IMPACT OF ACUTE AND CHRONIC HYPERGLYCEMIA ON VASOCONSTRICTOR RESPONSES OF RETINAL VENULES: ROLE OF REVERSE-MODE SODIUM-CALCIUM EXCHANGER

A Dissertation

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ABSTRACT

Hyperglycemia, a hallmark of diabetes, is associated with increased retinal fluid filtration, a process heavily influenced by the postcapillary pressure and venous constriction. In hyperglycemia/diabetes, the serum level of vasoconstrictors endothelin-1 (ET-1) and thromboxane A2 (TXA_2) are elevated, and ET-1 has been implicated in retinal venous pathologies such as diabetic retinopathy and retinal vein occlusion. However, their impacts and mechanistic actions on venular vasomotor activity in the retina with hyperglycemic/diabetic insult remain unclear. First, we characterized the mechanistic action of ET-1 on isolated porcine retinal venules by examining 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 vasoconstriction. We found that 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 endothelin A receptors (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. Second, we found that chronic hyperglycemia (diabetes) and acute hyperglycemia (incubating the vessels with 25 mM glucose for 2 hours) significantly enhanced retinal venular constriction to ET-1, norepinephrine, and TXA_2 analogue U46619. Angiotensin II and phenylephrine elicited insignificant constriction of retinal venules. Moreover, the receptor mRNA expressions for ET-1, TXA_2, and α_{2A}-adrenoceptor were not altered in diabetic venules, but the ET-1 level in vitreous humor was elevated in diabetic animals. After exposure to a high glucose (25 mM), the isolated human retinal arterioles also exhibited enhanced constriction to ET-1. Administration of both reverse-mode sodium-calcium exchanger (NCX) inhibitor KB-R7943 and sodium hydrogen exchanger (NHE) blocker cariporide did not affect the vasoconstriction to ET-1 in normal vessels but abolished the enhanced constriction to ET-1 in diabetic venules. Overall, our

findings indicate that both chronic and acute hyperglycemia augment constriction of retinal venules to ET-1, U46619, and norepinephrine, via activation of both reverse-mode NCX and NHE. It is speculated that the enhanced venular response to these vasoconstrictors may increase capillary filtration by elevating postcapillary venular pressure during the early stage of diabetes. These pathophysiological changes may consequently promote tissue edema and development of diabetic retinopathy, in addition to the reduction of ocular perfusion. Blockade of reverse-mode NCX and NHE in retinal venules might provide a new therapeutic strategy to mitigate the diabetes-induced impairment of retinal microvascular function.

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I. INTRODUCTION

Purpose

Hyperglycemia, a hallmark of diabetes, is associated with increased retinal fluid filtration,[1] a process primarily governed by the postcapillary pressure and venous resistance, which is dictated by the vasomotor activity of muscular venules and veins. The constriction of venous vessels could lead to elevated venous resistance and capillary pressure, consequently facilitating fluid filtration.[2,3] Without appropriate treatment and control, the changes in venous circulation would consequently promote vision impairment due to edema and other complications. The clinical evidence has shown increased retinal venous pressure and decreased retinal blood flow in patients with diabetic retinopathy.[4,5] However, it is unclear whether diabetes or hyperglycemia alters vasomotor responses of retinal venules to endogenous vasoconstrictors. Accumulating evidence has shown elevated endothelin-1 (ET-1) levels in the vitreous humor and plasma in human patients with proliferative diabetic retinopathy,[6] and in retinal tissue of diabetic rats.[7,8] Elevated expression levels of other vasoconstrictors, such as thromboxane A_2 (TXA_2),[9] norepinephrine[10] and angiotensin II,[11] have been detected in plasma/serum or retinal tissue of experimental models of diabetes. Therefore, it is important to investigate the effects of these endogenous vasoconstrictors in the vasomotor response of retinal venules during diabetes/hyperglycemia.

It is generally accepted that Ca^{2+} entry plays an important role in the vascular smooth muscle cells triggering vasoconstriction.[12] In some blood vessels, activation of L-type voltage-operated calcium channels (L-VOCCs)[12] and calcium release-activated calcium (CRAC) channels[13] have been demonstrated in this process. However, it remains unclear whether these channels contribute to the constriction of retinal venules. Another possible route for Ca^{2+} entry is

the activation of the reverse mode sodium-calcium exchanger (NCX).[14] Interestingly, the study in cardiomyocytes has demonstrated that ET-1 can exert an inotropic effect by eliciting extracellular Ca^{2+} influx through coupling of the sodium-hydrogen exchanger (NHE) with reverse-mode NCX.[14] Moreover, exposure of cardiomyocytes to a high level of glucose can activate the reverse-mode NCX and lead to increased Ca^{2+} entry.[15] Activation of NHE in diabetes appears to have an adverse impact on the retinal arterial circulation.[16] However, the putative role of NCX and NHE-NCX in mediating venular pathophysiology, especially under hyperglycemic/diabetic insult, in the retina remains to be determined. Although clinical evidence has shown increased retinal venous pressure in human patients with diabetic retinopathy,[4] there is no information on how venular reactivity to vasoconstrictors is influenced by hyperglycemia in the human retina. Herein, the goals of these studies were to investigate the mechanistic action of ET-1 and other vasoconstrictors (U46619, norepinephrine, phenylephrine, and angiotensin II) on isolated porcine retinal venules under normal and diabetic/hyperglycemic conditions, and to examine whether the reverse mode NCX and NHE are involved in the vasomotor regulation. The vasomotor reactivity of human retinal venules in response to ET-1 was also investigated in vitro under normal and hyperglycemic conditions, as an attempt to support the use of the porcine model for studying the human retinal microcirculation.

Significance

The venular vasculature is the most effective vessel in the circulation, in terms of regulating capillary pressure and tissue fluid homeostasis, by altering its activity, i.e., resistance, through constriction or dilation.[17] However, there are few studies focusing on the vasomotor responses and the mechanistic actions of vasoconstrictors in retinal venules. Overproduction of vasoconstrictors is known to contribute to the pathogenesis of many diseases, including diabetes.[7,18-21] Diabetic pathogenesis is noted to be associated with increased retinal venous pressure and reduced retinal blood flow.[5,22] It is clinically important to understand the vasomotor responses of retinal venules to ET-1 because the concentration and expression of this potent vasoconstrictor are elevated in the blood circulation[23] and retinal tissues.[6,24] However, there is no information concerning the vasomotor action of ET-1, as well as other endogenous vasoconstrictors (TXA_2, norepinephrine and angiotensin II), on retinal venules and its pathophysiological contribution remains undetermined. A better characterization of the mechanisms involved in retinal venular reactivity and its abnormal reaction is critical in understanding the pathogenesis of retinal disorders, especially in the development of retinopathy. The present study is the first to systematically examine this important issue. I documented that the constrictions of retinal venules to ET-1, TXA_2 and norepinephrine are augmented by chronic hyperglycemia (diabetes) and acute hyperglycemia (2-hour exposure to high glucose) through activation of the NHE-NCX pathway. In view that the venular circulation plays cardinal roles in determining capillary pressure and transcapillary fluid exchange, the enhanced vasomotor activity of the venules in diabetes can directly influence the fluid homeostasis of the tissue and promote the development of retinopathy. The results of my study may provide novel therapeutic strategies to mitigate diabetic retinopathy or diabetes-induced retinal tissue edema by targeting the NHE and NCX pathway.

This dissertation follows the style of *Investigative Ophthalmology and Visual Sciences*.

Function of Retinal Venules

The ocular circulation is important in providing necessary nutrients and oxygen, removing metabolic waste, and thus maintaining normal vision.[25] Within the ocular circulation, numerous investigations have demonstrated the crucial role of the retinal arteriolar system in the regulation of retinal blood flow.[2,3,25] Most studies on the retinal venous system were about its architecture and basic function in the drainage pathway,[25] and there is no report on the regulation of vasomotor activity of retinal venules under health or disease. It has been shown that the wall of the retinal vein (porcine model) is thin and consists of a single layer of endothelial cells and only a few smooth muscle cells.[26] Moreover, the circumferential orientation of smooth muscle cells in retinal venules (non-circularly oriented) is different from retinal arterioles (circularly oriented).[26] The existence of pericytes appearing on the surface of the retinal venule remains to be investigated. The potential contribution of the retinal venous circulation to the development of diabetic retinopathy is generally associated with augmented vascular permeability, retinal edema, and retinal vein occlusion,[4,18,20,27-29] with the thought of breaking down of the blood-retinal barrier (BRB) in the venous vasculature.[1,27-29] However, the contribution of altered vasomotor responses of retinal venules was unknown and received less attention.

Most investigators, based on the isogravimetric/isovolumetric whole-organ preparations, accept the following formula for determining capillary pressure (P_c) :[2]

$$P_{c_{iso}} = \frac{[(R_v/R_a) \cdot P_a + P_v]}{[1 + (R_v/R_a)]}$$

where P_{ciso} is isogravimetric/isovolumetric capillary pressure, P_a is mean arterial pressure, P_v is venous pressure, R_a is arterial resistance, and R_v is venous resistance. In general, the value of R_v/R_a is less than 1 (value ≈ 0.3[30]). Many studies primarily supported that P_c is tightly regulated in response to alteration in P_a, while P_v is held constant or remains essentially unchanged. However, considering the formula mentioned above with changed P_v, the venular vasculature becomes the most effective vessel in the circulation in terms of regulating P_c and fluid homeostasis. It has been shown experimentally that the change in venous pressure is about 5 to 10 times more effective in changing capillary pressure than a similar change of pressure applied from the arterial side.[17] It was calculated that about 80%-85% of a venous pressure increment is transmitted to the capillaries,[17,31] with a greater emphasis on the critical role of venous constriction and dilation in determining capillary pressure.[32] Therefore, characterizing the vasomotor action of retinal venules is important toward the understanding of pathogenesis of retinal vascular diseases. There had been no study focusing on vasomotor function of retinal venule until Yu and colleagues demonstrated that ET-1 and adenosine caused local vasoreactivity of porcine retinal veins.[26] In addition, in an epidemiological study, Cybulska-Heinrich and colleagues found a significant association between increased retinal venous pressure (RVP) and diabetic retinopathy.[4] However, it remains unknown whether diabetes alters the retinal venular response to endogenous vasoconstrictor ET-1. In the following sections, I will first discuss the vasomotor action of endogenous vasoconstrictors, ET-1, norepinephrine, TXA_2, and angiotensin II in the ocular circulation and then discuss the underlying mechanisms, gathered from other vascular beds, contributing to these vasoconstrictions.

Vasoactive Responses to Vasoconstrictors in the Ocular Circulation

In general consideration, one of the dominant effects of vasoconstrictors is the narrowing of the blood vessels resulting from contraction of smooth muscle cells in the vascular wall, in addition to evoking other biological activities, including cell proliferation, migration and inflammation. This process is extremely important in the event of alleviating hemorrhage and acute blood loss, and regulating perfusion and distribution of blood flow. Different vasoconstrictors may act differently in different tissues and vessel types, and the sensitivity and responsiveness may be vessel-size dependent.[33] In this study, I will focus on the vasoconstrictors ET-1, TXA_2, angiotensin II and norepinephrine, and will elucidate their vasomotor impacts in the retina because upregulation of these vasoconstrictors has been suggested to play a role in disease development.

ET-1

ET-1 is a potent vasoactive peptide comprised of 21 amino acids and plays important roles in many biological functions, including vasoconstriction, cardiovascular remodeling, tissue inflammation, and cell proliferation.[13,34-38] With human ocular investigation, a reduced retinal blood flow has been shown after intravenous administration of ET-1 in healthy subjects.[39] ET-1 and ET-3 were found in most human and porcine ocular tissues via immunoreactivity study or high-pressure liquid chromatography.[40,41] Moreover, reduced retinal blood flow and retinal artery constriction in rats has been shown after intravitreal injection of ET-1.[21] Interestingly, the retinal arterial constrictions evoked by isometric exercise in healthy humans was attenuated by blocking ET-1 type A receptor (ET_A), suggesting the involvement of ET-1 in retinal blood flow regulation during systemic blood pressure changes.[42] A number of studies have demonstrated a potent vasoconstrictor effect of ET-1 in isolated porcine[43-45] and human[46] retinal arterioles, suggesting that ET-1 is capable of reducing arterial blood flow in the retina via vasoconstriction. In contrast to the arterioles, the action of ET-1 in the venous circulation is not well studied, and

there is only one report demonstrating the constriction of retinal venules to ET-1 in the porcine eye.[26] However, the underlying mechanism and the impact of hyperglycemia/diabetes on this venular constriction remains unknown.

TXA_2

TXA_2 is one of the metabolites generated from arachidonic acid via cyclooxygenase/prostaglandin H_2 synthase.[47] Prostaglandin H_2 is then converted to TXA_2 by thromboxane-A synthase.[47] The activated platelets are the major source of TXA_2, which has pro-thrombotic properties by promoting platelet aggregation and plays pathophysiological roles during tissue injury and inflammation.[47,48] TXA_2 is unstable in aqueous solution because it is hydrolyzed within few seconds to the biologically inactive thromboxane B_2 (TXB_2).[49] Therefore, the stable TXA_2 synthetic analog U46619 (9,11-dideoxy-9α,11α-methanoepoxy PGF2α) is widely used to assess the biological action of TXA_2.[50] In the ocular system, TXB_2 expression was detected in the human vitreous humor.[51] Chen and colleagues showed that TXA_2 receptors are mainly expressed in non-pigmented epithelial cells of the ciliary body, photoreceptors, and the endothelium of posterior ciliary arteries.[52] The TXA_2 analog U46619 evokes significant constriction of choroidal vessels in newborn pigs.[53] In isolated arterial ring segments from the bovine retina, U46619 has been shown to be the most potent vasoconstrictor compared to the other arachidonic acid derivatives, i.e., prostaglandin F2α and prostaglandin E2.[54] Furthermore, U46619 elicits moderate constriction of isolated porcine retinal arterioles with attached perivascular tissue.[55,56] However, the response of retinal venules to TXA_2 remains to be determined.

Angiotensin II

The renin-angiotensin system (RAS) plays important roles in regulating blood pressure and sodium retention in kidney.[57,58] The hormone angiotensin II is the main bioactive molecule

of the RAS converted from angiotensin I by the enzyme angiotensin-converting enzyme (ACE).[57] Angiotensin II can increase vasopressin production from the central nervous system and also causes vasoconstriction by acting on the angiotensin II receptors in the vasculature.[57] In the ocular system, angiotensin II was detected in the anterior uveal tract, neural retina and retinal pigment epithelium of pigs,[59] and in the porcine vitreous fluid.[59] Administration of angiotensin II caused contraction of pericytes[60] in the rat retina and reduced pericyte-containing capillary diameter,[61] and elicited voltage change in abluminal cells of the rat retinal microvasculature.[62] In the feline retinal circulation, continuous infusion of angiotensin II through a transvitreal cannula placed near the retinal surface or optic nerve resulted in either transient or sustained constrictions of the retinal arteries.[63] However, angiotensin II failed to evoke any vasomotor activity in isolated bovine retinal arterial rings before or after inhibition of prostaglandins and nitric oxide (NO), or after endothelium removal, but the vasoconstriction was apparent in isolated posterior ciliary arteries.[64] In the isolated bovine retinal vascular network perfused with oxygenated physiological salt solution, topical application of angiotensin II (0.1 mM) elicited moderate constriction of the arterial and venous vascular beds with more pronounced constriction in the microvessels, i.e., ~15% constriction of arterioles and ~7% constriction of venules from their basal resting diameters.[65] Interestingly, the vasoconstriction to angiotensin II was enhanced by inhibiting endogenous synthesis of prostacyclin and NO.[65] In contrast, intraluminal administration of angiotensin II caused marked dilation of the same vascular network.[65] In retinal vascular beds, discrepancies on vasomotor response to angiotensin II exist, depending upon animal species, experimental approach, and methodology used. It appears that the vasodilators NO and prostacyclin released from the endothelium, under angiotensin II stimulation, may confound the contractile action of the vessel, especially in the retinal microcirculation. The action of angiotensin II on the retinal vasculature deserves further investigation.

Norepinephrine

Norepinephrine, also called noradrenaline or noradrenalin, is an organic chemical classified in the catecholamine family and a neurotransmitter released by the sympathetic nerves. It is also the main hormone secreted by the adrenal medulla to regulate cardiovascular function.[66] Many researchers have demonstrated the important role of norepinephrine in regulating physiological processes and the development of neurological, endocrine, and cardiovascular diseases mainly via activation of α-adrenergic receptors.[66] Norepinephrine is synthesized from tyrosine by a series of enzymatic reactions and then converted from dopamine by dopamine β-monooxygenase.[66,67] The metabolic synthesis of norepinephrine is presented below:

Phenylalanine → Tyrosine → L-DOPA → Dopamine → Norepinephrine

Catecholamines (dopamine, norepinephrine, and epinephrine) and their metabolites are detectable in human vitreous humor and their metabolites have been considered as biomarkers to identify antemortem cold stress and fatal hypothermia in forensic examination.[68,69] Among α-adrenergic receptors, predominance of the α_{2A} subtype has been reported in human ocular tissue homogenates.[70] In agreement with the human data above, porcine retinal arterioles appear to express mainly α_{2A} receptor message and the α_1 subtype was reported to be undetectable.[71] The existence of adrenergic retinal neurons in rats and monkeys,[72] and the expression/distribution of α-adrenergic receptor subtypes in the ocular tissue have been documented in various animal species, including humans.[70,73] Although the specific adrenergic binding sites also have been identified in retinal vessels,[71,74,75] their function in regulation of retinal blood flow remains to be determined.[71,73] It has been shown that activation of α_2-adrenergic receptors by brimonidine evokes constriction of small retinal arterioles (< 60 μm in diameter) but dilation of upstream larger arterioles via an endothelial NO-mediated

mechanism.[71] In addition, norepinephrine can elicit a transient and unrepeatable constriction of isolated bovine retinal arteries.[76] Interestingly, the constriction of isolated porcine retinal arterioles to norepinephrine appears to be greater when the agent is applied intraluminally vs. extraluminally.[77] The explanation for the inconsistent findings on the vasoconstrictor characteristics between bovine and porcine studies above is unclear. Nevertheless, the constriction of small retinal arterioles to adrenergic receptor activation is rather moderate[33,77] and its physiological and pathophysiological roles remain to be determined.

The α_1-adrenergic receptor subtypes ($\alpha_{1A,}$ α_{1B} and α_{1D}) in retinal arteriole have been detected in mice.[74] Activation of the α_1-adrenergic receptor by phenylephrine evokes constriction of rabbit retinal arteries and causes glutamate-induced neuronal cell death.[78] In enucleated bovine eyes, application of phenylephrine directly onto the surface of the vessel wall caused constriction of main retinal arteries and veins, as well as some of their small branches.[79] However, in this in-situ preparation, the vasoconstrictions were observed in the absence of vascular lumenal pressure and under a supra-pharmacological concentration (0.1 mM) of phenylephrine. The physiological interpretation of this vasoconstrictor effect is uncertain. On the other hand, the main retinal arterial segments isolated from the bovine eye had no response to phenylephrine and only a small and inconsistent transient constriction was observed with higher phenylephrine concentrations (1 μM and 10 μM).[80] Interestingly, the extent of vasoconstriction to phenylephrine was increased after a circumferential stretch was imposed on the vascular wall or a high concentration of membrane depolarization agent KCl (125 mM) was used to preconstrict the vessel.[80] It appears that pre-activation of the vascular smooth muscle with a physical force (stretch) or chemical (depolarization agent) is required to evoke the constriction of bovine retinal arteries to phenylephrine. In a human study, instillation of eye drops containing phenylephrine caused a reduction of vessel density (assessed by optical coherence tomography angiography) in the peripapillary area, but not in the macular area of the retina.[81] This clinical study may suggest a site-dependent retinal vessel constriction (i.e., reducing vascular perfusion

density) to α_1-adrenergic activation, although the explanation for this heterogenous vascular responsiveness is unclear. Interestingly, topical instillation of phenylephrine can reduce blood velocity in the optic nerve head of monkeys but not in the central retinal artery of humans.[82] Although inconsistent vasomotor responses of retinal arteries to phenylephrine are noted, the vasoconstriction, if it occurs, appears to be small compared to that evoked by TXA_2, norepinephrine, and ET-1 as discussed above. Nevertheless, it remains unclear whether retinal venules respond to phenylephrine.

Mechanistic Pathway and Calcium-Dependent Vasoconstriction

Vasoconstrictor agonists may have similar or different mechanistic pathways mediating smooth muscle activity in different vascular beds (tissue/organ), types (artery vs. vein), or size (conduit vs. resistance vessel). Although it is not completely understood, in this section the G-protein-coupled receptors-dependent vasoconstrictions to ET-1, TXA_2, angiotensin II, norepinephrine, and phenylephrine, will be discussed based on the available literature concerning the general signal transduction pathways and specific vasoconstriction mechanisms.

ET-1

Previous studies in different organ systems and vascular beds have suggested that vasoconstriction to ET-1 is mediated by the activation of its receptors (ET_A and/or ET_B), elevation of intracellular Ca^{2+}, and signaling through Rho kinase (ROCK) and/or protein kinase C (PKC).[13,35,36] The detailed signaling mechanisms are described as follows (Figure 1). In general, ET_BRs expressed in endothelial cells can exert vasomotor function via production of vasodilators (NO or PGI_2) and via clearance of ET-1 to mitigate excessive ET_AR-dependent activity.[13,35] In porcine retinal arterioles, ET-1 predominantly elicits vasoconstriction via the activation of ET_ARs in smooth muscle.[43] The component of vasodilation via endothelial released NO by ET_BR activation is rather minor, and activation of ET_BR in the smooth muscle cells

exclusively produces vasoconstriction.[43] With current knowledge, stimulation of ET_ARs with ET-1 causes contraction of vascular smooth muscle cells through $G_{q/11}$ protein-mediated and $G_{12/13}$ protein-mediated activation.[13] Upon $G_{q/11}$ protein-mediated activation, phospholipase C (PLC) is activated and sequentially leads to inositol triphosphate (IP_3) and diacylglycerol (DAG) production, which then results in increased intracellular Ca^{2+} release and PKC activation, respectively.[13] The binding of Ca^{2+} with calmodulin, in association with activation of PKC isoforms, ultimately activates myosin light chain kinase (MLCK) and inhibits myosin light chain phosphatase (MLCP), respectively, leading to vasocontraction.[35,83] On the other hand, upon $G_{12/13}$ protein-mediated activation, the Rho-GTPase is activated by several different guanine nucleotide exchange factors for Rho. Subsequently, Rho-GTPase activates Rho kinase (ROCK) and promotes smooth muscle contraction by inhibiting myosin light chain phosphatase.[13,83]

It is generally thought that sustained vasoconstriction to ET-1 requires elevation of cytosolic Ca^{2+} via extracellular influx and/or intracellular release.[13,35,44,84-86] As mentioned above, the elevation of cellular IP_3 and DAG, via G_q protein-linked PLC-β, could subsequently activate Ca^{2+} channels in a voltage-dependent and/or -independent manner.[13] Binding of IP_3 to its receptor on the endoplasmic reticulum (ER) triggers the release of Ca^{2+} from this store. It is widely accepted that the depletion of Ca^{2+} within the ER produces a sustained increase in cytosolic Ca^{2+} signaling through the activation of store-operated calcium channels (SOCs), such as Ca^{2+} release-activated calcium (CRAC) channels.[13,35] The DAG can also activate receptor-operated Ca^{2+} channels for extracellular Ca^{2+} entry.[13] Moreover, transient receptor potential canonical (TRPC) channels, permeable to Ca^{2+} in addition to Na^+, can function as SOCs.[13,87,88] It has been shown that ET-1 can trigger Na^+ influx (membrane depolarization) via the opening of TRPC,[13] and consequently activate L-VOCC and possibly the reverse-mode NCX for Ca^{2+} entry.[13,87,89,90] Several studies have demonstrated that vascular smooth muscle cell L-VOCC currents are positively modulated by PKC activity.[91-94] However, the roles of ET_AR, ET_BR, PKC,

ROCK, L-VOCC, and reverse-mode NCX in mediating vasomotor responses to ET-1 in retinal venules remain unknown.

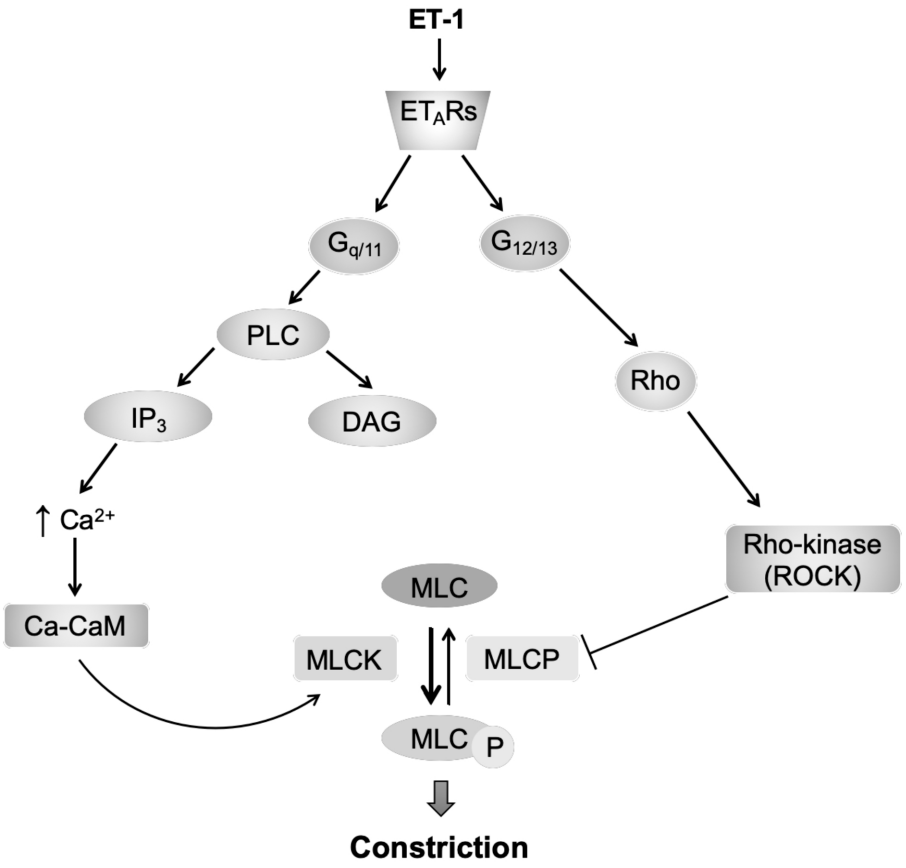

Figure 1. Role of ET_AR-mediated signaling in the regulation of vascular tone. PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; CaM, calmodulin; MLCK, myosin light chain kinase; MLC, myosin light chain; MLC-P, phosphorylated MLC; MLCP, myosin light chain phosphatase; ROCK, Rho-associated protein kinase (Figure modified from Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. J Pharmacol Sci. 2013;123:85-101[13]).

