Local Modulation of Retinal Vein Tone

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PURPOSE. To determine whether vascular tone of isolated porcine retinal veins can be modulated by tissue-generated vasoactive factors such as endothelin-1 and adenosine. Such information may be useful in understanding the role of the retinal veins in regulating blood flow, and also provide a model for investigating new hypotheses suggesting a role for vasoactive factors in retinal vascular diseases such as retinal vein occlusion.

METHODS. An isolated perfused retinal vein preparation was used for this study. Segments of porcine retinal veins were dissected, cannulated, and perfused, and their diameter was monitored during vasoactive agent application of increasing doses of endothelin-1 (10^{-12}–10^{-8} M) or adenosine (10^{-10}–10^{-4} M). Adenosine (10^{-6} M) was also applied on veins during preconstriction with endothelin-1 (10^{-8} M). The significance of any induced change in vessel diameter was assessed in relation to the baseline vessel diameter prior to any drug delivery.

RESULTS. Dose-dependent vasocontractile responses were induced by endothelin-1 administration. Endothelin-1 produced a significant contraction at doses of 10^{-11} M and above. At 10^{-8} M the maximal endothelin-1–induced contractions were to 70.2 ± 2.1% of baseline. Adenosine produced a dose-dependent dilation reaching 113.0 ± 2.4% at 10^{-4} M. Adenosine (10^{-6} M) induced a significant dilation in endothelin-1 (10^{-8} M)–contracted vessels.

CONCLUSIONS. Porcine retinal veins can be modulated by both vasocontraction and vasodilation agents, suggesting that the retinal veins may play a regulatory role in the retinal circulation, particularly in regard to the capillary pressure upstream from the draining retinal veins. To our knowledge, this is the first study of vasoactivity in isolated perfused retinal veins, providing an opportunity to study the direct vasoactive effects of specific vasoactive agents.

Keywords: vasoactivity, retinal veins, endothelium, porcine, endothelin-1, adenosine

The retinal circulation plays a vital role in the survival and functioning of the retinal neurons.[1,2] Although the retinal circulation is responsible for supplying a high metabolic rate tissue, it must be anatomically sparse to minimize optical interference with the light path to the photoreceptors. These requirements result in a limited flow circulation, with a very high arteriovenous oxygen tension difference. A further unusual feature of the retinal circulation is that it has no autonomic innervation,[3] so total reliance must be placed on local vascular control mechanisms to regulate retinal blood flow. There is no controversy about the regulatory ability of the retinal circulation, and it has long been accepted that the retinal circulation has powerful regulatory mechanisms.[4,5] Human and animal data demonstrate that flow in the major vessels is regulated and that the circulation regulates both blood pressure and systemic oxygen changes.[6-8]

Generally, in microcirculation systems, arteries with normal vascular tone are responsible for approximately 50% to 60% of the total pressure drop and vascular resistance across the vascular system.[9,10] The importance of retinal arteries in blood flow regulation is well recognized. The retinal arteries have a continuous layer of smooth muscle cells that generate the vasoactive response to local stimuli.[11-14] In contrast, the wall of the retinal veins is very thin and consists of a single layer of endothelial cells and only a few smooth muscle cells.[15] The retinal vein has largely been considered to be incapable of regulating vascular tone, and there has been little attention paid to possible effects of vasoactivity of retinal veins. In vivo studies

of changes in retinal vein diameter in response to altered retinal metabolism induced by flicker stimuli[16] are not able to distinguish between direct effect on retinal vein tone and passive response of the vein to blood flow changes due to vasoactivity further upstream.

The retinal veins are the only drainage pathways for blood flowing in the retinal circulation and they normally operate in a positive pressure environment due to the intraocular pressure. Intravascular pressure in retinal veins is close to intraocular pressure.[15] Retinal vein resistance and diameter changes could potentially affect vein drainage resistance and therefore upstream pressure in the retinal capillaries. Capillary hydrostatic pressure is a key determinant of fluid exchange between blood and tissue, and since capillary pressure is much closer to venous pressure than to arterial pressure, changes in venous resistance would have a significant effect on capillary pressure. It would therefore be interesting to determine whether retinal vein vasoactivity can be modulated locally as a means of influencing retinal blood flow and retinal capillary pressure.

