Published in final edited form as:

Invest Ophthalmol Vis Sci. 2009 July ; 50(7): 3329–3336. doi:10.1167/iovs.08-3129.

Functional and Molecular Characterization of the Endothelin System in Retinal Arterioles

Travis W. Hein^{1,2}, Yi Ren³, Zhaoxu Yuan^{1,2}, Wenjuan Xu^{1,2}, Sonal Somvanshi³, Taiji Nagaoka⁴, Akitoshi Yoshida⁴, and Lih Kuo^{1,2,3}

¹Department of Ophthalmology, Scott and White Eye Institute, Temple, Texas

²Department of Surgery, Scott and White Eye Institute, Temple, Texas

³Department of Systems Biology and Translational Medicine, College of Medicine, Texas A&M Health Science Center, Temple, Texas

⁴Department of Ophthalmology, Asahikawa Medical College, Asahikawa, Japan

Abstract

Purpose—Activation of the endothelin (ET) system has been implicated in the pathogenesis of retinal ischemic disease. Although ET-1, the predominant endogenous isoform of ET, has been shown to cause constriction of retinal vessels, the expression and functional significance of its synthesis and the involved specific ET receptors in retinal arterioles remain unknown. The authors examined the roles of ET_A and ET_B receptors and of endothelin-converting enzyme (ECE)-1 in ET-1–induced vasomotor responses of single retinal arterioles.

Methods—To exclude systemic confounding effects, porcine retinal arterioles were isolated for vasoreactivity and molecular studies.

Results—Isolated and pressurized retinal arterioles developed basal tone and constricted in a manner dependent on concentration to ET-1. ET-1 precursor big ET-1 elicited time-dependent vasoconstriction over 20 minutes, which was blocked by the ECE-1 inhibitor phosphoramidon. ET_A receptor antagonist BQ123 inhibited most (approximately 90%) of vasoconstrictions to ET-1 and big ET-1. ET_B receptor agonist sarafotoxin also elicited concentration-dependent constriction of retinal arterioles but with significantly less potency than ET-1. ET_B receptor antagonist BQ788 abolished vasoconstriction to sarafotoxin but only slightly reduced responses to ET-1 and big ET-1. Protein and mRNA expressions of ET_A, ET_B, and ECE-1 were detected in retinal arterioles. Immunohistochemistry revealed ET_A and ET_B receptors predominantly in smooth muscle and ECE-1 predominantly in endothelium and smooth muscle.

Conclusions—ET-1 elicits constriction of retinal arterioles predominantly through the activation of smooth muscle ET_A receptors. Endogenous production of ET-1 from vascular ECE-1 is sufficient to evoke ET_A receptor–dependent constriction in retinal arterioles.

Endothelin (ET) was originally discovered as a potent vasoconstrictor peptide produced by and released from vascular endothelial cells.¹ However, accumulating evidence also shows that ET can be synthesized from vascular smooth muscle cells in nonocular vasculature^{2–4} and from nonvascular cells such as neuronal and glial cells in the retina.⁵ Three isoforms of ET—ET-1, ET-2, and ET-3—have been identified, with ET-1 the predominant biologically active peptide.

Corresponding author: Travis W. Hein, Departments of Ophthalmology and Surgery, Scott and White Eye Institute, 702 Southwest H. K. Dodgen Loop, Temple, TX 76504; thein@tamu.edu.

Disclosure: T.W. Hein, None; Y. Ren, None; Z. Yuan, None; W. Xu, None; S. Somvanshi, None; T. Nagaoka, None; A. Yoshida, None; L. Kuo, None

⁶ Cells initially produce the ET-1 precursor, preproendothelin-1, which is subsequently processed to yield a biologically inactive intermediate, big ET-1.⁶ This precursor peptide is proteolytically cleaved by endothelin-converting enzyme (ECE)-1 to generate ET-1.^{6–8} The functional effects of ET-1 are mediated by activation of two receptors, ET_A and ET_B. Activation of both receptors on the vascular smooth muscle leads to sustained vasoconstriction,¹ whereas the activation of endothelial ET_B receptors promotes vasodilation.^{9,10}

Several in vitro (bovine, porcine, human)^{11–13} and in vivo (rabbit, rat, cat, human)^{14–19} studies have demonstrated potent ET-1-mediated vasoconstriction in the normal retinal circulation. Enhanced activation of the ET system has been implicated in the reduction of retinal vessel diameter during isometric exercise²⁰ and of retinal blood flow under conditions of retinal hyperoxia,^{21–23} ischemia,²⁴ and diabetes.^{19,25} Furthermore, several ocular diseases are associated with ET system activation; elevated levels of ET-1 have been observed in the plasma of patients with retinal occlusions of the ischemic type^{26,27} and with progressive open-angle glaucoma²⁸ and in the vitreous humor of patients with diabetic retinopathy.^{29,30} Although autoradiography and immunoreactivity of ET receptors and ECE-1 have been suggested in the retinal tissue of animals and humans,^{31–33} the cellular localization/expression and the functional significance of specific ET receptors and ECE-1 in small retinal arterioles, the site for flow regulation, have not been established. To address these issues directly without the confounding influences of metabolic, hemodynamic, humoral, and glial/neuronal factors associated with in vivo preparations, porcine retinal arterioles were isolated and pressurized without flow for in vitro functional and molecular studies. We examined the vasomotor action of ET-1 and big ET-1 and elucidated the relative functional role and molecular distribution of ET_A/ET_B receptors and ECE-1 in single retinal arterioles.