TXA_2

In humans, but not in rodents, there are two major G protein-coupled receptor isoforms, TP_α and TP_β, involved in the signaling of TXA_2 by activating and inhibiting adenylyl cyclase, respectively.[95] In most cell types, the expression of TP_α mRNA is more predominant than TP_β.[96] The TXA_2/TP interaction involves mainly two types of G-proteins: G_q and $G_{12/13}$.[97,98] TP receptors are coupled with G_q, which initiates the PLC-IP_3/PKC signaling cascade.[98] On the other hand, TP-coupled $G_{12/13}$ activates Rho kinase as a major signaling pathway leading to contraction of smooth muscle cells.[98] Previous studies have suggested that L-type Ca^{2+} channels and SOC channels contribute to extracellular Ca^{2+} influx leading to TXA_2 receptor-mediated contraction of smooth muscle in rodent models.[99] There is sparse information on the vasomotor action of TXA_2 in the retinal circulation. Nevertheless, TXA_2 analogue, U46619, appears to constrict bovine[100] and human[101] retinal arterioles. However, whether retinal venules react to TXA_2 and the potential underlying signaling mechanisms remain unclear.

Angiotensin II

Angiotensin II acts through at least two types of receptors, termed AT_1 (vasoconstriction) and AT_2 (vasodilation).[58,102] AT_1 receptors have been suggested to mediate most of the physiological actions of angiotensin II, and it is the predominant receptor subtype responsible for the control of vascular functions.[58] The interaction of angiotensin II and AT_1 mainly involves two types of G-proteins: G_q and $G_{12/13}$.[58] AT_1 receptors coupled with G_q initiates the PLC-IP_3/PKC signaling cascade.[58] On the other hand, AT_1 receptors coupled with $G_{12/13}$ activates Rho kinase as a major signaling pathway leading to contraction of smooth muscle cells.[103] These signaling pathways are similar to that elicited by TXA_2/TP stimulation, i.e., the PLC-IP_3/PKC signaling cascade.[58,103] It appears that IP_3 triggers Ca^{2+} release from the ER and subsequently contributes to vasoconstriction.[58] Interestingly, DAG, through PKC signaling cascade, might elevate cytosolic Ca^{2+} for vasoconstriction via activation of reverse-mode NCX

in a manner linking to NHE and/or Na^+/Mg^{2+} exchanger.[58] In addition, Arun and colleagues have shown the relevant impact of L- VOCC in angiotensin II-induced vasoconstriction.[104] It remains unclear whether the signaling pathways discussed above contribute to the retinal venular constriction to angiotensin II.

Norepinephrine

Two families of adrenergic receptors have been identified as α (α_1 and α_2 subtype) and β (β_1, β_2, and β_3 subtype) in response to the binding of norepinephrine.[105] Phenylephrine is a selective α_1-adrenergic receptor agonist.[105] The G proteins involved in adrenergic receptor (AR) signaling include G_s, which couples with β-ARs to activate adenylyl cyclase and elevate cAMP. The coupling of G_i with α_2-ARs can inhibit adenylyl cyclase and decrease cAMP. For α_1-ARs, the $G_{q/11}$ family can mediate the activation of PLC-β and subsequently elevate the intracellular Ca^{2+} concentration, leading to contraction of vascular smooth muscle. The activation of α_2-ARs/G_i could inhibit cAMP-PKA signaling and result in vascular smooth muscle contraction.[105] On the other hand, β-ARs with G_s coupling activate the cAMP-PKA signaling cascade and elicit K^+ channel-dependent hyperpolarization, which, in turn, promotes the closure of L-VOCC and subsequently leads to smooth muscle relaxation or vasodilation due to reduction of cytosolic Ca^{2+}.[105] The signaling pathway responsible for the adrenergic receptor activation by norepinephrine in the retinal venule remains unclear.

Retinal Blood Flow in Diabetic Retinopathy

From the metabolic viewpoint, the neural retina is a highly oxygen demanding tissue, and adequate ocular blood flow is required for its normal function.[25] The retinal circulation provides nutritional needs and waste removal of the inner two-thirds of the highly metabolically active retina, and the choroidal circulation supplies the remaining retina.[25] Numerous studies have shown that alterations in retinal blood flow are associated with open-angle glaucoma or diabetic retinopathy,[5,106-108] which is the most common cause of vision impairment or loss among people with diabetes in working-age adults. Diabetic retinopathy is primarily classified in four stages (mild, moderate, and severe non-proliferative and proliferative).[109] Intriguingly, previous studies have reported reduced, increased or unchanged retinal blood flow in human studies with diabetic retinopathy. These different patterns may be attributed to the measurement techniques, type of diabetes, gender of subjects, or different stages of diabetic retinopathy. The literature describing a link between retinal blood flow and diabetic retinopathy in humans are organized in chronological order in Table 1. In general, the reduction of retinal arteriolar and venular diameters and decrease of retinal blood flow are associated with the early stage of diabetes in humans, in which the results are consistent with the rodent type I diabetes studies (STZ injection).[110,111] The manifestation of retinal blood flow reduction in early diabetes is likely to be a main culprit to promote development of retinopathy and retinal edema if the venular circulation is impacted by the development of the disease. A better understanding of the alteration of retinal blood flow regulation is essential and important to the management of progression of diabetic retinopathy and the development of strategies for therapeutic treatment. Therefore, it is important to characterize the alteration of vasomotor activity of the retinal circulation in early diabetes. Herein, I will discuss below on the alteration of retinal vascular reactivity in response to several endogenous vasoconstrictors mentioned earlier in this section and also focus on the venular responsiveness if the literature is available.

Table 1. Summary of retinal blood flow or retinal vascular change in human studies with diabetic retinopathy (DR)

Author(s), Year	Subjects & DR Stages	Measurement Technique	Retinal Blood Flow (RBF)	Retinal Vascular Diameter
Yoshida et al., 1983[22]	◙ No DR ◙ Mild DR ◙ Moderate DR ◙ Severe DR	Fluorescein mean circulation times measurement	◙ Increased RBF with the progression of background DR ◙ Decreased RBF in diabetic patients with no retinopathy compared with controls	◙ No different in arterial widths ◙ Increased venous widths in Moderate and severe DR
Grunwald et al., 1984[112]	◙ Background DR ◙ PDR	Laser Doppler velocimetry	◙ No significantly different	No change
Patel et al., 1992[113]	◙ Background DR ◙ Pre-PDR ◙ PDR	Laser Doppler velocimetry	◙ Increased RBF in diabetic patients with background DR, pre-PDR, and PDR	
Bursell et al., 1996[114]	IDDM	Video-fluorescein angiography	◙ Decreased RBF in IDDM with no DR	
Konno et al., 1996[115]	Type 1 diabetes	Laser Doppler velocimetry	◙ The RBF initially decreases, and it begins to increase with increasing duration of diabetes	
Nagaoka et al., 2010[116]	Type 2 diabetes: ◙ No DR ◙ Mild NPDR	Laser Doppler velocimetry	◙ Decreased RBF in patients with type 2 diabetes without retinopathy and in those with mild NPDR	
Roy et al., 2011[117]	African Americans with Type 1 diabetes	Fundus photography		The wider diameter of retinal venules is an early indicator of progression to PDR, not retinal arterioles

Table 1. Continued

Author(s), Year	Subjects & DR Stages	Measurement Technique	Retinal Blood Flow (RBF)	Retinal Vascular Diameter
Klein et al., 2012[118]	Type 1 diabetes & Type 2 diabetes	Fundus photography		The widening of retinal venules but not arteriolar diameter was associated with progression of DR
Frydkjaer-Olsen et al., 2016[119]	Type 2 diabetes	Fundus photography		The widening of central retinal vein but not central retinal artery diameter was associated with progression of DR
Nguyen et al., 2016[120]	Type 1 diabetes: ◙ No DR ◙ NPDR ◙ PDR (treated)	Laser Doppler velocimetry	◙ Increased RBF in patients with treated PDR, not untreated NPDR	
Srinivas et al., 2017[108]	◙ Severe NPDR ◙ PDR	Doppler Fourier-domain optical coherence tomography (FD-OCT)	◙ Lower total RBF in eyes with severe NPDR and PDR compared with normal eyes	
Lee et al., 2017[107]	◙ DME with or without DR ◙ Mild NPDR ◙ Moderate NPDR ◙ Severe NPDR ◙ PDR	En-face Doppler OCT	◙ Diabetic eyes with DME exhibited lower total RBF than healthy eyes	
Pechauer et al., 2018[5]	◙ Mild/Moderate NPDR ◙ Severe NPDR ◙ PDR	En-face Doppler OCT	◙ Reduced total RBF was correlated with DR disease severity	

DR, diabetic retinopathy; DME, diabetes macular edema; IDDM, Insulin-dependent diabetes mellitus; NPDR, non-proliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy; RBF, retinal blood flow; OCT, optical coherence tomography; FD-OCT, Fourier-domain optical coherence tomography.

Involvement of Vasoconstrictors in the Pathogenesis of Diabetic Retinopathy

The ocular expression of several endogenous vasoconstrictors (ET-1, TXA_2, angiotensin II, and norepinephrine) have been demonstrated in various animal models, including humans.

ET-1

Accumulating evidence has shown that aqueous humor[7], vitreous humor[8] and plasma[8] levels of ET-1 are elevated in diabetes, especially in diabetic patients with proliferative diabetic retinopathy. It has been speculated that the elevated ET-1 is closely associated with the reduced retinal blood flow in patients with non-proliferative diabetic retinopathy.[41,121,122] In cellular and molecular studies, high-glucose treatment increases permeability of endothelial cells through upregulated ET-1 expression, possibly through PKC isoform activation.[123] Activation of PKC-β and PKC-δ can enhance the expression of ET-1 with high-glucose in bovine retinal capillary endothelial cells and bovine retinal pericytes.[124] In addition, the bovine retinal pericytes treated with a high concentration of glucose (25 mM) showed a reduction of ET-1-stimulated Ca^{2+} influx from the L-type Ca^{2+} channels.[125] However, it is unclear whether the endothelium and vascular smooth muscle are affected by the high glucose, and it remains unknow whether the vasomotor response of retinal venules to ET-1 is altered in early diabetes. In STZ-induced diabetic rats, intravitreal injection of ET_AR or ET_BR antagonist attenuated retinal inflammation and reduced retinal vascular leakage.[126] The diabetes-dependent reduction of

retinal blood flow can be prevented by administration of an ET_AR antagonist.[110] These studies suggest the pathophysiological role of ET-1, as well as the involved ET receptors, in the development of diabetic retinopathy. Interestingly, intravitreal injection of ET-1 causes retinal arterial constriction and slows down the retinal circulation (i.e., increased flow resistance), which are both attenuated in the animals after induction of diabetes by STZ for one week.[21] This in-vivo study appears to suggest that the increase of blood flow and relaxation of retinal vasculature by ET-1 in diabetes, possibly due to desensitization of ET receptors in the vascular smooth muscle cells that are pre-exposed to the elevated level of endogenous ET-1 in diabetes.[21] In retinal arterioles isolated from type I diabetic pigs, the endothelium-dependent NO-mediated vasodilator function was found to be compromised,[127,128] but the ability of these diseased vessels to constrict to ET-1 was unaffected.[128] It remains to be determined whether the vasomotor response of retinal venules to ET-1 is affected by diabetes.

TXA_2

The increased expression level of TXA_2 in plasma or serum with diabetes mellitus has been shown in epidemiological and animal studies.[9,49,129-132] Interestingly, the TXA_2 and PGI_2 productions have been shown to link to the pathological development of diabetic retinopathy.[133,134] Application of thromboxane receptor antagonist in mice attenuates diabetes-induced retinal vasoconstriction without altering the retinal levels of thromboxane receptor and thromboxane synthase.[111] Moreover, incubation of cultured bovine retinal endothelial cells with high levels of glucose increases the production of TXA_2.[130] Although the TXB_2 (a stable TXA_2 metabolite) level is detectable in the human vitreous humor[51] and the location of TXA_2 receptor expression in human eyes has been identified,[52] the information on the expression and function of TXA_2 in the retina vasculature is limited and the effect of hyperglycemia and diabetes is unclear.

Angiotensin II

Angiotensin II is implicated as one of the contributing factors in the vascular complication during diabetes.[135] The elevated expression level of angiotensin II has been shown in the retinal tissue and vitreous humor of experimental models of diabetes,[11,136] and in the vitreous humor of patients with proliferative diabetic retinopathy.[137,138] ACE inhibition ameliorates diabetes-induced retinal hyperpermeability[139] and abnormal oscillatory potential amplitudes of the electroretinogram.[140] Moreover, application of ACE inhibition or angiotensin AT_1 receptor antagonism in rats restores diabetes-induced attenuation of retinal blood flow.[141] Although the angiotensin II level is detectable in the vitreous humor of human[137,138] or experimental animal models,[11,136] the information on vasomotor function of the retinal vasculature to angiotensin II during euglycemia or diabetes is limited.

Norepinephrine

The α_1-adrenergic and α_2-adrenergic receptors are conventionally thought to mediate vasoconstriction in vascular system. Interestingly, elevation of serum levels of α- and β-adrenergic receptors agonist norepinephrine (nanomolar level in normal plasma) has been documented in diabetic rats.[10] In addition, activation of β-adrenergic signaling appears to decrease VEGF levels,[142] and attenuate pericyte loss[143] in the diabetic retina, suggesting the protective role of β-adrenergic activation in diabetic retinopathy. A previous study examining the retinal arterial reaction to an α_2-adrenergic receptor agonist (brimonidine) showed dilation of large retinal arterioles (> 60 μm in diameter) but constriction of small retinal arterioles (< 60 μm in diameter).[71] It appears that α-adrenergic activation might elicit heterogeneous vasomotor responses in the retinal vascular network. Although one study has showed a decreased systemic vasopressor response to norepinephrine in rats at 4 weeks of diabetes induced by STZ,[144] it remains unclear whether diabetes has any impact on the vasomotor response of retinal venules to phenylephrine and norepinephrine stimulation.

Perspective on NCX-NHE Pathway

Cytosolic Ca^{2+}, the important molecule for smooth muscle contraction, can be elevated via several pathways, including the transmembrane exchange mechanisms. The forward mode of NCX prevents Ca^{2+} overload of the cells by using energy, i.e., electrochemical influx of Na^+ to counter transport of Ca^{2+} across the plasma membrane with 3 Na^+ to 1 Ca^{2+} ratio.[145] However, when intracellular levels of Na^+ rise beyond a critical point, the reverse-mode NCX begins importing Ca^{2+}.[145] The biological roles of NCX have been demonstrated in various tissues/organs[146-149] and its participation in the development of cardiovascular disease,[150-152] possibly related to hyperglycemia and vascular disorders as suggested recently.[149,153-156] On the other hand, the impact of NCX on the vasculature is less understood. Szewczyk et al. have demonstrated mRNA and protein expressions of NCX1 in the endothelium and smooth muscle of porcine coronary arteries,[157] but the functional role of NCX remains to be determined. A reverse-mode NCX inhibitor, KB-R7943, has been shown to inhibit ET-1-induced contractions of cardiac papillary muscle in cats and vena cava in rats.[14] Takai et al. reported that KB-R7943 inhibited the extracellular Na^+-dependent change in $Ca^{2+}{}_i$ in rat carotid arterial myocytes, which was presumably related to the reverse mode of NCX.[158] Interestingly, a recent study demonstrated that the reverse-mode NCX is involved in intracellular Ca^{2+} mobilization and predominantly mediates ET-1-induced contraction of the rat vena cava.[159] However, the possible effect of ET-1 in which $Na^+{}_i$ mediates Ca^{2+} influx via reverse-mode NCX has not been examined in retinal venules under diabetic insults. Activation of vascular smooth muscle NCX1 has been reported to be involved in the maintenance of blood pressure by using smooth muscle-specific knockout of NCX1,[160] and in the initiation of salt-sensitive hypertension.[161] These studies suggest the critical role of NCX in maintaining vascular homeostasis and possibly contributing to vascular disorders by enhancing vasoconstriction. For example, the NCX inhibitor has been reported to protect endothelial function from hyperglycemic insults.[153]

However, little is known about the mechanism of regulating NCX-dependent constriction of retinal venules under hyperglycemic insults.

Namekata and colleagues have shown that ET-1-induced positive inotropy is mediated by NHE (Na^+_i-dependent) and that negative inotropy is related to the activity of NCX in mouse ventricular myocardium.[162] This study suggests an interaction of NHE and NCX in regulating myocardial function. This context is supported by the involvement of PKC-dependent and NHE-dependent activation of reverse-mode NCX in ET-1-induced positive inotropic effects of cat papillary muscles.[14] Because NHE mediates the transmembrane exchange of one sodium for one hydrogen via the electrochemical gradient,[163] the elevated cytosolic sodium by NHE can potentially activate reverse-mode NCX in the disease state, i.e., hyperglycemic insult, and consequently alter vascular reactivity to endogenous vasoconstrictors. This context is supported by the observed elevation of cytosolic Ca^{2+} by enhanced reverse-mode NCX activity after sodium overload, leading to a positive inotropic effect of cardiomyocytes (Figure 2)[36] and smooth muscle cell contraction in response to ET-1.[13] The contribution of NHE-NCX to disease development is also evident by the elevation of NHE mRNA in the diabetic retina [16] and in agonists-induced myocardial hypertrophy.[164,165] Interestingly, p38 MAPK activity in mesangial cells, under ET-1 stimulation, was found to be enhanced under hyperglycemia.[166] Because p38 MAPK-dependent activation of NHE has also been demonstrated under ET-1 stimulation,[167] the signaling from p38 MAPK may be important in modulating NHE-NCX activity in disease states. Moreover, inhibition of NHE activity has been shown to preserve coronary endothelial function[168] and reduce resistivity index of the central retinal artery in diabetic rats.[16] It is likely that activation of p38 MAPK linking to NHE-NCX can contribute to retinal vascular dysfunction in early diabetes.

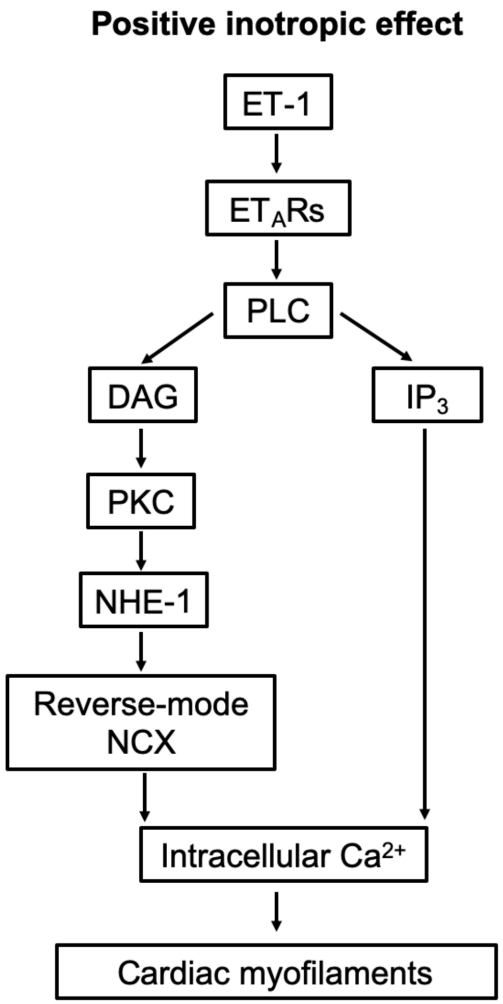

Figure 2. Summary of signal transduction mechanisms involved in ET-1-mediated positive inotropic effects of cardiomyocytes (Figure modified from Brunner F, Brás-Silva C, Cerdeira AS, Leite-Moreira AF. Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. Pharmacol Ther. 2006;111:508-31[36]).

Summary

The venular vasculature is the most effective vessel segment in the circulation in terms of regulating capillary pressure and fluid homeostasis by altering its activity, i.e., constriction or dilation. However, the research of retinal venules in response to endogenous vasoconstrictors is sparse. In this dissertation, I characterized the vasomotor response of isolated porcine retinal venule to ET-1 and other vasoconstrictors (norepinephrine, phenylephrine, TXA_2, and angiotensin II) with and without hyperglycemic/diabetic insults. I will pay special attention to the determination of signaling mechanisms (Figure 3) responsible for the vasoconstriction to ET-1 (Chapter II) because this peptide has been consistently shown to be involved in the pathogenesis of diabetic retinopathy as discussed above. In the pig model of chronic hyperglycemia/diabetes (induced by STZ) and acute hyperglycemia (exposure of the vessel to a high level of glucose), I examined whether these insults can alter retinal venular responses to the aforementioned vasoconstrictors and determined whether activation of p38 MAPK and NHE-NCX contribute to the observed retinal venular dysfunction (Chapter III). Furthermore, in order to support the porcine retinal venous vessels as a viable model resembling the human ocular microcirculation, I also investigated the vasomotor responses of human retinal venules to ET-1 and norepinephrine (Chapter IV). My detailed investigations are described in the following chapters.

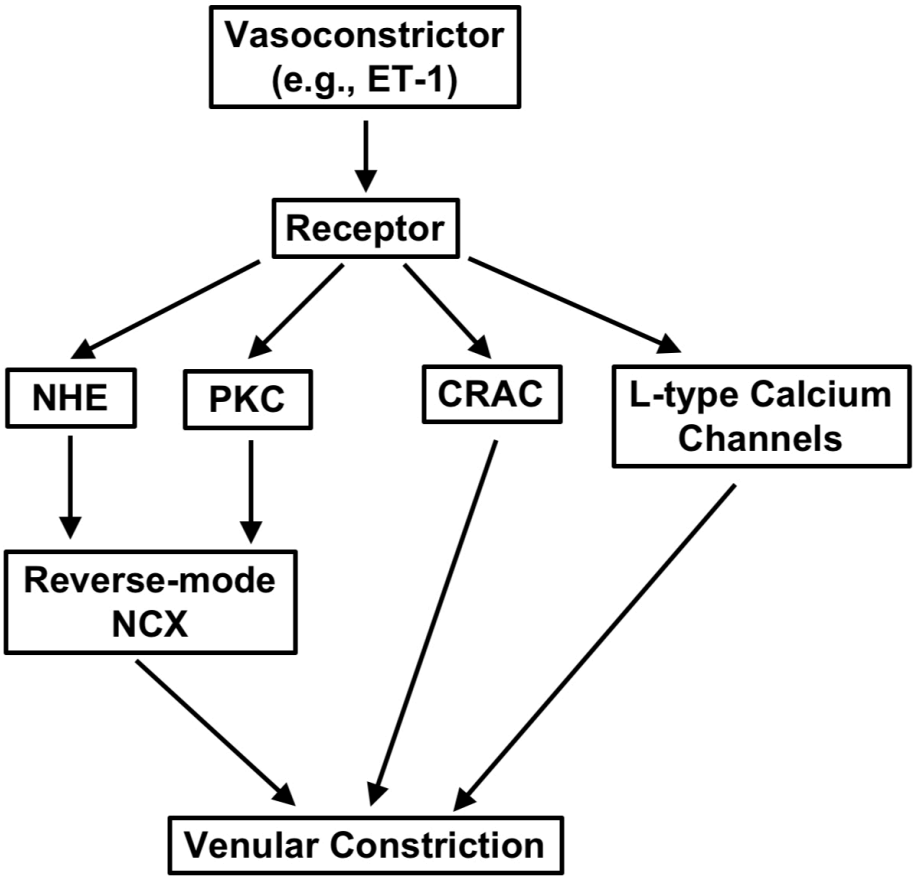

Figure 3. A proposed multi-pathway signaling to vasoconstrictor-induced constriction of retinal venules.

References

1. Lutty GA. Effects of diabetes on the eye. *Invest Ophthalmol Vis Sci.* 2013;54:ORSF81-ORSF87.
2. 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.
3. 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.
4. Cybulska-Heinrich AK, Baertschi M, Loesche CC, et al. Patients with diabetic retinopathy have high retinal venous pressure. *EPMA J.* 2015;6:5.
5. 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.
6. 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.
7. 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.
8. 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.

9. De La Cruz JP, Moreno A, Ruiz-Ruiz MI, Sanchez De La Cuesta F. Effect of DT-TX 30, a combined thromboxane synthase inhibitor and thromboxane receptor antagonist, on retinal vascularity in experimental diabetes mellitus. *Thromb Res.* 2000;97:125-131.

10. Paulson DJ, Light KE. Elevation of serum and ventricular norepinephrine content in the diabetic rat. *Res Commun Chem Pathol Pharmacol.* 1981;33:559-562.

11. Nagai N, Izumi-Nagai K, Oike Y, et al. Suppression of diabetes-induced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-kappaB pathway. *Invest Ophthalmol Vis Sci.* 2007;48:4342-4350.

12. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *J Physiol.* 2008;586:5047-5061.

13. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci.* 2013;123:85-101.

14. Aiello EA, Villa-Abrille MC, Dulce RA, Cingolani HE, Perez NG. Endothelin-1 stimulates the Na^+/Ca^{2+} exchanger reverse mode through intracellular Na^+ (Na^+_i)-dependent and Na^+_i-independent pathways. *Hypertension.* 2005;45:288-293.

15. Nishio S, Teshima Y, Takahashi N, et al. Activation of CaMKII as a key regulator of reactive oxygen species production in diabetic rat heart. *J Mol Cell Cardiol.* 2012;52:1103-1111.

16. Cukiernik M, Hileeto D, Downey D, et al. The role of the sodium hydrogen exchanger-1 in mediating diabetes-induced changes in the retina. *Diabetes Metab Res Rev.* 2004;20:61-71.

17. Pappenheimer JR, Soto-Rivera A. Effective osmotic pressure of the plasma proteins and other quantities associated with the capillary circulation in the hindlimbs of cats and dogs. *Am J Physiol.* 1948;152:471-491.

18. 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.

19. 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.

20. Flammer J, Konieczka K. Retinal venous pressure: the role of endothelin. *EPMA J.* 2015;6:21.

21. 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.

22. Yoshida A, Feke GT, Morales-Stoppello J, Collas GD, Goger DG, McMeel JW. Retinal blood flow alterations during progression of diabetic retinopathy. *Arch Ophthalmol.* 1983;101:225-227.

23. Schneider JG, Tilly N, Hierl T, et al. Elevated plasma endothelin-1 levels in diabetes mellitus. *Am J Hypertens.* 2002;15:967-972.

24. 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.

25. Kiel JW. *The Ocular Circulation*. 1st ed. San Rafael, CA: Morgan & Claypool Life Sciences; 2010:1-69.

26. 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.

27. Barber AJ, Antonetti DA. Mapping the blood vessels with paracellular permeability in the retinas of diabetic rats. *Invest Ophthalmol Vis Sci.* 2003;44:5410-5416.

28. Qaum T, Xu Q, Joussen AM, et al. VEGF-initiated blood-retinal barrier breakdown in early diabetes. *Invest Ophthalmol Vis Sci.* 2001;42:2408-2413.

29. Klaassen I, Van Noorden CJ, Schlingemann RO. Molecular basis of the inner blood-retinal barrier and its breakdown in diabetic macular edema and other pathological conditions. *Prog Retin Eye Res.* 2013;34:19-48.

30. Tatara T. Context-sensitive fluid therapy in critical illness. *J Intensive Care.* 2016;4:20.

31. Folkow B, Lundgren O, Wallentin I. Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat. *Acta Physiol Scand.* 1963;57:270-283.

32. Johnson PC. Effect of venous pressure on mean capillary pressure and vascular resistance in the intestine. *Circ Res.* 1965;16:294-300.

33. 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.

