Physiology and Pharmacology

Constriction of Retinal Venules to Endothelin-1: Obligatory Roles of ET_A Receptors, Extracellular Calcium Entry, and Rho Kinase

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Purpose. Endothelin-1 (ET-1) is a potent vasoconstrictor peptide implicated in retinal venous pathologies such as diabetic retinopathy and retinal vein occlusion. However, underlying mechanisms contributing to venular constriction remain unknown. Thus, we examined the roles of ET-1 receptors, extracellular calcium (Ca^{2+}), L-type voltage-operated calcium channels (L-VOCCs), Rho kinase (ROCK), and protein kinase C (PKC) in ET-1-induced constriction of retinal venules.

Methods. Porcine retinal venules were isolated and pressurized for vasoreactivity study using videomicroscopic techniques. Protein and mRNA were analyzed using molecular tools.

Results. Retinal venules developed basal tone and constricted concentration-dependently to ET-1. The ET_A receptor (ET_AR) antagonist BQ123 abolished venular constriction to ET-1, but ET_B receptor (ET_BR) antagonist BQ788 had no effect on vasoconstriction. The ET_BR agonist sarafotoxin S6c did not elicit vasomotor activity. In the absence of extracellular Ca^{2+}, venules lost basal tone and ET-1–induced constriction was nearly abolished. Although L-VOCC inhibitor nifedipine also reduced basal tone and blocked vasoconstriction to L-VOCC activator Bay K8644, constriction of venules to ET-1 remained. The ROCK inhibitor H-1152 but not PKC inhibitor Gö 6983 prevented ET-1-induced vasoconstriction. Protein and mRNA expressions of ET_ARs and ET_BRs, along with ROCK1 and ROCK2 isoforms, were detected in retinal venules.

Conclusions. Extracellular Ca^{2+} entry via L-VOCCs is essential for developing and maintaining basal tone of porcine retinal venules. ET-1 causes significant constriction of retinal venules by activating ET_ARs and extracellular Ca^{2+} entry independent of L-VOCCs. Activation of ROCK signaling, without involvement of PKC, appears to mediate venular constriction to ET-1 in the porcine retina.

Keywords: retinal microcirculation, vasoconstriction, endothelin receptors

The light-sensitive retinal tissue exhibits a high metabolic rate, which requires the retinal circulation to provide sufficient oxygen and nutrients to maintain its proper function.[1] In the microcirculation, two factors that impact optimal tissue perfusion are the arteriolar resistance for controlling blood flow and the venular resistance for regulating hydrostatic pressure and fluid homeostasis at the level of the capillaries.[2,3] Because the retinal circulation lacks autonomic innervation,[4] local vascular control mechanisms appear to dominate the regulation of retinal vascular resistance.[5] Although the importance of the intrinsic ability of retinal arterioles to regulate their resting diameter is well established,[5] there is a paucity of information on vasomotor function of retinal venules. In vivo studies have reported an increase in retinal venular diameter during flickering light stimulation[6] or a reduction in retinal venular diameter in response to hyperoxia,[7] suggesting these vessels play an active role in flow regulation. However, it is difficult to distinguish the direct influence on retinal venular tone from a response secondary to the upstream changes in pressure and/or flow[8] using a whole-organ approach or during systemic intervention. A recent report has shown that isolated porcine retinal veins are reactive to local vasoactive factors such as vasodilator adenosine and vasoconstrictor endothelin-1 (ET-1).[9] It is unclear, however, whether smaller upstream venules exhibit similar reactivity, especially vasoconstriction, which dictates not only blood drainage but also capillary pressure and fluid exchange.

ET-1 is a potent vasoactive peptide composed of 21 amino acids and plays important roles in many biological functions, including vasoconstriction, cardiovascular remodeling, tissue inflammation, and cell proliferation.[10–17] In the eye, ET-1 is distributed locally in the retina,[18,19] causes retinal vasoconstriction in vivo,[20–23] and reduces retinal blood flow.[23–25] Our previous studies have demonstrated the cell signaling mechanisms contributing to ET-1–induced constriction of porcine and human retinal arterioles.[26–29] Although others have shown that isolated porcine retinal veins are capable of constricting to ET-1,[9] the underlying mechanisms contributing to this response have not been established. Accumulating evidence has shown that vitreous humor, plasma, and ocular levels of ET-1 are

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elevated in diabetes,[23,24,30,31] especially in patients with diabetic retinopathy.[31,32] The increased plasma ET-1 level also has been implicated in the pathogenesis of retinal vein occlusion (RVO).[33,34] Because alteration in function of the retinal microcirculation, including tissue edema, is regarded as a key event contributing to visual impairment and blindness with diabetes and RVO, greater insight into the mechanisms controlling retinal venular vasomotor activity would advance our understanding of these retinal pathologies.

Previous studies in different organ systems and vascular beds have suggested that vasoconstriction to ET-1 can be mediated by the activation of its receptors (ET_A and/or ET_B),[11,12,26] elevation of intracellular calcium (Ca^{2+}),[29,35–37] and signaling through Rho kinase (ROCK) and/or protein kinase C (PKC).[12,29,38] However, the contributions of specific ET-1 receptors, extracellular Ca^{2+} entry, and intracellular signaling pathways to ET-1–induced constriction of retinal venules remain unknown. To address these issues directly without the influence of metabolic, hemodynamic, and neuronal/glial factors commonly inherent in in-vivo preparations, porcine retinal venules were isolated and pressurized for in vitro study. The relative functional roles of ET_ARs, ET_BRs, ROCK, PKC, and extracellular Ca^{2+} entry through L-type voltage-operated calcium channels (L-VOCCs) in the ET-1–induced constriction of porcine retinal venules were investigated. We also assessed ET_AR, ET_BR, and ROCK isoform (ROCK1 and ROCK2) expression in porcine retinal venules to assist functional interpretation.

Methods

Animal Preparation

All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Baylor Scott & White Health Institutional Animal Care and Use Committee. Pigs (*Sus scrofa domesticus*, Yorkshire) of either sex (age range, 8–12 weeks; weight range, 10–20 kg) purchased from Real Farms (San Antonio, TX, USA) were sedated with Telazol (4–8 mg/kg, intramuscularly), anesthetized with 2% to 5% isoflurane, and intubated. The procedure used for harvesting eyes has been described previously.[39,40]