Materials and 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 Scott and White Institutional Animal Care and Use Committee. Pigs of either sex (age range, 8–12 weeks; weight range, 7–10 kg) purchased from Barfield Farms (Rogers, TX) were sedated with telazol (4.4 mg/kg, intramuscularly) and xylazine (2.2 mg/kg, intramuscularly), anesthetized with sodium pentobarbital (30 mg/kg, intravenously), intubated, and ventilated with room air. Heparin (1000 U/kg) was administered into the marginal ear vein to prevent clotting. Eyes were enucleated and immediately placed in a moist chamber on ice.

Isolation and Cannulation of Microvessels

Techniques for identifying and isolating retinal microvessels were described previously.^{34–36} After enucleation, the eyecup was placed in a cooled dissection chamber (approximately 8°C) containing a physiological salt solution (PSS; 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂, 1.17 mM MgSO₄, 1.2 mM NaH₂PO₄, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, and 3.0 mM MOPS) with 0.1% albumin (USB, Cleveland, OH). Single second-order retinal arterioles (ranges: 40–60 μm internal diameter in situ, 0.6–1.0 mm length) were carefully dissected with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY). After the removal of any remaining neural/connective tissues, the arteriole was transferred to a polymethylmethacrylate vessel chamber containing PSS-albumin solution equilibrated with room air at ambient temperature. One end of the arteriole was cannulated using a glass micropipette filled with PSS containing 0.1% albumin, and the outside of the arteriole was securely tied to the pipette with 11-0 ophthalmic suture (Alcon, Fort Worth, TX). The other end of the vessel was cannulated and secured with suture. After cannulation, the vessel and pipettes were transferred to the stage of an inverted microscope (model CKX41; Olympus)

coupled to a video camera (DXC-190; Sony, Tokyo, Japan), video micrometer (Cardiovascular Research Institute, Texas A&M Health Science Center, College Station, TX), and data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO) for recording of internal diameter.³⁴ The vessels were pressurized to 55 cm H₂O (40 mm Hg) intraluminal pressure without flow by two independent pressure reservoirs. This level of pressure was used based on pressure ranges documented in retinal arterioles in vivo³⁷ and in the isolated, perfused retinal microcirculation,³⁸ and was consistent with estimated ocular perfusion pressure in humans, as reported previously.³⁹

Experimental Protocols

Cannulated arterioles were bathed in PSS-albumin at 36°C to 37°C to allow development (approximately 60 minutes) of basal tone. In one series of studies, the vasomotor response to cumulative administration of ET-1 (0.1 pM to 10 nM), selective ET_B receptor agonist sarafotoxin S6c (0.1 pM to 10 nM; Tocris Cookson, Ellisville, MO),⁴⁰ or ET-1 precursor big ET-1 (10 nM, 50 nM, 100 nM) was evaluated. Concentrations of ET-1 used in the present study were within the clinical and experimental range reported for vitreous fluid (picomolar range)³⁰ and the predicted level for local vasculature (nanomolar range).⁴¹ Retinal arterioles were exposed to each concentration of ET receptor agonists for 5 minutes or to big ET-1 for 20 minutes until a stable diameter was established. Because the vasoconstrictor actions of ET-1, sarafotoxin, and big ET-1 were maintained after washout, only one dose-response curve was constructed in each vessel for these drugs.

Relative roles of ET_A and ET_B receptors in the retinal arteriolar response to ET agonists were evaluated after incubation of the vessels with respective antagonists BQ123 (1 μM)^{42,43} and BQ788 (0.1 μM).^{43,44} In another set of vessels, the specificity of the ET receptor antagonists was assessed by examining their ineffectiveness on vasoconstriction to thrombin (0.1 U/mL). To differentiate the vasomotor effect of ET-1 from big ET-1 and to determine the functional importance of endogenously synthesized ET-1 for vasoconstriction, another series of experiments was performed in the presence of ECE-1 inhibitor phosphoramidon (50 μM), which can prevent the conversion of big ET-1 to ET-1.^{4,45} Given that phosphoramidon can also inhibit neutral endopeptidase (NEP), some vessels were examined in the presence of the selective NEP inhibitor thiorphan (10 μM)⁴⁶ for comparison. All vessels were pretreated with antagonists or inhibitors extraluminally for at least 30 minutes.

Chemicals

Drugs were obtained from Sigma-Aldrich (St. Louis, MO) except where specifically stated otherwise. ET-1, big ET-1, sarafotoxin, BQ788, phosphoramidon, and thrombin were dissolved in water, whereas BQ123 and thiorphan were dissolved in ethanol. Subsequent concentrations of these drugs were diluted in PSS. The final concentration of ethanol in the vessel bath was less than 0.1%. Vehicle control studies indicated that this final concentration of solvent had no effect on the arteriolar function.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was isolated from retinal arterioles (12–16 vessels/sample pooled from both eyes) and neural retina tissue, as described previously.³⁶ Sets of primers specific for ET_A receptor (GenBank accession no. NM214229; sense, 1102–1125 bp; antisense, 1321–1344 bp), ET_B receptor (GenBank accession no. L06623; sense, 1200–1223 bp; antisense, 1521–1544 bp), ECE-1 (GenBank accession no. NM001397; sense, 2041–2064 bp; antisense, 2290–2313 bp), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GenBank accession no. U48832; sense, 106–129 bp; antisense, 393–416 bp) genes were engineered (Sigma-Genosys, The Woodlands, TX). With the use of 0.1 and 0.3 μg total RNA for GAPDH and ET receptor/ECE-1 samples, respectively, RT-PCR was conducted as described previously.³⁶ The PCR reaction

was optimized and run for 35 cycles for all genes. The level of expression of ET receptor or ECE-1 transcripts was normalized to that of GAPDH transcripts. For some samples, the relative purity of vessels and neural retina was verified by detection of endothelial nitric oxide synthase (eNOS) and α_1 -adrenergic receptor (α_1 -AR) transcripts, respectively, as shown in our previous study.³⁶