34. Salvatore S, Vingolo EM. Endothelin-1 role in human eye: a review. *J Ophthalmol.* 2010;2010:354645.

35. Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol.* 2016;77:143-175.

36. Brunner F, Bras-Silva C, Cerdeira AS, Leite-Moreira AF. Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. *Pharmacol Ther.* 2006;111:508-531.

37. 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.

38. 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.

39. 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.

40. Chakravarthy U, Douglas AJ, Bailie JR, McKibben B, Archer DB. Immunoreactive endothelin distribution in ocular tissues. *Invest Ophthalmol Vis Sci.* 1994;35:2448-2454.

41. Pang IH, Yorio T. Ocular actions of endothelins. *Proc Soc Exp Biol Med.* 1997;215:21-34.

42. 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:H1693-1698.

43. 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.

44. 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.

45. 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.

46. 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.

47. Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP, Versteeg HH. Prostanoids and prostanoid receptors in signal transduction. *Int J Biochem Cell Biol.* 2004;36:1187-1205.

48. Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol.* 2011;31:986-1000.

49. Halushka PV, Rogers RC, Loadholt CB, et al. Increased platelet prostaglandin and thromboxane synthesis in diabetes mellitus. *Horm Metab Res Suppl.* 1981;11:7-11.

50. Coleman RA, Humphrey PP, Kennedy I, Levy GP, Lumley P. Comparison of the actions of U46619, a prostaglandin H_2-analogue, with those of prostaglandin H_2 and thromboxane A_2 on some isolated smooth muscle preparations. *Br J Pharmacol.* 1981;73:773-778.

51. Thomas MA, O'Grady GE, Swartz SL. Prostaglandin levels in human vitreous. *Br J Ophthalmol.* 1985;69:275-279.

52. Chen Z, Prasad S, Cynader M. Localisation of thromboxane A_2 receptors and the corresponding mRNAs in human eye tissue. *Br J Ophthalmol.* 1994;78:921-926.

53. Abran D, Varma DR, Chemtob S. Regulation of prostanoid vasomotor effects and receptors in choroidal vessels of newborn pigs. *Am J Physiol.* 1997;272:R995-1001.

54. Nielsen PJ, Nyborg NC. Contractile and relaxing effects of arachidonic acid derivatives on isolated bovine retinal resistance arteries. *Exp Eye Res.* 1990;50:305-311.

55. Holmgaard K, Aalkjaer C, Lambert JD, Hessellund A, Bek T. The relaxing effect of perivascular tissue on porcine retinal arterioles in vitro is mimicked by N-methyl-D-aspartate and is blocked by prostaglandin synthesis inhibition. *Acta Ophthalmol.* 2008;86:26-33.

56. Torring MS, Aalkjaer C, Bek T. Constriction of porcine retinal arterioles induced by endothelin-1 and the thromboxane analogue U46619 in vitro decreases with increasing vascular branching level. *Acta Ophthalmol.* 2014;92:232-237.

57. Paul M, Poyan Mehr A, Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev.* 2006;86:747-803.

58. Touyz RM, Schiffrin EL. Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells. *Pharmacol Rev.* 2000;52:639-672.

59. Danser AH, Derkx FH, Admiraal PJ, Deinum J, de Jong PT, Schalekamp MA. Angiotensin levels in the eye. *Invest Ophthalmol Vis Sci.* 1994;35:1008-1018.

60. Schonfelder U, Hofer A, Paul M, Funk RH. In situ observation of living pericytes in rat retinal capillaries. *Microvasc Res.* 1998;56:22-29.

61. Kawamura H, Kobayashi M, Li Q, et al. Effects of angiotensin II on the pericyte-containing microvasculature of the rat retina. *J Physiol.* 2004;561:671-683.

62. Zhang T, Wu DM, Xu GZ, Puro DG. The electrotonic architecture of the retinal microvasculature: modulation by angiotensin II. *J Physiol.* 2011;589:2383-2399.

63. Rockwood EJ, Fantes F, Davis EB, Anderson DR. The response of retinal vasculature to angiotensin. *Invest Ophthalmol Vis Sci.* 1987;28:676-682.

64. Nyborg NC, Nielsen PJ, Prieto D, Benedito S. Angiotensin II does not contract bovine retinal resistance arteries in vitro. *Exp Eye Res.* 1990;50:469-474.

65. Kulkarni PS, Hamid H, Barati M, Butulija D. Angiotensin II-induced constrictions are masked by bovine retinal vessels. *Invest Ophthalmol Vis Sci.* 1999;40:721-728.

66. Eisenhofer G, Kopin IJ, Goldstein DS. Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol Rev.* 2004;56:331-349.

67. Flatmark T. Catecholamine biosynthesis and physiological regulation in neuroendocrine cells. *Acta Physiol Scand.* 2000;168:1-17.

68. Hervet T, Teresinski G, Hejna P, Descloux E, Grouzmann E, Palmiere C. Catecholamines and their O-methylated metabolites in vitreous humor in hypothermia cases. *Forensic Sci Med Pathol.* 2016;12:163-169.

69. Wilke N, Janssen H, Fahrenhorst C, et al. Postmortem determination of concentrations of stress hormones in various body fluids--is there a dependency between adrenaline/noradrenaline quotient, cause of death and agony time? *Int J Legal Med.* 2007;121:385-394.

70. Bylund DB, Chacko DM. Characterization of α_2 adrenergic receptor subtypes in human ocular tissue homogenates. *Invest Ophthalmol Vis Sci.* 1999;40:2299-2306.

71. 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:H231-238.

72. Ehinger B, Falck B. Morphological and pharmacohistochemical characteristics of adrenergic retinal neurons of some mammals. *Albrecht Von Graefes Arch Klin Exp Ophthalmol.* 1969;178:295-305.

73. McAuliffe-Curtin D, Buckley C. Review of α adrenoceptor function in the eye. *Eye (Lond).* 1989;3:472-476.

74. Bohmer T, Manicam C, Steege A, Michel MC, Pfeiffer N, Gericke A. The α_{2B}-adrenoceptor subtype mediates adrenergic vasoconstriction in mouse retinal arterioles with damaged endothelium. *Br J Pharmacol.* 2014;171:3858-3867.

75. Forster BA, Ferrari-Dileo G, Anderson DR. Adrenergic α_1 and α_2 binding sites are present in bovine retinal blood vessels. *Invest Ophthalmol Vis Sci.* 1987;28:1741-1746.

76. Josefsson A, Sigurdsson SB, Bang K, Eysteinsson T. Dorzolamide induces vasodilatation in isolated pre-contracted bovine retinal arteries. *Exp Eye Res.* 2004;78:215-221.

77. Yu DY, Alder VA, Cringle SJ, Su EN, Yu PK. Vasoactivity of intraluminal and extraluminal agonists in perfused retinal arteries. *Invest Ophthalmol Vis Sci.* 1994;35:4087-4099.

78. Hara H, Ichikawa M, Oku H, Shimazawa M, Araie M. Bunazosin, a selective α_1-adrenoceptor antagonist, as an anti-glaucoma drug: effects on ocular circulation and retinal neuronal damage. *Cardiovasc Drug Rev.* 2005;23:43-56.

79. Ferrari-Dileo G, Davis EB, Anderson DR. Response of retinal vasculature to phenylephrine. *Invest Ophthalmol Vis Sci.* 1990;31:1181-1182.

80. Hoste AM, Boels PJ, Brutsaert DL, De Laey JJ. Effect of α1 and β agonists on contraction of bovine retinal resistance arteries in vitro. *Invest Ophthalmol Vis Sci.* 1989;30:44-50.

81. Cheng J, Yu J, Jiang C, Sun X. Phenylephrine affects peripapillary retinal vasculature-an optic coherence tomography angiography study. *Front Physiol.* 2017;8:996.

82. Takayama J, Mishima A, Ishii K. Effects of topical phenylephrine on blood flow in the posterior segments of monkey and aged human eyes. *Jpn J Ophthalmol.* 2004;48:243-248.

83. Puetz S, Lubomirov LT, Pfitzer G. Regulation of smooth muscle contraction by small GTPases. *Physiology (Bethesda).* 2009;24:342-356.

84. 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.

85. 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.

86. 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.

87. Reading SA, Earley S, Waldron BJ, Welsh DG, Brayden JE. TRPC3 mediates pyrimidine receptor-induced depolarization of cerebral arteries. *Am J Physiol Heart Circ Physiol.* 2005;288:H2055-H2061.

88. Onohara N, Nishida M, Inoue R, et al. TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J.* 2006;25:5305-5316.

89. Lemos VS, Poburko D, Liao CH, Cole WC, van Breemen C. Na^{+} entry via TRPC6 causes Ca^{2+} entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem Biophys Res Commun.* 2007;352:130-134.

90. Eder P, Probst D, Rosker C, et al. Phospholipase C-dependent control of cardiac calcium homeostasis involves a TRPC3-NCX1 signaling complex. *Cardiovasc Res.* 2007;73:111-119.

91. Chik CL, Li B, Ogiwara T, Ho AK, Karpinski E. PACAP modulates L-type Ca^{2+} channel currents in vascular smooth muscle cells: involvement of PKC and PKA. *FASEB J.* 1996;10:1310-1317.

92. Obejero-Paz CA, Auslender M, Scarpa A. PKC activity modulates availability and long openings of L-type Ca^{2+} channels in A7r5 cells. *Am J Physiol.* 1998;275:C535-543.

93. Viard P, Exner T, Maier U, Mironneau J, Nurnberg B, Macrez N. Gbetagamma dimers stimulate vascular L-type Ca^{2+} channels via phosphoinositide 3-kinase. *FASEB J.* 1999;13:685-694.

94. Ren C, Zhang J, Philipson KD, Kotlikoff MI, Blaustein MP, Matteson DR. Activation of L-type Ca^{2+} channels by protein kinase C is reduced in smooth muscle-specific Na^{+}/Ca^{2+} exchanger knockout mice. *Am J Physiol Heart Circ Physiol.* 2010;298:H1484-1491.

95. Hirata T, Ushikubi F, Kakizuka A, Okuma M, Narumiya S. Two thromboxane A_2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation. *J Clin Invest.* 1996;97:949-956.

96. Miggin SM, Kinsella BT. Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) alpha and beta isoforms. *Biochim Biophys Acta.* 1998;1425:543-559.

97. Shenker A, Goldsmith P, Unson CG, Spiegel AM. The G protein coupled to the thromboxane A_2 receptor in human platelets is a member of the novel G_q family. *Trans Assoc Am Physicians.* 1991;104:11-20.

98. Chen H. Role of thromboxane A_2 signaling in endothelium-dependent contractions of arteries. *Prostaglandins Other Lipid Mediat.* 2018;134:32-37.

99. Tosun M, Paul RJ, Rapoport RM. Role of extracellular Ca^{2+} influx via L-type and non-L-type Ca^{2+} channels in thromboxane A_2 receptor-mediated contraction in rat aorta. *J Pharmacol Exp Ther.* 1998;284:921-928.

100. Kulkarni P, Payne S. Eicosanoids in bovine retinal microcirculation. *J Ocul Pharmacol Ther.* 1997;13:139-149.

101. Spada CS, Nieves AL, Woodward DF. Vascular activities of prostaglandins and selective prostanoid receptor agonists in human retinal microvessels. *Exp Eye Res.* 2002;75:155-163.

102. Fletcher EL, Phipps JA, Ward MM, Vessey KA, Wilkinson-Berka JL. The renin-angiotensin system in retinal health and disease: its influence on neurons, glia and the vasculature. *Prog Retin Eye Res.* 2010;29:284-311.

103. Kimura K, Eguchi S. Angiotensin II type-1 receptor regulates RhoA and Rho-kinase/ROCK activation via multiple mechanisms. Focus on "Angiotensin II induces RhoA activation through SHP2-dependent dephosphorylation of the RhoGAP p190A in vascular smooth muscle cells". *Am J Physiol Cell Physiol.* 2009;297:C1059-1061.

104. Arun KH, Kaul CL, Ramarao P. AT_1 receptors and L-type calcium channels: functional coupling in supersensitivity to angiotensin II in diabetic rats. *Cardiovasc Res.* 2005;65:374-386.

105. Finch AM, Sarramegna V, Graham RM. Ligand binding, activation, and agonist trafficking. In: Perez DM, ed. *The Adrenergic Receptors: In the 21ST Century*. Totowa, NJ: Humana Press; 2006:25-86.

106. Feke GT, Pasquale LR. Retinal blood flow response to posture change in glaucoma patients compared with healthy subjects. *Ophthalmology.* 2008;115:246-252.

107. 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.

108. 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.

109. Wong TY, Cheung CM, Larsen M, Sharma S, Simo R. Diabetic retinopathy. *Nat Rev Dis Primers.* 2016;2:16012.

110. 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.

111. Wright WS, Messina JE, Harris NR. Attenuation of diabetes-induced retinal vasoconstriction by a thromboxane receptor antagonist. *Exp Eye Res.* 2009;88:106-112.

112. Grunwald JE, Riva CE, Brucker AJ, Sinclair SH, Petrig BL. Altered retinal vascular response to 100% oxygen breathing in diabetes mellitus. *Ophthalmology.* 1984;91:1447-1452.

113. Patel V, Rassam S, Newsom R, Wiek J, Kohner E. Retinal blood flow in diabetic retinopathy. *BMJ.* 1992;305:678-683.

114. Bursell SE, Clermont AC, Kinsley BT, Simonson DC, Aiello LM, Wolpert HA. Retinal blood flow changes in patients with insulin-dependent diabetes mellitus and no diabetic retinopathy. *Invest Ophthalmol Vis Sci.* 1996;37:886-897.

115. Konno S, Feke GT, Yoshida A, Fujio N, Goger DG, Buzney SM. Retinal blood flow changes in type I diabetes. A long-term follow-up study. *Invest Ophthalmol Vis Sci.* 1996;37:1140-1148.

116. Nagaoka T, Sato E, Takahashi A, Yokota H, Sogawa K, Yoshida A. Impaired retinal circulation in patients with type 2 diabetes mellitus: retinal laser Doppler velocimetry study. *Invest Ophthalmol Vis Sci.* 2010;51:6729-6734.

117. Roy MS, Klein R, Janal MN. Retinal venular diameter as an early indicator of progression to proliferative diabetic retinopathy with and without high-risk characteristics in African Americans with type 1 diabetes mellitus. *Arch Ophthalmol.* 2011;129:8-15.

118. Klein R, Myers CE, Lee KE, Gangnon R, Klein BE. Changes in retinal vessel diameter and incidence and progression of diabetic retinopathy. *Arch Ophthalmol.* 2012;130:749-755.

119. Frydkjaer-Olsen U, Soegaard Hansen R, Simo R, et al. Correlation between retinal vessel calibre and neurodegeneration in patients with type 2 diabetes mellitus in the european consortium for the early treatment of diabetic retinopathy (eurocondor). *Ophthalmic Res.* 2016;56:10-16.

120. Nguyen HT, van Duinkerken E, Verbraak FD, et al. Retinal blood flow is increased in type 1 diabetes mellitus patients with advanced stages of retinopathy. *BMC Endocr Disord.* 2016;16:25.

121. 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.

122. Granstam E, Wang L, Bill A. Ocular effects of endothelin-1 in the cat. *Curr Eye Res.* 1992;11:325-332.

123. Chen S, Apostolova MD, Cherian MG, Chakrabarti S. Interaction of endothelin-1 with vasoactive factors in mediating glucose-induced increased permeability in endothelial cells. *Lab Invest.* 2000;80:1311-1321.

124. Park JY, Takahara N, Gabriele A, et al. Induction of endothelin-1 expression by glucose: an effect of protein kinase C activation. *Diabetes.* 2000;49:1239-1248.

125. McGinty A, Scholfield CN, Liu WH, Anderson P, Hoey DE, Trimble ER. Effect of glucose on endothelin-1-induced calcium transients in cultured bovine retinal pericytes. *J Biol Chem.* 1999;274:25250-25253.

126. Alrashdi SF, Deliyanti D, Wilkinson-Berka JL. Intravitreal administration of endothelin type A receptor or endothelin type B receptor antagonists attenuates hypertensive and diabetic retinopathy in rats. *Exp Eye Res.* 2018;176:1-9.

127. Hein TW, Xu W, Xu X, Kuo L. Acute and chronic hyperglycemia elicit JIP1/JNK-mediated endothelial vasodilator dysfunction of retinal arterioles. *Invest Ophthalmol Vis Sci.* 2016;57:4333-4340.

128. 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.

129. Moreno A, De La Cruz JP, Garcia Campos J, Sanchez De La Cuesta F. Prostacyclin-thromboxane balance and retinal vascular pattern in rats with experimentally induced diabetes. *Can J Ophthalmol.* 1995;30:117-123.

130. Sone H, Okuda Y, Kawakami Y, Yamashita K. Effects of high glucose concentration and a thromboxane synthase inhibitor on the production of thromboxane A_2 and prostaglandin I_2 and E_2 by retinal endothelial cells. *Life Sci.* 1996;58:239-243.

131. Hishinuma T, Tsukamoto H, Suzuki K, Mizugaki M. Relationship between thromboxane/prostacyclin ratio and diabetic vascular complications. *Prostaglandins Leukot Essent Fatty Acids.* 2001;65:191-196.

132. Thomas G, Skrinska V, Lucas FV, Schumacher OP. Platelet glutathione and thromboxane synthesis in diabetes. *Diabetes.* 1985;34:951-954.

133. Naveh-Floman N, Moisseiev J. Prostanoids and thromboxane A_2 involvement in diabetic retinopathy. *Metab Pediatr Syst Ophthalmol.* 1982;6:321-325.

134. Lane LS, Jansen PD, Lahav M, Rudy C. Circulating prostacyclin and thromboxane levels in patients with diabetic retinopathy. *Ophthalmology.* 1982;89:763-766.

135. Schoonmaker GC, Fallet RW, Carmines PK. Superoxide anion curbs nitric oxide modulation of afferent arteriolar ANG II responsiveness in diabetes mellitus. *Am J Physiol Renal Physiol.* 2000;278:F302-309.

136. Byon IS, Lee DH, Jun ES, Shin MK, Park SW, Lee JE. Effect of angiotensin II type 1 receptor blocker and angiotensin converting enzyme inhibitor on the intraocular growth

factors and their receptors in streptozotocin-induced diabetic rats. *Int J Ophthalmol.* 2017;10:896-901.

137. Funatsu H, Yamashita H, Nakanishi Y, Hori S. Angiotensin II and vascular endothelial growth factor in the vitreous fluid of patients with proliferative diabetic retinopathy. *Br J Ophthalmol.* 2002;86:311-315.

138. Funatsu H, Yamashita H, Noma H, Mimura T, Sakata K, Hori S. Risk evaluation of outcome of vitreous surgery for proliferative diabetic retinopathy based on vitreous level of vascular endothelial growth factor and angiotensin II. *Br J Ophthalmol.* 2004;88:1064-1068.

139. Gilbert RE, Kelly DJ, Cox AJ, et al. Angiotensin converting enzyme inhibition reduces retinal overexpression of vascular endothelial growth factor and hyperpermeability in experimental diabetes. *Diabetologia.* 2000;43:1360-1367.

140. Bui BV, Armitage JA, Tolcos M, Cooper ME, Vingrys AJ. ACE inhibition salvages the visual loss caused by diabetes. *Diabetologia.* 2003;46:401-408.

141. Horio N, Clermont AC, Abiko A, et al. Angiotensin AT_1 receptor antagonism normalizes retinal blood flow and acetylcholine-induced vasodilatation in normotensive diabetic rats. *Diabetologia.* 2004;47:113-123.

142. Jiang Y, Zhang Q, Steinle JJ. β-adrenergic receptor agonist decreases VEGF levels through altered eNOS and PKC signaling in diabetic retina. *Growth Factors.* 2015;33:192-199.

143. Yun JH, Jeong HS, Kim KJ, et al. β-adrenergic receptor agonists attenuate pericyte loss in diabetic retinas through Akt activation. *FASEB J.* 2018;32:2324-2338.

144. Hong E, Huang F, Villafana S. Effect of early diabetes on the response to norepinephrine and dopamine in pithed Wistar Kyoto and spontaneously hypertensive rats. *Clin Exp Hypertens.* 2010;32:390-394.

145. Khananshvili D. Sodium-calcium exchangers (NCX): molecular hallmarks underlying the tissue-specific and systemic functions. *Pflugers Arch.* 2014;466:43-60.

146. Shattock MJ, Ottolia M, Bers DM, et al. Na^+/Ca^{2+} exchange and Na^+/K^+-ATPase in the heart. *J Physiol.* 2015;593:1361-1382.

147. Liao QS, Du Q, Lou J, Xu JY, Xie R. Roles of Na^+/Ca^{2+} exchanger 1 in digestive system physiology and pathophysiology. *World J Gastroenterol.* 2019;25:287-299.

148. Shenoda B. The role of Na^+/Ca^{2+} exchanger subtypes in neuronal ischemic injury. *Transl Stroke Res.* 2015;6:181-190.

149. Khananshvili D. The SLC8 gene family of sodium-calcium exchangers (NCX) - structure, function, and regulation in health and disease. *Mol Aspects Med.* 2013;34:220-235.

150. Iwamoto T. Sodium-calcium exchange inhibitors: therapeutic potential in cardiovascular diseases. *Future Cardiol.* 2005;1:519-529.

151. Pignataro G, Sirabella R, Anzilotti S, Di Renzo G, Annunziato L. Does Na^+/Ca^{2+} exchanger, NCX, represent a new druggable target in stroke intervention? *Transl Stroke Res.* 2014;5:145-155.

152. Ottolia M, Torres N, Bridge JH, Philipson KD, Goldhaber JI. Na/Ca exchange and contraction of the heart. *J Mol Cell Cardiol.* 2013;61:28-33.

153. Liu D, Cui KZ, Sun YM, Liu JW, Li YB, Su Y. Protective effects of the sodium/calcium exchanger inhibitor on endothelial dysfunction induced by high glucose. *Exp Clin Endocrinol Diabetes.* 2015;123:7-10.

154. Fernandez-Velasco M, Ruiz-Hurtado G, Gomez AM, Rueda A. Ca^{2+} handling alterations and vascular dysfunction in diabetes. *Cell Calcium.* 2014;56:397-407.

155. Singh RM, Waqar T, Howarth FC, Adeghate E, Bidasee K, Singh J. Hyperglycemia-induced cardiac contractile dysfunction in the diabetic heart. *Heart Fail Rev.* 2018;23:37-54.

156. Cui KZ, Liu D, Liu JW, et al. Role of the Na^+/Ca^{2+} exchanger on the development of diabetes mellitus and its chronic complications. *Biochem Biophys Res Commun.* 2012;426:445-447.

157. Szewczyk MM, Davis KA, Samson SE, Simpson F, Rangachari PK, Grover AK. Ca^{2+}-pumps and Na^+-Ca^{2+}-exchangers in coronary artery endothelium versus smooth muscle. *J Cell Mol Med.* 2007;11:129-138.

158. Takai N, Yamada A, Muraki K, Watanabe M, Imaizumi Y. KB-R7943 reveals possible involvement of Na^+-Ca^{2+} exchanger in elevation of intracellular Ca^{2+} in rat carotid arterial myocytes. *J Smooth Muscle Res.* 2004;40:35-42.

159. Tykocki NR, Jackson WF, Watts SW. Reverse-mode Na^+/Ca^{2+} exchange is an important mediator of venous contraction. *Pharmacol Res.* 2012;66:544-554.

160. Zhang J, Ren C, Chen L, et al. Knockout of Na^+/Ca^{2+} exchanger in smooth muscle attenuates vasoconstriction and L-type Ca^{2+} channel current and lowers blood pressure. *Am J Physiol Heart Circ Physiol.* 2010;298:H1472-1483.

161. Iwamoto T, Kita S, Zhang J, et al. Salt-sensitive hypertension is triggered by Ca^{2+} entry via Na^+/Ca^{2+} exchanger type-1 in vascular smooth muscle. *Nat Med.* 2004;10:1193-1199.

162. Namekata I, Fujiki S, Kawakami Y, et al. Intracellular mechanisms and receptor types for endothelin-1-induced positive and negative inotropy in mouse ventricular myocardium. *Naunyn Schmiedebergs Arch Pharmacol.* 2008;376:385-395.

163. Slepkov ER, Rainey JK, Sykes BD, Fliegel L. Structural and functional analysis of the Na+/H+ exchanger. *Biochem J.* 2007;401:623-633.

164. Karmazyn M, Kilic A, Javadov S. The role of NHE-1 in myocardial hypertrophy and remodelling. *J Mol Cell Cardiol.* 2008;44:647-653.

165. Stanbouly S, Kirshenbaum LA, Jones DL, Karmazyn M. Sodium hydrogen exchange 1 (NHE-1) regulates connexin 43 expression in cardiomyocytes via reverse mode sodium

calcium exchange and c-Jun NH2-terminal kinase-dependent pathways. *J Pharmacol Exp Ther.* 2008;327:105-113.

166. Tsiani E, Lekas P, Fantus IG, Dlugosz J, Whiteside C. High glucose-enhanced activation of mesangial cell p38 MAPK by ET-1, ANG II, and platelet-derived growth factor. *Am J Physiol Endocrinol Metab.* 2002;282:E161-169.

167. Horinouchi T, Asano H, Higa T, et al. Differential coupling of human endothelin type A receptor to $G_{q/11}$ and G_{12} proteins: the functional significance of receptor expression level in generating multiple receptor signaling. *J Pharmacol Sci.* 2009;111:338-351.

168. Vial G, Dubouchaud H, Couturier K, Lanson M, Leverve X, Demaison L. Na^+/H^+ exchange inhibition with cariporide prevents alterations of coronary endothelial function in streptozotocin-induced diabetes. *Mol Cell Biochem.* 2008;310:93-102.

II. CONSTRICTION OF RETINAL VENULES TO ENDOTHELIN-1: OBLIGATORY ROLES OF ET_A RECEPTORS, EXTRACELLULAR CALCIUM ENTRY, AND RHO KINASE *

Introduction

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.[15] 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.[16,17] Because the retinal circulation lacks autonomic innervation,[131] local vascular control mechanisms appear to dominate the regulation of retinal vascular resistance.[132] Although the importance of the intrinsic ability of retinal arterioles to regulate their resting diameter is well established,[132] there is paucity of information on vasomotor function of retinal venules. *In vivo* studies have reported an increase in retinal venular diameter during flickering light stimulation[133] or a reduction in retinal venular diameter in response to hyperoxia,[134] 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[135] 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).[18] It is unclear, however, whether smaller upstream venules exhibit similar

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reactivity, especially vasoconstriction, which dictates not only blood drainage but also capillary pressure and fluid exchange.