Furthermore, retinal vein occlusion (RVO) is the second most common retinal vascular disorder, often leading to severe loss of visual function. For many decades the question of the pathogenesis of RVO has been discussed, but a clear answer has not yet been obtained.[17] It would be interesting to investigate whether a local venous constriction induced by vasoconstrictive molecules is involved in addition to other pathogenic factors in RVO.[18]
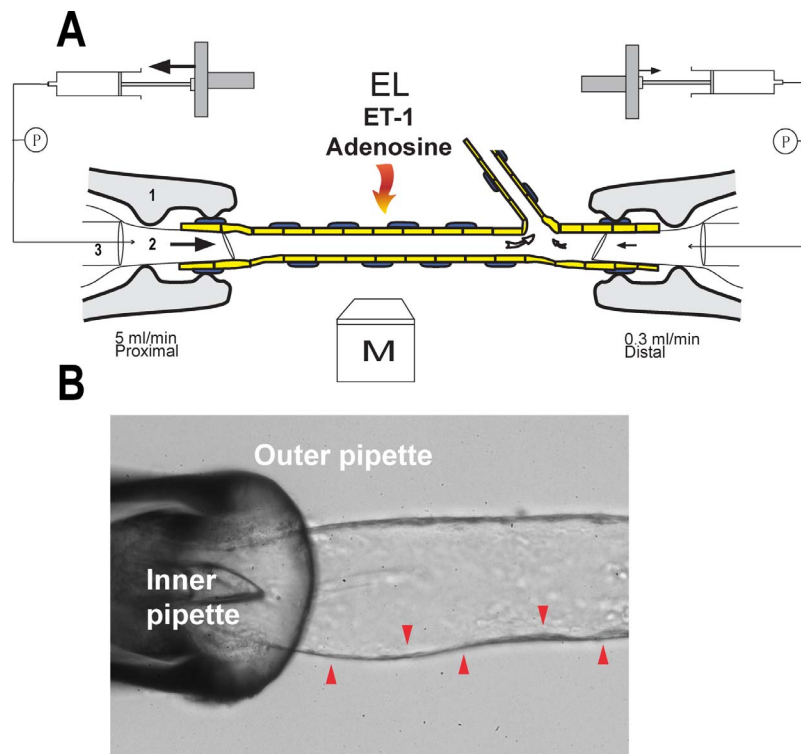
FIGURE 1. The isolated perfused retinal vein experimental setup. (A) Schematic representation of the isolated perfused vessel system. The isolated pig retinal vein is cannulated at both ends and perfused intraluminally in a temperature-controlled bath on the stage of an inverted microscope. Drugs, including ET-1 and adenosine, can be administered into the bath as required. The retinal vein is firmly held between the outer (*1*) and inner (*2*) pipettes. A third pipette (*3*) is used for intraluminal perfusion. Vessel diameter is monitored every 2 seconds by an automated frame-grabbing and vessel diameter measurement routine. (B) Microphotograph of a cannulated retinal vein held by the outer and inner pipettes. The vein wall is thin, indicated by *arrowheads*, and a thin layer of the endothelium is clearly located along the inside of the vessel wall. However, smooth muscle cells cannot be easily identified.

In this study, we determine for the first time whether retinal vein diameter can be modulated by vasoactive agents that are known to be locally generated such as endothelin-1 (ET-1) and adenosine, using our recently developed isolated perfused porcine retinal vein preparation.

METHODS

Isolated Perfused Retinal Vein Preparation

The dissection, cannulation, perfusion, monitoring, and vessel diameter measuring system are fully described in our previous publications using isolated perfused retinal arteries[11,12,19,20] and will be only briefly described here. Pig eyes were obtained from a local abattoir. Following enucleation, the eyes were placed in a sealed bottle of oxygenated Krebs solution and kept on ice during transfer to the laboratory (∼60 minutes). All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Ethics Committee of the University of Western Australia.

Dissection and Cannulation of Vessels

The eyes were sectioned at pars plana ciliaris, separating the anterior segment and adherent vitreous body from the posterior pole with the aid of a dissecting microscope. The retina, choroid, and sclera were divided into quadrants. The retina was then separated from the underlying choroid and sclera. A quadrant of retina was placed on a hollowed glass slide containing Krebs solution, and individual first-order retinal veins were dissected free of retinal tissue with a micropipette. A segment of retinal vein (∼150-μm outer diameter) approximately 800 to 1500 μm long and containing only one relatively large side branch was selected. The vein segment was then relocated to an incubation chamber (PDMI-2; Medical System Corp., New York, NY, USA) mounted on the stage of an inverted microscope (Nikon Diaphot-TMD; Tokyo, Japan). The chamber contained 5 mL Krebs solution. Temperature was maintained at 37°C and the incubating solution equilibrated with 95% O_2, 5% CO_2 so as to maintain PO_2, PCO_2, and pH of the incubating solution.