Western Blot Analysis

Retinal arterioles (12–16 vessels/sample) and neural retina tissue were isolated and sonicated in lysis buffer. Porcine lung tissue was used as positive control for the detection of ET receptors and ECE-1. The protein content of each lysate was determined with the BCA protein assay kit (Pierce, Rockford, IL). Equal amounts of protein (10 μ g) were separated by Tris-glycine SDS-PAGE (4%–15% Tris-HCl Ready Gels; Bio-Rad, Hercules, CA), transferred onto a nitrocellulose membrane, and incubated with rabbit anti-ET_A or anti-ET_B receptor polyclonal antibody (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) or with rat anti-ECE-1 monoclonal antibody (1:250; R&D Systems, Minneapolis, MN). Membranes were stripped and reprobed with rabbit anti- α -smooth muscle actin antibody (1:1000; Sigma-Aldrich) to verify the relative purity of samples or with rabbit anti-p38 antibody (1:1000; Santa Cruz Biotechnology), which has been shown to be highly expressed in retinal homogenates.⁴⁷ After incubation with appropriate secondary antibody, the membranes were washed and developed by enhanced chemiluminescence (Pierce). Densitometric analyses of immunoblots were performed by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). Results for total ET_A and ET_B in vessel samples were normalized to total α -smooth muscle actin.

Immunohistochemistry

To identify and localize vascular ET system components, retinal arterioles were embedded and frozen in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA). Frozen sections (10- μ m thick) were fixed in 4% paraformaldehyde and immunolabeled with specific primary antibodies (ET_A and ET_B [Sigma-Aldrich]; ECE-1 [Santa Cruz Biotechnology]) and either an anti- α -smooth muscle actin antibody (Sigma-Aldrich) or an anti-eNOS (Santa Cruz Biotechnology) antibody. Afterward, the slides were incubated with rhodamine red-labeled (Jackson Laboratory, Bar Harbor, ME) or FITC-labeled (Jackson Laboratory) secondary antibodies. Staining control tissues were exposed for the same duration to nonimmune serum (Jackson Laboratory) in place of primary antibody. Slides were observed for red (rhodamine red) and green (FITC) images, and were analyzed under a fluorescence microscope (Axiovert 200; Zeiss, Thornwood, NY). Merged images were created with ImageJ software (NIH).

Data Analysis

At the end of each functional experiment, the vessel was relaxed with 0.1 mM sodium nitroprusside in EDTA (1 mM)-calcium-free PSS to obtain its maximal diameter at 55 cm H₂O intraluminal pressure.³⁴ Diameter changes in response to the ET agonists were normalized to the resting diameter and expressed as percentage changes in diameter. The median effective concentration (EC₅₀) value for the response to ET-1 was calculated (Prism software; GraphPad, San Diego, CA). Data are reported as mean $\pm$ SEM, and *n* represents the number of vessels (one per pig) studied. One-way analysis of variance (ANOVA) followed by Dunnett post hoc comparison was used to determine the significance of changes in baseline diameter by different concentrations of ET agonists. Student's *t*-test or two-way ANOVA followed by Bonferroni multiple-range test was used to determine the significance of experimental interventions and gene/protein expression, as appropriate. *P* < 0.05 was considered significant.

Results

Vasomotor Response to ET Receptor Activation

In this study, all vessels developed a similar level of basal tone (constricted to $61\% \pm 1\%$ of maximal diameter) at 36°C to 37°C bath temperature with 55 cm H_2O intraluminal pressure. Average resting and maximal diameters of the vessels were $55 \pm 1 \mu\text{m}$ (range, 31–78 μm) and $90 \pm 1 \mu\text{m}$ (range, 48–110 μm), respectively. Administration of ET-1 (1 nM) produced rapid (initiated within 1 minute) and sustained constriction of an isolated arteriole from the baseline diameter of 62 μm to 36 μm (Fig. 1A). Further study showed that ET-1 produced concentration-dependent constriction of retinal arterioles with threshold response at 1 pM. The EC_{50} of ET-1 was 0.14 nM, and the highest concentration (10 nM) of ET-1 caused a 75% reduction in resting diameter (Fig. 1B). The ET_A receptor antagonist BQ123 abolished the vasoconstriction, but 15% vasoconstriction remained at the highest concentration of ET-1 (10 nM; Fig. 1B). In vessels treated with BQ123, lower concentrations of ET-1 (1–100 pM) tended to cause vasodilation (5%–10% increase in resting diameter) but did not reach statistical significance. In another group of vessels, the ET_B receptor antagonist BQ788 only attenuated the vasoconstriction to higher concentrations of ET-1 (i.e., 1 nM and 10 nM) by approximately 10% to 15% (Fig. 1B). In the presence of BQ123 and BQ788, the vasomotor responses to ET-1 were abolished (Fig. 1B). Inhibitory effects appeared to be specific for ET receptor activation because, in the presence of both antagonists, the vasoconstriction to thrombin (0.1 U/mL) was not altered (control, $46\% \pm 5\%$; BQ123 + BQ788, $44\% \pm 2\%$; $n = 4$; $P = 0.74$, unpaired t -test). We also found that administration of the selective ET_B agonist sarafotoxin caused a concentration-dependent constriction of retinal arterioles with a threshold response at 10 pM, but the response was modest, and the highest concentration (10 nM) only caused approximately 15% reduction in resting diameter (Fig. 2). The vasoconstrictor response was abolished by BQ788 (Fig. 2) but unaffected by BQ123 (Fig. 2).