ET-1 is a potent vasoactive peptide comprised of 21 amino acids and plays important roles in many biological functions, including vasoconstriction, cardiovascular remodeling, tissue inflammation, and cell proliferation.[22-27,136,137] In the eye, ET-1 is distributed locally in the retina,[31,138] causes retinal vasoconstriction *in vivo*,[3,32,33,88] and reduces retinal blood flow.[32,100,101] Our previous studies have demonstrated the cell signaling mechanisms contributing to ET-1-induced constriction of porcine and human retinal arterioles.[28,29,34,35] Although others have shown that isolated porcine retinal veins are capable of constricting to ET-1,[18] 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 elevated in diabetes,[2,32,99,100] especially in patients with diabetic retinopathy.[2,139] The increased plasma ET-1 level also has been implicated in the pathogenesis of retinal vein occlusion (RVO).[11,12] 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),[23,24,28] elevation of intracellular Ca^{2+},[29,30,69,70] and signaling through Rho kinase (ROCK) and/or protein kinase C (PKC).[23,29,68] 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 Ca^{2+} 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 (ROCK 1 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 and 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.[140,141]

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.[140-142] 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 pipette 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 cmH_2O (4 mmHg) 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 mmHg.[143] Vasomotor activity of isolated venules was recorded using videomicroscopic techniques throughout the experiments.[140]

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),[28] selective ET_BR agonist sarafotoxin S6c (10 pM to 0.1 μM; Tocris Cookson, Ellisville, MO),[28] 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_A receptors (ET_ARs) and ET_B receptors (ET_BRs) in the retinal venule responses to ET-1 were evaluated after treatment of the vessel with respective antagonists BQ123 (1 μM)[28] and BQ788 (0.1 μM).[28] 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 Ca^{2+} channel antagonist nifedipine (1 μM).[29,70] 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).[144] 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)[29,35] 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.[28] The final concentration of dimethyl sulfoxide or ethanol in the vessel bath 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-Polymerase Chain Reaction 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.[145] 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 polymerase chain reaction (PCR) reagents (PowerUp SYBR Green Master Mix, Thermo Fisher Scientific). In order 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.[146]

Western Blot Analysis

Western blot analysis was performed as described in our previous study of retinal arterioles with slight modification.[28,147] 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; Santa Cruz Biotechnology, Santa Cruz, CA, USA; Catalog # sc-33535 and sc-33537),[28] or mouse anti-ROCK1 or anti-ROCK2 monoclonal antibody (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, CA; Catalog # sc-17794 and sc-398519). Membranes were stripped and reprobed with rabbit anti-p38 antibody (1:1000; Santa Cruz Biotechnology; Catalog #sc-535), which we previously have shown is highly expressed in neural retina tissue.[28] After incubation with an appropriate secondary antibody (anti-rabbit or anti-mouse IgG, 1:1000; Cell Signaling Technology, Danvers, MA; Catalog # 7074s and 7076s), 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[140] at 5 cmH_2O intraluminal pressure. Diameter changes in response to agonists were

normalized to the resting diameter and expressed as percentage changes in diameter.[28] 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 two-way analysis of variance 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 cmH_2O with average maximum diameter of 133 $\pm$ 2 μm. These vessels developed stable basal tone by constricting to about 92% of maximum diameter within 30 minutes at 36-37°C. In one cohort, administration of ET-1 caused constriction of retinal venules in a concentration dependent manner (Figure 4A). The threshold concentration of ET-1 for venular constriction was about 1 pM and the vessels constricted to about 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 4B.

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 about 10% constriction remaining at the highest concentration (10 nM) of ET-1 (Figure 4A). In the presence of ET_BR antagonist BQ788, neither the resting diameter nor the vasoconstriction to ET-1 were altered (Figure 4A). The retinal venules failed to respond to the challenge of ET_BR agonist sarafotoxin S6c throughout the concentrations tested (Figure 4C).

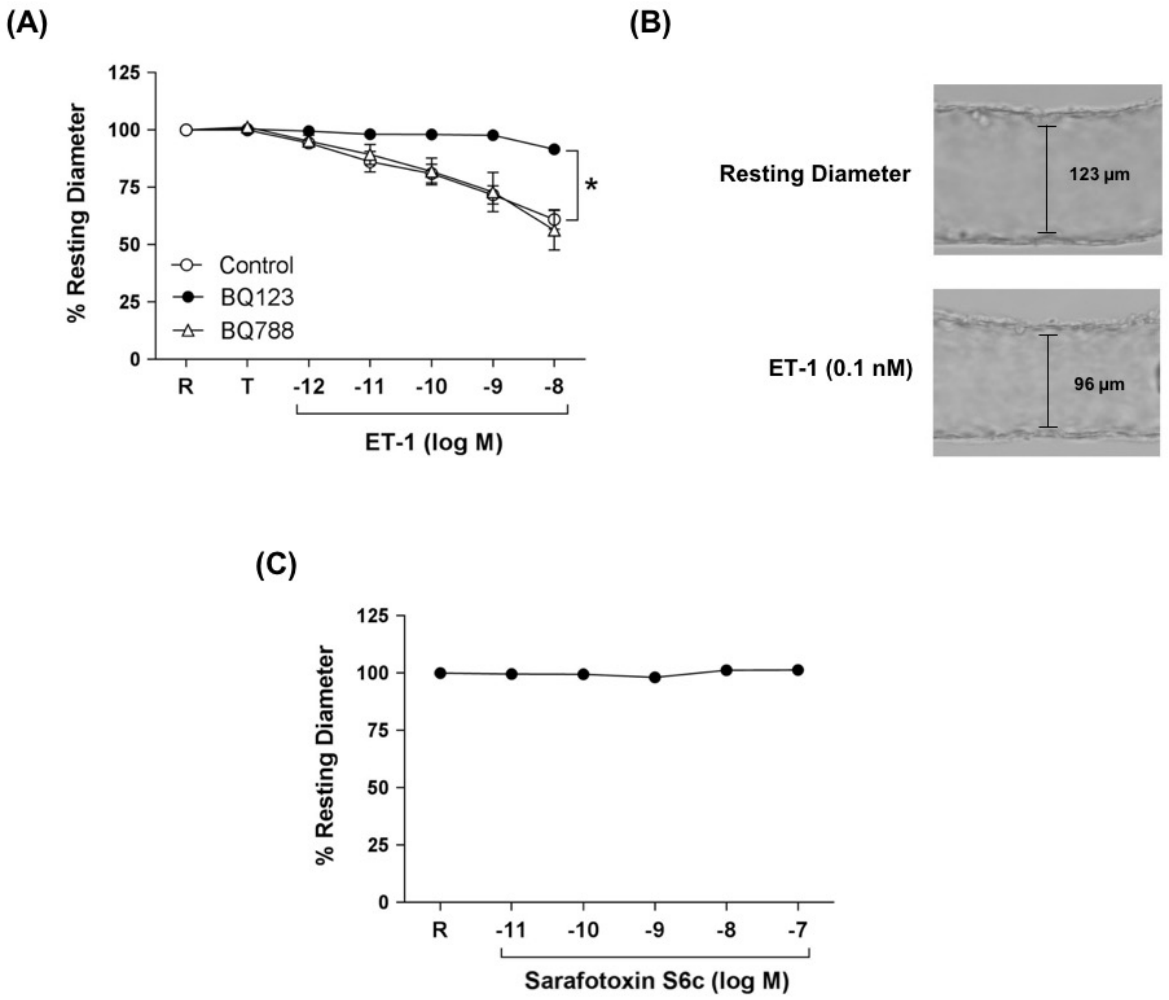


Figure 4. Vasomotor response of isolated and pressurized porcine retinal venules to ET-1. (A) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with an ET-1 receptor antagonist. In the absence of receptor antagonist, retinal venules constricted to ET-1 in 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 vs. 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). Reprinted with permission from Yen-Lin Chen, Invest Opthalmol Vis Sci, Copyright 2018.

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 (Figure 5A). The neural retina tissue expressed 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, while only modest expression of both ET-1 receptors was detected in neural retina tissue (Figure 5B).

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 (Figure 6A). At 10 nM ET-1, the retinal venules constricted to about 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 (Figure 6A). Furthermore, the L-VOCC agonist Bay K8644 elicited a modest but significant constriction (8% reduction in resting diameter) of retinal venules (Figure 6B), which was prevented in the presence of nifedipine (Figure 6B).

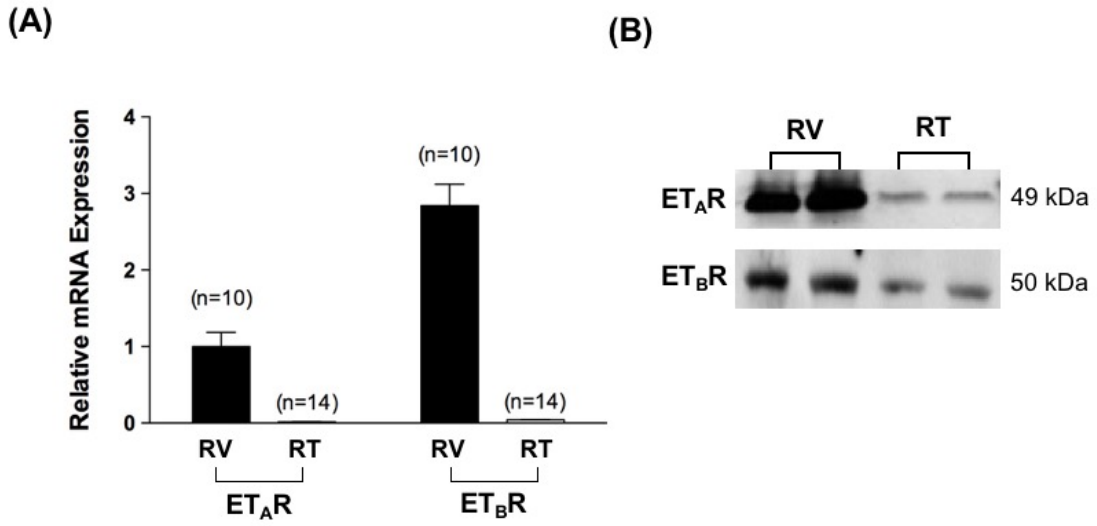


Figure 5. Molecular analyses of ET-1 receptors in porcine retinal venules. (A) Equal amounts of total RNA isolated from porcine retinal venules (RV) and neural retina tissue (RT) were reverse transcribed and then analyzed by real-time PCR for detection of ET_AR, ET_BR, and GAPDH mRNAs. The ET_AR and 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 retinal venules (RV) and neural retina tissue (RT) from pigs. Data represent 4 independent experiments. Reprinted with permission from Yen-Lin Chen, Invest Opthalmol Vis Sci, Copyright 2018.

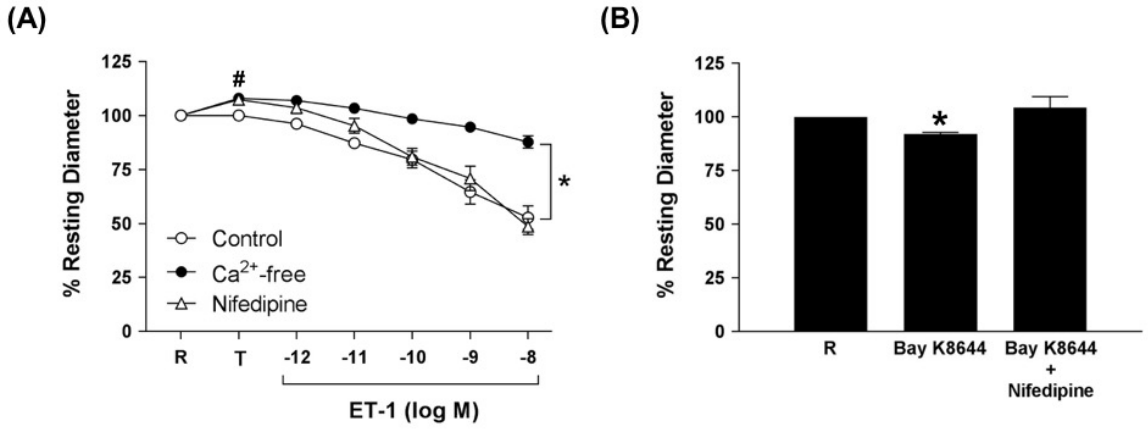


Figure 6. Roles of extracellular Ca^{2+} and L-VOCCs in vasoconstriction of isolated retinal venules to ET-1. (A) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) of the vessel with Ca^{2+}-free solution (*n*=10) or L-VOCC inhibitor nifedipine (1 μM; *n*=7). 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 vs. R; *P < 0.05 vs. 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 vs. R.

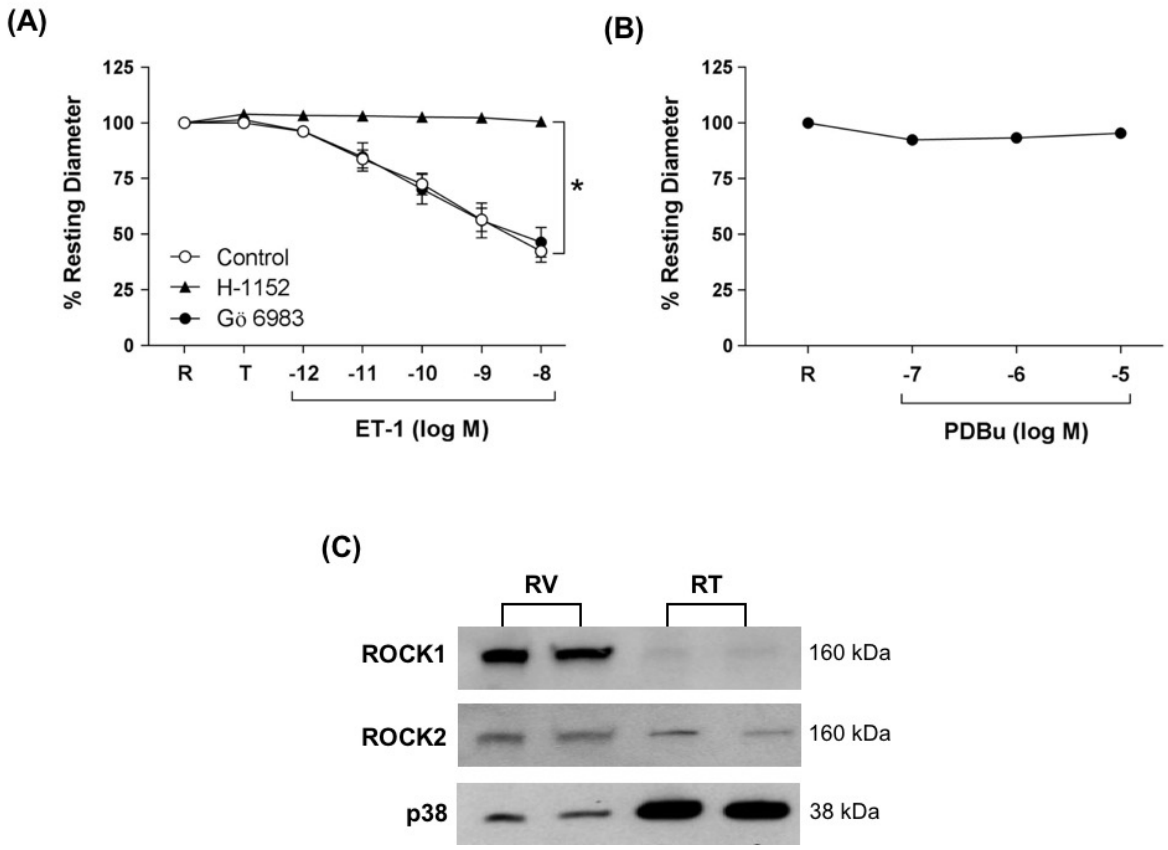


Figure 7. Roles of ROCK and PKC in vasoconstriction of isolated retinal venules to ET-1. (A) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with ROCK inhibitor H-1152 (3 μM; *n*=6) or PKC inhibitor Gö 6983 (10 μM; *n*=7). In the absence of treatment (Control; *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 vs. 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 4 independent experiments. **Re**printed with permission from Yen-Lin Chen, Invest Opthalmol Vis Sci, Copyright 2018.

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 (Figure 7A). In contrast, PKC inhibitor Gö 6983 affected neither resting diameter nor vasoconstriction to ET-1 (Figure 7A). Cumulative addition of PKC activator PDBu to the vessel bath also did not significantly affect the resting diameter of retinal venules (Figure 7B). Immunoblotting showed that both ROCK1 and ROCK2 isoforms were strongly expressed in retinal venules, while only faint expression of these proteins was detected in neural retina tissue (Figure 7C). Sufficient neural retina protein was present in these samples because p38 protein expression was strikingly stronger in neural retina tissue than in venules (Figure 7C).

Conclusions

Although venules are known to play a critical role in regulation of postcapillary pressure and capillary fluid exchange,[16,17] 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.

Studies using isolated vessel preparations have shown that ET-1 directly causes constriction of small arterioles[28] and veins[18] 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 about 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[135] and significantly less than the 50-60% tone

developed in porcine and human retinal arterioles.[35] 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.[28] 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 (Figure 4). This venular responsiveness is comparable to that observed in a recent ET-1 study in isolated retinal veins from pigs[18] and is consistent with the report of the greater amount of smooth muscle in retinal arteries than retinal veins.[18] 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)[148,149] and the estimated level at the local microvasculature (nanomolar range).[150] 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 (Figure 4). These results are consistent with the major contribution of ET_ARs in retinal arteriolar constriction to ET-1 reported in our previous studies.[28] 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 (Figure 4). 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).[28] 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,[28,34] 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 (Figure 5). 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[151] and rodents,[152-154] 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, i.e., endothelial vs. 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.[155,156] 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.[23] 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+} (Figure 6). 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 K 8644 (Figure 6). 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[157] 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,[158] which is modulated by Ca^{2+}-dependent activity of MLC kinase[68,159,160] and by MLC phosphatase.[161] Protein kinases such as ROCK and PKC can inhibit MLC phosphatase leading to enhanced MLC phosphorylation[162-164] and subsequent contraction of vascular smooth muscle.[68,161] 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.[165,166] 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 (Figure 7). 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 (Figure 7). This observation contrasts with potent PDBu-induced constriction of porcine retinal arterioles, which was blocked by PKC inhibitor Gö 6983 in our previous studies.[29,34] 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,[167] 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.[13,139,168] A complication of RVO[169] and late stage diabetic retinopathy[170] 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.[13] This notion is supported by clinical studies reporting elevation of retinal venous pressure and reduced retinal blood flow in patients with RVO[171,172] and diabetic retinopathy,[10,14,86] including those with diabetic macular edema.[85] 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.[11,13,14,168] Evaluation of the impact of experimental diabetes[105] 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

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 USA.* 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 Pt 2:177-185.

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.

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.

III. ACUTE AND CHRONIC HYPERGLYCEMIA ENHANCE CONSTRICTION OF RETINAL VENULES VIA ACTIVATION OF REVERSE-MODE SODIUM-CALCIUM EXCHANGER AND SODIUM-HYDROGEN EXCHANGER

Introduction

Retinopathy is one of the most common microvascular complications of diabetes mellitus and a major cause of vision impairment and blindness in working-age adults.[1] Hyperglycemia, a hallmark of diabetes, is closely linked to the retinal pathology including progression from early disruption of retinal perfusion for proper oxygen supply to the development of edema and fluid accumulation during the late stages of the disease.[2] Although alteration of microcirculatory function in the retina is considered a critical event contributing to tissue edema with diabetes, the precise vasomotor regulatory mechanisms remain unclear. It is well recognized that venular resistance influences hydrostatic pressure and fluid homeostasis at the level of the capillaries.[3,4] Constriction of retinal venules is expected to increase retinal venous resistance and pressure, which could consequently reduce retinal perfusion and promote edema under disease states such as diabetes.[5,6] In accord with this idea, clinical evidence has shown increased retinal venous pressure and decreased retinal blood flow in patients with diabetic retinopathy,[7,8] including those with diabetic macular edema.[9] However, it is unclear whether diabetes or hyperglycemia affects responses of retinal venules to endogenous vasoconstrictors. The potent vasoconstrictor endothelin-1 (ET-1) has been implicated in the pathogenesis of diabetic retinopathy based in part on elevated levels of this peptide in the retinal tissue[10,11] of diabetic rats and vitreous fluid[12] of human patients with diabetic retinopathy. Elevated plasma/serum or retinal tissue levels of other vasoconstrictors, such as thromboxane A_2 (TXA_2),[13] norepinephrine[14] and angiotensin II,[15] have also been detected in experimental

diabetes. Therefore, it is important to investigate whether diabetes/hyperglycemia alters venular reactivity to these endogenous vasoconstrictors in the retina.

It is generally accepted that Ca^{2+} entry into the vascular smooth muscle cells triggers vasoconstriction.[16] In some blood vessels, this process is mediated by activation of L-type voltage-operated Ca^{2+} channels (L-VOCCs),[16] calcium release-activated calcium (CRAC) channels,[17] or transient receptor potential-canonical (TRPC) channels.[17-20] However, our recent study shows that L-VOCCs play no role in retinal venular constriction to ET-1 despite the fact that extracellular Ca^{2+} entry is required.[21] An additional mechanism for Ca^{2+} entry is activation of the sodium-calcium exchanger (NCX).[22] The antiporter membrane protein, NCX, can operate in both forward and reverse modes. The forward-mode NCX prevents Ca^{2+} overload in cells and uses the energy that is stored in the electrochemical gradient of sodium by allowing three sodium ions to flow down their gradient across membranes in exchange for export of one Ca^{2+} ion.[23] However, when intracellular levels of sodium ions rise beyond a critical point, especially under pathological conditions, NCX begins importing Ca^{2+} ions in the reverse mode.[23] Interestingly, previous studies demonstrated that ET-1 can exert both inotropic and hypertrophic effects on cardiomyocytes by promoting extracellular Ca^{2+} influx through the sodium-hydrogen exchanger (NHE)-dependent or protein kinase C (PKC)-dependent reverse-mode NCX.[22,24,25] The activation of reverse-mode NCX also has been shown to cause increased Ca^{2+} entry in cultured cardiomyocytes exposed to high levels of glucose.[26]

Mitogen-activated protein kinases (MAPKs) are a highly conserved family of serine/threonine protein kinases, which are believed to play important roles in vasoactive responses.[27-29] There are three isoforms: c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal–regulated kinases (ERK). Previous evidence shows that JNK signaling contributes in part to norepinephrine- and ET-1-induced contraction of rat aorta.[27,28] In addition, thromboxane A_2 (TXA_2) receptor agonist U46619 increased JNK signaling in rat aortic smooth muscle cells.[30] High glucose can increase activity of NHE in canine distal nephron cells through

enhanced p38 MAPK and ERK signaling.[31] The activation of p38 MAPK-has also been shown to contribute retinal endothelial and neural retina dysfunction in diabetic rats.[32,33] However, whether NHE and reverse-mode NCX linked to MAPK and/or PKC signaling contribute to agonist-induced constriction of retinal venules under normal and diabetic/hyperglycemic conditions have not been investigated. Therefore, we addressed these issues by examining the direct response of isolated retinal venules to ET-1 and other vasoconstrictors (U46619, norepinephrine, phenylephrine, and angiotensin II) and investigating whether acute and chronic hyperglycemia influence these vasoconstrictions via NHE-dependent or PKC-dependent reverse-mode NCX activation. In addition, the contribution of MAPK pathways to vasoactive responses of retinal venules to ET-1 under euglycemia and chronic hyperglycemia were evaluated. We also assessed the impact of hyperglycemia on vitreous humor levels of vasoconstrictor substances and mRNA expression of the target receptors for vasoconstriction in retinal venules using biochemical and molecular tools.

Methods

Porcine Diabetes Model

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. Domestic (Yorkshire) male pigs (age range, 6-10 wk; weight range, 9-20 kg) were purchased from Real Farms (San Antonio, TX). Diabetes (i.e., chronic hyperglycemia) was induced by selective ablation of pancreatic β-cells with intravenous injection of streptozocin (STZ, Zanosar®, 200 mg/kg in saline) via an ear vein (48 pigs), as described in detail in our previous study.[34,35] The control euglycemic group was intravenously injected with saline (59 pigs). Fasting blood glucose levels were obtained every other day using a Bayer Contour glucometer (Bayer Corporation, Pittsburgh, PA). Pigs were treated with insulin (Humulin® 70/30, 2-8 units; Lilly, Indianapolis, IN) if blood glucose was

sustained above 600 mg/dl to keep the level between 250 and 600 mg/dl. After 2 weeks, pigs were sedated with Telazol (4-8 mg/kg, intramuscularly), anesthetized with 2-5% isoflurane, and intubated. The eyes were harvested as described previously.[36]

Isolation and Cannulation of Retinal Venules

Single second-order retinal venules (1 to 1.5 mm in length without side branches) were dissected from surrounding neural/connective tissues and then cannulated on each end with glass micropipettes containing physiological saline solution (PSS) with 5 mM D-glucose and 1% albumin.[21] In another cohort, 25 mM D-glucose in the PSS was used. Vessels were pressurized to 5 cmH_2O intraluminal pressure without flow by two independent pressure reservoirs and their inner diameter was recorded using videomicroscopic techniques throughout the experiments, as described previously.[21]

Study of Vasomotor Function

Cannulated, pressurized retinal venules were bathed in PSS-albumin at 36-37°C to allow development of basal tone (stable within 60-90 minutes). To evaluate the effect of chronic hyperglycemia on vasomotor function, diameter changes to cumulative administration of ET-1 (1 pM to 10 nM; Bachem, Torrance, CA), TXA_2 receptor agonist U46619 (0.1 nM to 1 μM; Cayman Chemical, Ann Arbor, MI), angiotensin II (0.1 nM to 10 μM; MilliporeSigma, St. Louis, MO), non-selective α-adrenergic receptor (AR) agonist norepinephrine (1 nM to 10 μM; Cayman Chemical), or α_1-AR agonist phenylephrine (1 nM to 10 μM; Cayman Chemical) were recorded and compared in vessels isolated from diabetic and control pigs. Retinal venules were exposed to each concentration of agonist for 10 minutes until a stable diameter was attained. The contribution of ET_A receptors (ET_ARs) in vasoconstriction to ET-1 was evaluated after incubation with respective antagonist BQ123 (1 μM).[21] The contribution of TXA_2 receptors (TXA_2Rs) in vasoconstriction to U46619 was examined in the presence of respective antagonist SQ29548 (10 μM).[37] The relative roles of α_1-ARs and α_2-ARs in vasoconstriction to norepinephrine or

phenylephrine were evaluated in the presence of selective α_1-AR antagonist prazosin hydrochloride (10 μM)[38] or α_2-AR antagonist atipamezole (10 μM).[39] The contributions of TRPC3 and CRAC channels in vasoconstriction to ET-1 were evaluated after incubation with TRPC3 inhibitor Pyr3 (10 μM)[40] and CRAC channel inhibitor BTP2 (1 μM; EMD Millipore, Billerica, MA, USA),[41] respectively. The role of reverse-mode NCX in agonist-induced constrictions was examined in the presence of its selective inhibitors KB-R7943 (10 μM)[42] or SEA0400 (10 μM).[43] The contributions of PKC and NHE in vasoconstriction to ET-1 were evaluated after incubation with PKC antagonist Gö 6983 (10 μM; EMD Millipore, Billerica, MA, USA)[44] and NHE inhibitor cariporide (1 μM).[45] Vasomotor response to PKC activator phorbol-12,13-dibutyrate (PDBu; 0.1 μM to 10 μM) was also evaluated. In some vessels, the role of L-VOCCs in the vasoconstriction to ET-1 was evaluated in the presence of L-type Ca^{2+} channel antagonist nifedipine (1 μM).[21] To assess the involvement of MAPKs in the chronic hyperglycemia-induced effect, the vasomotor responses of retinal venules to ET-1 were examined following treatment with p38 kinase inhibitor SB203580 (0.1 μM; EMD Millipore, Billerica, MA, USA),[46] ERK inhibitor PD98059 (10 μM; EMD Millipore, Billerica, MA, USA),[47] or JNK inhibitor SP600125 (10 μM; EMD Millipore, Billerica, MA, USA).[27] All vessels were pretreated with antagonists or inhibitors extraluminally for at least 30 minutes. In order to assess the activity of reverse-mode NCX in retinal vessels, resting diameter of venules was measured during exposure to low-Na^+ (1.4 mM) PSS for 20 minutes and then in the presence of normal-Na^+ (146.2 mM) PSS for 20 minutes. To control for changes in osmolarity, N-methyl D-glucamine was substituted for NaCl in low-Na^+ PSS.