The vein segment was cannulated at both ends using a customized pipette and manipulating system shown schematically in Figure 1A. The vessel was then perfused through the proximal end in the orthograde direction at a constant flow of 5 μL min⁻¹. The distal end was perfused at 0.3 μL min⁻¹ in the retrograde direction to avoid drug entrapment. Both flows exited through the side branch. The choice of 5 μL/min as a baseline perfusion flow rate was based on in vivo measurements using laser Doppler velocimetry[21] and validated when we initially built the isolated perfused retinal vessel preparation.[11]

With constant flow within the physiological range, the retinal vein was under a basal or baseline tone. Figure 1B shows an image of a cannulated retinal vein held by the outer and inner pipettes in an incubation bath. The endothelial cell layer with few smooth muscle cells in the thin vein wall can be seen under the microscope.

The vessel was visualized on a video monitor, and a preprogrammed computer algorithm was used to measure the external vessel diameter at user-selected locations from a

frame-grabbed image at 2-second intervals. The vessel was left to stabilize for 30 minutes prior to any drug study. Drugs can be reliably delivered in the bath as required in order to determine ET-1- or adenosine-induced vasoactive changes. The concentration range of ET-1 used was 10^{-12} to 10^{-8} M, and the concentration range of adenosine was 10^{-10} to 10^{-4} M. These concentration ranges are comparable with those used for ET-1 or adenosine vasoactivity studies in retinal arteries and short posterior artery preparations.[12,22] Adenosine (10^{-6} M) was also applied on veins preconstricted with ET-1 (10^{-8} M). The significance of any induced change in vessel diameter was assessed in relation to the baseline vessel diameter. All data are presented as normalized vessel diameter percentage (%), where the data are normalized to the diameter of the vessel prior to any drug administration.

Solutions and Agents

Vessels were usually bathed and perfused with normal Krebs solution of composition (in mM) NaCl 119; KCl 4.6; $CaCl_2$ 1.5; $MgCl_2$ 1.2; $NaHCO_3$ 15; NaH_2PO_4 1.2; and glucose 6. Ca^{2+}-free solution was prepared by omitting the $CaCl_2$ and adding ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA).[23]

All chemicals and vasoactive agents used were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Stock solutions were stored at $-70°C$ and fresh dilutions were made daily.

Experimental Protocol

The effect of a wide range of adenosine concentration (10^{-10}–10^{-4} M) and ET-1 concentration (10^{-12}–10^{-8} M) was determined in isolated perfused retinal veins. When application of ET-1 (10^{-8} M) was used to precontract the vessels, the ET-1 remained in the bath during adenosine (10^{-6} M) administration. Precontracted vessels can be sustained over the experimental period, so any increase in vessel diameter can be attributed to the effects of adenosine.[12,13]

Fixation Protocols for the Confocal Imaging of Retinal Veins

Pig eyes were cannulated at one of the major cilia-retinal arteries supplying the retinal vasculature within an hour of delivery. The standard protocol of perfusion fixation and intravascular staining was followed as detailed in previous publications.[24,25] Briefly, blood cells were washed out from the superior retinal circulation by intravascular perfusion of oxygenated Ringer's with 1% bovine serum albumin for 20 minutes. A semiconcave lens was used in conjunction with an operating microscope to visualize the clearing of blood cells from the superior hemisphere through the pupil and cornea. Subsequent to the Ringer's wash, 4% paraformaldehyde in 0.1 M phosphate buffer was perfused for 15 minutes. This was followed by 20 minutes wash using 0.1 M phosphate buffer, then 5 minutes permeation using 0.1% Triton X-100 made up in 0.1 M phosphate buffer. A further wash for 20 minutes using 0.1 M phosphate buffer, 30 U phalloidin conjugated with Alexa Fluor 647 or 546 (A22283 and A22287; Invitrogen, Carlsbad, CA, USA) made up in 0.1 M phosphate buffer, was perfused through the vasculature over a period of 2 hours; a final wash in phosphate buffer for 30 minutes followed. The globe was then unmounted from the cannula and the anterior chamber removed using a razor blade cutting just posterior to the equator. The posterior chamber was immersion fixed in 4% paraformaldehyde overnight before the retina was flat mounted for confocal imaging.