Vasoconstriction of Retinal Arterioles to Big ET-1

To determine whether retinal arterioles exhibit functional ECE-1 activity, vasomotor response to the ECE-1 substrate big ET-1 was examined. Figure 3A shows a representative tracing of the vasoconstrictor response of an isolated retinal arteriole to big ET-1. Big ET-1 (100 nM) elicited relatively slow vasoconstriction (initiated within 5 minutes) from the baseline diameter of 50 μm and stabilized at 25 μm after 15 to 20 minutes (Fig. 3A). Figure 3B shows concentration-dependent constriction of retinal arterioles to big ET-1. Vasoconstrictor responses to 50 nM and 100 nM big ET-1 were significantly attenuated by the ECE-1 inhibitor phosphoramidon and by BQ123. By contrast, BQ788 reduced vasoconstriction to 100 nM big ET-1 by approximately 10% without affecting the response to 50 nM big ET-1 (Fig. 3B). Because the selective NEP inhibitor thiorphan did not alter vasoconstriction to big ET-1 (100 nM; control, $56\% \pm 4\%$, $n = 9$; thiorphan, $48\% \pm 5\%$, $n = 5$; $P = 0.32$, unpaired t -test) and phosphoramidon did not alter vasoconstriction to ET-1 (10 nM; control, $75\% \pm 4\%$, $n = 8$; phosphoramidon, $65\% \pm 8\%$, $n = 5$; $P = 0.24$, unpaired t -test), these results suggest that phosphoramidon selectively blocked the action of ECE-1.

ET Receptor and ECE-1 mRNA Expression in Retinal Arterioles

Figure 4 shows that ET_A , ET_B , and ECE-1 mRNA were detected in retinal arterioles and in the neural retina tissue devoid of retinal vessels. Samples were relatively homogeneous because eNOS transcripts were detected only in the retinal arterioles, whereas α_1 -AR was observed only in the neural retina tissue (Fig. 4A). The expression of ET_B receptor mRNA in retinal arterioles and neural retina, after normalization with GAPDH, was consistently higher than was the corresponding ET_A receptor expression (Fig. 4B). Normalization of ECE-1 and both ET receptors showed greater expression of all three transcripts in retinal arterioles than in neural retina tissue (Fig. 4B).

ET Receptor and ECE-1 Protein Expression and Localization in Retinal Arterioles

At the protein level, immunoblotting showed that ET_A and ET_B receptors were expressed in retinal arterioles and in neural retina tissue, with a markedly higher amount in the former (Fig. 5A). Moreover, ET_A expression was significantly higher than ET_B expression in retinal arterioles ($P = 0.03$; Fig. 5B). ECE-1 protein was detected in retinal arterioles (Fig. 5A), but a greater amount (50 μ g vs. 10 μ g) was required to detect ECE-1 in neural retina than in the vessels (data not shown). Vascular and neural tissue samples were relatively homogeneous because α -smooth muscle actin protein was detected only in retinal arterioles (Fig. 5A). Sufficient neural retina protein was present because p38 expression was consistently higher in neural retina tissue than in retinal arterioles (Fig. 5A). For cellular localization of proteins, immunohistochemical data show that eNOS and α -smooth muscle actin were distinctly distributed in the endothelium and smooth muscle, respectively, as evident by the lack of overlap in the merged image (Fig. 6A). ET_A receptor protein appeared to be localized exclusively to the smooth muscle layer, as evident by the overlap with α -smooth muscle actin (yellow staining in merged image; Fig. 6B) but not with eNOS (Fig. 6C). Modest ET_B receptor staining was observed in the outer aspect of the smooth muscle layer (Fig. 6D), and only slight overlap with eNOS was detected. On the other hand, ECE-1 staining was detected in smooth muscle and endothelial layers, with the latter showing overlap with eNOS (yellow staining in merged image; Fig. 6E).

Discussion

ET-1 is a potent vasoactive peptide that has been implicated in modulating retinal microvascular tone. The putative regulatory role of ET-1 is based on observations in animal and human studies showing that intravitreal^{14–17,19} or intravenous¹⁸ administration of ET-1 constricts retinal arteries and reduces retinal blood flow, respectively, in vivo. In addition, direct evidence for the vasoconstrictor action of ET-1 has been described in isolated bovine retinal conduit arteries¹² and large porcine arterioles (first-order feeding arterioles without tone).¹¹ However, the sensitivity and threshold concentrations of ET-1 and the receptor subtypes responsible for its vasomotor action have not been documented. It is also unclear whether retinal arterioles are capable of synthesizing ET-1 to exert vasomotor activity. Our present results demonstrate that exposure of isolated small porcine retinal arterioles to ET-1 causes constriction, predominantly through smooth muscle ET_A receptor activation, with sensitivity and threshold levels of 0.14 nM and 1 pM, respectively. We also provide the first direct evidence that retinal arterioles have ECE-1 and that its activation is sufficient to elicit ET-1-mediated, ET_A-dependent constriction of retinal arterioles.

Although previous in vivo studies have shown ET-1 causes constriction and decreases blood flow,^{15,16,18} the unequivocal determination of the direct role of ET-1 in vascular regulation in vivo is difficult because diameter changes and blood flow regulation under this situation are influenced by hemodynamic (i.e., myogenic and flow-induced responses) and local metabolic control mechanisms.^{48,49} Additional limitations of these in vivo studies are the inability to measure diameter changes in small retinal arterioles, which play a predominant role in retinal blood flow regulation, because of the limited resolution of the detection instrument^{15,16,18} and the inability to distinguish between vascular and neural/glial ET system components. In the present study, we used an isolated vessel approach to study the direct vasomotor influence of ET receptor and ECE-1 activation on small retinal arterioles and to identify the molecular distribution of the ET system in these microvessels.