The acute effect of high glucose on vasoconstrictions to ET-1, U46619, and norepinephrine was evaluated after intraluminal incubation of vessels from nondiabetic pigs (49 pigs) with 25 mM D-glucose for 2 hours,[34] and the results were compared with the treatment with normal 5 mM D-glucose as control. The contribution of NCX in agonist-induced vasoconstrictions was evaluated after co-incubation of 5 mM or 25 mM glucose-PSS with KB-R7943 (10 μM) for 2 hours. In some vessels, vasoconstriction to ET-1 (0.1 nM) was assessed in

the presence of 20 mM L-glucose (MilliporeSigma) plus 5 mM D-glucose to determine the stereospecific impact of glucose on these responses. Osmolarity in all the 25 mM glucose solutions was balanced to 290 mOsm by reducing the NaCl concentration to avoid a potential hyperosmolarity effect.

Chemicals

Drugs were obtained from Tocris Cookson (Ellisville, MO) except as specifically stated otherwise. ET-1, angiotensin II and PDBu were dissolved in water; BQ123 and nifedipine were dissolved in ethanol; and phenylephrine, norepinephrine, U46619, SQ29548, prazosin hydrochloride, atipamezole, Pyr3, BTP2, Gö 6983 KB-R7943, SEA0400, cariporide, SB203580, PD98059, and SP600125 were dissolved in dimethyl sulfoxide. Subsequent concentrations of these drugs were diluted in PSS. The final concentration of dimethyl sulfoxide or ethanol in the vessel bath did not exceed 0.1% by volume. Vehicle control studies indicated that this final concentration for these two solvents had no effect on vessel viability, vasoconstrictor responses, or maintenance of basal tone (data not shown).

mRNA Isolation and Real-Time Polymerase Chain Reaction (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 previously.[21,48] To perform real-time PCR experiments, specific primer sets presented in the Table were used. The same amount of total RNA for each sample was used to synthesize cDNA followed by real-time PCR with the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Life Technologies Corporation, Carlsbad, CA), as described previously.[21]

Measurements of ET-1 and TXB_2 in the Vitreous Humor

Vitreous humor was obtained after harvesting the eyes from control and diabetic pigs. Undiluted vitreous samples were centrifuged at 12,000 rpm for 10 minutes at 4°C, and the supernatants were then stored at -80°C for subsequent analysis within 3 months. Levels of ET-1 protein and TXB_2, a stable TXA_2 lipid metabolite, in the vitreous were measured using an ET-1

ELISA kit (ADI-900-020A; Enzo Life Sciences Inc., Farmingdale, NY) and a TXB_2 ELISA kit (ADI-900-002; Enzo Life Sciences Inc.), respectively, according to the manufacturer's instructions.

Data Analysis

At the end of each functional experiment, the vessel was relaxed with 0.1 mM sodium nitroprusside in EDTA (1 mM)-Ca^{2+}-free PSS to obtain its maximum diameter at 5 cmH_2O intraluminal pressure.[21] Diameter changes in response to agonists were normalized to the resting diameter and expressed as percentage changes in diameter.[21] Data are reported as mean ± SEM, and *n* represents number of vessels (1 per pig for each agonist and treatment groups for functional studies) or pigs (for molecular/biochemical studies). Student's t-test or repeated measures two-way analysis of variance 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). $P < 0.05$ was considered significant.

Table 2. Primer sets used for real-time PCR

	PCR Primers (upper)	PCR Primers (lower)
ET_A Receptor	5'-AAGCAGGACAGCCCATTAAG-3'	5'-CTGCTAGCTGAAGTACTCGGAAC-3'
TXA_2 Receptor	5'-GGGCCACATGGTGCATAC-3'	5'-CAGAGAGGGGCTGAGAAACTC-3'
α_1-Adrenergic Receptor	5'-ATGGGTCCTGGCGACTAAG-3'	5'-TTCCAAGCCTTGCTTTTGTC-3'
α_{2A}-Adrenergic Receptor	5'-CAACGTGCTTGTCATCATT-3'	5'-TGGGTGATGGACCAGTAA-3'
NCX1 (SLC8A1)	5'-CAGGGCACAGGTCTCCTTT-3'	5'-TGCAGAGTGGCCAAACCT-3'
NCX2 (SLC8A2)	5'-ACAGAAGCACCCGGACAA-3'	5'-GGTGCAGCAACGCATAGTAG-3'
NCX3 (SLC8A3)	5'-TGTCGGTCATCAGGACAATG-3'	5'-CCCCTTCTTGAGGACCTCTC-3'

ET_A Receptor, endothelin A receptor; TXA_2 Receptor, thromboxane A_2 receptor; NCX1, 2, 3, sodium-calcium exchanger 1, 2, 3, respectively.

Results

Agonist-induced Constriction of Retinal Venules from Euglycemic Pigs

Porcine retinal venules (total 100 vessels in Fig. 8) were isolated and pressurized at 5 cmH_2O with an average maximum diameter of 130 $\pm$ 1 μm. All vessels in this study developed similar levels of stable basal tone by constricting to about 93% of maximum diameter. Administration of ET-1, U46619, or norepinephrine caused concentration-dependent constriction of retinal venules from nondiabetic pigs (Fig. 8). Threshold concentrations of ET-1 and U46619 for venular constrictions were about 1 pM and 0.1 nM, respectively. Maximum vasoconstrictions were about 50% of resting diameters at the highest concentrations of 10 nM ET-1 and 1 μM U46619 (Fig. 8). The threshold concentration of norepinephrine for venular constriction was about 0.1 μM, and the maximum constriction was about 75% of resting diameter at the highest concentration of 10 μM (Fig. 8). However, administration of angiotensin II or phenylephrine constricted to about only 90% of resting diameter at the highest concentration of 10 μM (Fig. 8).

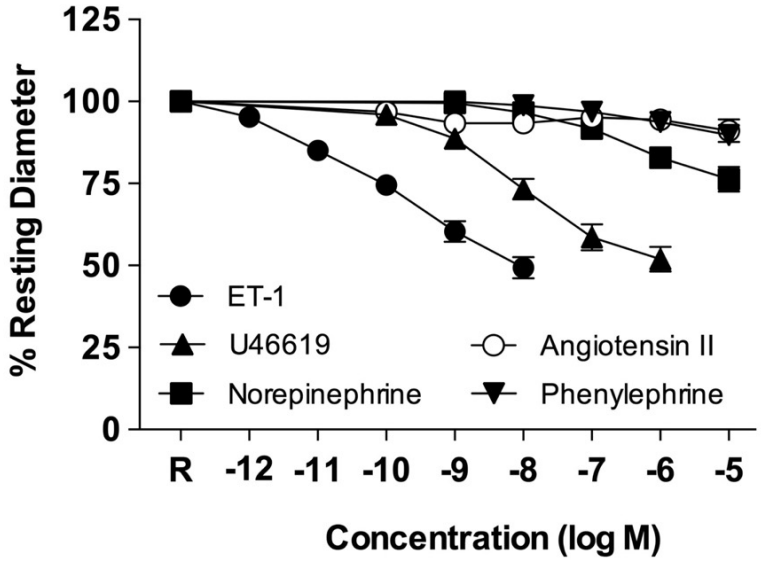


Figure 8. Vasomotor response of isolated and pressurized porcine retinal venules to vasoconstrictors. Venular diameters were recorded before (R: resting diameter) and after administration of vasoconstrictor agonists. ET-1 (n = 36), U46619 (n = 33), and norepinephrine (n = 13) significantly constricted retinal venules in a concentration-dependent manner. Angiotensin II (n = 5) and phenylephrine (n = 13) caused only slight constriction in retinal venules at the highest concentration used, 10 μM.

Effect of Chronic Hyperglycemia on Retinal Venular Constriction

Retinal venules isolated from the 2-week diabetic pigs (chronic hyperglycemia: 449 ± 19 mg/dl) and age-matched control pigs (euglycemia: 97 ± 3 mg/dl) developed a comparable level of basal tone by constricting to about 93% of maximum diameter. However, retinal venules from diabetic pigs exhibited enhanced constrictions to ET-1, U46619, and norepinephrine (Fig. 9A). Maximum constrictions to the highest concentrations of these three agonists was about 25-30% greater in diabetic than control venules. In contrast, angiotensin II and phenylephrine did not alter basal tone of retinal venules except for modest constriction (about 10%) observed at 10 μM, and there was no enhanced vasoconstriction with diabetes (data not shown).

Effect of Acute Hyperglycemia on Retinal Venular Constriction

Retinal venules isolated from nondiabetic pigs developed a comparable level of basal tone after intraluminal exposure to normal (5 mM) or high (25 mM) level of D-glucose for 2 hours. Vasoconstrictions to ET-1, U46619, and norepinephrine (Fig. 9B) were significantly increased by high D-glucose with increased maximum constriction about 15-25% compared to the vessels exposed to normal D-glucose. The high L-glucose solution (20 mM L-glucose plus 5 mM D-glucose) did not enhance constriction of retinal venules to 0.1 nM ET-1 (A. 1).

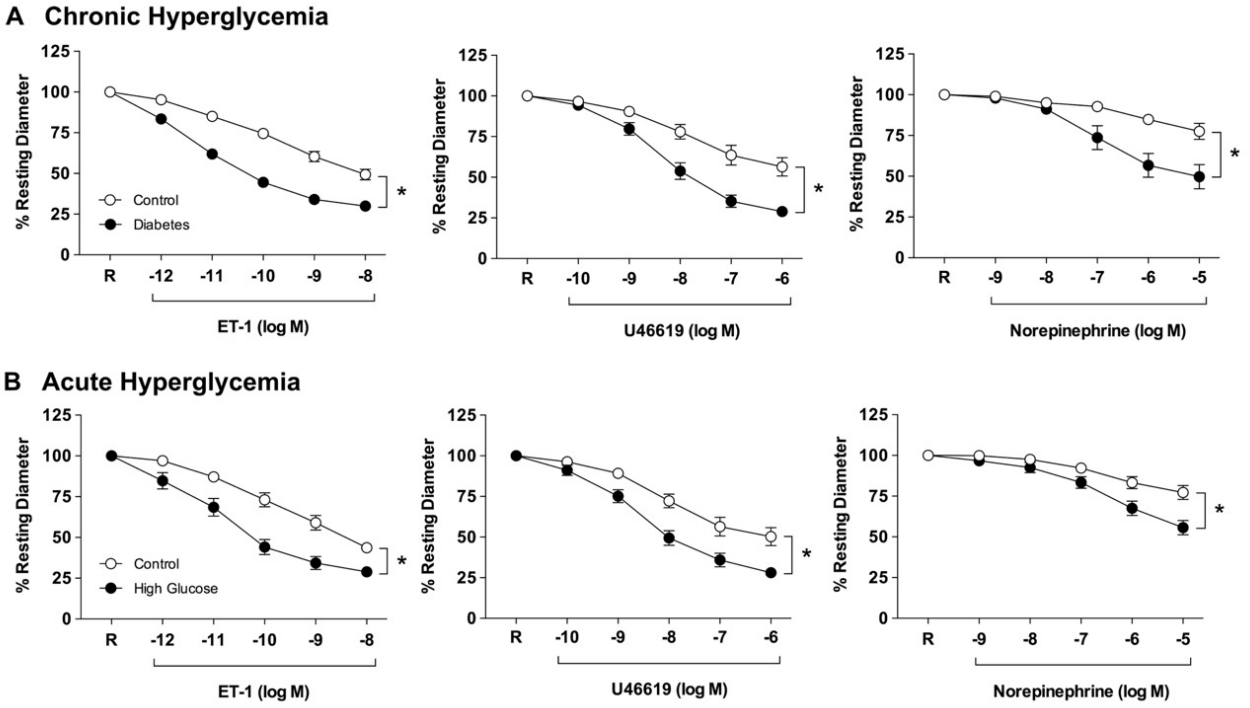


Figure 9. Effect of chronic and acute hyperglycemia on vasomotor reactivity of isolated retinal venules to vasoconstrictors. Venular diameters were recorded before (R: resting diameter) and after administration of vasoconstrictor agonists. (A) Chronic hyperglycemia for 2 weeks in diabetic pigs significantly enhanced ET-1- (Control, n = 36; Diabetes, n = 34), U46619- (Control, n = 15; Diabetes, n = 14), and norepinephrine-induced (Control, n = 6; Diabetes, n = 7) constrictions of retinal venules. *P < 0.05 vs. Control. (B) For retinal venules isolated from euglycemic pigs, concentration-dependent constrictions to ET-1 (Control, n = 14; High Glucose, n = 12), U46619 (Control, n = 15; High Glucose, n = 15), and norepinephrine (Control, n = 11; High Glucose, n = 10) were significantly greater after 2-hour intraluminal exposure to high glucose (25 mM) than to control normal glucose (5 mM). *P < 0.05 vs. Control.

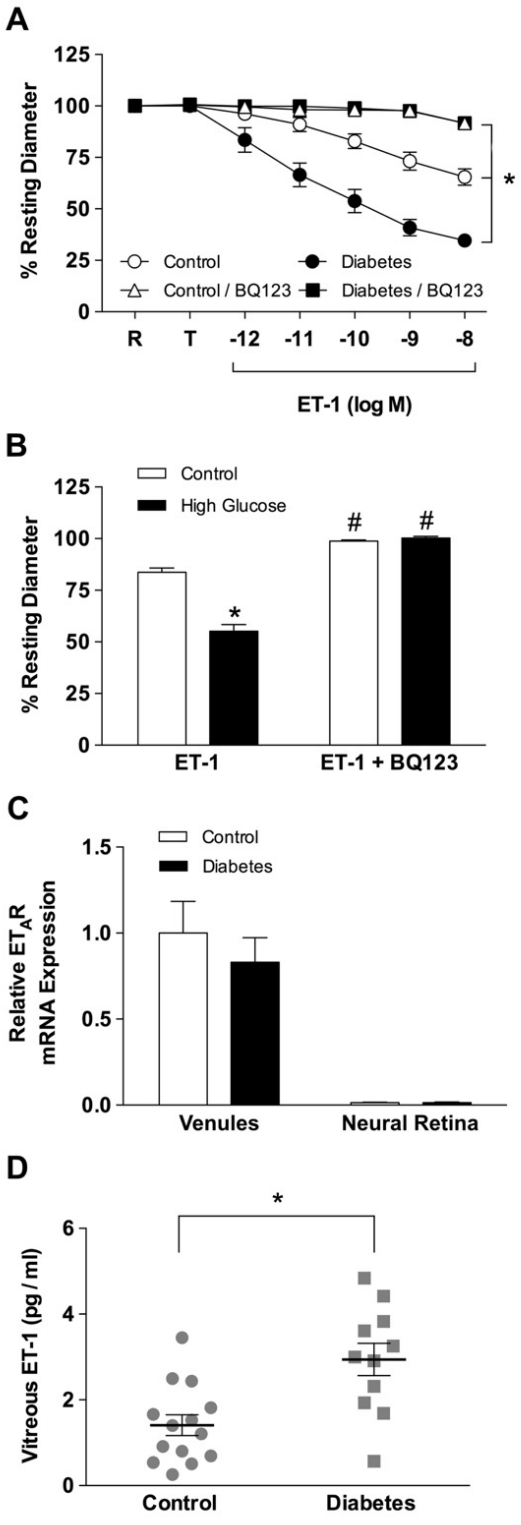


Figure 10. Effects of hyperglycemia on ET_AR-mediated constriction to ET-1, ET_AR expression, and vitreous levels of ET-1. (A) Venular diameters were recorded before (R: resting diameter)

and after 30-minute treatment (T) with ET_AR antagonist BQ123 prior to administration of ET-1. In the absence of BQ123, chronic hyperglycemia for 2 weeks in diabetic pigs significantly enhanced ET-1-induced constriction of retinal venules (Control, n = 7; Diabetes, n = 8). BQ123 (1 μM) nearly abolished both of these responses. *P < 0.05 vs. Control. (B) Constriction of retinal venules from euglycemic pigs to ET-1 (0.1 nM) was significantly greater after 2-hour intraluminal exposure to high glucose (25 mM; n = 8) than to control normal glucose (5 mM; n = 7). BQ123 (1 μM) abolished ET-1-induced constriction of retinal venules under control and high glucose exposures. *P < 0.05 vs. Control; #P < 0.05 vs. ET-1 only. Equal amounts of total RNA isolated from porcine retinal venules and neural retina tissue after 2 weeks of euglycemia (Control) and chronic hyperglycemia (Diabetes) were reverse transcribed and then analyzed by real-time PCR for detection of ET_AR and GAPDH mRNAs. (C) After normalization with GAPDH, the ET_AR was not differentially expressed (presented as relative mRNA expression) between Control (n = 10) and Diabetes (n = 12) in retinal venules. The ET_AR expression in neural tissue was not different between Control (n = 14) and Diabetes (n = 13) but was less than in venules. n = number of pigs studied. *P < 0.05 vs. Venules. (D) Chronic hyperglycemia for 2 weeks in diabetic pigs significantly increased expression level of ET-1 in the vitreous humor (Control, n = 14; Diabetes, n = 11). *P < 0.05 vs. Control.

Venular Receptors and Vitreous Humor Level of ET-1 and TXB_2

The ET-1-induced constriction of retinal venules exposed to euglycemia or hyperglycemia (chronic and acute) was abolished in the presence of ET_AR antagonist BQ123 (Figs. 10A and 10B). There was a greater amount of ET_AR mRNA expressed in retinal venules than in neural retina under control and diabetic conditions, but the expression in both tissues was not influenced by chronic hyperglycemia (Fig. 10C). The ET-1 protein level was greater in the vitreous isolated from diabetic (2.9 ± 0.4 pg/ml ≈ 1.2 ± 0.2 pM) compared to control (1.4 ± 0.2 pg/ml ≈ 0.6 ± 0.1 pM) pigs (Fig. 10D).

Constriction of retinal venules from control pigs to U46619 was completely blocked in the presence of TXA_2R antagonist SQ29548 (Fig. 11A). The TXA_2R mRNA expression in retinal venules was greater than that in neural retina, but in both tissues it was not altered by diabetes (Fig. 11B). Concentrations of TXB_2, the stable TXA_2 metabolite, were comparable in the vitreous isolated from control (24 ± 4 pg/ml ≈ 65 ± 9 pM) and diabetic (17 ± 3 pg/ml ≈ 47 ± 9 pM) pigs (Fig. 11C).

The α_2-AR antagonist atipamezole abolished norepinephrine-induced constriction of retinal venules from control pigs, whereas α_1-AR antagonist prazosin did not alter this vasoconstriction (Fig. 12A). Expression of α_1-AR mRNA in retinal venules and neural retina tissue was unaffected by diabetes, but it was less in neural retina than in venules only after diabetes (Fig. 12B). The α_{2A}-AR mRNA in venules was greater than that in neural retina and in both tissues it was unaffected by diabetes (Fig. 12C).

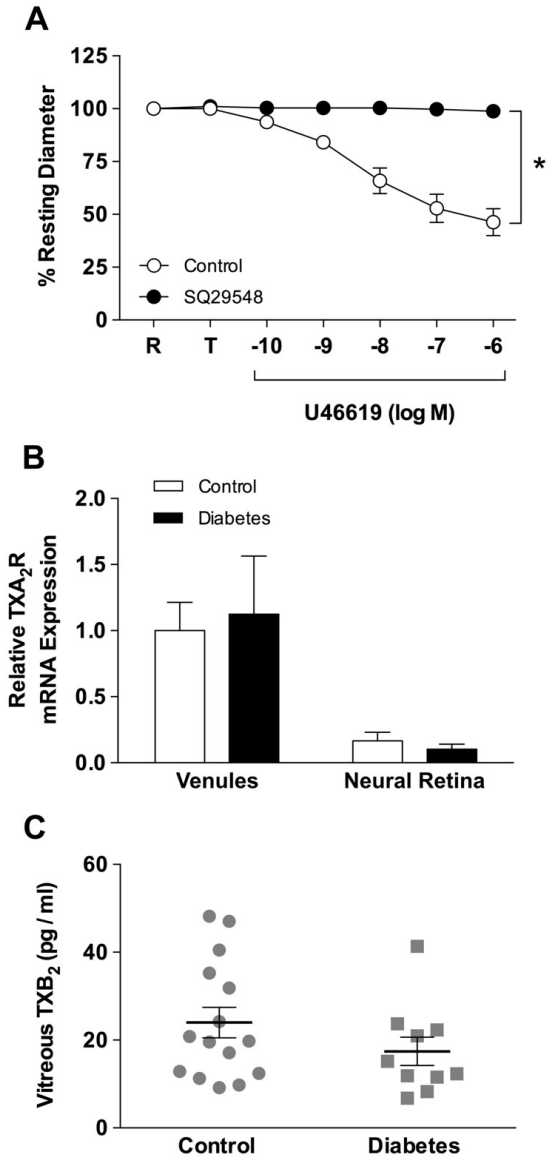


Figure 11. Role of TXA_2R activation in U46619-induced constriction of retinal venules and effects of chronic hyperglycemia on TXA_2R expression and vitreous levels of TXB_2. (A) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with TXA_2R antagonist SQ29548 prior to administration of U46619. In the absence of SQ29548, retinal venules from euglycemic pigs constricted to U46619 in a concentration dependent manner (Control; n = 7). SQ29548 (10 μM) abolished venular constriction to U46619 (n = 7). *P < 0.05 vs. Control. (B) Equal amounts of total RNA isolated from porcine retinal venules and

neural retina tissue after 2 weeks of euglycemia (Control) and chronic hyperglycemia (Diabetes) were reverse transcribed and then analyzed by real-time PCR for detection of TXA_2R and GAPDH mRNAs. The TXA_2R transcript was normalized to GAPDH expression and presented as relative mRNA expression. The TXA_2R expression was not different between Control (n = 9) and Diabetes (n = 7) in retinal venules or in neural retina tissue (Control, n = 10; Diabetes, n = 10), but it was less in neural retina than in venules. n = number of pigs studied. *P < 0.05 vs. Venules. (C) A similar expression level of TXB_2 (stable TXA_2 metabolite) was detected in the vitreous humor after 2 weeks of euglycemia (Control, n = 15) and chronic hyperglycemia (Diabetes, n = 10). n = number of pigs studied.

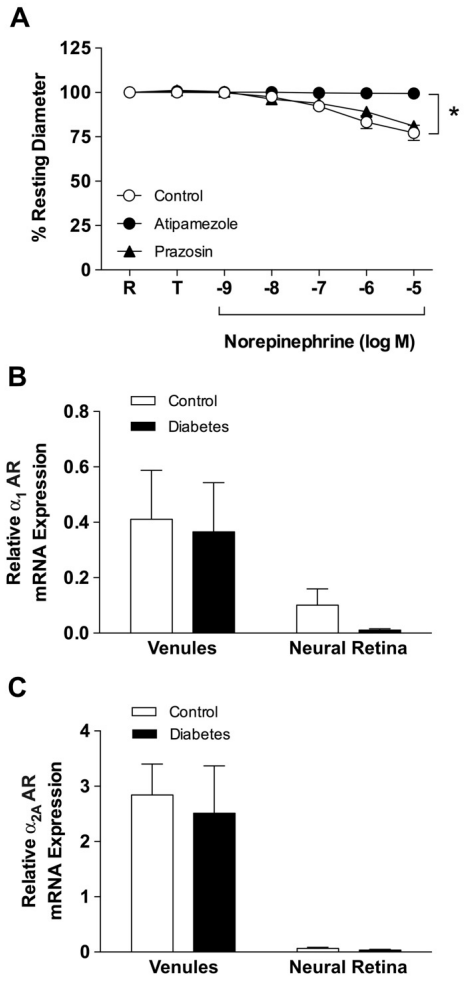


Figure 12. Role of α-AR activation in norepinephrine-induced constriction of retinal venules and effects of chronic hyperglycemia on α-AR expression. (A) Venular diameters were recorded

before (R: resting diameter) and after 30-minute treatment (T) with an α-AR antagonist prior to administration of norepinephrine. In the absence of receptor antagonist, retinal venules from euglycemic pigs constricted to norepinephrine in a concentration dependent manner (Control; n = 11). α_2-AR antagonist atipamezole (10 μM; n = 5), but not α_1-AR antagonist prazosin (10 μM; n = 6), inhibited venular constriction to norepinephrine. *P < 0.05 vs. Control. Equal amounts of total RNA isolated from porcine retinal venules and neural retina tissue after 2 weeks of euglycemia (Control) and chronic hyperglycemia (Diabetes) were reverse transcribed and then analyzed by real-time PCR for detection of α-ARs and GAPDH mRNAs. The α-AR transcript was normalized to GAPDH expression and presented as relative mRNA expression. (B) The α_1-AR mRNA expression was not different between Control (n = 9) and Diabetes (n = 8) in retinal venules or in neural retina tissue (Control, n = 10; Diabetes, n = 10), but it was less in neural retina than in venules after diabetes. *P < 0.05 vs. Venules. (C) The α_{2A}-AR mRNA was not differentially expressed between Control (n = 9) and Diabetes (n = 8) in retinal venules or in neural retina tissue (Control, n = 10; Diabetes, n = 9), but it was less in neural retina than in venules under both conditions. n = number of pigs studied. *P < 0.05 vs. Venules.

Role of Reverse-mode NCX in Hyperglycemia-enhanced Venular Constriction

The low Na^+ PSS induced reverse-mode NCX inhibitor KB-R7943-dependent constriction of retinal venules (A. 2). In the presence of reverse-mode NCX inhibitor KB-R7943, retinal venules exposed to euglycemia or hyperglycemia (chronic and acute) lost about 50% of basal tone (Fig. 13). The KB-R7943 treatment also prevented the enhanced constrictions of retinal venules to ET-1 (Figs. 13A and 13B), U46619 (Figs. 13A and 13B) and norepinephrine (Figs. 13A and 13B) under hyperglycemia (chronic and acute) without altering normal vasoconstrictor responses in euglycemia. An additional reverse-mode NCX inhibitor SEA0400 also reduced basal tone nearly 44% and blocked the enhanced venular constriction to 0.1 nM ET-1 under chronic hyperglycemia, but it did not affect ET-1-induced vasoconstriction under euglycemia (A. 3). The L-VOCC inhibitor nifedipine reduced basal tone of venules exposed to euglycemia or hyperglycemia (acute and chronic) to the same level induced by KB-R7943, but it did not affect the enhanced vasoconstrictions to ET-1 by acute or chronic hyperglycemia (A. 4). The ET-1-induced constriction of retinal venules under euglycemia was also not altered in the presence of TRPC3 inhibitor Pyr3 or CRAC channel inhibitor BTP2 (A. 5). The amount of NCX1-3 isoform mRNA expressions in retinal venules (A. 6A) was greater than that in neural retina tissue (A. 6B) but was unaltered by chronic hyperglycemia.