Confocal imaging was carried out on the Nikon C1 in combination with Nikon 90i microscope. Depending on the probe used, laser line 551 nm and 635 nm were used to excite the corresponding Alexa Fluor fluorophores for visualization on the imaging system. The retinal vessels were imaged using $\times$20 plan apo objection lens with the depth of the vessel wall imaged at 0.30-μm intervals. Selected range of optical images was projected to obtain a two-dimensional merged image of the phalloidin-labeled vessel through its z-depth as shown in Figures 2C and 2D.

Statistics

All statistical testing was performed using the statistics program SigmaStat (Jandel Scientific Software, San Raphael, CA, USA). The significance of any drug-induced concentration-dependent changes was tested using 1-way ANOVA, with significance acceptance level of $P < 0.05$ for the F value. When appropriate, Student's t-test was employed. All mean data are expressed as mean $\pm$ standard error, and all error bars on graphs are also standard errors.

RESULTS

General

In all, 66 vein segments were studied. The average diameter at baseline was 124.6 ± 2.37 μm. Marked differences were noted between the thickness of the vessel wall in the retinal veins compared to that of retinal arteries of similar order that we had previously studied. Figures 2A and 2B are trans-illumination microphotographs of an isolated perfused retinal artery and vein, and show a comparison of vessel wall structure between the retinal artery (Fig. 2A) and vein (Fig. 2B) under baseline conditions before any drug administration. The focus level of all images is at the site of the vessel wall. The diameter of the retinal vein is always slightly larger than that of the retinal artery. However, the thinness of the vein wall is remarkable in comparison to that of the retinal artery. Retinal arteries, like most terminal arteries, are composed of an almost complete layer of circularly oriented smooth muscle cells evidenced by continuously arranged cross sections of smooth muscle cells in the artery wall, while the endothelium lines the inside of the retinal artery wall and appears as a thin dark line at the inner wall (arrowheads). Some defocused longitudinally arranged cells along the artery can be seen and are very likely endothelial cells and their nuclei. However, in Figure 2B, the vein wall is much thinner and some endothelial nuclei can be found the inside of the vessel wall, but smooth muscle cells are difficult to identify. Some wider cells with blurred edge can be seen, which could be defocused endothelial cells. To further illustrate the structure of the vessel wall, particularly smooth muscle cells, intravascular microperfusion, fixation, and labeling in isolated preparations of retinal arteries and veins were used. Figures 2C and 2D are projected confocal images from a retinal artery and vein stained for f-actin to label smooth muscle and endothelial cell cytoskeleton. Endothelial cells in the retinal arteries and veins have been described in our previous publications.[2,24–27] With f-actin staining and confocal microscopy (Figs. 2C, 2D), the relative thinness of the vein wall (Fig. 2D) is even more apparent compared to that in the retinal artery (Fig. 2C), which is consistent with the microscope images taken during the experiments. Dense circularly oriented smooth muscle cells can be clearly seen as spider-like cells (Fig. 2C). However, no circularly oriented spider-like cells can be seen in the tunica media of the retinal
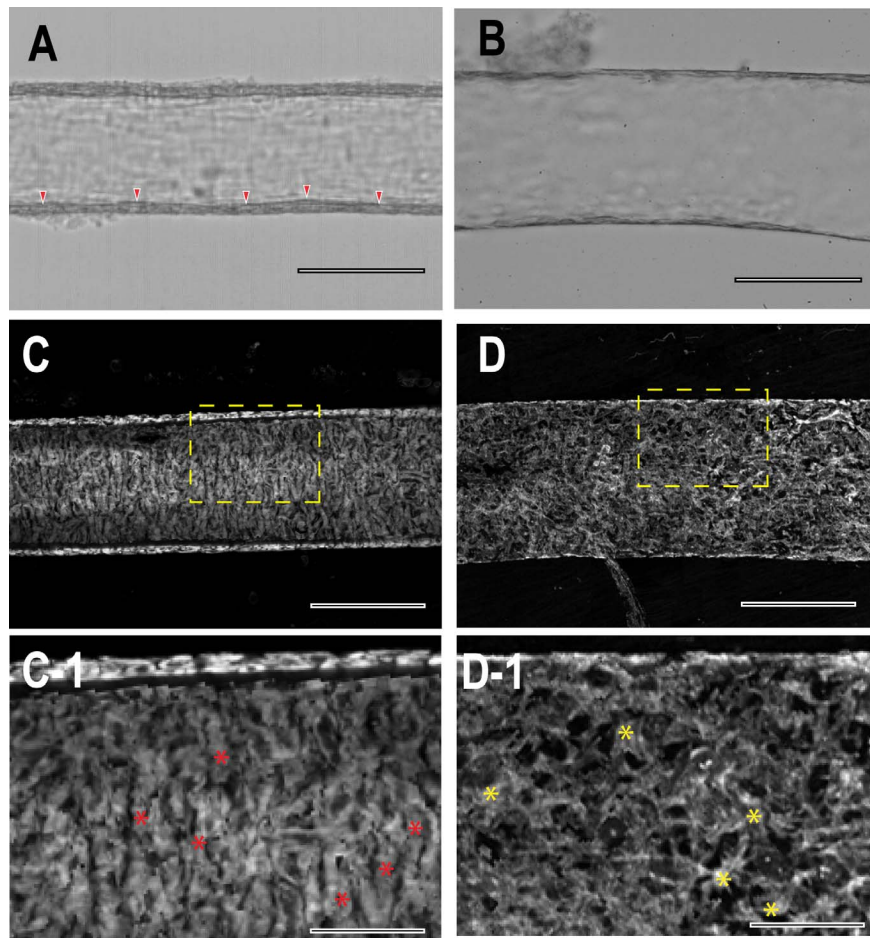
FIGURE 2. Comparison of vessel wall structure between the retinal artery and vein. (**A**, **B**) Transillumination microphotographs of an isolated perfused retinal artery and vein taken during experiments before vasoactive agent administration; (**C**, **D**) confocal images of a retinal artery and vein, respectively, from pig eyes that have been perfused, fixed, and labeled with f-actin. The focus level of all images is at the site of the vessel wall. In (**A**, **B**), the wall of the retinal artery is much thicker than that of the retinal vein. Retinal arteries are composed of an almost complete layer of circularly-oriented smooth muscle cells, and the endothelium lines the inside of the retinal artery wall and appears as a dark line (*arrows*). However, in (**B**), the vein wall is much thinner and some endothelial nuclei can be found on the inside of the vessel wall, but smooth muscle cells cannot be clearly identified. In (**C**), spider-like smooth muscle cells can frequently be seen in the tunica media. (**C-1**) An enlarged image from the highlighted area of (**C**); some spider-like cells of smooth muscle cells are closely arranged (indicated by *red asterisks*). In (**D**), no circularly oriented spider-like cells can be seen in the tunica media. (**D-1**) An enlarged image from the highlighted area of (**D**); most cells have an irregular shape and various orientations with relatively large spaces between these cells. *Scale bars*: 100 μm (**A–D**); 20 μm (**C-1**, **D-1**).