To characterize the functional role of specific ET receptor subtypes, we examined the vascular response to ET-1, a non-selective ET_A and ET_B agonist, and to sarafotoxin, a selective ET_B agonist. Both agonists caused concentration-dependent constriction; however, the maximal response to ET-1 was approximately fivefold greater. ET-1-induced vasoconstriction appeared

to be mediated primarily by the ET_A receptor subtype because selective ET_A blocker BQ123 abolished the vasoconstriction except at the highest concentration of ET-1. Given that the ET_B antagonist BQ788 completely inhibited sarafotoxin-induced vasoconstriction and only slightly attenuated constriction in response to the higher concentrations of ET-1 (1 and 10 nM), it appears that high concentrations of ET-1 also activate ET_B receptors for vasoconstriction. The vasoconstriction in response to ET-1 in the range of 1 pM to 1 nM observed in the present study is considered pathophysiologically relevant because the level of ET-1 in the vitreous and in the vascular wall can reach picomolar³⁰ and nanomolar⁴¹ ranges, respectively. The EC₅₀ (0.14 nM) of ET-1 was comparable to that reported for isolated, pressurized resistance arteries in other tissues, such as coronary (0.57 nM)⁵⁰ and intracerebral (4.8 pM)⁵¹ arterioles. Our results are also consistent with the estimated EC₅₀ (0.50 nM) of ET-1 in the retinal circulation in vivo.¹⁵ Interestingly, a recent clinical study in healthy subjects has shown that intravenous administration of ET-1 did not alter the diameters of large retinal arteries but significantly reduced retinal blood flow,¹⁸ suggesting that ET-1 exerts its vasoconstrictor action primarily on downstream small retinal arterioles. Consistent with BQ123 administration blunting the ET-1-induced reduction in human retinal blood flow,¹⁸ our findings support the predominant role of ET_A receptors in mediating ET-1-induced constriction of small retinal arterioles.

Previous results in other vascular beds indicate that ET_B receptors are located mainly on endothelial cells^{9,52} and that activation of these receptors mediate vasodilation by the release of nitric oxide.^{9,10,52} In the present study, we only observed a tendency for some retinal arterioles to increase (5%–10%) resting diameter in response to ET-1 (1–100 pM) in the presence of ET_A receptor blockade (Fig. 1), suggesting a minor role of ET_B receptor in vasomotor regulation of retinal arterioles. Moreover, in the presence of ET_A receptor blocker, the activation of ET_B receptors by sarafotoxin only elicited vasoconstriction (Fig. 2). It appears that the component of endothelial ET_B receptor-mediated vasodilation contributes little, if at all, to the vasomotor action of ET-1 in the porcine retinal circulation.

With the use of autoradiography and pharmacologic binding analysis, previous studies demonstrated that ET_A receptors are present in human and rabbit retinal blood vessels and that ET_B receptors are localized in the neural/glial cells of both species.³¹ On the other hand, ET_A and ET_B receptors have been detected in the neural retina tissue of rats.⁵³ Unfortunately, the size and type (i.e., arteries or veins) of blood vessels studied in those previous reports are unclear, as is the molecular distribution of the ET receptors in the retinal microcirculation. In the present study, we showed the expression of ET_A and ET_B receptor mRNA and protein in retinal arterioles, which provided additional evidence for the functional roles of these two subtypes in ET-1-induced vasomotor responses. The mRNA and the protein expression of both ET receptor subtypes was also detected in neural retina tissue but was strikingly lower, especially at the protein level, than in retinal arterioles. Because ET_B expression was higher than ET_A expression at the mRNA but not the protein level in retinal arterioles, it appeared that functional ET receptors might have been regulated at the translational level. Interestingly, it has been reported that ET_B receptor-mediated vasoconstriction can be enhanced after ischemic stress,⁵⁴ possibly through a mechanism that selectively regulates ET_B receptor expression.⁵⁵ Although ET_B-mediated retinal arteriolar constriction to ET-1 was moderate in the present study, its pathophysiologic potential may be implicated during disease development.

Immunohistochemical analysis of single intact retinal arterioles indicated that the ET_A receptors are selectively expressed in the smooth muscle layer, whereas the ET_B receptors are located mainly at the outer smooth muscle cells. There was only punctate, overlap staining for ET_B with eNOS, indicating sparse expression of ET_B in the endothelium and supporting the lack of a significant endothelial ET_B-mediated dilation in response to ET-1 or sarafotoxin.

Collectively, our molecular data corroborate the functional data by showing distinct cellular distribution of ET_A and ET_B receptors in the porcine retinal arteriolar wall.

Previous studies have shown that ECE-1 protein expression and activity are present in human³² and bovine³³ retinal tissue, respectively. The present results, to our knowledge, provide the first evidence of ECE-1 mRNA/protein expression and ECE-1 activity in the retinal microvasculature. Support for functional ECE-1 activity was evident by the ability of ET-1 precursor, big ET-1, to constrict isolated retinal arterioles. The initiation of the vasoconstrictor response to big ET-1 was slower (5 minutes) than that to ET-1 (1–2 minutes), which is consistent with the required conversion of big ET-1 to ET-1 by ECE-1 to exert vasomotor activity. This conclusion was supported by the ability of the ECE-1 inhibitor phosphoramidon to inhibit vasoconstriction to big ET-1 but not to ET-1. The prevention of retinal arteriolar constriction to big ET-1 by BQ123 and the weak inhibitory effect of BQ788 indicates that ET_A receptor activation is primarily responsible for the observed vasomotor activity after ECE-1 activation. The functional role of vascular ECE-1 is further supported by our molecular findings showing ECE-1 mRNA and protein expression in isolated retinal arterioles. We also detected ECE-1 mRNA and protein in neural retina tissue; however, the expression levels were significantly less than those in the retinal vessels. Interestingly, immunohistochemical analysis showed ECE-1 staining in endothelial and smooth muscle layers. It is speculated that the widespread distribution of ECE-1 in the vascular wall may contribute to the high susceptibility of the vasculature to pathophysiological insults during retinal disease development/progression in association with ET-1 elevation.^{19,25,26,28–30}

In summary, the present study demonstrates that a functional ET system is expressed in porcine retinal arterioles. Endogenous production of ET-1 from endothelial/smooth muscle ECE-1 is capable of evoking the constriction of retinal arterioles through smooth muscle ET_A receptor activation. Because of the potential influence of ET-1 on local retinal blood flow, better understanding of the ET system in the retinal circulation could lead to new therapeutic modalities for retinal vascular diseases elicited by ET-1.