Isolation and Cannulation of Retinal Venules

We previously described the techniques for isolating and cannulating porcine retinal arterioles, which were applied to retinal venules in the current study.[39–41] The ocular tissue was placed in a cooled dissection chamber (~8°C) containing a physiological salt solution (PSS; 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, and 3.0 mM MOPS) with 0.1% albumin. The retinal venules (1 to 1.5 mm in length without side branches) were identified based on the dark-red deoxygenated blood in the lumen and their thin vascular wall compared with the parallel arterioles containing bright-red oxygenated blood and a thick vascular wall. The second-order venules were carefully dissected with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY, USA). After removal of any remaining neural/connective tissues, the venule was transferred with a capillary micropipette (Wiretrol II; Drummond Scientific Company, Broomall, PA, USA) to a polymethylmethacrylate vessel chamber containing PSS-albumin solution equilibrated with room air at ambient temperature. Both ends of the venule were cannulated using glass micropipettes filled with PSS containing 1% albumin, and the outside of the venules were securely tied to the pipettes with 11-0 ophthalmic suture (Alcon, Fort Worth, TX, USA). Vessels were pressurized to 5 cm H_2O (4 mm Hg) intraluminal pressure by two independent pressure reservoirs and allowed to develop basal tone before study. This intraluminal pressure was chosen based on a study reporting porcine retinal transmural venous pressure of 0 to 4 mm Hg.[42] Vasomotor activity of isolated venules was recorded using videomicroscopic techniques throughout the experiments.[39]

Experimental Protocols

Cannulated venules were bathed in PSS-albumin at 36° to 37°C to allow development (~90 minutes) of basal tone. In one series of studies, the vasomotor response to cumulative administration of ET-1 (1 pM to 10 nM; Bachem, Torrance, CA, USA),[26] selective ET_BR agonist sarafotoxin S6c (10 pM to 0.1 μM; Tocris Cookson, Ellisville, MO, USA),[26] or PKC activator phorbol-12,13-dibutyrate (PDBu; 0.1 μM to 10 μM)[29] was evaluated. Retinal venules were exposed to each concentration of ET-1, sarafotoxin, or PDBu for 10 minutes until a stable diameter was established. Because the vasoconstrictor action of ET-1 was maintained after washout, only one concentration-response curve was constructed in each vessel for this drug. The relative roles of ET_ARs and ET_BRs in the retinal venule responses to ET-1 were evaluated after treatment of the vessel with respective antagonists BQ123 (1 μM)[26] and BQ788 (0.1 μM).[26] To assess the contribution of extracellular Ca^{2+} entry to ET-1–induced vasoconstriction, vasomotor activity was examined in Ca^{2+}-free PSS-albumin (with 1 mM EDTA).[26] The role of L-VOCCs in ET-1–induced vasoconstriction was examined in the presence of L-type calcium channel antagonist nifedipine (1 μM).[29,37] In another set of vessels, the specificity and efficacy of nifedipine were assessed by examining its effectiveness on vasoconstriction to L-VOCC activator Bay K8644 (6 μM).[43] To determine the functional importance of endogenous ROCK and PKC in ET-1–induced vasoconstriction, experiments were performed in the presence of ROCK inhibitor H-1152 (3 μM)[27,29] or broad-spectrum PKC inhibitor Gö 6983 (10 μM).[29] Vessels were pretreated with antagonists or inhibitors extraluminally for at least 30 minutes.

Chemicals

Drugs for the functional studies were purchased from MilliporeSigma (St. Louis, MO, USA) except where specifically stated otherwise. ET-1, sarafotoxin, BQ788, and H-1152 were dissolved in water; BQ123, nifedipine, and Bay K8644 were dissolved in ethanol; and PDBu and Gö 6983 were dissolved in dimethyl sulfoxide. All subsequent concentrations of these drugs were diluted in PSS.[26] The final concentration of dimethyl sulfoxide or ethanol in the vessel bath was less than 0.1% by volume. Vehicle control studies indicated that this final concentration of solvent had no effect on vessel viability, vasoconstrictor responses, or maintenance of basal tone (data not shown).

mRNA Isolation and Real-Time PCR Analysis

Total RNA was isolated from retinal venules (sample pooled from both eyes) and neural retina tissue via RNeasy mini kit (QIAGEN, Crawley, UK), as described in our previous study.[44] The same amount of mRNA for each sample was used to synthesize cDNA with the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). RNA and cDNA were quantified using a Nanodrop spectrophotometer (BioTek, Winooski, VT, USA), and sequentially processed using real-time PCR reagents (PowerUp SYBR Green Master

Mix, Thermo Fisher Scientific). To perform the real-time PCR experiments, we used specific primer sets for ET_AR (forward primer: 5′-AAGCAGGACAGCCCATTAAG-3′, reverse primer: 5′-CTGCTAGCTGAAGTACTCGGAAC-3′), ET_BR (forward primer: 5′-GGAGTTGAGATGTGTAAGCTGGTG-3′, reverse primer: 5′-TCTGAGTAGGATGGAGCAAGCAGA-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward primer: 5′-CCACCCACGGCAAGTTCCACGGCA-3′, reverse primer: 5′-GGTGGTGCAGGAGGCATTGCTGAC-3′). Real-time PCR was performed by monitoring the increase in SYBR Green fluorescence using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA, USA). After an initial denaturing at 95°C for 2 minutes, reactions were carried out at 95°C for 1 second and 60°C for 30 seconds for 40 cycles. Relative fold change of each targeted mRNA was determined by the $2^{-\Delta\Delta Ct}$ method.[45]

Western Blot Analysis

Western blot analysis was performed as described in our previous study of retinal arterioles with slight modification.[26,46] Retinal venules (sample pooled from both eyes per pig) and neural retina tissue were isolated and sonicated in lysis buffer. The protein content of each lysate was determined with the BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein (2.5 μg for ET-1 receptors and 5 μg for ROCK isoforms and p38) were separated by Tris-glycine SDS-PAGE (4%–15% Tris-HCl Ready Gels; Bio-Rad, Hercules, CA, USA), transferred onto a nitrocellulose membrane, and incubated with rabbit anti-ET_AR or anti-ET_BR polyclonal antibody (1:250 dilution; Catalog nos. sc-33535 and sc-33537; Santa Cruz Biotechnology, Santa Cruz, CA, USA),[26] or mouse anti-ROCK1 or anti-ROCK2 monoclonal antibody (1:250 dilution; Catalog nos. sc-17794 and sc-398519; Santa Cruz Biotechnology). Membranes were stripped and reprobed with rabbit anti-p38 antibody (1:1000; Catalog no. sc-535; Santa Cruz Biotechnology), which we previously have shown is highly expressed in neural retina tissue.[26] After incubation with an appropriate secondary antibody (anti-rabbit or anti-mouse IgG, 1:1000; Catalog nos. 7074S and 7076S; Cell Signaling Technology, Danvers, MA, USA), the membranes were washed and developed by enhanced chemiluminescence (Pierce).