vein wall (Fig. 2D). Most cells found in the tunica media of the vein wall have irregular shape and various orientations with relatively large spaces between these cells (Fig. 2D). With higher magnification of the selected regions from Figures 2C and 2D, individual spider-like smooth muscle cells with relatively high f-actin labeling can be seen in the tunica media of the artery (Fig. 2C-1, red asterisk). However, f-actin labeling in the vein (Fig. 2D-1) is much weaker in these irregularly shaped cells (yellow asterisk) with various orientations and relatively large spaces. Interestingly, there are dramatic differences of cellular morphologic appearances, density, and orientations in the vascular wall between the retinal arteries and veins.

ET-1–Induced Dose-Dependent Vasocontractile Responses in Basal Tone Retinal Veins

The effect of increasing doses of ET-1 on isolated perfused retinal veins is shown in Figure 3. The ET-1–induced contraction was dose dependent, becoming significant at 10^{-11} M ET-1 and above. At the maximum ET-1 concentration of 10^{-8} M, the contraction reached $70.2 \pm 2.1\%$ of the initial baseline diameter ($n = 30$).

Endothelin-1 produces a sustained retinal vein contraction in the isolated perfused retinal vein preparation for more than 20 minutes if no other agents are added.

To quantify the uncontracted retinal veins, that is, basal tone, normal perfusion solution was replaced by Ca^{2+}-free solution with the result that averaged diameter was significantly increased from 107.7 ± 4.0 to 133.2 ± 4.1 μm ($n = 9$, $P < 0.001$). This suggests that the basal tone in our preparation was $24.3 \pm 3.2\%$ from maximal passive diameter.