Acknowledgments

Supported by Scott and White Research Foundation (TWH), Retina Research Foundation (TWH, LK); National Institutes of Health/National Eye Institute Grant R01EY018420 (TWH); the Scott and White Research Foundation Ophthalmic Vascular Research Program (LK); and the Kruse Family Endowment Fund (LK).

References

1. Yanagisawa M, Kurihara H, Kimura S, et al. A novel vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988;332:411–415. [PubMed: 2451132]
2. Yu J, Davenport A. Secretion of endothelin-1 and endothelin-3 by human cultured vascular smooth muscle cells. *Br J Pharmacol* 1995;114:551–557. [PubMed: 7881755]
3. Maguire JJ, Johnson CM, Mockridge JW, Davenport AP. Endothelin converting enzyme (ECE) activity in human vascular smooth muscle. *Br J Pharmacol* 1997;122:1647–1654. [PubMed: 9422810]
4. McMahon E, Palomo M, Moore W, McDonald J, Stern M. Phosphoramidon blocks the pressor activity of porcine big endothelin-1(1–39) in vivo and conversion of big endothelin-1–(1–39) to endothelin-1–(1–21) in vitro. *Proc Nat Acad Sci U S A* 1991;88:703–707.
5. Ripodas A, de Juan J, Roldan-Pallares M, et al. Localisation of endothelin-1 mRNA expression and immunoreactivity in the retinal and optic nerve from human and porcine eye: evidence for endothelin-1 expression in astrocytes. *Brain Res* 2001;912:137–143. [PubMed: 11532429]
6. Masaki T. Historical review: endothelin. *Trends Pharmacol Sci* 2004;25:219–224. [PubMed: 15063086]
7. Xu D, Emoto N, Giaid A, et al. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 1994;78:473–485. [PubMed: 8062389]

8. Hioki Y, Okada K, Ito H, Matsuyama K, Yano M. Endothelin converting enzyme of bovine carotid artery smooth muscles. *Biochem Biophys Res Commun* 1991;174:446–451. [PubMed: 1899566]
9. Schilling L, Feger GI, Ehrenreich H, Wahl M. Endothelin-1-induced contraction and relaxation of isolated rat basilar artery: effect of the endothelium. *J Cardiovasc Pharmacol* 1995;26:S197–S199. [PubMed: 8587361]
10. Gurbanov K, Rubinstein I, Hoffman A, Abassi Z, Better OS, Winaver J. Differential regulation of renal regional blood flow by endothelin-1. *Am J Physiol* 1996;271:F1166–F1172. [PubMed: 8997390]
11. Yu DY, Su EN, Cringle S, Schoch C, Percicot C, Lambrou G. Comparison of the vasoactive effects of the docosonoid unoprostone and selected prostanoids on isolated perfused retinal arterioles. *Invest Ophthalmol Vis Sci* 2001;2001:1499–1504. [PubMed: 11381053]
12. Nyborg N, Prieto D, Benedito S, Nielsen P. Endothelin-1-induced contraction of bovine retinal small arteries is reversible and abolished by nitrendipine. *Invest Ophthalmol Vis Sci* 1991;32:27–31. [PubMed: 1987104]
13. Yu DY, Su EN, Cringle S, Alder V, Yu P, Desantis L. Effect of betaxolol, timolol and nimodipine on human and pig retinal arterioles. *Exp Eye Res* 1998;67:73–81. [PubMed: 9702180]
14. Takei K, Sato T, Nonoyama T, Hommura S, Miyauchi T, Goto K. Analysis of vasocontractile responses to endothelin-1 in rabbit retinal vessels using an ET_A receptor antagonist and an ET_B receptor agonist. *Life Sci* 1993;53:111–115.
15. Bursell S, Clermont A, Oren B, King G. The in vivo effect of endothelins on retinal circulation in nondiabetic and diabetic rats. *Invest Ophthalmol Vis Sci* 1995;36:596–607. [PubMed: 7890491]
16. Granstam E, Wang L, Bill A. Ocular effects of endothelin-1 in the cat. *Curr Eye Res* 1992;11:325–332. [PubMed: 1526164]
17. Butryn RK, Ruan H, Hull CM, Frank RN. Vasoactive agonists do not change the caliber of retinal capillaries of the rat. *Microvasc Res* 1995;50:80–93. [PubMed: 7476582]
18. 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. [PubMed: 12697427]
19. 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. [PubMed: 8933767]
20. Luksch A, Wimpissinger B, Polak K, Jandrasits K, Schmetterer L. ET_a-receptor blockade, but not ACE-inhibition, blunts retinal vessel response during isometric exercise. *Am J Physiol Heart Circ Physiol* 2006;290:H1693–H1698. [PubMed: 16327016]
21. Takagi C, King GL, Takagi H, Lin YW, Clermont AC, Bursell SE. Endothelin-1 action via endothelin receptors is a primary mechanism modulating retinal circulatory response to hyperoxia. *Invest Ophthalmol Vis Sci* 1996;37:2099–2109. [PubMed: 8814149]
22. Dallinger S, Dorner GT, Wenzel R, et al. Endothelin-1 contributes to hyperoxia-induced vasoconstriction in the human retina. *Invest Ophthalmol Vis Sci* 2000;41:864–869. [PubMed: 10711705]
23. Izumi N, Nagaoka T, Sato E, et al. Role of nitric oxide in regulation of retinal blood flow in response to hyperoxia in cats. *Invest Ophthalmol Vis Sci* 2008;49:4595–4603. [PubMed: 18552394]
24. Gidday JM, Zhu Y. Endothelium-dependent changes in retinal blood flow following ischemia. *Curr Eye Res* 1998;17:798–807. [PubMed: 9723995]
25. Deng D, Evans T, Mukherjee K, Downey D, Chakrabarti S. Diabetes-induced vascular dysfunction in the retina: role of endothelins. *Diabetologia* 1999;42:1228–1234. [PubMed: 10525664]
26. 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. [PubMed: 9713055]
27. Haufschild T, Prunte C, Messerli J, Flammer J. Increased endothelin-1 plasma level in young adults with retinal vascular occlusive diseases. *Klin Monatsbl Augenheilkd* 2004;221:357–359. [PubMed: 15162279]
28. Emre M, Orgül S, Haufschild T, Shaw S, Flammer J. Increased plasma endothelin-1 levels in patients with progressive open angle glaucoma. *Br J Ophthalmol* 2005;89:60–63. [PubMed: 15615748]