Data Analysis

The maximal diameter of retinal venules was obtained at the end of each functional experiment by relaxing vessels with 0.1 mM sodium nitroprusside in EDTA (1 mM)-Ca^{2+}-free PSS[39] at 5 cm H_2O intraluminal pressure. Diameter changes in response to agonists were normalized to the resting diameter and expressed as percentage changes in diameter.[26] Data are reported as mean ± SEM, and *n* represents the number of animals (1–2 vessels per pig per treatment group for functional studies). Student's *t*-test or repeated measures 2-way ANOVA followed by Bonferroni multiple-range test was used to determine the significance of experimental interventions, as appropriate (GraphPad Prism, Version 6.0; GraphPad Software, La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Retinal Venular Constriction to ET-1 and the Role of ET-1 Receptors

Porcine retinal venules (total 50 vessels, 1–2 per pig) were isolated and pressurized at 5 cm H_2O with average maximum diameter of 133 ± 2 μm. These vessels developed stable basal tone by constricting to approximately 92% of maximum diameter within 30 minutes at 36°C to 37°C. In one cohort, administration of ET-1 caused constriction of retinal venules in a concentration-dependent manner (Fig. 1A). The threshold concentration of ET-1 for venular constriction was approximately 1 pM and the vessels constricted to approximately 65% of their resting diameters at 10 nM, the highest ET-1 concentration tested. Images of the retinal venular constriction in response to 0.1 nM ET-1 are shown in Figure 1B.

Incubation of the retinal venules with the ET_AR antagonist BQ123 did not alter the resting vascular diameter but the vasoconstriction elicited by ET-1 was abolished, except for approximately 10% constriction remaining at the highest concentration (10 nM) of ET-1 (Fig. 1A). In the presence of ET_BR antagonist BQ788, neither the resting diameter nor the vasoconstriction to ET-1 was altered (Fig. 1A). The retinal venules failed to respond to the challenge of ET_BR agonist sarafotoxin S6c throughout the concentrations tested (Fig. 1C).

ET-1 Receptor mRNA and Protein Expressions in Porcine Retinal Venules

ET_AR and ET_BR mRNAs were detected in retinal venules and in neural retina tissue devoid of retinal vessels (Fig. 2A). The neural retina tissue expressed a relatively low amount of ET-1 receptor mRNA compared with the venular tissue. At the protein level, immunoblotting showed that ET_ARs and ET_BRs were strongly expressed in retinal venules, whereas only modest expression of both ET-1 receptors was detected in neural retina tissue (Fig. 2B).

Roles of Extracellular Ca^{2+} Entry and L-VOCCs in ET-1-Induced Venular Constriction

In the presence of a Ca^{2+}-free solution, retinal venules completely lost basal tone, and constriction of these vessels to ET-1 was nearly abolished (Fig. 3A). At 10 nM ET-1, the retinal venules constricted to approximately 88% of their resting diameter in the absence of extracellular Ca^{2+} versus 65% in the presence of extravascular Ca^{2+}. The L-VOCC inhibitor nifedipine also reduced venular basal tone to the same level as in the Ca^{2+}-free solution, but it did not affect the constriction of these vessels to ET-1 (Fig. 3A). Furthermore, the L-VOCC agonist Bay K8644 elicited a modest but significant constriction (8% reduction in resting diameter) of retinal venules (Fig. 3B), which was prevented in the presence of nifedipine (Fig. 3B).

Roles of Rho Kinase and PKC in ET-1-Induced Venular Constriction

The Rho kinase inhibitor H-1152 did not significantly alter the resting diameter, but it abolished ET-1–induced constriction of retinal venules (Fig. 4A). In contrast, PKC inhibitor Gö 6983 affected neither resting diameter nor vasoconstriction to ET-1 (Fig. 4A). Cumulative addition of PKC activator PDBu to the vessel bath also did not significantly affect the resting diameter of retinal venules (Fig. 4B). Immunoblotting showed that both ROCK1 and ROCK2 isoforms were strongly expressed in retinal venules, whereas only faint expression of these proteins was detected in neural retina tissue (Fig. 4C). Sufficient neural retina protein was present in these samples because p38 protein expression was strikingly stronger in neural retina tissue than in venules (Fig. 4C).

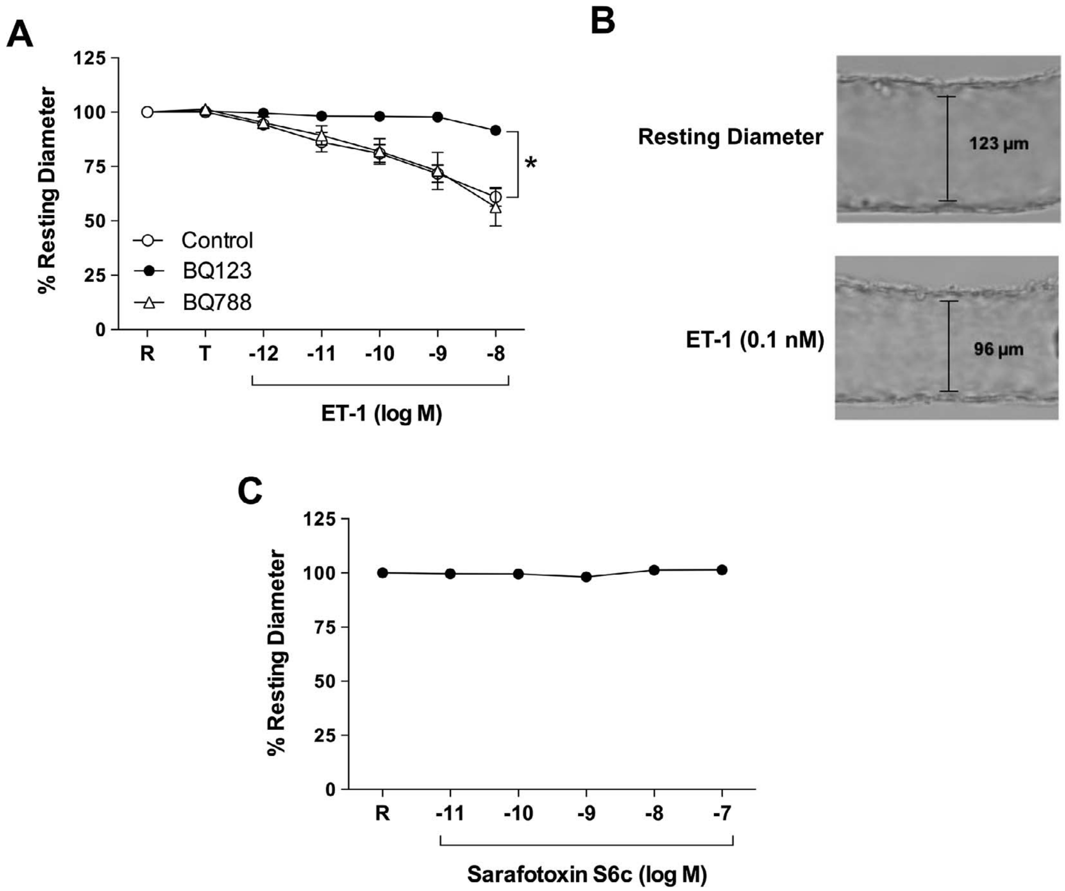

FIGURE 1. Vasomotor response of isolated and pressurized porcine retinal venules to ET-1. (**A**) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with an ET-1 receptor antagonist. In the absence of receptor antagonist, retinal venules constricted to ET-1 in a concentration-dependent manner (Control; $n = 9$). ET_AR antagonist BQ123 (1 µM; $n = 5$), but not ET_BR antagonist BQ788 (0.1 µM; $n = 5$), inhibited venular constriction to ET-1. Both antagonists did not alter resting basal tone. *$P < 0.05$ versus Control. (**B**) Representative images of a porcine retinal venule at resting diameter and after constriction in response to ET-1 (0.1 nM). (**C**) The response of retinal venules to ET_BR agonist sarafotoxin S6c was examined ($n = 6$).