Vasodilatory Responses Were Induced by Adenosine in Basal Tone Retinal Veins

The effect of increasing doses of adenosine on isolated perfused retinal veins is shown in Figure 4. The adenosine-induced dilation was dose dependent, becoming significant at 10^{-6} M adenosine and above. At the maximum adenosine
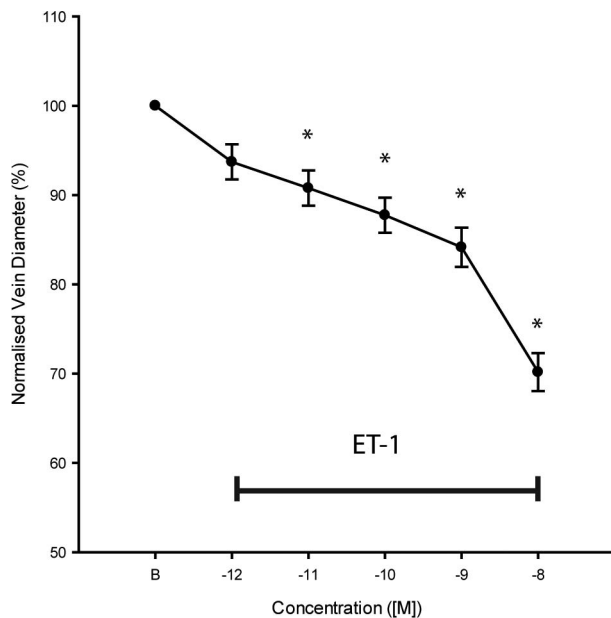
FIGURE 3. Average vein diameter in normal tone in response to increasing doses of ET-1 (10^{-12}–10^{-8} M) administration. Significant vasoconstriction was seen at ET-1 10^{-11} M, with contraction increasing at higher concentrations. *Asterisk* denotes a significant contraction compared to initial baseline ($P < 0.05$).

FIGURE 4. Average vein diameter in normal tone in response to increasing doses of adenosine (10^{-8}–10^{-4} M) administration. Significant vasodilation was seen at adenosine 10^{-6} M, with vasodilation increasing further at higher concentrations. *Asterisk* denotes a significant dilation compared to initial baseline ($P < 0.05$).

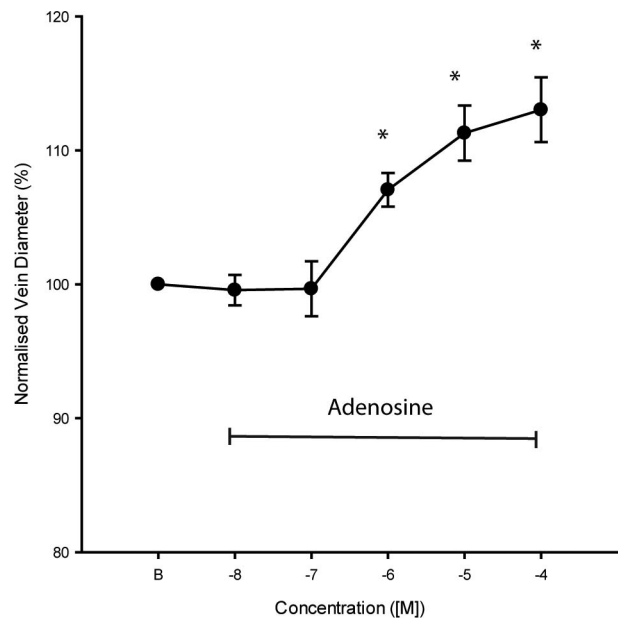concentration of 10^{-4} M, the dilation reached $113.0 \pm 2.4\%$ of the initial baseline diameter ($n = 24$).

Adenosine Dilation of ET-1–Constricted Retinal Veins

The effect of 10^{-6} M adenosine on isolated perfused retinal veins precontracted with 10^{-8} M ET-1 is shown in Figure 5. The adenosine-induced dilation was significant, completely overcoming the ET-1–induced contraction to reach $107.3 \pm 2.0\%$ ($n = 12$), which was significantly larger than the original baseline. Endothelin-1 10^{-8} M was added to the bath after basal tone had been stable for at least 3 minutes. Adenosine was added after the retinal vein contraction induced by ET-1 became stable, which could often take more than 5 minutes. Adenosine-induced retinal vein dilation was measured after a stable vessel diameter was reached, which could be slightly longer depending on the concentration level and equalization time in the bath solution. Increased vasomotion spikes could be often be seen after adenosine was applied. Similar vasomotion spikes also occurred in retinal arteries after higher concentrations of adenosine.[12,22]

DISCUSSION

Using the isolated perfused retinal vein preparation, we have found that ET-1 can induce dose-dependent vasocontractile responses in basal tone retinal veins, that vasodilatory responses were induced by adenosine in basal tone retinal veins, and that adenosine can dilate ET-1–constricted retinal veins. Therefore, we have demonstrated that the retinal vein can be modulated by common, locally produced, vasoactive agents such as ET-1 and adenosine.