29. 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. [PubMed: 11756889]
30. 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. [PubMed: 16030497]
31. MacCumber MW, D'Anna SA. Endothelin receptor-binding subtypes in the human retina and choroid. *Arch Ophthalmol* 1994;112:1231–1235. [PubMed: 8085966]
32. Wollensak G, Löffler BM, Beyermann B, Ihling C. An immunohistochemical study of endothelin-1 converting enzyme in the human eye. *Curr Eye Res* 2002;24:6–11. [PubMed: 12187489]
33. Dibas A, Prasanna G, Yorio T. Localization of endothelin-converting enzyme in bovine optic nerve and retina. *J Ocul Pharmacol Ther* 2005;21:288–297. [PubMed: 16117692]
34. 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. [PubMed: 15914631]
35. 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. [PubMed: 16431969]
36. 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;H231–238. [PubMed: 16489103]
37. Glucksberg MR, Dunn R. Direct measurement of retinal microvascular pressure in the live, anesthetized cat. *Microvasc Res* 1993;45:158–165. [PubMed: 8361399]
38. Kulkarni P, Joshua IG, Roberts AM, Barnes G. A novel method to assess reactivities of retinal microcirculation. *Microvasc Res* 1994;48:39–49. [PubMed: 7990722]
39. Nagaoka T, Takahashi A, Sato E, et al. Effect of systemic administration of simvastatin on retinal circulation. *Arch Ophthalmol* 2006;124:665–670. [PubMed: 16682588]
40. Williams DL Jr, Jones KL, Pettibone DJ, Lis EV, Clineschmidt BV. Sarafotoxin S6c: an agonist which distinguishes between endothelin receptor subtypes. *Biochem Biophys Res Commun* 1991;175:556–561. [PubMed: 1850245]
41. Masaki T, Yanagisawa M, Goto K. Physiology and pharmacology of endothelins. *Med Res Rev* 1992;12:391–421. [PubMed: 1528080]
42. Ihara M, Noguchi K, Saeki T, et al. Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor. *Life Sci* 1992;50:247–255. [PubMed: 1310132]
43. Donato A, Lesniewski L, Delp M. The effects of aging and exercise training on endothelin-1 vasoconstrictor responses in rat skeletal muscle arterioles. *Cardiovasc Res* 2005;66:393–401. [PubMed: 15820208]
44. Ishikawa K, Ihara M, Noguchi K, et al. Biochemical and pharmacological profile of a potent and selective endothelin B-receptor antagonist, BQ-788. *Proc Natl Acad Sci U S A* 1994;91:4892–4896. [PubMed: 8197152]
45. Ikegawa R, Matsumura Y, Tsukahara Y, Takaoka M, Morimoto S. Phosphoramidon, a metalloproteinase inhibitor, suppresses the secretion of endothelin-1 from cultured endothelial cells by inhibiting a big endothelin-1 converting enzyme. *Biochem Biophys Res Commun* 1990;171:669–675. [PubMed: 2206130]
46. Watts S, Thakali K, Smark C, Rondelli C, Fink G. Big ET-1 processing into vasoactive peptides in arteries and veins. *Vasc Pharmacol* 2007;47:302–312.
47. Manabe S, Lipton SA. Divergent NMDA signals leading to proapoptotic and antiapoptotic pathways in the rat retina. *Invest Ophthalmol Vis Sci* 2003;44:385–392. [PubMed: 12506100]
48. Pournaras CJ, Rungger-Brandle E, Riva CE, Hardarson SH, Stefansson E. Regulation of retinal blood flow in health and disease. *Prog Retin Eye Res* 2008;27:284–330. [PubMed: 18448380]
49. Nagaoka T, Sakamoto T, Mori F, Sato E, Yoshida A. The effect of nitric oxide on retinal blood flow during hypoxia in cats. *Invest Ophthalmol Vis Sci* 2002;43:3037–3044. [PubMed: 12202527]

