodilators may play a vital role in precapillary arterioles and postcapillary venules. The cyclooxygenase inhibitor flurbiprofen alone did not cause vasoconstriction of any retinal vessels. However, 1-NOARG, a NO synthase inhibitor, significantly caused vasoconstriction of all vessels, whereas combined treatment with both inhibitors resulted in significantly greater vasoconstriction of only small arterioles at the A3 level. From these results it appears that PGI2 is probably of no significance in larger vessels and that vasodilatory NO is probably the only, and most important, substance in this respect. Thus, bovine retinal vessels mask Ang II-induced vasoconstriction by releasing both PG and NO. Furthermore, direct evidence to support that Ang II stimulates the release of PG (PGI2 in this case) and NO was provided from isolated bovine retinal vessels. This showed that Ang II increased the release of cGMP and 6-keto-PG-F1α (markers for NO and PGI2, respectively) in a dose-dependent manner.

In an earlier study, it was reported that vasoactive compounds like norepinephrine, histamine, and Ang II (which are water soluble) did not cause vasoconstriction; whereas fatsoluble papaverine caused vasodilation (i.e., increased blood flow) in cat retinal circulation when injected intra-arterially.11 It was suggested that the water-soluble compounds do not cross the blood-retinal barriers and, therefore, do not affect retinal blood flow. However, it is now known that Ang II causes the release of vasodilators such as PGs and NO from retinal vessels.

### Table 2. Effects of Ang II in Release of NO and PGI2 from Isolated Bovine Retinal Vessels

<table>
<thead>
<tr>
<th>Ang II Dose, M</th>
<th>Control</th>
<th>10⁻⁸</th>
<th>10⁻⁷</th>
<th>10⁻⁶</th>
<th>10⁻⁵</th>
<th>10⁻⁵ + 10⁻⁶ M</th>
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<tr>
<td></td>
<td>cGMP fM/mg protein</td>
<td>3.75 ± 0.6</td>
<td>4.7 ± 0.5</td>
<td>5.2 ± 0.5*</td>
<td>5.4 ± 0.3*</td>
<td>7.7 ± 0.8*</td>
</tr>
<tr>
<td></td>
<td>6-keto-PG-F₁₀ nM/mg protein</td>
<td>2.5 ± 0.5</td>
<td>2.6 ± 0.3</td>
<td>3.6 ± 0.4*</td>
<td>7.3 ± 0.5*</td>
<td>4.4 ± 0.6*</td>
</tr>
</tbody>
</table>

Numbers in columns represent cGMP or 6-keto-PG-F₁₀ content ± SEM, which were used as an index to determine the release of NO and PGI2, respectively. The number of preparations used was seven, one for a control and one for each dose of Ang II. N/A, not available.

*P < 0.05. Significance was determined by comparing values of experimental (Ang II-treated) and control vessels.
vascular endothelial cells that are able to cross the blood-retinal barrier. We tested the effects of Ang II in an ex vivo perfused bovine microcirculation preparation in which the blood-retinal barrier is intact. Interestingly, in this study Ang II administered intraluminally caused only vasodilation, even at the highest dose (10^{-4} M) tested, suggesting that Ang II does not cross the retinal barrier and that it probably stimulates the release of endothelial-derived vasodilators like NO and PGs. This study may be further explored using inhibitors of PGs and NO. Although earlier studies have shown that Ang II does not cause vasocostriction of the cat retinal vessels, those studies did not report whether intra-arterial injections of this polypeptide increased the retinal blood flow. It is possible that the difference in the observations made in cat versus bovine studies could be due to in vitro versus in vivo studies; difference of species; or differences in the technique used.

In 1982, the differential effects of Ang II on larger and smaller rat cremaster muscle arteriolar vessels were reported. Angiotensin II did not cause vasoconstriction of the larger arterioles (>130 μm in diameter), whereas the smaller arterioles (<17 μm in diameter) showed vasoconstriction. In the perfused retinal microcirculation preparation we observed the effects of Ang II on the larger and smaller vessels simultaneously. The smallest arterioles and venules were significantly more sensitive to Ang II than the larger vessels, especially after PG and NO inhibition. This observation may be significant because in vascular diseases and diabetic retinopathy the blood-retinal barrier is disrupted, and hyperglycemic conditions affect vascular NO/PG synthesis. At such states, sensitivity of the smallest vessels to Ang II is extremely important because resultant vasoconstriction of the vessels will disrupt blood flow and thus the nutritional supply to the retinal neural tissue.

CONCLUSIONS

As of today, the effects of Ang II on retinal vessels, in vivo or in vitro, are controversial. The reason is that in addition to vasoconstriction, Ang II also releases vasodilators such as NO and PGI2 from the vascular endothelium. Thus, the net result is little or no response. This study shows that NO and PGI2 synthesis inhibition unmask Ang II-induced vasoconstrictions of the bovine retinal arterioles and venules. However, it appears that NO is perhaps the only substance to play a regulatory role in the entire retinal microcirculation, whereas PGI2 probably only contributes to smaller arterioles.

The smallest bovine retinal arterioles (i.e., precapillary vessels) are more sensitive than the larger ones. This is important because these precapillary vessels are the ones more responsible for tissue blood supply.

Angiotensin II causes vasoconstriction of retinal venules (this is the first study to show this; most veins and venules do not respond to this polypeptide). Retinal vascular endothelial cells play a primary role in local regulation by releasing vasodilatory NO and PGs, which is important because retinal vessels are not innervated by autonomic nerves. These findings are important to not only understand the physiological effects of Ang II on vascular smooth muscle and endothelium but also pathophysiological conditions such as hypertension (high levels of circulating Ang II) and diabetes (dysfunctional vascular endothelium) in which Ang II may affect retinal vessels.

References