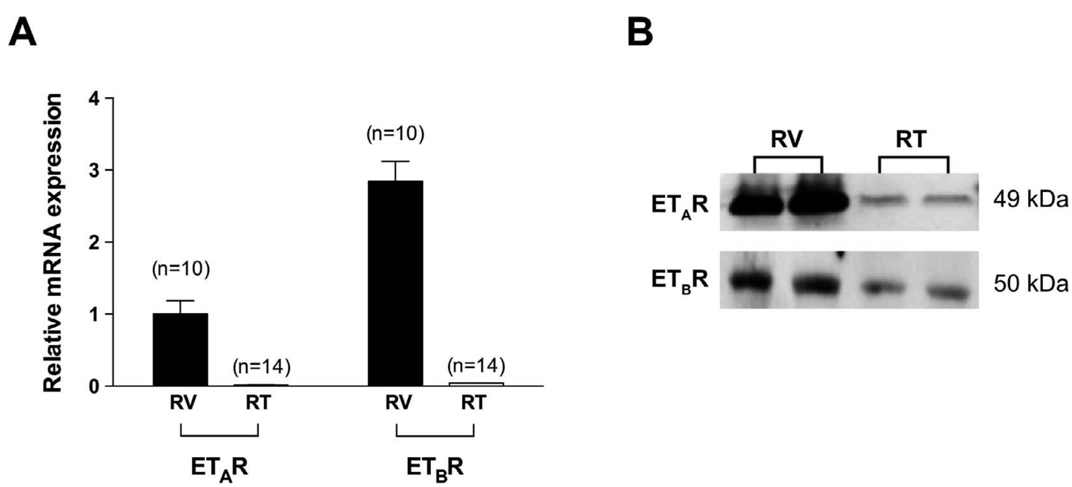

FIGURE 2. Molecular analyses of ET-1 receptors in porcine retinal venules. (**A**) Equal amounts of total RNA isolated from porcine retinal venules (RV) and neural retina tissue (RT) were reverse transcribed and then analyzed by real-time PCR for detection of ET_AR, ET_BR, and GAPDH mRNAs. The ET_AR and ET_BR transcripts were normalized to GAPDH expression and presented as relative mRNA expression. n = number of pigs studied. (**B**) Equal amount of protein was loaded for Western blot analyses of ET_AR and ET_BR in RVs and neural RT from pigs. Data represent four independent experiments.

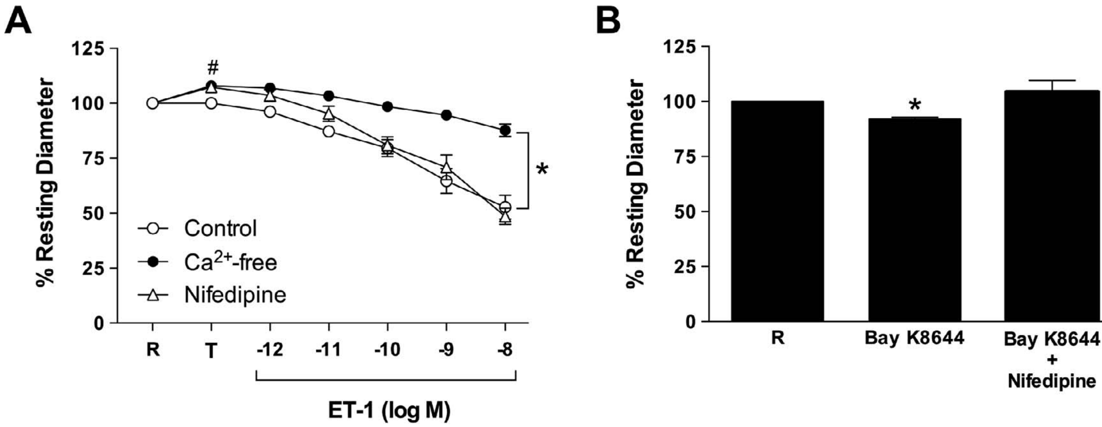

Figure 3. Roles of extracellular calcium and L-VOCCs in vasoconstriction of isolated retinal venules to ET-1. (**A**) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) of the vessel with Ca^{2+}-free solution ($n = 10$) or L-VOCC inhibitor nifedipine (1 μM; $n = 7$). In the absence of treatment (Control; $n = 12$), retinal venules constricted to ET-1 in a concentration-dependent manner. Both Ca^{2+}-free solution and nifedipine abolished resting tone, and the venular constriction to ET-1 was attenuated in the Ca^{2+}-free solution. The vasoconstrictor response to ET-1 remained intact in the presence of nifedipine. #$P < 0.05$ versus R; *$P < 0.05$ versus Control. (**B**) The effect of L-VOCC agonist Bay K8644 (6 μM) on resting diameter (R) was examined in the absence ($n = 3$) or presence of nifedipine (1 μM; $n = 3$). *$P < 0.05$ versus R.

Discussion

Although venules are known to play a critical role in regulation of postcapillary pressure and capillary fluid exchange,[2,3] there is limited study on the direct vasomotor function and cognate mechanisms of retinal venules. The salient findings of the present study are that pressurized porcine retinal venules exhibit basal tone and constrict to ET-1 through ET_AR activation. The entry of extracellular Ca^{2+} and activation of ROCK signaling, independent of L-VOCC and PKC pathways, appear to mediate the venular constriction to ET-1. We also found that extracellular Ca^{2+} is important in maintenance of basal tone, linking to L-VOCC activity.