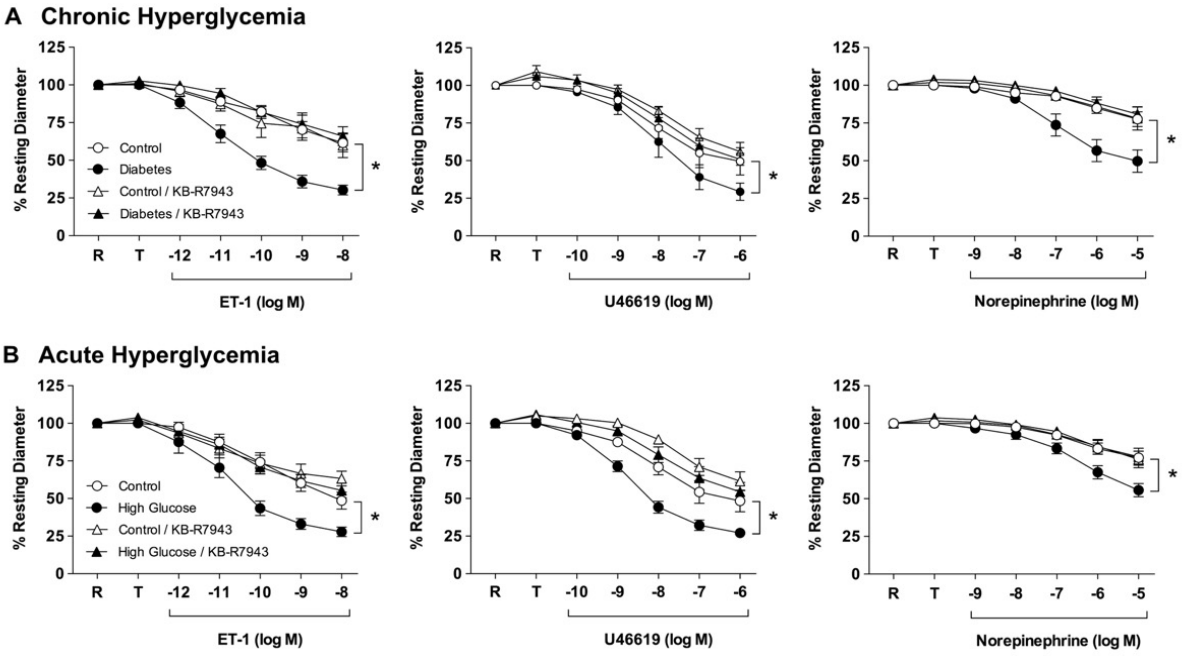


Figure 13. Role of reverse-mode NCX in enhanced agonist-induced constriction of isolated retinal venules during chronic and acute hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 60-minute treatment (T) with reverse-mode inhibitor KB-R7943 prior to administration of vasoconstrictor agonists. (A) In the absence of KB-R7943, chronic hyperglycemia for 2 weeks in diabetic pigs significantly increased ET-1-, U46619- and norepinephrine-induced constrictions of retinal venules (Control, n = 6-10 for each agonist; Diabetes, n = 6-12 for each agonist). KB-R7943 (10 μM) prevented the enhanced constriction of retinal venules to ET-1 (Diabetes / KB-R7943, n = 8), U46619 (Diabetes / KB-R7943, n = 5), and norepinephrine (Diabetes / KB-R7943, n = 5) after chronic hyperglycemia (Diabetes), but it did not alter these agonist-induced vasoconstrictor responses during euglycemia (Control / KB-R7943, n = 5-7 for each agonist). *P < 0.05 vs. Control. (B) In the absence of KB-R7943, 2-hour intraluminal exposure of retinal venules isolated from euglycemic pigs to high glucose (25 mM) significantly increased ET-1-, U46619- and norepinephrine-induced constrictions (Control, n = 6-11 for each agonist; High Glucose, n = 7-10 for each agonist). KB-R7943 (10 μM) prevented the

enhanced constrictions to ET-1 (High Glucose / KB-R7943, n = 6), U46619 (High Glucose / KB-R7943, n = 7) and norepinephrine (High Glucose / KB-R7943, n = 9) after acute hyperglycemia, but it did not alter these agonist-induced vasoconstrictor responses in the presence of control normal (5 mM) glucose (Control / KB-R7943, n= 6-10 for each agonist). *P < 0.05 vs. Control.

Role of PKC and NHE in Hyperglycemia-enhanced Venular Constriction

The PKC inhibitor Gö 6983 did not affect basal tone of venules or their vasoconstrictions to ET-1 after euglycemia or chronic hyperglycemia (Fig. 14A). In addition, exposure of vessels to the PKC activator PDBu did not significantly alter resting diameter under euglycemia or chronic hyperglycemia (Fig. 14B). In the presence of NHE inhibitor cariporide, retinal venules exposed to euglycemia or hyperglycemia (chronic and acute) lost about 50% of basal tone. The cariporide treatment prevented the enhanced constrictions of retinal venules to ET-1 (Fig. 15A) and U46619 (Fig. 15B) under chronic hyperglycemia without altering normal vasoconstrictor responses in euglycemia.

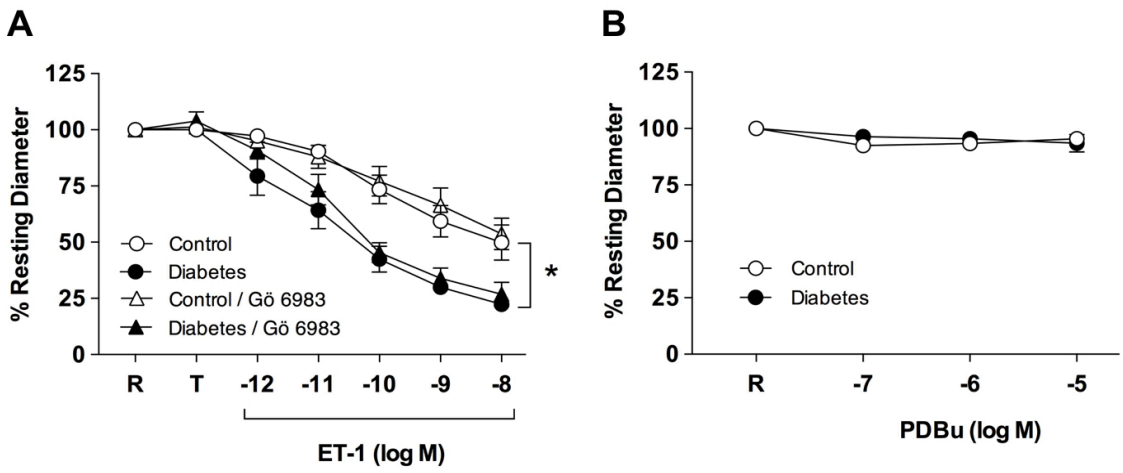


Figure 14. Role of PKC in ET-1-induced constriction of retinal venules after chronic hyperglycemia. (A) Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with PKC inhibitor Gö 6983 (10 μM) prior to administration of ET-1. The retinal venules from euglycemic pigs constricted concentration-dependently to ET-1 (Control, n = 8), and the vasoconstriction to ET-1 was enhanced by chronic hyperglycemia (Diabetes, n = 8). Gö 6983 did not alter the constriction to ET-1 in vessels from euglycemic pigs (Control / Gö 6983, n = 7) or the enhanced ET-1-induced vasoconstriction in chronic hyperglycemic pigs (Diabetes / Gö 6983, n = 5). *P < 0.05 vs. Control. (B) The response of retinal venules to PKC agonist PDBu was examined during euglycemia (Control, n = 7) and chronic hyperglycemia (Diabetes, n = 6).

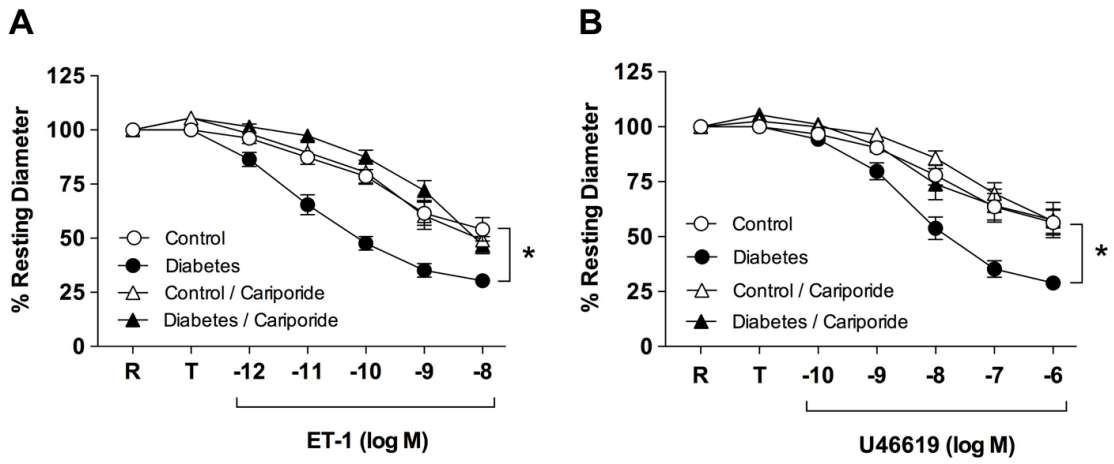


Figure 15. Role of NHE in enhanced ET-1-induced and U46619-induced constriction of isolated retinal venules during chronic hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 60-minute treatment (T) with NHE inhibitor cariporide prior to administration of vasoconstrictor agonists. (A) In the absence of cariporide, chronic hyperglycemia for 2 weeks in diabetic pigs significantly increased ET-1-induced constrictions of retinal venules (Control, n = 14; Diabetes, n = 17). Cariporide (1 μM) prevented the enhanced constriction of retinal venules to ET-1 (Diabetes / Cariporide, n = 8) after chronic hyperglycemia (Diabetes), but it did not alter ET-1-induced vasoconstrictor response during euglycemia (Control / Cariporide, n = 9). *P < 0.05 vs. Control. (B) In the absence of cariporide, chronic hyperglycemia significantly increased U46619-induced constrictions of retinal venules (Control, n = 15; Diabetes, n = 14). Cariporide (1 μM) prevented the enhanced constriction of retinal venules to U46619 (Diabetes / Cariporide, n = 7) after chronic hyperglycemia (Diabetes), but it did not alter U46619-induced vasoconstrictor response during euglycemia (Control / Cariporide, n = 7). *P < 0.05 vs. Control.

Role of MAPKs in Hyperglycemia-enhanced Venular Constriction

The p38 MAPK inhibitor SB203580 did not influence basal tone (Fig. 16). However, it prevented the enhanced constrictions of retinal venules to ET-1 under chronic hyperglycemia without altering normal vasoconstrictor responses in euglycemia (Fig. 16). The ERK inhibitor PD98059 did not affect basal tone of venules or their vasoconstrictions to ET-1 after euglycemia or chronic hyperglycemia (Fig. 17). In the presence of JNK inhibitor SP600125, retinal venules lost about 70% of basal tone, and ET-1-induced vasoconstriction was attenuated during euglycemia and chronic hyperglycemia (Fig. 18). However, the ET-1-induced constriction after SP600125 treatment remained greater for vessels exposed to chronic hyperglycemia than to euglycemia (Fig. 18). In the presence of cariporide under chronic hyperglycemia, the additional treatment of KB-R7943 or SB203580 did not further alter ET-1-induced vasoconstriction (Fig. 19).

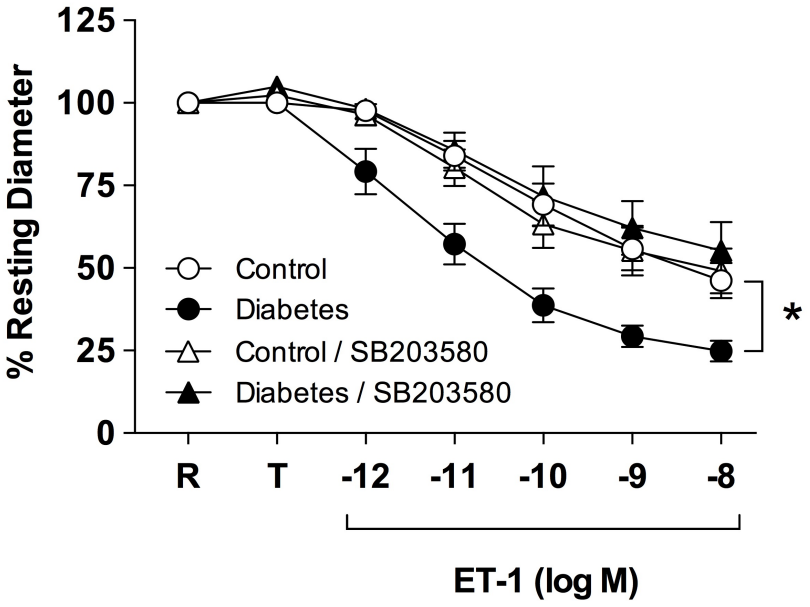


Figure 16. Role of p38 MAPK in enhanced ET-1-induced constriction of isolated retinal venules during chronic hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with p38 MAPK inhibitor SB203580 (0.1 μM) prior to administration of ET-1. In the absence of SB203580, chronic hyperglycemia for 2 weeks in diabetic pigs significantly increased ET-1-induced constrictions of retinal venules (Control, n = 7; Diabetes, n = 7). SB203580 prevented the enhanced constriction of retinal venules to ET-1 (Diabetes / SB203580, n = 6) after chronic hyperglycemia (Diabetes), but it did not alter ET-1-induced vasoconstrictor response during euglycemia (Control / SB203580, n = 8). *P < 0.05 vs. Control.

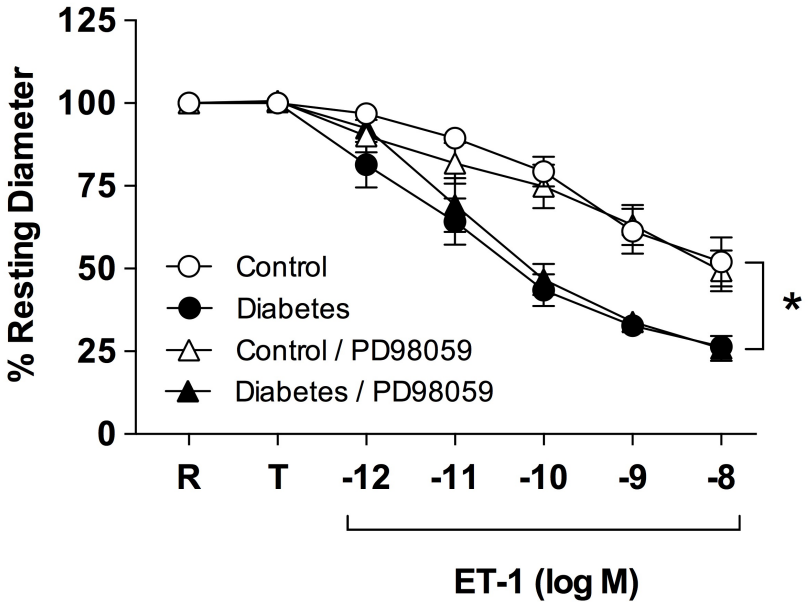


Figure 17. Role of ERK in enhanced ET-1-induced constriction of isolated retinal venules after chronic hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with ERK inhibitor PD98059 (10 μM) prior to administration of ET-1. The retinal venules from euglycemic pigs constricted concentration-dependently to ET-1 (Control, n = 8), and the vasoconstriction to ET-1 was enhanced by chronic hyperglycemia (Diabetes, n = 10). PD98059 did not alter the constriction to ET-1 in vessels under euglycemia (Control / PD98059, n = 10) or the enhanced ET-1-induced vasoconstriction after chronic hyperglycemia (Diabetes / PD98059, n = 6). *P < 0.05 vs. Control.

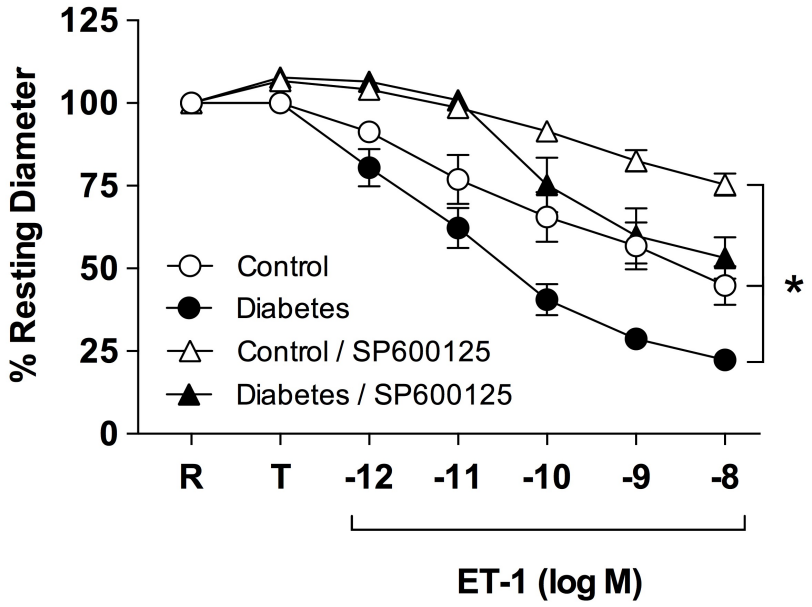


Figure 18. Role of JNK in enhanced ET-1-induced constriction of isolated retinal venules during chronic hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with JNK inhibitor SP600125 (10 μM) prior to administration of ET-1. In the absence of SP600125, chronic hyperglycemia for 2 weeks in diabetic pigs significantly increased ET-1-induced constrictions of retinal venules (Control, n = 8; Diabetes, n = 12). SP600125 attenuated basal tone and constriction of retinal venules to ET-1 after euglycemia (Control / SP600125, n = 8) or chronic hyperglycemia (Diabetes / SP600125, n = 6). In the presence of SP600125, chronic hyperglycemia still significantly increased ET-1-induced constrictions of retinal venules. *P < 0.05 vs. Control.

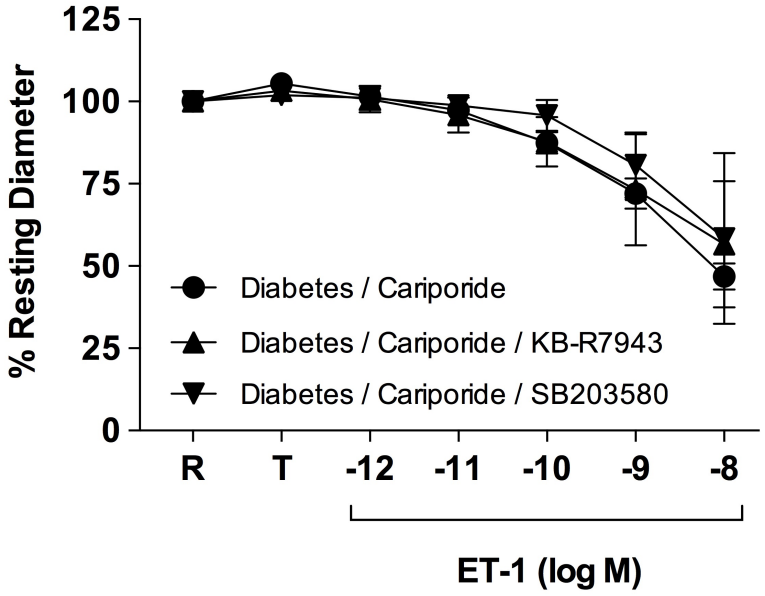


Figure 19. Influence of combined blockade of NHE and NCX or p38 MAPK on ET-1-induced constriction of isolated retinal venules during chronic hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 60-minute treatment (T) with NHE inhibitor cariporide (1 μM; n = 8) alone, cariporide and KB-R7943 (10 μM), or cariporide and SB203580 (0.1 μM) prior to administration of ET-1. KB-7943 (n = 5) or SB203580 (n = 3) did not further alter ET-1-induced vasoconstriction with cariporide under chronic hyperglycemia.

Conclusions

In the microcirculation, venular pressure plays an important role in governing fluid exchange across capillaries by regulating postcapillary resistance.[3,4] Disruption of this homeostatic process can contribute to tissue edema during the development of diseases, including diabetic retinopathy. Recent evidence of high retinal venous pressure in patients with diabetic retinopathy[7] suggests that elevation of vascular tone (i.e., vasoconstriction) in the venous microcirculation may promote retinal pathology. However, there is little information regarding vasomotor reactivity of retinal venules under normal and hyperglycemic states, especially the ability to constrict to endogenous substances. The main findings of the present study are that porcine retinal venules constrict in response to ET-1, U46619, and norepinephrine but not to angiotensin II and phenylephrine; vasoconstrictions to ET-1, U46619, and norepinephrine are augmented during acute and chronic hyperglycemia; and activation of the reverse-mode NCX and NHE appear to mediate these enhanced vasoconstrictor responses.

Direct evidence for vasoconstrictor function of retinal veins is limited to two recent studies showing that ET-1 elicits constriction of these vessels in pigs.[21,49] To further our understanding of vasomotor regulation of retinal venules, we evaluated herein the responsiveness of these microvessels to ET-1 and other endogenous vasoconstrictors (Fig. 8). As shown previously, ET-1 elicited potent constriction of isolated retinal venules with threshold concentration of about 1 pM.[21] We extended these findings to show for the first time the ability of retinal venules to constrict in response to activation of $TBXA_2R$ (U46619) and α-AR (norepinephrine) agonists. We also documented for the first time that hyperglycemia (both acute and chronic) enhances venular responses to these vasoconstrictors (Fig. 9). In contrast to other peripheral tissues,[50,51] retinal venules appear to lack sufficient signaling for vasoconstriction via angiotensin II receptors and α_1-ARs, because their specific agonists (angiotensin II and phenylephrine) evoked nominal changes in venular tone.

We have recently shown that ET-1 elicits constriction of retinal venules by activating ET_ARs.[21] The present study corroborates and extends this finding by demonstrating that pharmacological blockade of ET_ARs prevents constriction of retinal venules to ET-1 during hyperglycemia (Fig. 10A). In addition, both acute and chronic hyperglycemia enhanced ET-1-induced constriction of retinal venules in a similar manner without altering the ET_AR mRNA expression level (Fig. 10A-10C). It seems that hyperglycemia, while increasing vascular reactivity to ET-1, does not alter ET_AR expression at least at the transcription level (Fig. 10C). Furthermore, the ability of acute exposure to high D-glucose but not high L-glucose to enhance ET-1-induced vasoconstriction indicates that the venular reactivity was specifically influenced by the functional hyperglycemia (A. 1). The combination of increased vasomotor activity of retinal venules to ET-1 and elevated ET-1 concentration in the vitreous of diabetic pigs in our study (Fig. 10D) suggests the potential contribution of this vasoactive peptide to the retinal venous pathology. Enhanced ET-1-induced constriction of retinal venules during diabetes could potentially increase retinal venous pressure and promote both edema and reduction in retinal perfusion pressure.[5] This contention is supported by previous reports in diabetic patients of increased levels of ET-1 in the vitreous,[12] elevated retinal venous pressure,[7] and reduced retinal blood flow with macular edema.[9] Furthermore, diminished blood flow in retinal veins of diabetic mice can be improved by treatment with an ET_AR antagonist.[52] Collectively, these previous observations along with our current findings suggest that enhanced retinal venular constriction to ET-1, via ET_AR, may contribute to development of diabetic retinopathy.

TXA_2 is a lipid product of arachidonic metabolism synthesized from thromboxane synthase by activated platelets and vascular cells.[53,54] After cellular release, TXA_2 can activate vascular smooth muscle TXA_2Rs leading to vasoconstriction. Because TXA_2 is quickly and spontaneously converted to the stable inactive TXB_2 in aqueous solution, experimental studies commonly evaluate TXA_2 function with the TXA_2 analog U46619. This stable TXA_2R agonist causes constriction of retinal arterioles,[55] but its vasomotor influence on retinal venules has not

yet been determined. In the current study, U46619 elicited constriction of retinal venules with threshold concentration of about 0.1 nM under euglycemic conditions. Pharmacological blockade of TXA_2Rs abolished U46619-induced vasoconstriction, indicating a functional vasomotor role for these receptors in porcine retinal venules (Fig. 11A). Moreover, acute and chronic hyperglycemia had no effect on the threshold concentration of U46619 to constrict retinal venules, but they both enhanced the maximum vasoconstriction (Fig. 9). Chronic hyperglycemia caused this augmented response without altering transcription of TXA_2R mRNA (Fig. 11B). To our knowledge, this is the first evidence of TXA_2R expression in the retinal microcirculation, and it extends previous reports detecting this receptor in human[56] and rodent[57] neural retina tissue. It also appears that local production of TXA_2 (estimated from the levels of stable TXA_2 metabolite TXB_2) within the vitreous is not altered within 2 weeks of diabetes (Fig. 11C). However, we are unable to rule out the potential contribution from the vascular or serum level of TXA_2 with hyperglycemia in view that elevated production of TXB_2 from cultured bovine retinal endothelial cells and rabbit aorta after acute exposure to high glucose[53] and chronic hyperglycemia,[58] respectively, has been reported. Experimental type 1 diabetes in rats also significantly increases the platelet-generated TXB_2 levels in the serum within 2 weeks of hyperglycemia.[13] Moreover, intravenous administration of a TXA_2 synthase inhibitor or a TXA_2R antagonist can reverse the diminished retinal blood flow after 3-4 weeks of type 1 diabetes in rodents.[59,60] Taken together, our present findings and previous evidence from other investigators support a potential role for TXA_2R activation in retinal vascular disturbances during diabetes.