Systemically the venous system is considered to be more complex than the arterial system in many respects,[28] and venous diseases are 10 times more frequent than arterial diseases.[29] The main homeostatic significance of veins or venules in microcirculation may be in stabilizing capillary hydrostatic pressure, which is a key determinant of fluid exchange between blood and tissue.[9] The hemodynamic role of the veins and venules may be critical, but this issue has not been sufficiently addressed. Most studies on regulatory roles in the microcirculation have been focused on the arterial system rather than on veins.

Retinal circulation has some unique features. Due to the optical nature of the eye, high metabolic rate of the retina can be supplied only by anatomically sparse retinal vasculature. To meet such unusual requirements, the retinal circulation has powerful and local regulatory capability. To achieve local regulatory mechanism, signal communications between retinal vasculature and tissue-released factors play critical roles.[2,12,30-32]

The retinal veins are the only drainage pathway for blood flowing in the retinal circulation. The hemodynamic role of the retinal veins may be critical, but this issue has not been sufficiently addressed. There is no doubt that RVO is a common and sight-threatening disease. The sequelae of RVO include persistent retinal edema, ischemia, hemorrhage, and neovascularization. Most therapies target only the secondary effects of RVO, particularly macular edema, and do not address the underlying causal pathology. Numerous theories abound concerning the pathogenesis of RVO and remain controversial. Mechanical compression of the vessel wall or thrombotic occlusion and rheologic disorders are assumed. Recent studies have identified location-specific phenotypic differences of endothelium at the level of venous microvasculature. Studies of endothelial cell morphology in the central and branch retinal veins in human donor eyes[33,34] have found site-specific changes at these locations known to be vulnerable to venous occlusive diseases. A concept of a local venous constriction induced by vasoconstrictive molecules diffusing from neighboring diseased arteries and/or from other neighboring (hypoxic) tissues has been proposed.[18] It is important to know whether retinal vein could be modulated like retinal
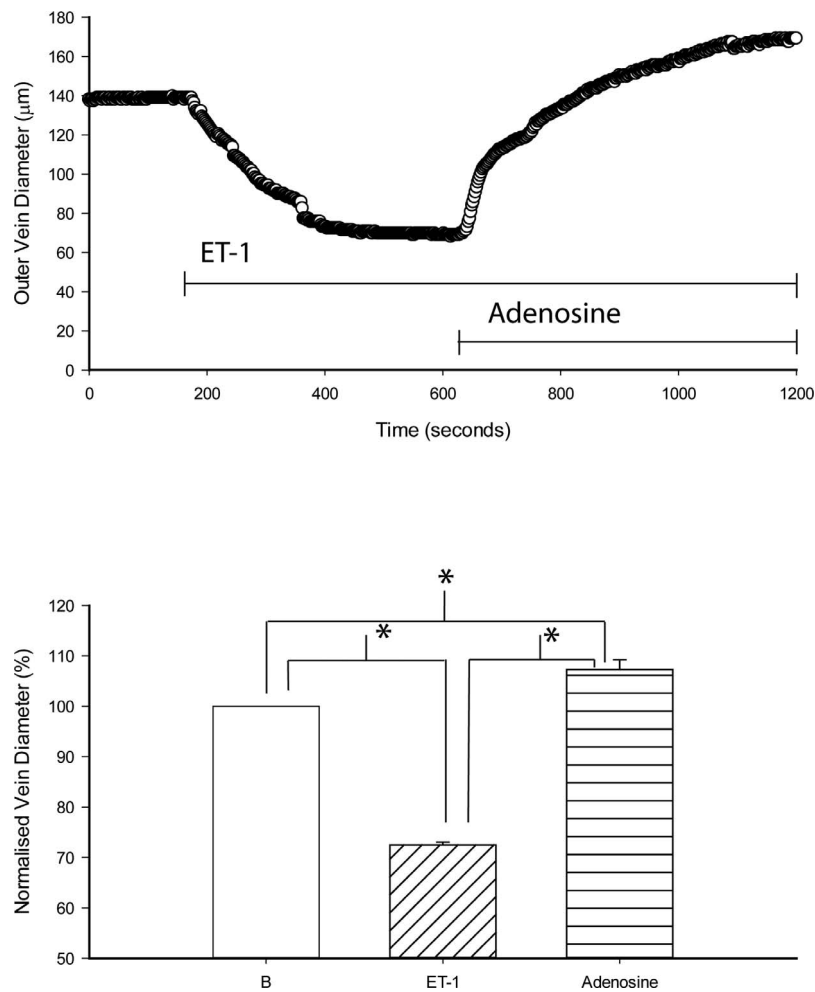
FIGURE 5. Adenosine induced dilation in ET-1 precontracted retinal veins. (**A**) Raw data showed that the vein diameter was reduced after ET-1 10^{-8} M administration and dilated after adenosine 10^{-6} M application. (**B**) Average vessel diameter was reduced by ET-1 10^{-8} M administration and dilated after adenosine 10^{-6} M application. *Asterisk* denotes a significant difference between a significant contraction by ET-1 and dilation by adenosine compared to initial baseline ($P < 0.05$).