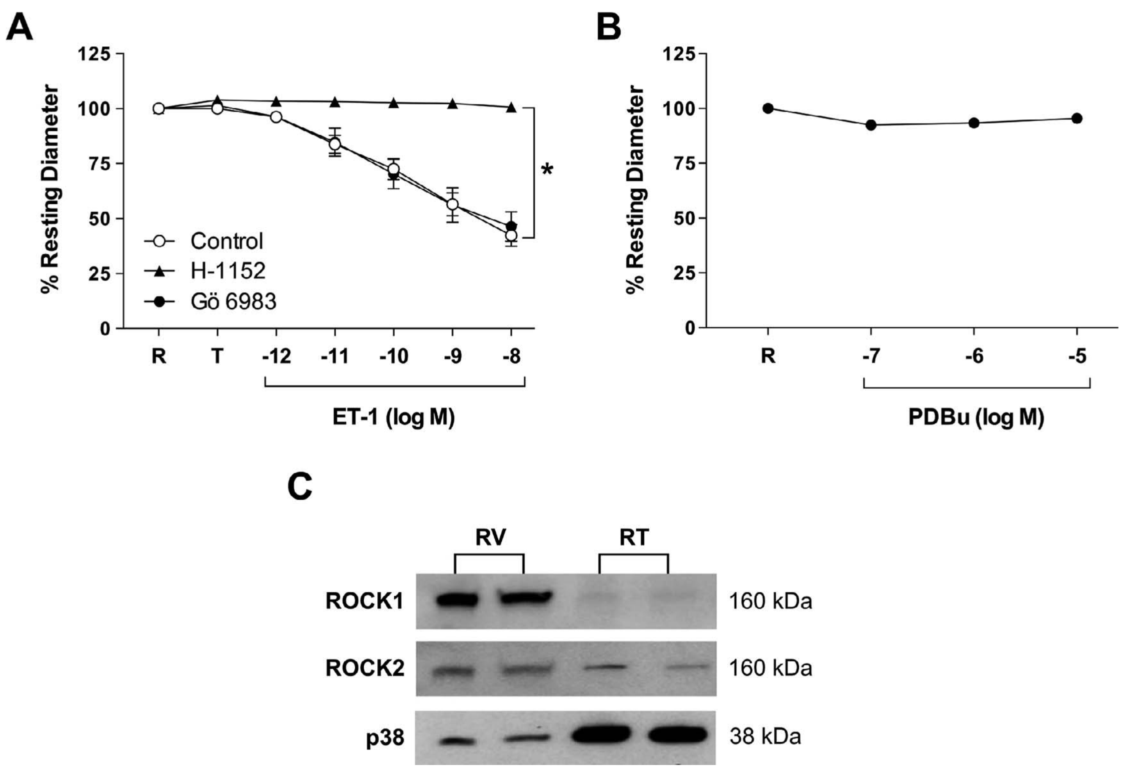

Figure 4. Roles of ROCK and PKC in vasoconstriction of isolated retinal venules to ET-1. (**A**) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with ROCK inhibitor H-1152 (3 μM; $n = 6$) or PKC inhibitor Gö 6983 (10 μM; $n = 7$). In the absence of treatment (Control; $n = 12$), retinal venules constricted to ET-1 in a concentration-dependent manner. The resting diameter of retinal venules was not altered by either drug treatment. The ET-1–induced constriction was abolished by H-1152 but not Gö 6983. *$P < 0.05$ versus Control. (**B**) Venular diameters were recorded under resting conditions (R) and at different concentrations of PKC activator PDBu ($n = 6$). (**C**) Equal amount of protein was loaded for Western blot analyses of ROCK isoforms (ROCK1 and ROCK2) and p38 in retinal venules (RV) and neural retina tissue (RT). Data represent three independent experiments.

Studies using isolated vessel preparations have shown that ET-1 directly causes constriction of small arterioles[26] and veins[9] isolated from the porcine retina. Our current study corroborates the latter report on porcine retinal veins, as well as extends this earlier finding by demonstrating venular constriction to ET-1 and characterizing the underlying mechanism. Our findings show that porcine retinal venules develop stable basal tone of approximately 8% reduction in diameter from its maximum value. This level of basal tone is slightly less than the nearly 20% tone reported in isolated porcine coronary venules[8] and significantly less than the 50% to 60% tone developed in porcine and human retinal arterioles.[27] Our previous study in isolated retinal arterioles from pigs showed that the threshold concentration for ET-1–induced constriction is 1 pM, and the 10 nM concentration elicits a 75% reduction in resting diameter.[26] In the present study, retinal venules exhibited a similar threshold concentration of 1 pM in response to ET-1, but constricted less robustly than the arterioles with only 35% reduction in resting diameter with 10 nM ET-1 (Fig. 1). This venular responsiveness is comparable to that observed in a recent ET-1 study in isolated retinal veins from pigs[9] and is consistent with the report of the greater amount of smooth muscle in retinal arteries than retinal veins.[9] It appears that vascular smooth muscle, albeit a thin layer, in the porcine retinal venule is sufficient to exert an active response to ET-1. These vasoconstrictor responses appear pathophysiologically relevant, because the concentrations of ET-1 used in the current study were within the clinical and experimental range reported for vitreous fluid (picomolar range)[47,48] and the estimated level at the local microvasculature (nanomolar range).[49] The ability of retinal venules to develop basal tone and to constrict in response to ET-1 suggests that these vessels may contribute to the physiological and/or pathophysiological regulation of flow resistance, local pressure, and fluid exchange in the retinal microcirculation.

In the current study, the functional role of specific ET-1 receptor subtypes was characterized by examining the vascular response to ET-1 in the presence of ET_AR or ET_BR antagonist. The concentration-dependent constriction of retinal venules was nearly abolished by pharmacologic ET_AR blockade (Fig. 1). These results are consistent with the major contribution of ET_ARs in retinal arteriolar constriction to ET-1 reported in our previous studies.[26] By contrast, ET_BR blockade (BQ778) did not affect ET-1–induced venular constriction, suggesting that ET_BR does not contribute to this vasomotor response. This contention is supported by the efficacy of BQ778 and the observed unresponsiveness of the vessel to the ET_BR agonist sarafotoxin. Sarafotoxin did not alter resting vascular tone or cause vasoconstriction in the present study (Fig. 1). Moreover, the same concentrations of sarafotoxin were reported in our previous study to elicit significant constriction of porcine retinal arterioles in a manner sensitive to ET_BR blockade (BQ778).[26] It appears that ET_BR plays little role in retinal venular constriction to ET-1, a mechanism that differs from their upstream arterioles, in which both ET_ARs and ET_BRs contribute to the vasoconstriction to ET-1,[26,28] at least under normal physiological conditions. At the molecular level, we showed expression of both ET_AR and ET_BR mRNA and protein in isolated porcine retinal venules (Fig. 2). The mRNA and protein expressions of both ET-1 receptor subtypes were also detected in the neural retina tissue but were strikingly lower than those in the retinal venules. Expression of both ET-1 receptor subtypes in the neural retina layers is also supported by the immunohistochemical data in pigs[50] and rodents,[51–53] but the quantitative comparison with retinal vascular tissue is not available. Although we can detect the mRNA and protein levels in retinal venules, the distinct cellular distribution, that is, endothelial versus smooth muscle cells, of ET_ARs and ET_BRs in the retinal venules requires further investigation. Collectively, our functional and molecular findings support the dominant role of ET_ARs in mediating ET-1–induced constriction of porcine retinal venules.