Although the retinal microcirculation apparently lacks autonomic innervation,[61] the existence of α-ARs in retinal microvessels has been suggested based on pharmacological studies. The α_1-AR agonist phenylephrine has been shown to constrict retinal arterioles in mice, cows, and rabbits.[38,62,63] However, our findings showed that phenylephrine only elicited minor constriction of porcine retinal venules (Fig. 8), indicating that these vessels contain few or no

functional α_1-ARs. Brimonidine, an α_2-adrenergic receptor agonist, evokes constriction of isolated second-order arterioles from pigs.[48] Comparably, we found constriction of second-order retinal venules to non-selective α-AR agonist norepinephrine, in a manner sensitive to the blockade of α_2-ARs but not of α_1-ARs (Fig. 12A). These results indicate that α_2-ARs are responsible for constrictor action of norepinephrine in retinal venules. At the molecular level, we identified for the first time the expression of α_1- and α_{2A}-AR mRNAs in retinal venules (Fig. 12B-12C). The α_{2A}-AR expression in these vessels is consistent with the dominant expression of this α-AR subtype in porcine retinal arterioles[48] and human retinal tissue.[64] Similar to its effects on ET-1 and U46619, chronic hyperglycemia augmented norepinephrine-induced constriction of retinal venules without modifying expression of α-AR (α_1 and α_{2A}) mRNA levels (Fig. 12B-12C). This appears to be the first report of the direct impact of hyperglycemia on constriction of venules to norepinephrine during diabetes, and it is consistent with the increased norepinephrine-induced contraction of the large portal vein isolated from diabetic rats.[65]

A common mechanism for constriction of blood vessels involves entry of extracellular Ca^{2+} into the vascular smooth muscle.[16] The transmembrane proteins that have been shown to contribute to regulation of Ca^{2+} entry into cells are reverse-mode NCXs,[22] L-VOCCs,[16] TRPC,[18-20] and CRAC channels.[17] In our present study, the TRPC3 and CRAC channels did not appear to be involved in ET-1-induced vasoconstriction under euglycemia (A. 5). However, because the commercially available inhibitors of TRPC and CRAC channels are relatively non-selective, the involvement of TRPC or CRAC channels in vasoconstrictor responses to ET-1 remains unclear. On the other hand, the ability of low Na^+ PSS exposure to cause constriction of retinal venules under euglycemia indicated that the reverse-mode NCX could be stimulated in these vessels (A. 2). We proceeded to determine whether increased activity of reverse-mode NCX or L-VOCC contributes to the enhanced agonist-induced constriction of retinal venules during hyperglycemia by treating vessels with their cognate inhibitors. The pharmacological blockade of reverse-mode NCX (Fig. 13) prevented the enhanced constriction of retinal venules to ET-1,

U46619 and norepinephrine after exposure to acute and chronic hyperglycemia, suggesting possible role of NCX activation during diabetes. This conclusion is supported by the ability of two structurally different selective inhibitors of reverse-mode NCX, KB-R7943[42] (Fig. 13) and SEA0400[43] (A. 3), to block the hyperglycemia-enhanced constriction of retinal venules to ET-1. In contrast, L-VOCC blockade did not prevent the enhanced vasoconstriction, indicating that these channels were not involved in the altered vascular reactivity by hyperglycemia (A. 4). It does not appear that NCX contributes to agonist-induced venular constrictions during euglycemia, because NCX blockade had no effect on these responses. Interestingly, our data suggest that both L-VOCC and NCX contribute to the maintenance of basal tone of retinal venules, because their respective antagonists reduced the resting diameter to the same degree. At the molecular level, mRNAs for all three NCX isoforms (NCX1-3) were detected in porcine retinal venules, but chronic hyperglycemia did not affect their expression (A. 6). Future functional studies are warranted to evaluate whether a specific NCX isoform is activated in retinal venules by hyperglycemia.

A possible mechanism triggered by hyperglycemia upstream of NCX is activation of the NHE and PKC. PKC has been associated with vascular alteration during hyperglycemia.[66] In our present study, we demonstrated that PKC was not involved in hyperglycemia-enhanced constriction of retinal venules to ET-1 (Fig. 14). On the other hand, because hyperglycemia can lead to intracellular acidosis and elevation of hydrogen ions, this could increase activity of the NHE to remove these ions from the cell in exchange for entry of sodium ions.[67] As a result, intracellular sodium ion concentration may rise sufficiently to activate the reverse-mode NCX. In support of this notion, we found that NHE inhibitor cariporide abolished the enhanced constriction of retinal venules to ET-1 and U46619 under chronic hyperglycemia (Fig. 15). It appears that NHE and NCX are acting in series during chronic hyperglycemia because the combined treatment with their cognate inhibitors did not cause greater reduction in ET-1-induced vasoconstriction than either treatment alone. Therefore, our findings support the ability

of hyperglycemia to activate an NHE/reverse-mode NCX pathway to enhance agonist-induced constriction of retinal venules. Our findings may provide insight into the ability of cariporide to prevent the apparent diabetes-induced increase in retinal microvascular resistance.[45]

The p38 MAPK activity of mesangial cells to ET-1 was enhanced after exposure to hyperglycemia,[68] and the pathway linking ET-1-induced activity of NHE via p38 MAPK has also been demonstrated in Chinese hamster ovary cells.[69] Our findings seem to support the mechanism of p38 MAPK-dependent activation of NHE in hyperglycemia-enhanced constriction of retinal venules to ET-1 (Fig. 16). However, we need to perform additional molecular investigations to confirm ET-1-induced activation of p38 linking to downstream NHE and NCX signaling in retina venules under hyperglycemia. Based on our current findings that pharmacological blockade of reverse-mode NCX and NHE prevented the increased constriction of retinal venules to multiple endogenous receptor pathways, therapeutic targeting of these specific proteins may help mitigate enhanced vasoconstrictors-induced constriction of retinal venules under diabetes and alleviate retinal complications during diabetes.

References

1. Lutty GA. Effects of diabetes on the eye. *Invest Ophthalmol Vis Sci.* 2013;54:ORSF81-ORSF87.
2. Bandello F, Battaglia Parodi M, Lanzetta P, et al. Diabetic macular edema. *Dev Ophthalmol.* 2017;58:102-138.
3. 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.
4. 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.
5. Flammer J, Konieczka K. Retinal venous pressure: the role of endothelin. *EPMA J.* 2015;6:21.
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. Cybulska-Heinrich AK, Baertschi M, Loesche CC, et al. Patients with diabetic retinopathy have high retinal venous pressure. *EPMA J.* 2015;6:5.
8. 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.
9. 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.

10. Chakravarthy U, Hayes RG, Stitt AW, Douglas A. Endothelin expression in ocular tissues of diabetic and insulin-treated rats. *Invest Ophthalmol Vis Sci.* 1997;38:2144-2151.

11. 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.

12. 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.

13. De La Cruz JP, Moreno A, Ruiz-Ruiz MI, Sanchez De La Cuesta F. Effect of DT-TX 30, a combined thromboxane synthase inhibitor and thromboxane receptor antagonist, on retinal vascularity in experimental diabetes mellitus. *Thromb Res.* 2000;97:125-131.

14. Paulson DJ, Light KE. Elevation of serum and ventricular norepinephrine content in the diabetic rat. *Res Commun Chem Pathol Pharmacol.* 1981;33:559-562.

15. Nagai N, Izumi-Nagai K, Oike Y, et al. Suppression of diabetes-induced retinal inflammation by blocking the angiotensin II type 1 receptor or its downstream nuclear factor-kappaB pathway. *Invest Ophthalmol Vis Sci.* 2007;48:4342-4350.

16. Berridge MJ. Smooth muscle cell calcium activation mechanisms. *J Physiol.* 2008;586:5047-5061.

17. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci.* 2013;123:85-101.

18. Lemos VS, Poburko D, Liao CH, Cole WC, van Breemen C. Na^+ entry via TRPC6 causes Ca^{2+} entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem Biophys Res Commun.* 2007;352:130-134.

19. Eder P, Probst D, Rosker C, et al. Phospholipase C-dependent control of cardiac calcium homeostasis involves a TRPC3-NCX1 signaling complex. *Cardiovasc Res.* 2007;73:111-119.

20. Onohara N, Nishida M, Inoue R, et al. TRPC3 and TRPC6 are essential for angiotensin II-induced cardiac hypertrophy. *EMBO J.* 2006;25:5305-5316.

21. Chen YL, Ren Y, Xu W, Rosa RH, Jr., Kuo L, Hein TW. Constriction of retinal venules to endothelin-1: obligatory roles of ET_A receptors, extracellular calcium entry, and Rho kinase. *Invest Ophthalmol Vis Sci.* 2018;59:5167-5175.

22. Aiello EA, Villa-Abrille MC, Dulce RA, Cingolani HE, Perez NG. Endothelin-1 stimulates the Na^+/Ca^{2+} exchanger reverse mode through intracellular Na^+ (Na^+_i)-dependent and Na^+_i-independent pathways. *Hypertension.* 2005;45:288-293.

23. Giladi M, Shor R, Lisnyansky M, Khananshvili D. Structure-functional basis of ion transport in sodium-calcium exchanger (NCX) proteins. *Int J Mol Sci.* 2016;17:1949.

24. Bupha-Intr T, Haizlip KM, Janssen PM. Role of endothelin in the induction of cardiac hypertrophy in vitro. *PLoS One.* 2012;7:e43179.

25. Dulce RA, Hurtado C, Ennis IL, et al. Endothelin-1 induced hypertrophic effect in neonatal rat cardiomyocytes: involvement of Na^+/H^+ and Na^+/Ca^{2+} exchangers. *J Mol Cell Cardiol.* 2006;41:807-815.

26. Nishio S, Teshima Y, Takahashi N, et al. Activation of CaMKII as a key regulator of reactive oxygen species production in diabetic rat heart. *J Mol Cell Cardiol.* 2012;52:1103-1111.

27. Lee YR, Lee CK, Park HJ, et al. c-Jun N-terminal kinase contributes to norepinephrine-induced contraction through phosphorylation of caldesmon in rat aortic smooth muscle. *J Pharmacol Sci.* 2006;100:119-125.

28. Zhou MS, Schulman IH, Chadipiralla K, Raij L. Role of c-Jun N-terminal kinase in the regulation of vascular tone. *J Cardiovasc Pharmacol Ther.* 2010;15:78-83.

29. Massett MP, Ungvari Z, Csiszar A, Kaley G, Koller A. Different roles of PKC and MAP kinases in arteriolar constrictions to pressure and agonists. *Am J Physiol Heart Circ Physiol.* 2002;283:H2282-2287.

30. Bayat H, Xu S, Pimentel D, Cohen RA, Jiang B. Activation of thromboxane receptor upregulates interleukin (IL)-1β-induced VCAM-1 expression through JNK signaling. *Arterioscler Thromb Vasc Biol.* 2008;28:127-134.

31. da Costa-Pessoa JM, Damasceno RS, Machado UF, Beloto-Silva O, Oliveira-Souza M. High glucose concentration stimulates NHE-1 activity in distal nephron cells: the role of the Mek/Erk1/2/p90RSK and p38MAPK signaling pathways. *Cell Physiol Biochem.* 2014;33:333-343.

32. Du Y, Tang J, Li G, et al. Effects of p38 MAPK inhibition on early stages of diabetic retinopathy and sensory nerve function. *Invest Ophthalmol Vis Sci.* 2010;51:2158-2164.

33. Sun Y, Liu YX. LncRNA HOTTIP improves diabetic retinopathy by regulating the p38 MAPK pathway. *Eur Rev Med Pharmacol Sci.* 2018;22:2941-2948.

34. Hein TW, Xu W, Xu X, Kuo L. Acute and chronic hyperglycemia elicit JIP1/JNK-mediated endothelial vasodilator dysfunction of retinal arterioles. *Invest Ophthalmol Vis Sci.* 2016;57:4333-4340.

35. 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.

36. 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.

37. Dorn GW, 2nd, Becker MW, Davis MG. Dissociation of the contractile and hypertrophic effects of vasoconstrictor prostanoids in vascular smooth muscle. *J Biol Chem.* 1992;267:24897-24905.

38. Bohmer T, Manicam C, Steege A, Michel MC, Pfeiffer N, Gericke A. The α_{1B}-adrenoceptor subtype mediates adrenergic vasoconstriction in mouse retinal arterioles with damaged endothelium. *Br J Pharmacol.* 2014;171:3858-3867.

39. Teng B, Muir WW, 3rd. Effects of xylazine on canine coronary artery vascular rings. *Am J Vet Res.* 2004;65:431-435.

40. Alvarez-Miguel I, Cidad P, Perez-Garcia MT, Lopez-Lopez JR. Differences in TRPC3 and TRPC6 channels assembly in mesenteric vascular smooth muscle cells in essential hypertension. *J Physiol.* 2017;595:1497-1513.

41. Mauban JR, Wilkinson K, Schach C, Yuan JX. Histamine-mediated increases in cytosolic Ca^{2+} involve different mechanisms in human pulmonary artery smooth muscle and endothelial cells. *Am J Physiol Cell Physiol.* 2006;290:C325-336.

42. Iwamoto T, Watano T, Shigekawa M. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. *J Biol Chem.* 1996;271:22391-22397.

43. Christ T, Kovacs PP, Acsai K, et al. Block of Na^+/Ca^{2+} exchanger by SEA0400 in human right atrial preparations from patients in sinus rhythm and in atrial fibrillation. *Eur J Pharmacol.* 2016;788:286-293.

44. 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.

45. Cukiernik M, Hileeto D, Downey D, et al. The role of the sodium hydrogen exchanger-1 in mediating diabetes-induced changes in the retina. *Diabetes Metab Res Rev.* 2004;20:61-71.

46. Nagaoka T, Kuo L, Ren Y, Yoshida A, Hein TW. C-reactive protein inhibits endothelium-dependent nitric oxide-mediated dilation of retinal arterioles via enhanced superoxide production. *Invest Ophthalmol Vis Sci.* 2008;49:2053-2060.

47. Lei Y, Cao Y, Zhang Y, Edvinsson L, Xu CB. Enhanced airway smooth muscle cell thromboxane receptor signaling via activation of JNK MAPK and extracellular calcium influx. *Eur J Pharmacol.* 2011;650:629-638.

48. 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.

49. 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.

50. Hara H, Ichikawa M, Oku H, Shimazawa M, Araie M. Bunazosin, a selective α_1-adrenoceptor antagonist, as an anti-glaucoma drug: effects on ocular circulation and retinal neuronal damage. *Cardiovasc Drug Rev.* 2005;23:43-56.

51. Arun KH, Kaul CL, Ramarao P. AT_1 receptors and L-type calcium channels: functional coupling in supersensitivity to angiotensin II in diabetic rats. *Cardiovasc Res.* 2005;65:374-386.

52. 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.

53. Sone H, Okuda Y, Kawakami Y, Yamashita K. Effects of high glucose concentration and a thromboxane synthase inhibitor on the production of thromboxane A_2 and prostaglandin I_2 and E_2 by retinal endothelial cells. *Life Sci.* 1996;58:239-243.

54. Chen H. Role of thromboxane A_2 signaling in endothelium-dependent contractions of arteries. *Prostaglandins Other Lipid Mediat.* 2018;134:32-37.

55. Torring MS, Aalkjaer C, Bek T. Constriction of porcine retinal arterioles induced by endothelin-1 and the thromboxane analogue U46619 in vitro decreases with increasing vascular branching level. *Acta Ophthalmol.* 2014;92:232-237.

56. Chen Z, Prasad S, Cynader M. Localisation of thromboxane A_2 receptors and the corresponding mRNAs in human eye tissue. *Br J Ophthalmol.* 1994;78:921-926.

57. Wright WS, McElhatten RM, Harris NR. Expression of thromboxane synthase and the thromboxane-prostanoid receptor in the mouse and rat retina. *Exp Eye Res.* 2009;89:532-537.

58. Tesfamariam B, Jakubowski JA, Cohen RA. Contraction of diabetic rabbit aorta caused by endothelium-derived PGH_2-TXA_2. *Am J Physiol.* 1989;257:H1327-H1333.

59. Wright WS, Messina JE, Harris NR. Attenuation of diabetes-induced retinal vasoconstriction by a thromboxane receptor antagonist. *Exp Eye Res.* 2009;88:106-112.

60. Lee S, Morgan GA, Harris NR. Ozagrel reverses streptozotocin-induced constriction of arterioles in rat retina. *Microvasc Res.* 2008;76:217-223.

61. Ye XD, Laties AM, Stone RA. Peptidergic innervation of the retinal vasculature and optic nerve head. *Invest Ophthalmol Vis Sci.* 1990;31:1731-1737.

62. Ferrari-Dileo G, Davis EB, Anderson DR. Response of retinal vasculature to phenylephrine. *Invest Ophthalmol Vis Sci.* 1990;31:1181-1182.

63. Ichikawa M, Okada Y, Asai Y, Hara H, Ishii K, Araie M. Effects of topically instilled bunazosin, an α_1-adrenoceptor antagonist, on constrictions induced by phenylephrine and ET-1 in rabbit retinal arteries. *Invest Ophthalmol Vis Sci.* 2004;45:4041-4048.

64. Bylund DB, Chacko DM. Characterization of α_2 adrenergic receptor subtypes in human ocular tissue homogenates. *Invest Ophthalmol Vis Sci.* 1999;40:2299-2306.

65. Fiol de Cuneo M, Ruiz RD, Lacuara JL, Santillan de Torres R. Contractility and pharmacological reactivity of isolated vascular smooth muscle from diabetic rats. *Pharmacology.* 1988;36:228-237.

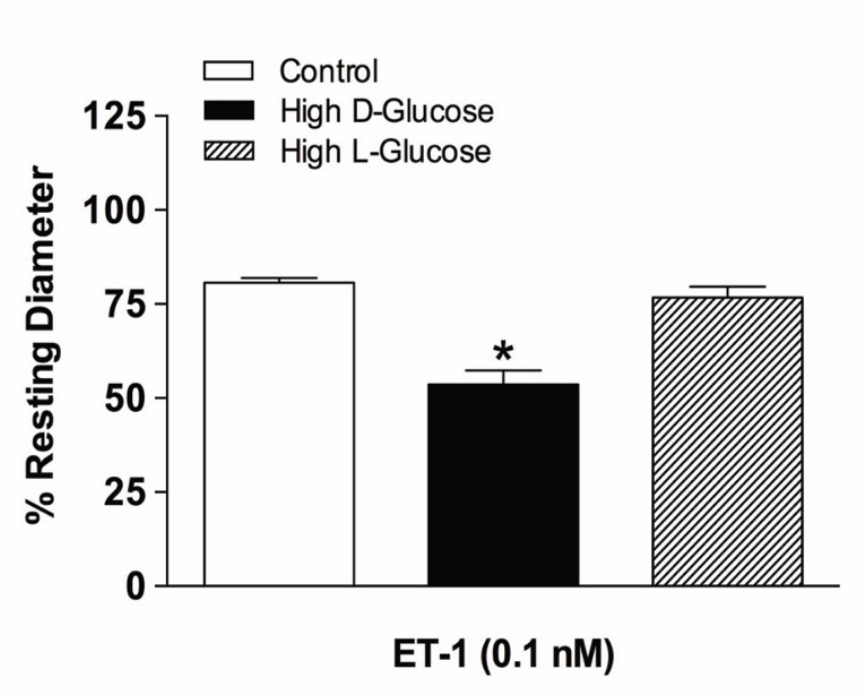
66. Geraldes P, King GL. Activation of protein kinase C isoforms and its impact on diabetic complications. *Circ Res.* 2010;106:1319-1331.

67. Zhang HM, Liu MY, Lu JX, et al. Intracellular acidosis via activation of Akt-Girdin signaling promotes post ischemic angiogenesis during hyperglycemia. *Int J Cardiol.* 2018.

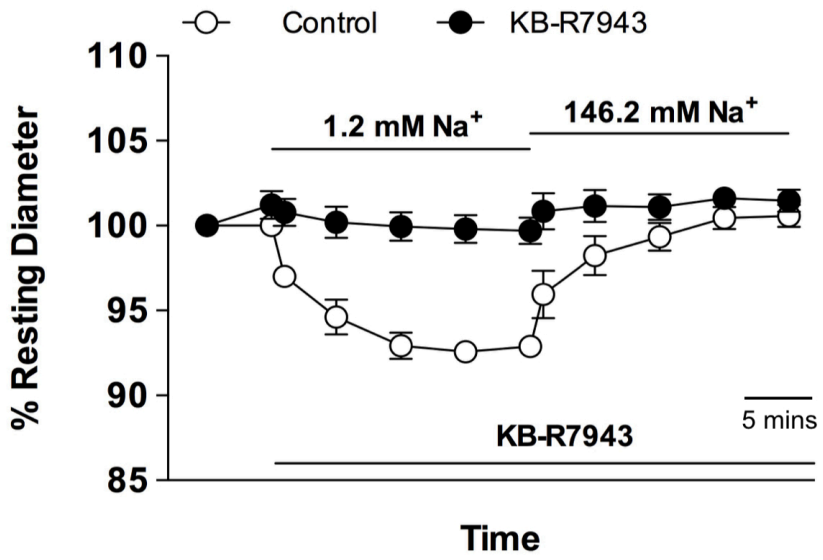
68. Tsiani E, Lekas P, Fantus IG, Dlugosz J, Whiteside C. High glucose-enhanced activation of mesangial cell p38 MAPK by ET-1, ANG II, and platelet-derived growth factor. *Am J Physiol Endocrinol Metab.* 2002;282:E161-169.

69. Horinouchi T, Asano H, Higa T, et al. Differential coupling of human endothelin type A receptor to $G_{q/11}$ and G_{12} proteins: the functional significance of receptor expression level in generating multiple receptor signaling. *J Pharmacol Sci.* 2009;111:338-351.

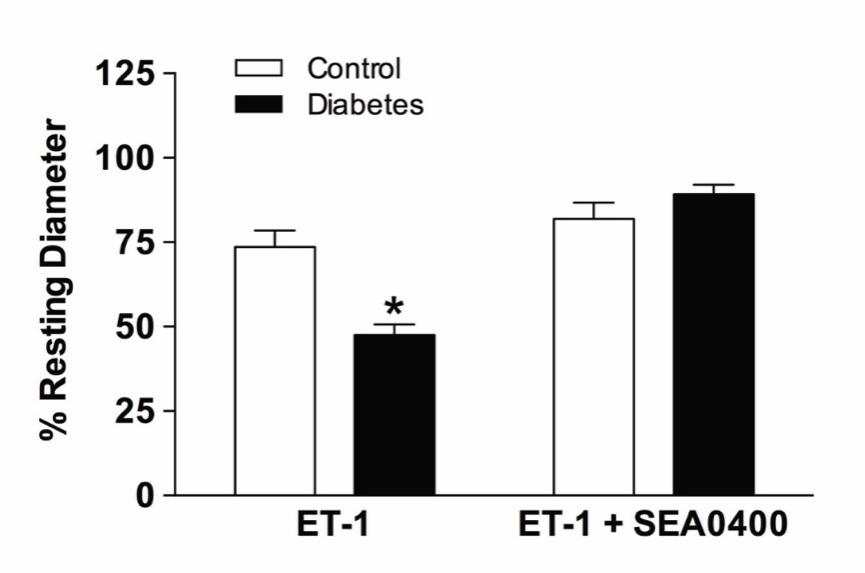
Appendix



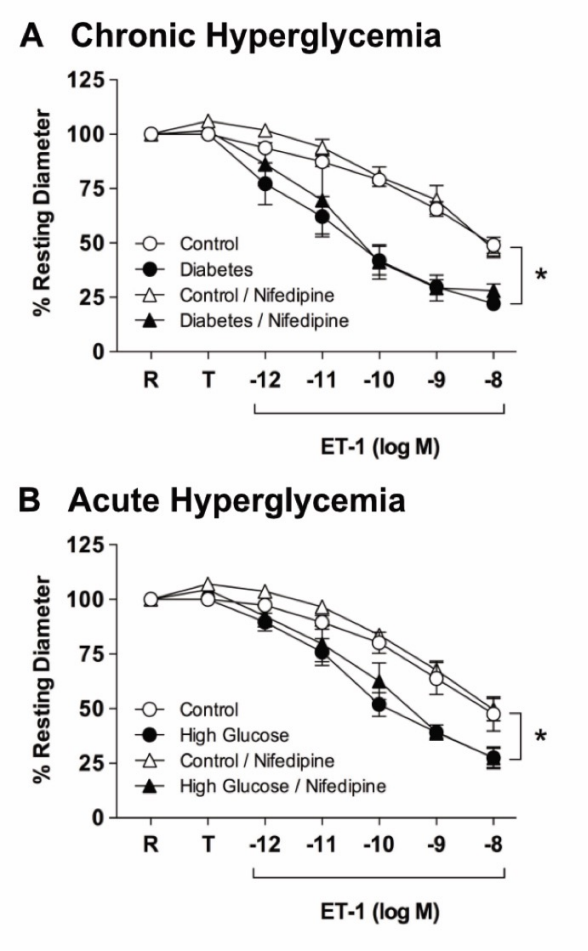
A. 1. Stereospecific effect of glucose on ET-1-induced constriction of isolated porcine retinal venules. Constriction of retinal venules to ET-1 (0.1 nM) was significantly increased after intraluminal exposure to high D-glucose (25 mM; n = 8) for 2 hours. After intraluminal incubation with high L-glucose (20 mM L-glucose + 5 mM D-glucose; n = 4) for 2 hours, ET-1-induced constriction was comparable to exposure to normal (5 mM) D-glucose (Control; n = 6). *P < 0.05 vs. Control.



A. 2. Tracings of retinal venular constriction in response to rapid exposure to low-Na^+ (~1.2 mM) physiological salt solution. KB-R7943 (10 μM; n = 5) abolished low-Na^+-induced vasoconstriction (Control; n = 7).

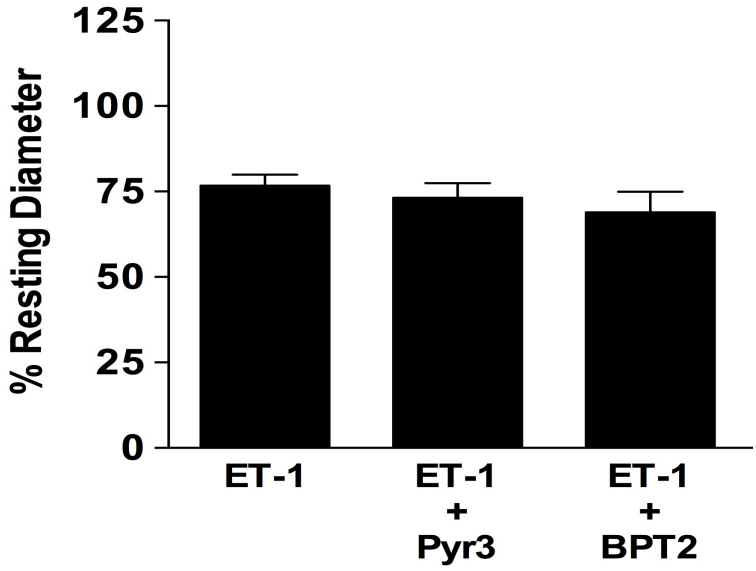


A. 3. Role of reverse-mode NCX in enhanced ET-1-induced constriction of isolated retinal venules after chronic hyperglycemia. Constriction of retinal venules to ET-1 (0.1 nM) was significantly increased after 2 weeks of diabetes in pigs. Reverse-mode NCX inhibitor SEA0400 (10 μM) prevented the enhanced constriction of retinal venules to ET-1 after chronic hyperglycemia (Diabetes; n = 9), but it did not alter ET-1-induced vasoconstriction after euglycemia (Control; n = 8). *P < 0.05 vs. Control.