artery and whether we can modulate the retinal vein to regulate retinal circulation.

In this study, we selected two vasoactive agents, ET-1 and adenosine, to determine whether retinal vein could be modulated. Adenosine is a signaling molecule in the retina.[35] Adenosine, along with the excitatory amino acids glutamate and aspartate, is known to be released from ischemic and hypoxic neural tissue.[36,37] We have demonstrated an asymmetrical response to exogenous adenosine in retinal arteries in that extraluminal administration of adenosine produces a dose-dependent dilation, whereas intraluminal adenosine fails to produce a significant dilation response.[22] Adenosine has extensively been studied.[38-44] Endothelins are endogenous vasocontracting peptide agents. Endothelin-1, ET-2, and ET-3 are produced in a variety of tissues, where they act as modulators of important cell processes. Endothelin-1 is very clearly an important vascular hormone. Endothelin-1 has been shown to be one of the most potent vasocontractors in the ocular vasculature.[12,45-47] Significant reduction in the blood flow in the retina, choroid, and optic nerve head by exogenously administered ET-1 has been reported in humans and in a number of animal species.[30,48-50] Endothelin-1 has also been studied in the retina and optic nerve.[51-62] In this study, ET-1–induced retinal vein contraction has been demonstrated,

although ET-1–induced dose-dependent vasocontraction in retinal vein is less potent than that in arteries with a similar range of dosage.[12,14] At the maximum ET-1 concentration of 10^{-8} M, the contraction reached 70% of the initial baseline diameter. Interestingly, adenosine is not only able to induce a concentration-dependent dilation; increased vasomotion spikes could also occur with higher concentrations of adenosine in a similar manner to that observed in retinal arteries.[22] In addition, adenosine can dilate ET-1 precontracted retinal vein, indicating that retinal vein could actively be modulated by these vasoactive agents.

Our findings support the results from in vivo experiments performed by Campochiaro and Sen.[42] Interestingly, the dilation of the retinal vein was induced by adenosine and its agonists, which were injected intravitreally in the rabbits. In fact, intravitreal injection of adenosine is similar to our extraluminal administration. A different time course of adenosine-induced dilation of the retinal vein between their in vivo experiments and our in vitro studies could be explained by relatively slow diffusion of adenosine through the vitreous.

Although the wall of the retinal vein is very thin, the wall of the retinal vein has been described as consisting of a single layer of endothelial cells, a subendothelial coat of connective tissue, and a few smooth muscle cells, as well as a thin adventitia of

connective tissue.[15] In fact, we are still attempting to understand the morphologic appearance and functional activity of these cells in the retinal vein wall. In the retinal artery, smooth muscle cells are dense, well aligned, and circularly oriented. They are capable of strong vasoactive responses. However, these cells in the retinal vein are weakly stained, irregularly shaped, and poorly oriented, which would suggest only a weak vasoactive capability. However, the distinct vasoactive responses seen in the present study suggest otherwise. It is therefore interesting to investigate how different cell types contribute to the vasoactivity of the retinal vein.

Acknowledgments

Disclosure: **D.-Y. Yu**, None; **E.-N. Su**, None; **S.J. Cringle**, None; **W.H. Morgan**, None; **I.L. McAllister**, None; **P.K. Yu**, None

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