Vascular smooth muscle contraction is regulated by changes in intracellular Ca^{2+} with Ca^{2+} entry occurring through several types of channels, including L-VOCCs.[54,55] Although activation of ET_ARs elicits Ca^{2+} signaling in blood vessels, the specific channels involved are diverse, depending on the vascular bed and species.[12] The potential roles of extracellular Ca^{2+} and L-VOCCs in basal tone and ET-1–induced constriction of retinal venules have not been explored previously. In the present study, the resting basal tone was abolished in the absence of extracellular Ca^{2+} or during exposure to nifedipine, suggesting that extracellular Ca^{2+} and L-VOCCs are indispensable for maintenance of basal tone at physiologic intraluminal pressure. The constriction of retinal venules to ET-1 also required Ca^{2+} entry, because ET-1 failed to elicit vasoconstriction in the absence of extracellular Ca^{2+} (Fig. 3). However, the inability of nifedipine to prevent the ET-1–induced vasoconstriction indicates that L-VOCCs do not mediate the extracellular Ca^{2+} entry. The efficacy of nifedipine in blocking L-VOCCs was supported by the obliteration of the venular constriction to L-VOCC activator Bay K8644 (Fig. 3). Our finding with ET-1 is consistent with the lack of an effect of L-VOCC blockade on the ET-1–induced contraction of rat mesenteric veins[56] and our previous results with porcine retinal arterioles.[29] It seems that the pathway of Ca^{2+} entry for vasoconstriction in response to ET-1 in retinal venules is distinct from that used for basal tone maintenance in that the former does not involve L-VOCCs. The specific smooth muscle channels responsible for ET-1–induced constriction in retinal venules remains unclear, and it will be the subject of future investigation.

It is generally accepted that the process of smooth muscle contraction is coupled to the level of myosin light chain (MLC) phosphorylation,[57] which is modulated by Ca^{2+}-dependent activity of MLC kinase[38,58,59] and by MLC phosphatase.[60] Protein kinases such as ROCK and PKC can inhibit MLC phosphatase, leading to enhanced MLC phosphorylation[61–63] and subsequent contraction of vascular smooth muscle.[38,60] Both ROCK and PKC have been shown to be possible signaling molecules modulating contractile myofilament sensitivity to Ca^{2+}, thus regulating the force of smooth muscle contraction.[64,65] However, it is unknown whether ET-1 also uses these signaling molecules in the retinal venules to exert its contractile action. We found that specific pharmacological blockade of ROCK prevented constriction of porcine retinal venules to ET-1 without altering basal tone (Fig. 4). In the presence of PKC inhibitor Gö 6983, both resting tone and ET-1–induced vasoconstriction remained intact. Moreover, the retinal venules were not responsive to the PKC activator PDBu (Fig. 4). This observation contrasts with potent PDBu-induced constriction of porcine retinal arterioles, which was blocked by PKC inhibitor Gö 6983 in our previous studies.[28,29] Overall, these results suggest that activation of ROCK, but not PKC, signaling is involved in the venular constriction to ET-1. Furthermore, we detected both ROCK isoforms (ROCK1 and ROCK2) at the protein level in retinal venules. Although the ROCK2 isoform has been suggested to play a major role in smooth muscle contraction,[66] the cellular distributions and their individual functional linking to extracellular Ca^{2+} entry in retinal venules remain unknown and warrant further investigation.

A fundamental understanding of vasomotor regulation mechanisms of retinal venules in response to ET-1 is important, because increased local or plasma levels of this peptide in the retina have been implicated in the pathogenesis of RVO and diabetic retinopathy.[32,67,68] A complication of RVO[69] and latestage diabetic retinopathy[70] is the onset of edema and fluid accumulation in the retina contributing to neural retina dysfunction and blindness. Constriction of retinal veins by ET-1 may increase retinal venous pressure, which could promote edema and reduction in retinal perfusion pressure under disease states.[68] This notion is supported by clinical studies reporting elevation of retinal venous pressure and reduced retinal blood flow in patients with RVO[71,72] and diabetic retinopathy,[73–75] including those with diabetic macular edema.[76] Taken together, these clinical observations along with our current findings underpin the concept that retinal vein constriction to elevated levels of ET-1 contributes to the pathogenesis of retinal diseases, such as RVO and diabetes.[33,67,68,73] Evaluation of the impact of experimental diabetes[77] on ET-1–induced constriction of retinal venules could help corroborate this hypothesis.

In summary, we found that isolated porcine retinal venules develop modest basal tone and constrict markedly to ET-1 in an extracellular Ca^{2+}-dependent manner. Although L-VOCCs play a critical role in maintaining basal tone of retinal venules, they do not contribute to the ET-1–induced vasoconstriction. Retinal venules express both ET_ARs and ET_BRs, but ET_ARs play a dominant role in vasoconstriction to ET-1. It appears that activation of ROCK but not PKC signaling mediates the venular constriction to ET-1. This study provides important insight into the mechanisms of ET-1–induced constriction of retinal venules and lays a foundation for future research to better understand vasomotor regulation of these microvessels under physiological and pathophysiological conditions in the retina.