50. Shipley RD, Muller-Delp JM. Aging decreases vasoconstrictor responses of coronary resistance arterioles through endothelium-dependent mechanisms. *Cardiovasc Res* 2005;66:374–383. [PubMed: 15820206]
51. Ogura K, Takayasu M, Dacey RG Jr. Differential effects of intra- and extraluminal endothelin on cerebral arterioles. *Am J Physiol Heart Circ Physiol* 1991;261:H531–H537.
52. Higashi T, Ishizaki T, Shigemori K, Yamamura T, Nakai T. Pharmacological characterization of endothelin-induced rat pulmonary arterial dilatation. *Br J Pharmacol* 1997;121:782–786. [PubMed: 9208148]
53. de Juan J, Moya F, Fernandez-Cruz A, Fernandez-Durango R. Identification of endothelin receptor subtypes in rat retina using subtype-selective ligands. *Brain Res* 1995;690:25–33. [PubMed: 7496803]
54. Stenman E, Malmström M, Uddman E, Gidö G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. *Stroke* 2002;33:2311–2316. [PubMed: 12215604]
55. Henriksson M, Stenman E, Edvinsson L. Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. *Stroke* 2003;34:1479–1483. [PubMed: 12750545]

Figure 1.

Vasomotor response of isolated retinal arterioles to ET-1. **(A)** Representative tracing shows ET-1 (1 nM)-induced constriction of retinal arterioles initiated within 1 minute. **(B)** Dose-dependent change in resting diameter from baseline (B) in response to ET-1 was examined in the absence (control, $n = 8$) or presence of ET_B receptor antagonist BQ788 ($0.1 \mu\text{M}$, $n = 8$), ET_A receptor antagonist BQ123 ($1 \mu\text{M}$, $n = 8$), or both BQ123 and BQ788 ($n = 5$). * $P < 0.05$ versus baseline. † $P < 0.05$ versus control.

Figure 2.

Vasomotor response of isolated retinal arterioles to sarafotoxin. Dose-dependent change in resting diameter from baseline (B) in response to ET_B receptor agonist sarafotoxin was examined in the absence (control, $n = 8$) or presence of ET_B receptor antagonist BQ788 (0.1 μ M, $n = 6$) or ET_A receptor antagonist BQ123 (1 μ M, $n = 5$). * $P < 0.05$ versus baseline. † $P < 0.05$ versus control.

Figure 3.

Vasomotor response of isolated retinal arterioles to big ET-1. **(A)** Representative tracing shows big ET-1 (100 nM)-induced constriction of retinal arterioles initiated within 5 minutes. **(B)** Dose-dependent change in resting diameter from baseline (B) in response to big ET-1 was examined in the absence (control, $n = 9$) or presence of ECE-1 inhibitor phosphoramidon (50 μM , $n = 5$), ET_B receptor antagonist BQ788 (0.1 μM , $n = 5$), or ET_A receptor antagonist BQ123 (1 μM , $n = 5$). * $P < 0.05$ versus baseline. † $P < 0.05$ versus control.

Figure 4.

RT/PCR analyses of ET receptors and ECE-1 mRNA expression in porcine retinal arterioles. (**A**, *upper*) Equal amounts of total RNA isolated from normal retinal arterioles and neural retina tissue were reversed transcribed using gene-specific primers for ET_A receptor (243 bp), ET_B receptor (345 bp), and ECE-1 (273 bp) mRNA. After 35 cycles of PCR, gene products were electrophoresed on a 1.8% agarose gel and visualized with ethidium bromide staining. *Hae*III-restricted ϕ X174-DNA was used as a size marker. Data are representative of three independent experiments. *Lower*: RT/PCR gene products for eNOS (342 bp) or α_1 -AR receptor (178 bp) mRNA from retinal arterioles and neural retina tissue. (**B**) ET_{A/B} and ECE-1 transcripts for neural retina tissue and retinal arterioles were normalized with corresponding GAPDH transcripts. * $P < 0.05$ versus neural retina tissue.

Figure 5.

Western blot analyses of ET receptors and ECE-1 protein expression in porcine retinal arterioles. **(A)** Immunoblots show detection of ET_A and ET_B in retinal arterioles (RA) and in neural retina tissue (RT) but ECE-1 expression only in retinal arterioles. ET receptors and ECE-1 were also detected in the porcine lung tissue (positive control). α -Smooth muscle actin (SMA) was detected only in retinal arterioles; greater expression of p38 was present in neural retina than in retinal arterioles. **(B)** ET receptors for retinal arterioles were normalized with corresponding SMA. Data represent three independent experiments. * $P < 0.05$ versus ET_A.

Figure 6.

Immunohistochemical detection of ET receptors and ECE-1 in isolated retinal arterioles. (A) In the presence of anti-eNOS (red) or anti- α -smooth muscle actin (SMA, green) antibodies, high levels of immunostaining were detected in endothelial (arrowhead) and smooth muscle (arrow) layers, respectively. The merged image shows the lack of overlap staining. (B) In the presence of anti-ET_A receptor (green) or anti-SMA (red) antibodies, high levels of immunostaining were detected in the smooth muscle for both proteins, which was confirmed by overlap staining (yellow). (C) In the presence of anti-ET_A receptor (green) or anti-eNOS (red) antibodies, selective immunostaining was detected in smooth muscle for ET_A receptors and endothelium for eNOS, respectively. The merged image shows the lack of overlap staining.

(D) In the presence of anti-ET_B receptor (*green*) or anti-eNOS (*red*) antibodies, immunostaining was detected in smooth muscle and endothelium for ET_B receptor and in endothelium for eNOS. The merged image shows slight overlap staining. **(E)** In the presence of anti-ECE-1 (*green*) or anti-eNOS (*red*) antibodies, immunostaining was detected in the endothelium and smooth muscle for ECE-1 and in the endothelium for eNOS, respectively. The merged image shows overlap staining (*yellow*) in the endothelial layer. *White arrowheads*: endothelial cells. *Arrows*: vascular smooth muscle cells. Data shown are representative of three separate experiments.