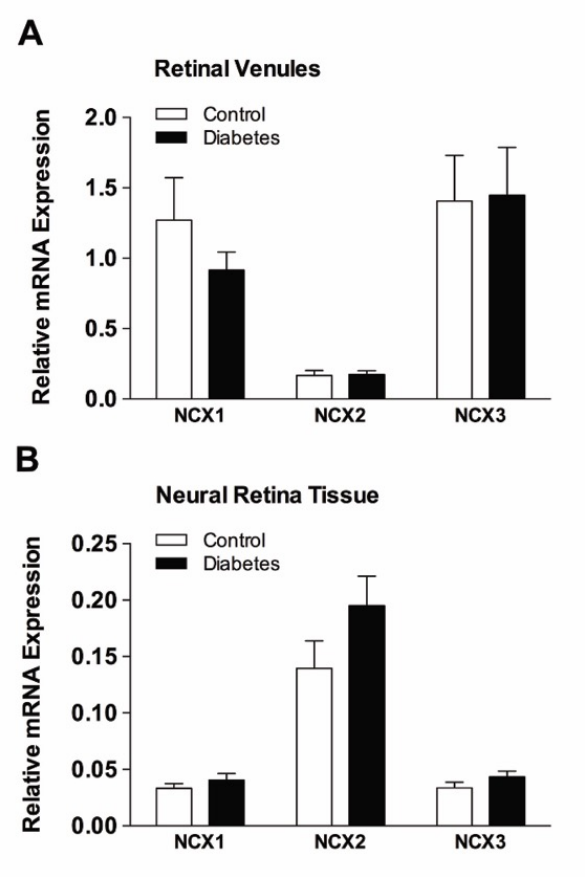


A. 4. Role of L-VOCCs in ET-1-induced constriction of retinal venules after chronic and acute hyperglycemia. Venular diameters were recorded before (R: resting diameter) and after 30-minute treatment (T) with L-VOCC inhibitor nifedipine prior to administration of ET-1. (A) The retinal venules from euglycemic pigs constricted concentration-dependently to ET-1 (Control, n = 10), and the vasoconstriction to ET-1 was enhanced by chronic hyperglycemia (Diabetes, n = 7). Nifedipine (1 μM) did not alter the constriction to ET-1 in vessels from euglycemic pigs (Control / Nifedipine, n = 6) or the enhanced ET-1-induced vasoconstriction in chronic hyperglycemic pigs (Diabetes / Nifedipine, n = 4). *P < 0.05 vs. Control. (B) For retinal venules isolated from euglycemic pigs, the constriction to ET-1 in normal 5 mM D-glucose (Control, n = 8) was significantly enhanced by acute exposure to 25 mM D-glucose (High Glucose, n = 7). Nifedipine did not affect either the vasoconstriction to ET-1 in normal level of glucose (Control /

Nifedipine, n = 7) or the enhanced vasoconstriction in acute hyperglycemia (High Glucose / Nifedipine, n = 6). *P < 0.05 vs. Control.



A. 5. Roles of TRPC3 and CRAC channels in vasoconstriction of isolated retinal venules to ET-1. Administration of TRPC3 antagonist Pyr3 (10 μM; *n*=3) or CRAC channels inhibitor BPT2 (1 μM; *n*=6) did not alter ET-1-induced (0.1 nM) constriction of retinal venules during euglycemia.



A. 6. Real-time PCR analysis of mRNA expression of NCX isoforms in porcine retinal venules and neural retina tissue. Equal amounts of total RNA isolated from porcine retinal venules and neural retina tissue after 2 weeks of euglycemia (Control) and chronic hyperglycemia (Diabetes) were reverse transcribed and then analyzed by real-time PCR for detection of NCX1-3 isoforms and GAPDH mRNAs. The NCX1-3 isoforms transcripts were normalized to GAPDH expression and presented as relative mRNA expression. (A) NCX 1-3 isoform expressions in retinal venules were not different between control (n = 9 for each isoform) and diabetes (n = 12 for each isoform) samples. (B) The NCX isoform expressions in neural retina tissue were not altered by diabetes (Control, n = 11 for each isoform; Diabetes, n = 10 for each isoform). n = number of pigs studied.

IV. ACUTE HYPERGLYCEMIA ENHANCES ENDOTHELIN-1-INDUCED CONSTRICTION OF HUMAN RETINAL VENULES

Introduction

Diabetes mellitus is a global epidemic and leads to a range of macrovascular and microvascular complications. Diabetic retinopathy is one of the specific microvascular complications of diabetes and is a leading cause of vision impairment and blindness in working-age adults.[1,2] Accumulating evidence has shown that vitreous humor, plasma and ocular levels of endothelin-1 (ET-1) are elevated in diabetes, especially in diabetic patients with proliferative diabetic retinopathy.[3-5]

ET-1 is a potent vasoactive peptide comprised of 21 amino acids and plays important roles in many biological functions, including vasoconstriction, cardiovascular remodeling, tissue inflammation, and cell proliferation.[3,6-9] Since overproduction of ET-1 is known to contribute to the pathogenesis of many diseases, it is clinically important to understand the mechanism of retinal venular constriction to ET-1 in physiology and pathophysiology. Although the retinal microcirculation lacks extrinsic innervation,[10] the identification of autonomic receptors on the walls of retinal vessels has been demonstrated in many species, including human.[11-13] Among those receptors, α_1 adrenergic and α_2 adrenergic receptors generally mediate vasoconstriction in the vascular system. Our previous studies demonstrated that norepinephrine elicited α_2 adrenergic receptor-dependent constriction in porcine retinal venules (Chapter III). However, there is no study related to norepinephrine-induced vasomotor activity in human retinal venules.

In the retinal microcirculation, the arterioles through changes in their vasomotor tone and resistance play an important role in governing retinal blood flow.[14] At the level of retinal venules, changes venular resistance influence hydrostatic pressure and fluid homeostasis of upstream capillaries.[15,16] Constriction of retinal venules leads to increased retinal venous

resistance and pressure, which could subsequently reduce retinal perfusion and promote edema under disease states such as diabetes.[17,18] However, in contrast to retinal arterioles, our understanding of factors affecting vasomotor activity of retinal venules is rather sparse.[19,20] Our previous study found that ET-1 causes marked constriction of porcine retinal venules through ET_ARs and extracellular Ca^{2+} influx independent of L-type voltage operated Ca^{2+} channels.[19] Also, we have shown that hyperglycemia augmented porcine venular constriction to ET-1, U46619, and norepinephrine (Chapter III). Intriguingly, there is a growing body of evidence indicating a connection between retinal venous pressure and diabetic retinopathy.[21] However, there has been no study addressing whether vasomotor function of human retinal venules is affected by hyperglycemia. We addressed these issues by examining the vasomotor response to ET-1 and norepinephrine in human retinal venules isolated from eyes donated by patients undergoing enucleation. Because elevated plasma glucose level is one of major risk factors and contributors to the development of diabetic retinopathy,[22,23] we also investigated whether exposure of human retinal venules to high glucose influences ET-1-induced vasoconstriction.

Methods

Human Subjects Study

Retinal tissues were obtained from 1 male (81 years old) and 4 female patients (45, 62, 69, and 69 years old) undergoing enucleation after informed consent and with approval from the Baylor Scott & White Health Institutional Review Board. The research followed the tenets of the Declaration of Helsinki. Eyes were enucleated from the 5 patients due to ocular melanoma. Immediately after enucleation, an eye cap was removed and transferred to a moist chamber on ice for microvessel isolation. The remaining ocular tissue with the tumor was placed in 10% neutral buffered formalin and submitted for histopathologic examination.

Isolation and Cannulation of Human Retinal Venules

Techniques for identifying and isolating human retinal microvessels were the same as our previous studies.[24-26] 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. Single retinal venules (1 to 1.5 mm in length without side branches) were carefully dissected with the aid of a stereomicroscope (model SZX12; Olympus, Melville, NY, USA). After the removal of any remaining neural/connective tissues, the venule was transferred 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 cmH_2O (4 mmHg) intraluminal pressure by two independent pressure reservoirs and allowed to develop basal tone before study. This intraluminal pressure was chosen based on our previous study in porcine retinal venules.[19] Vasomotor activity of isolated venules was recorded using videomicroscopic techniques throughout the experiments.

Experimental Protocols

Vasomotor Responses to ET-1 and Norepinephrine: Cannulated human retinal venules were bathed in PSS-albumin at 36° to 37°C to allow development (~90 minutes) of basal tone. The vasomotor response to cumulative administration of ET-1 (1 pM to 10 nM; Bachem, Torrance, CA)[27] or norepinephrine (1 nM to 10 μM; Cayman Chemical, Ann Arbor, MI) was then evaluated. Retinal venules were exposed to each concentration of ET-1 or norepinephrine for 10 minutes until a stable diameter was established.

High Glucose Study: To examine the influence of glucose on vasomotor responses, human retinal venules were isolated from enucleated patients and then the vessels were treated

with normal glucose (5 mM ≈ 90 mg/dl) or high glucose (25 mM ≈ 450 mg/dl) intraluminally for 2 hours as described in our previous study.[28] The vasomotor reactivity in response to ET-1 was then assessed after the 2-hour exposure to normal or high glucose. Osmolarity was maintained at a normal level by reducing the NaCl concentration in the high glucose-treated vessels.

Chemicals

ET-1 was dissolved in water, and norepinephrine was dissolved in dimethyl sulfoxide. Subsequent concentrations of these drugs were diluted in PSS. The final concentration of dimethyl sulfoxide was not more than 0.1%. 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).

Data Analysis

At the end of each functional experiment, the vessel was relaxed with 0.1 mM sodium nitroprusside in EDTA (1 mM)-Ca^{2+}-free PSS to obtain its maximal diameter at 5 cmH_2O intraluminal pressure. Diameter changes in response to agonists were normalized to the resting diameter and expressed as percentage changes in diameter. Data are reported as mean ± SEM, and *n* represents the number of vessels (1-2 vessels per patient per treatment group for functional studies). The repeated measures two-way analysis of variance 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

Human Retinal Venular Constriction to ET-1 and Norepinephrine

Human retinal venules (total 11 vessels in Figure 20) were isolated and pressurized at 5 cmH_2O with average maximum diameter of 64 ± 4 μm. These vessels developed stable basal tone by constricting to about 89% of maximum diameter within 60 minutes at 36-37°C. In one cohort, administration of ET-1 caused constriction of retinal venules in a concentration dependent manner (Figure 20A). In the other cohort, administration of norepinephrine also caused constriction of retinal venules in a concentration-dependent manner (Figure 20B). The threshold concentrations of ET-1 and norepinephrine for venular constriction were about 1 pM and 1 nM, respectively.

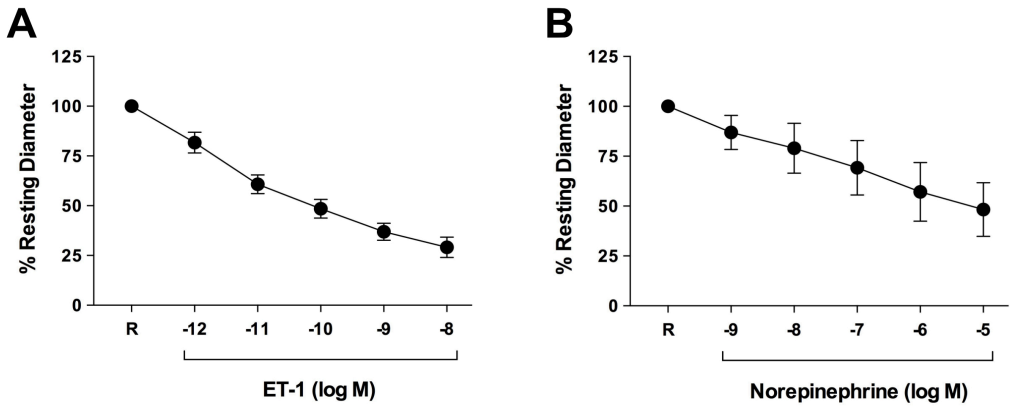


Figure 20. Vasomotor response of isolated and pressurized human retinal venules to ET-1 and norepinephrine. (A) ET-1 (*n* = 7) and (B) norepinephrine (*n* = 4) constricted retinal venules in a concentration-dependent manner.

High Glucose Enhances ET-1-Induced Constriction of Human Retinal Venules

Retinal venules exposed intraluminally to normal glucose and high glucose developed a comparable level of basal tone. The ET-1-induced constriction of venules from 10 pM to 1 nM was significantly increased after 2-hour exposure to high glucose (Figure 21).

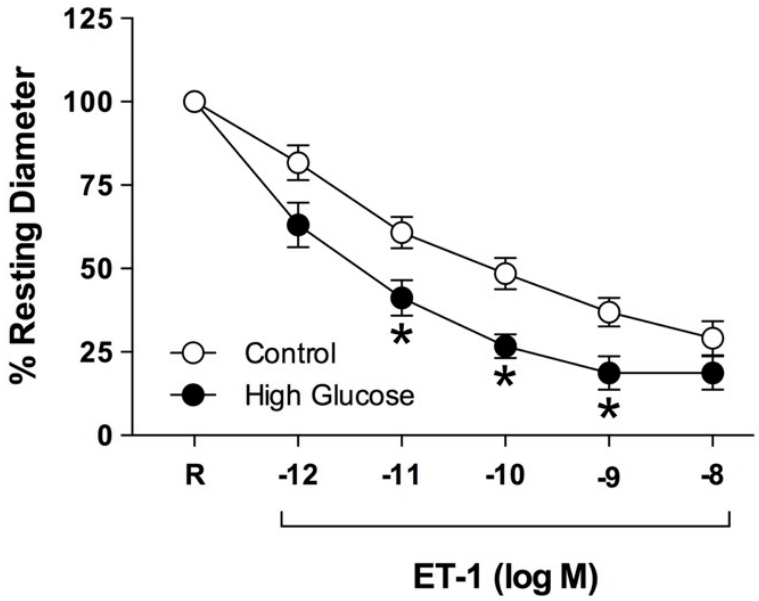


Figure 21. Effect of high glucose on the vasoconstrictor response of isolated and pressurized human retinal venules to ET-1. The vasoconstriction to ET-1 from 10 pM to 1 nM was significantly greater after 2-hour intraluminal exposure to high glucose (Control, *n* = 6; High Glucose, *n* = 5). *P < 0.05 vs. Control.

Conclusions

It is generally accepted that vasomotor activity of the arterioles primarily regulates retinal blood flow within the retinal circulation, and the venules maintain ocular fluid homeostasis and mediate postcapillary pressure linking to retinal pathogenesis, including retinal edema. There is an emerging concept depicting a connection between diabetic retinopathy and higher retinal venous pressure,[21] so abnormal vasoreactivity of retinal venules during retinal pathogenesis is conceivable. However, there is little information regarding the vasomotor reactivity of retinal venules, especially the vasoconstrictor responses of human retinal venules. Our previous study provided the information of vasomotor reactivity of porcine retinal venules to vasoconstrictors (ET-1, U46619, and norepinephrine) during acute and chronic hyperglycemia (Chapter III). The salient findings of the present study show similar vasoconstrictor responses of human retinal venules to ET-1 and norepinephrine (Figure 20), with enhanced vasoconstriction to ET-1 during short-term exposure to high glucose (Figure 21).

Despite the lack of extrinsic innervation in the retinal circulation,[10] autonomic receptors have been identified on the walls of retinal vessels.[11-13] Among these, α_{2A} adrenergic receptors are the predominant α-adrenergic receptor subtype in human ocular tissue homogenates,[34] and we observed predominantly α_2 adrenoceptor-dependent constriction of porcine retinal venules to norepinephrine in our previous study (Chapter III). Accumulating evidence has shown that vitreous humor, plasma and ocular levels of ET-1 are elevated in diabetes, especially in diabetic patients with proliferative diabetic retinopathy,[3,4,29,30] and associate with reduced retinal blood flow during the early stage of diabetes.[31-33] Our previous study also detected elevated ET-1 protein levels in the porcine vitreous humor, and ET-1 and norepinephrine elicited enhanced constriction of porcine retinal venules under hyperglycemia (Chapter III). The present investigation is the first to observe vasomotor function of isolated human retinal venules, with vasoconstrictions to ET-1 and norepinephrine. The ability of high glucose to enhance constriction of human retinal venules to ET-1, as observed previously with porcine vessels,

indicates the importance of a considering vasoreactivity of human retinal venules in ocular pathogenesis, especially diabetes.

The limitation of the present study is the inability to determine whether vascular disease or melanoma influences the results. Most patients were middle aged to elderly and had taken several types of medicine that could influence vascular function. Our previous study in human retinal arterioles addressed the potential influence of these factors (age, medicine-taken, and melanoma).[25] It is worth noting that we evaluated human retinal venules with an average maximum diameter of 64 μm in the present study. However, the maximum diameter of porcine retinal venules that we examined previously was about 130 μm.[19] The human retinal venules developed comparable but slightly greater tone (constricted to about 89% of maximum diameter) than did porcine retinal venules (constricted to about 93% of maximum diameter). In contrast, human retinal venules displayed similar vasoconstrictor responses to ET-1 and norepinephrine as observed with porcine retinal venules in our previous study (Chapter III), which strengthens use of the porcine eye to study the human retinal microcirculation.

In conclusion, we found that isolated human retinal venules develop stable basal tone and constrict in response to ET-1 and norepinephrine. We also showed that high glucose does not influence basal tone but enhances ET-1-induced constriction of human retinal venules. Our findings provide the initial insight into the impact of vasoconstrictor agonists on tone of retinal venules of humans under normal and high glucose conditions, which may indicate key therapeutic targets for patients with retinal complications of diabetes or other retinal diseases.

References

1. Lutty GA. Effects of diabetes on the eye. *Invest Ophthalmol Vis Sci.* 2013;54:ORSF81-ORSF87.
2. Wong TY, Cheung CM, Larsen M, Sharma S, Simo R. Diabetic retinopathy. *Nat Rev Dis Primers.* 2016;2:16012.
3. Salvatore S, Vingolo EM. Endothelin-1 role in human eye: a review. *J Ophthalmol.* 2010;2010:354645.
4. 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.
5. 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.
6. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci.* 2013;123:85-101.
7. Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol.* 2016;77:143-175.
8. Brunner F, Bras-Silva C, Cerdeira AS, Leite-Moreira AF. Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. *Pharmacol Ther.* 2006;111:508-531.
9. Iglarz M, Clozel M. At the heart of tissue: endothelin system and end-organ damage. *Clin Sci (Lond).* 2010;119:453-463.
10. Ye XD, Laties AM, Stone RA. Peptidergic innervation of the retinal vasculature and optic nerve head. *Invest Ophthalmol Vis Sci.* 1990;31:1731-1737.
11. McAuliffe-Curtin D, Buckley C. Review of alpha adrenoceptor function in the eye. *Eye (Lond).* 1989;3 (Pt 4):472-476.

12. Bohmer T, Manicam C, Steege A, Michel MC, Pfeiffer N, Gericke A. The α_{1B}-adrenoceptor subtype mediates adrenergic vasoconstriction in mouse retinal arterioles with damaged endothelium. *Br J Pharmacol.* 2014;171:3858-3867.
13. Jiang Y, Zhang Q, Steinle JJ. Beta-adrenergic receptor agonist decreases VEGF levels through altered eNOS and PKC signaling in diabetic retina. *Growth Factors.* 2015;33:192-199.
14. Needham M, McGahon MK, Bankhead P, et al. The role of K^+ and Cl^- channels in the regulation of retinal arteriolar tone and blood flow. *Invest Ophthalmol Vis Sci.* 2014;55:2157-2165.
15. 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.
16. 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.
17. Flammer J, Konieczka K. Retinal venous pressure: the role of endothelin. *EPMA J.* 2015;6:21.
18. 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.
19. Chen YL, Ren Y, Xu W, Rosa RH, Jr., Kuo L, Hein TW. Constriction of retinal venules to endothelin-1: obligatory roles of ET_A receptors, extracellular calcium entry, and Rho kinase. *Invest Ophthalmol Vis Sci.* 2018;59:5167-5175.
20. 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.

21. Cybulska-Heinrich AK, Baertschi M, Loesche CC, et al. Patients with diabetic retinopathy have high retinal venous pressure. *EPMA J.* 2015;6:5.

22. Xu J, Wei WB, Yuan MX, et al. Prevalence and risk factors for diabetic retinopathy: the Beijing Communities Diabetes Study 6. *Retina.* 2012;32:322-329.

23. Sasaki A, Horiuchi N, Hasewgawa K, Uehara M. Development of diabetic retinopathy and its associated risk factors in type 2 diabetic patients in Osaka district, Japan: a long-term prospective study. *Diabetes Res Clin Pract.* 1990;10:257-263.

24. 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.

25. 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.

26. 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.

27. 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.

28. Hein TW, Xu W, Xu X, Kuo L. Acute and chronic hyperglycemia elicit JIP1/JNK-mediated endothelial vasodilator dysfunction of retinal arterioles. *Invest Ophthalmol Vis Sci.* 2016;57:4333-4340.

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.

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.

31. 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.

32. 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.

33. 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.

34. Bylund DB, Chacko DM. Characterization of α_2 adrenergic receptor subtypes in human ocular tissue homogenates. *Invest Ophthalmol Vis Sci.* 1999;40:2299-2306.

V. SUMMARY

I demonstrated that 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. Retinal venules also constrict to norepinephrine and TXA_2 analog U46619. On the other hand, angiotensin II and α_1-adrenergic agonist phenylephrine fail to elicit vasomotor responses of retinal venules. Acute and chronic (diabetes) hyperglycemia enhance retinal venular constriction to ET-1, norepinephrine, and U46618 without altering the mRNA expression level of corresponding receptors. The enhanced vasoconstrictions appear to be mediated by the activation of reverse-mode NCX and the NHE through p38 MAPK signaling (Fig. 22). This enhanced responsiveness might be important for ET-1 because the vitreous concentration of this peptide is significantly elevated to the level sufficient to cause venular constriction in hyperglycemia/diabetes. The results from human retinal venular studies also support the alteration of venular reactivity to ET-1 when hyperglycemic insult is imposed. In conjunction with previous studies on the porcine retinal arterioles, the vasomotor activity of porcine retinal venules appears to be comparable to that of human, supporting use of the porcine eye for studying the human retinal microcirculation. The functional alteration of venular activity in response to several endogenous vasoconstrictors in early diabetes might aggravate the development of venous hypertension and retinal edema in the late stage of diabetic retinopathy, in addition to physical disruption of the retinal microcirculation. Consideration of correcting vasomotor function of retinal venules, as well as arterioles, with effective therapeutic strategies might be necessary to improve the retinal circulation and protect neural tissue during hyperglycemic insults.

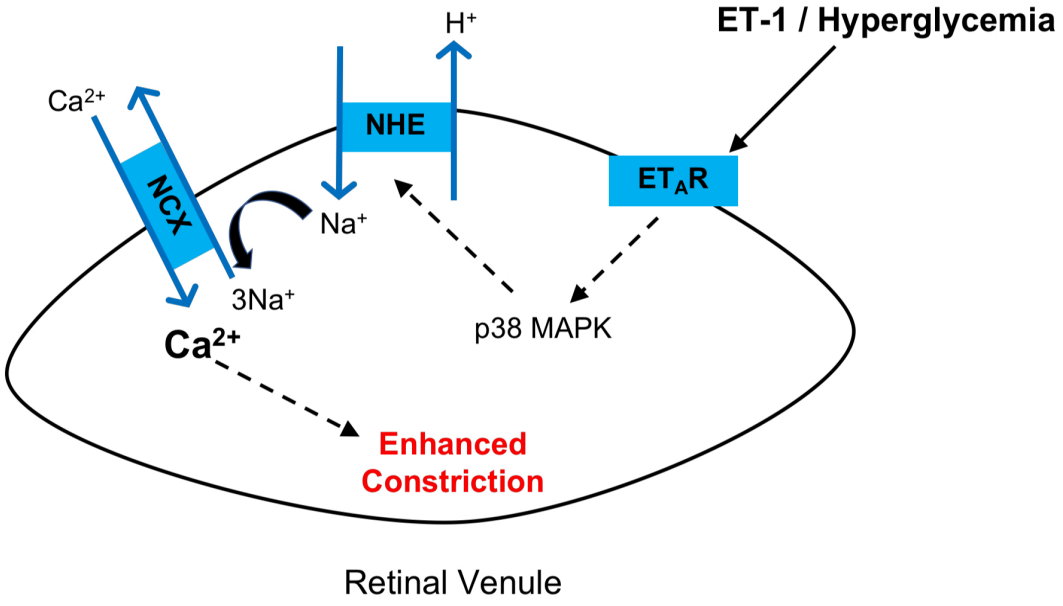


Figure 22. Schematic diagram of p38 MAPK-NHE-NCX pathway for enhanced constriction of retinal venules in response to ET-1 under hyperglycemia.

Future Directions

In my study, the p38 MAPK-dependent activation of NHE-NCX signaling is implicated in diabetes-related enhancement of constriction of retinal venules to ET-1 without the involvement of L-type Ca^{2+} channels. However, the p38 MAPK-dependent activity of NHE and the detailed signal transduction pathways involved in ET-1-induced constriction of retinal venules remain unknown. Moreover, our results showed that ET-1-elicited constriction of retinal venules is predominantly mediated by the activation of ET_AR. The ET_ARs, which belong to the G protein-coupled receptors (GPCRs) superfamily, can induce formation of second messengers, and subsequently lead to activation of Ca^{2+} channels (such as voltage-dependent cation channels and CRAC channels) accompanied by an influx of Ca^{2+} from the extracellular medium.[1,2] In addition, ET-1 can possibly stimulate Na^+ influx via the opening of TRPC, and consequently elevate cytoslic Na^+_i for membrane depolariation and then trigger the opening of L-VOCCs and activate the reverse-mode NCX.[3-5] Although these signaling events do not contribute to the vasoconstriction, their activations might modulate the vasomotor activity and reactivity to various agonists. Therefore, the detailed studies of the upstream signals on CRAC channels- ,TRPC-, or NCX-NHE- related vasomotor activity contributing to enhanced retinal venular responses to endogenous vasoconstrictors need to be further investigated. Although my studies suggest the link between NHE and NCX in enhanced vasoconstriction to ET-1, U46619 and norepinephrine, the corresponding changes in intracellular level of Na^+ and Ca^{2+} in diabetic vessels during ET-1 stimulation need to be verified. Moreover, the mechanism responsible for p38 MAPK activation during hyperglycemic insult remains to be explored. Although I did not examine whether the endothelial function, in terms of NO-mediated vasodilation, is altered by hyperglycemia, multiple reports have demonstrated the endothelial dysfunction in the arterial network of diabetic animals. It is worth investigating the impact of diabetes on the endothelial function of retinal venules because inflammatory leukocytes mainly interact with the vasculature, depending upon the endothelial integrity and function, in the venular circulation. The endothelial NO deficiency

apparently facilitates the development of inflammation and promotes activation of the immune system in the venular circulation. However, it is unclear whether hyperglycemia/diabetes could alter endothelial function in the venular circulation, in addition to the enhancement of vasoconstrictor function as I observed. It would be also interesting to know whether the altered vasomotor activity of retinal venules is reversible after hyperglycemic insult is removed. Answers to these issues could enhance our understanding on the pathogenesis of diabetic retinopathy, especially the alteration of retinal blood flow regulation and the disturbance of fluid homeostasis via the transvascular pathway.

References

1. Houde M, Desbiens L, D'Orleans-Juste P. Endothelin-1: biosynthesis, signaling and vasoreactivity. *Adv Pharmacol.* 2016;77:143-175.
2. Horinouchi T, Terada K, Higashi T, Miwa S. Endothelin receptor signaling: new insight into its regulatory mechanisms. *J Pharmacol Sci.* 2013;123:85-101.
3. Eder P, Probst D, Rosker C, et al. Phospholipase C-dependent control of cardiac calcium homeostasis involves a TRPC3-NCX1 signaling complex. *Cardiovasc Res.* 2007;73:111-119.
4. Reading SA, Earley S, Waldron BJ, Welsh DG, Brayden JE. TRPC3 mediates pyrimidine receptor-induced depolarization of cerebral arteries. *Am J Physiol Heart Circ Physiol.* 2005;288:H2055-H2061.
5. Lemos VS, Poburko D, Liao CH, Cole WC, van Breemen C. Na^+ entry via TRPC6 causes Ca^{2+} entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem Biophys Res Commun.* 2007;352:130-134.