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References

1. Kiel JW. *The Ocular Circulation*. 1st ed. San Rafael, CA: Morgan & Claypool Life Sciences; 2010:1–69.
2. Johnson PC. Overview of the microcirculation. In: Tuma RF, Duran WN, Ley K, eds. *Handbook of Physiology: The Cardiovascular System, Microcirculation*. 2nd ed. San Diego, CA: Academic Press; 2008:xi–xxiv.
3. Davis MJ, Hill MA, Kuo L. Local regulation of microvascular perfusion. In: Tuma RF, Duran WN, Ley K, eds. *Handbook of Physiology: The Cardiovascular System, Microcirculation (Section 2)*. 2nd ed. San Diego, CA: Academic Press; 2008: 161–284.
4. Ye XD, Laties AM, Stone RA. Peptidergic innervation of the retinal vasculature and optic nerve head. *Invest Ophthalmol Vis Sci*. 1990;31:1731–1737.
5. Tsai SH, Xie W, Zhao M, Rosa RH Jr, Hein TW, Kuo L. Alterations of ocular hemodynamics impair ophthalmic vascular and neuroretinal function. *Am J Pathol*. 2018;188: 818–827.
6. Garhofer G, Zawinka C, Resch H, Huemer KH, Dorner GT, Schmetterer L. Diffuse luminance flicker increases blood flow in major retinal arteries and veins. *Vision Res*. 2004;44:833–838.
7. Palkovits S, Told R, Boltz A, et al. Effect of increased oxygen tension on flicker-induced vasodilatation in the human retina. *J Cereb Blood Flow Metab*. 2014;34:1914–1918.
8. Kuo L, Arko F, Chilian WM, Davis MJ. Coronary venular responses to flow and pressure. *Circ Res*. 1993;72:607–615.
9. Yu DY, Su EN, Cringle SJ, Morgan WH, McAllister IL, Yu PK. Local modulation of retinal vein tone. *Invest Ophthalmol Vis Sci*. 2016;57:412–419.
10. Salvatore S, Vingolo EM. Endothelin-1 role in human eye: a review. *J Ophthalmol*. 2010;2010:354645.
11. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci*. 2013;123:85–101.
12. Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol*. 2016;77:143–175.
13. Brunner F, Bras-Silva C, Cerdeira AS, Leite-Moreira AF. Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. *Pharmacol Ther*. 2006;111:508–531.
14. Murphy JA, Archibald ML, Baldridge WH, Chauhan BC. Endothelin-1-induced proliferation is reduced and Ca^{2+} signaling is enhanced in endothelin B-deficient optic nerve head astrocytes. *Invest Ophthalmol Vis Sci*. 2011;52:7771–7777.
15. Thengchaisri N, Hein TW, Ren Y, Kuo L. Endothelin-1 impairs coronary arteriolar dilation: role of p38 kinase-mediated superoxide production from NADPH oxidase. *J Mol Cell Cardiol*. 2015;86:75–84.
16. Tsai SH, Lu G, Xu X, Ren Y, Hein TW, Kuo L. Enhanced endothelin-1/Rho-kinase signalling and coronary microvascular dysfunction in hypertensive myocardial hypertrophy. *Cardiovasc Res*. 2017;113:1329–1337.
17. Iglarz M, Clozel M. At the heart of tissue: endothelin system and end-organ damage. *Clin Sci (Lond)*. 2010;119:453–463.
18. Chakravarthy U, Douglas AJ, Bailie JR, McKibben B, Archer DB. Immunoreactive endothelin distribution in ocular tissues. *Invest Ophthalmol Vis Sci*. 1994;35:2448–2454.
19. Wollensak G, Schaefer HE, Ihling C. An immunohistochemical study of endothelin-1 in the human eye. *Curr Eye Res*. 1998; 17:541–545.
20. Bursell SE, Clermont AC, Oren B, King GL. The in vivo effect of endothelins on retinal circulation in nondiabetic and diabetic rats. *Invest Ophthalmol Vis Sci*. 1995;36:596–607.
21. Wang Z, Yadav AS, Leskova W, Harris NR. Attenuation of streptozotocin-induced microvascular changes in the mouse retina with the endothelin receptor A antagonist atrasentan. *Exp Eye Res*. 2010;91:670–675.
22. Polak K, Luksch A, Frank B, Jandrasits K, Polska E, Schmetterer L. Regulation of human retinal blood flow by endothelin-1. *Exp Eye Res*. 2003;76:633–640.
23. Pang IH, Yorio T. Ocular actions of endothelins. *Proc Soc Exp Biol Med*. 1997;215:21–34.
24. Takagi C, Bursell SE, Lin YW, et al. Regulation of retinal hemodynamics in diabetic rats by increased expression and action of endothelin-1. *Invest Ophthalmol Vis Sci*. 1996;37: 2504–2518.
25. Granstam E, Wang L, Bill A. Ocular effects of endothelin-1 in the cat. *Curr Eye Res*. 1992;11:325–332.
26. Hein TW, Ren Y, Yuan Z, et al. Functional and molecular characterization of the endothelin system in retinal arterioles. *Invest Ophthalmol Vis Sci*. 2009;50:3329–3336.
27. Hein TW, Rosa RH Jr, Yuan Z, Roberts E, Kuo L. Divergent roles of nitric oxide and Rho kinase in vasomotor regulation of human retinal arterioles. *Invest Ophthalmol Vis Sci*. 2010; 51:1583–1590.
28. Potts LB, Bradley PD, Xu W, Kuo L, Hein TW. Role of endothelium in vasomotor responses to endothelin system and protein kinase C activation in porcine retinal arterioles. *Invest Ophthalmol Vis Sci*. 2013;54:7587–7594.

29. Potts LB, Ren Y, Lu G, et al. Constriction of retinal arterioles to endothelin-1: requisite role of Rho kinase independent of protein kinase C and L-type calcium channels. *Invest Ophthalmol Vis Sci*. 2012;53:2904–2912.
30. Roldan-Pallares M, Rollin R, Martinez-Montero JC, Fernandez-Cruz A, Bravo-Llata C, Fernandez-Durango R. Immunoreactive endothelin-1 in the vitreous humor and epiretinal membranes of patients with proliferative diabetic retinopathy. *Retina*. 2007;27:222–235.
31. Khuu LA, Tayyari F, Sivak JM, et al. Aqueous humor endothelin-1 and total retinal blood flow in patients with non-proliferative diabetic retinopathy. *Eye (Lond)*. 2017;31:1443–1450.
32. Oku H, Kida T, Sugiyama T, Hamada J, Sato B, Ikeda T. Possible involvement of endothelin-1 and nitric oxide in the pathogenesis of proliferative diabetic retinopathy. *Retina*. 2001;21:647–651.
33. Fraenkl SA, Mozaffarieh M, Flammer J. Retinal vein occlusions: the potential impact of a dysregulation of the retinal veins. *EPMA J*. 2010;1:253–261.
34. Iannaccone A, Letizia C, Pazzaglia S, Vingolo EM, Clemente G, Pannarale MR. Plasma endothelin-1 concentrations in patients with retinal vein occlusions. *Br J Ophthalmol*. 1998;82:498–503.
35. Stewart M, Needham M, Bankhead P, et al. Feedback via Ca^{2+}-activated ion channels modulates endothelin 1 signaling in retinal arteriolar smooth muscle. *Invest Ophthalmol Vis Sci*. 2012;53:3059–3066.
36. Miwa S, Iwamuro Y, Zhang XF, et al. Ca^{2+} entry channels in rat thoracic aortic smooth muscle cells activated by endothelin-1. *Jpn J Pharmacol*. 1999;80:281–288.
37. Kawanabe Y, Hashimoto N, Masaki T. Characterization of Ca^{2+} channels involved in endothelin-1-induced contraction of rabbit basilar artery. *J Cardiovasc Pharmacol*. 2002;40:438–447.
38. Puetz S, Lubomirov LT, Pfitzer G. Regulation of smooth muscle contraction by small GTPases. *Physiology (Bethesda)*. 2009;24:342–356.
39. Hein TW, Yuan Z, Rosa RH Jr, Kuo L. Requisite roles of A_{2A} receptors, nitric oxide, and K_{ATP} channels in retinal arteriolar dilation in response to adenosine. *Invest Ophthalmol Vis Sci*. 2005;46:2113–2119.
40. Hein TW, Xu W, Kuo L. Dilation of retinal arterioles in response to lactate: role of nitric oxide, guanylyl cyclase, and ATP-sensitive potassium channels. *Invest Ophthalmol Vis Sci*. 2006;47:693–699.
41. Nagaoka T, Hein TW, Yoshida A, Kuo L. Simvastatin elicits dilation of isolated porcine retinal arterioles: role of nitric oxide and mevalonate-Rho kinase pathways. *Invest Ophthalmol Vis Sci*. 2007;48:825–832.
42. Westlake WH, Morgan WH, Yu DY. A pilot study of in vivo venous pressures in the pig retinal circulation. *Clin Exp Ophthalmol*. 2001;29:167–170.
43. Lawton BK, Brown NJ, Reilly CS, Brookes ZL. Role of L-type calcium channels in altered microvascular responses to propofol in hypertension. *Br J Anaesth*. 2012;108:929–935.
44. Rosa RH Jr, Hein TW, Yuan Z, et al. Brimonidine evokes heterogeneous vasomotor response of retinal arterioles: diminished nitric oxide-mediated vasodilation when size goes small. *Am J Physiol Heart Circ Physiol*. 2006;291:H231–H238.
45. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2_{-\Delta\Delta CT}$ method. *Methods*. 2001;25:402–408.
46. Hein TW, Rosa RH Jr, Ren Y, Xu W, Kuo L. VEGF receptor-2-linked PI3K/calpain/SIRT1 activation mediates retinal arteriolar dilations to VEGF and shear stress. *Invest Ophthalmol Vis Sci*. 2015;56:5381–5389.
47. Roldan-Pallares M, Rollin R, Mediero A, et al. Immunoreactive ET-1 in the vitreous humor and epiretinal membranes of patients with proliferative vitreoretinopathy. *Mol Vis*. 2005;11:461–471.
48. Adamiec-Mroczek J, Oficjalska-Mlynczak J, Misiuk-Hojlo M. Roles of endothelin-1 and selected proinflammatory cytokines in the pathogenesis of proliferative diabetic retinopathy: analysis of vitreous samples. *Cytokine*. 2010;49:269–274.
49. Masaki T, Yanagisawa M, Goto K. Physiology and pharmacology of endothelins. *Med Res Rev*. 1992;12:391–421.
50. Iandiev I, Uhlmann S, Pietsch UC, et al. Endothelin receptors in the detached retina of the pig. *Neurosci Lett*. 2005;384:72–75.
51. Torbidoni V, Iribarne M, Ogawa L, Prasanna G, Suburo AM. Endothelin-1 and endothelin receptors in light-induced retinal degeneration. *Exp Eye Res*. 2005;81:265–275.
52. Minton AZ, Phatak NR, Stankowska DL, et al. Endothelin B receptors contribute to retinal ganglion cell loss in a rat model of glaucoma. *PLoS One*. 2012;7:e43199.
53. McGrady NR, Minton AZ, Stankowska DL, He S, Jefferies HB, Krishnamoorthy RR. Upregulation of the endothelin A (ET_A) receptor and its association with neurodegeneration in a rodent model of glaucoma. *BMC Neurosci*. 2017;18:27.
54. Hill MA, Zou H, Potocnik SJ, Meininger GA, Davis MJ. Invited review: arteriolar smooth muscle mechanotransduction: Ca^{2+} signaling pathways underlying myogenic reactivity. *J Appl Physiol*. 2001;91:973–983.
55. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *J Physiol*. 2008;586:5047–5061.
56. Claing A, Shbaklo H, Plante M, Bkaily G, D'Orleans-Juste P. Comparison of the contractile and calcium-increasing properties of platelet-activating factor and endothelin-1 in the rat mesenteric artery and vein. *Br J Pharmacol*. 2002;135:433–443.
57. Isotani E, Zhi G, Lau KS, et al. Real-time evaluation of myosin light chain kinase activation in smooth muscle tissues from a transgenic calmodulin-biosensor mouse. *Proc Natl Acad Sci U S A*. 2004;101:6279–6284.
58. Mizuno Y, Isotani E, Huang J, Ding H, Stull JT, Kamm KE. Myosin light chain kinase activation and calcium sensitization in smooth muscle in vivo. *Am J Physiol Cell Physiol*. 2008;295:C358–C364.
59. He WQ, Peng YJ, Zhang WC, et al. Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. *Gastroenterology*. 2008;135:610–620.
60. Somlyo AP, Somlyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev*. 2003;83:1325–1358.
61. Amano M, Ito M, Kimura K, et al. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem*. 1996;271:20246–20249.
62. Kaneko-Kawano T, Takasu F, Naoki H, et al. Dynamic regulation of myosin light chain phosphorylation by Rho-kinase. *PLoS One*. 2012;7:e39269.
63. Ringvold HC, Khalil RA. Protein kinase C as regulator of vascular smooth muscle function and potential target in vascular disorders. *Adv Pharmacol*. 2017;78:203–301.
64. Somlyo AP, Somlyo AV. Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol*. 2000;522:177–185.
65. Woodsome TP, Eto M, Everett A, Brautigan DL, Kitazawa T. Expression of CPI-17 and myosin phosphatase correlates with Ca^{2+} sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. *J Physiol*. 2001;535:553–564.

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66. Wang Y, Zheng XR, Riddick N, et al. ROCK isoform regulation of myosin phosphatase and contractility in vascular smooth muscle cells. *Circ Res*. 2009;104:531–540.
67. Kida T. Mystery of retinal vein occlusion: vasoactivity of the vein and possible involvement of endothelin-1. *Biomed Res Int*. 2017;2017:4816527.
68. Flammer J, Konieczka K. Retinal venous pressure: the role of endothelin. *EPMA J*. 2015;6:21.
69. Jonas JB, Mones J, Glacet-Bernard A, Coscas G. Retinal vein occlusions. *Dev Ophthalmol*. 2017;58:139–167.
70. Bandello F, Battaglia Parodi M, Lanzetta P, et al. Diabetic macular edema. *Dev Ophthalmol*. 2017;58:102–138.
71. McAllister IL, Tan MH, Smithies LA, Wong WL. The effect of central retinal venous pressure in patients with central retinal vein occlusion and a high mean area of nonperfusion. *Ophthalmology*. 2014;121:2228–2236.
72. Jonas JB, Harder B. Ophthalmodynamometric differences between ischemic vs nonischemic retinal vein occlusion. *Am J Ophthalmol*. 2007;143:112–116.
73. Cybulska-Heinrich AK, Baertschi M, Loesche CC, et al. Patients with diabetic retinopathy have high retinal venous pressure. *EPMA J*. 2015;6:5.
74. Srinivas S, Tan O, Nittala MG, et al. Assessment of retinal blood flow in diabetic retinopathy using Doppler Fourier-domain optical coherence tomography. *Retina*. 2017;37:2001–2007.
75. Pechauer AD, Hwang TS, Hagag AM, et al. Assessing total retinal blood flow in diabetic retinopathy using multiplane en face Doppler optical coherence tomography. *Br J Ophthalmol*. 2018;102:126–130.
76. Lee B, Novais EA, Waheed NK, et al. En face Doppler optical coherence tomography measurement of total retinal blood flow in diabetic retinopathy and diabetic macular edema. *JAMA Ophthalmol*. 2017;135:244–251.
77. Hein TW, Potts LB, Xu W, Yuen JZ, Kuo L. Temporal development of retinal arteriolar endothelial dysfunction in porcine type 1 diabetes. *Invest Ophthalmol Vis Sci*. 2012;53:7943–7949.

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