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Intravitreal administration of endothelin type A receptor or endothelin type B receptor antagonists attenuates hypertensive and diabetic retinopathy in rats

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ABSTRACT

Hypertension is an independent risk factor for diabetic retinopathy, yet anti-hypertensive medications such as blockade of angiotensin II do not completely protect against vision-threatening vascular disease. We hypothesized that the potent vasoactive factor, endothelin (ET), is up-regulated in diabetic retinopathy and antagonism of the ET type A receptor (ETRA) or ET type B receptor (ETRB) ameliorates retinal vascular leakage independently of any blood pressure lowering effects. Spontaneously hypertensive rats (SHR) and their normotensive and genetic controls, Wistar Kyoto rats, were randomized to become diabetic or non-diabetic and studied for 8 weeks. Rats were further randomized to receive by intravitreal injection the ETRA antagonist, BQ123, the ETRB antagonist, BQ788, or vehicle, 5 days after the induction of streptozotocin diabetes and 4 weeks later. The treatments had no effect on systolic blood pressure which remained elevated in SHR. ET-1, ET-2, ETRA and ETRB were expressed in retina and retinal pigment epithelium (RPE)/choroid and increased by hypertension or diabetes. BQ123 reduced ET-1 and ET-2 expression in retina and RPE/choroid, while BQ788 had a similar effect but did not influence the mRNA levels of ET-1 in retina. Retinal vascular leakage and Müller cell stress as well as vascular endothelial growth factor (VEGF) expression in retina and RPE/choroid, were increased by hypertension or diabetes and there was an additive effect of these conditions. Treatment with BQ123 or BQ788 effectively reduced these events as well as the elevated levels of inflammatory factors in the retina. Our findings indicate that local ET systems exist in the retina and RPE/choroid that are up-regulated by hypertension and diabetes. The ability of locally delivered ET receptor antagonists to supress these overactive ET systems and reduce retinal vascular leakage and VEGF in the presence of hypertension indicate the potential of these approaches for the treatment of diabetic retinopathy.

1. Introduction

Hypertensive retinopathy is a disease of the retinal microvasculature associated with elevated blood pressure (Fraser-Bell et al., 2017). Narrowing of retinal arterioles occurs in the early stage of the disease, and with persistent hypertension further vascular pathology develops including vision-threatening breakdown of the blood-retinal barrier (BRB) (Fraser-Bell et al., 2017). Systemic hypertension is a risk factor for prevalent ocular diseases including age-related macular degeneration (Klein et al., 2003) and diabetic retinopathy (DR) (Klein et al., 1984a; b). Indeed, DR features damage to the retinal microvasculature (Fletcher et al., 2007) as well as the retinal pigment epithelium (RPE) and choroidal vasculature (Lutty, 2017). DR is a leading cause of vision loss and blindness in the working population throughout the world (Yau et al., 2012), yet preventative treatments are limited. As the global prevalence of diabetes increases to an estimated 642 million people by 2040 (Ogurtsova et al., 2017), there is an unmet medical need to identify new treatment targets to improve the outlook of patients with DR.

The vasoactive pathway, the renin-angiotensin aldosterone system, has a central role in regulating blood pressure and the development of DR. In particular, blood pressure lowering and blockade of angiotensin

Abbreviations: BRB, blood-retinal barrier; BQ123, endothelin type A receptor antagonist; BQ788, endothelin type B receptor antagonist; Diab, diabetic; ET-1, endothelin-1; ET-2, endothelin-2; ETRA, endothelin type A receptor; ETRB, endothelin type B receptor; DR, diabetic retinopathy; GFAP, glial fibrillary acidic protein; ICAM-1, intercellular adhesion-molecule-1; MCP-1, monocyte chemoattractant protein-1; NDiab, non-diabetic; RPE, retinal pigment epithelium; SHR, spontaneously hypertensive rat; TNFα, tumor necrosis factor-α; VEGF, vascular endothelial growth factor; WKY, Wistar Kyoto rat

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converting enzyme or the angiotensin type 1 receptor, improves DR in experimental models (Hamada et al., 1995; Miller et al., 2010; Phipps et al., 2007; Yamagishi et al., 2005) as well as retinopathy in subjects with type 1 and type 2 diabetes (Chaturvedi et al., 2008; Sjolie et al., 2011). However, these treatment approaches do not provide complete retinoprotection (Chaturvedi et al., 2008; Sjolie et al., 2011), suggesting that other vasoactive agents have a causal role. A potential candidate is endothelin (ET), a potent vasoconstrictor comprised of three isoforms designated ET-1, ET-2 and ET-3, whose actions are mediated by the ET type A receptor (ETRA) and ET type B receptor (ETRB) (Davenport et al., 2016; Yanagisawa et al., 1988). ET-1 and ET-2 bind to both receptors with similar affinity, while ET-3 binds only to the ETRB (Davenport et al., 2016). ET-1 is the most studied isoform due to its high expression in the vasculature, central role in the regulation of blood pressure (Davenport et al., 2016; Yanagisawa et al., 1988) and ability to influence a variety of cellular processes including cell proliferation and migration (Garrafa et al., 2012). ET-2 is less well studied due to its similar structure to ET-1, differing by only two amino acids in humans and three amino acids in rats and mice (Saida et al., 2000). Nonetheless, there is increasing interest in the actions of ET-2, which include the promotion of inflammation and vasculopathy (Alrashdi et al., 2017; Grimshaw et al., 2002). The development of ETRA and ETRB antagonists has assisted in understanding the contribution of ETs to physiological and pathological processes (Reichetzeder et al., 2014). Furthermore, inhibition of these receptors is of interest for the medical treatment of hypertension and diabetic complications, albeit systemic administration of ETRA inhibitors is controversial due to possible adverse cardiovascular events in patients (Mann et al., 2010; Reichetzeder et al., 2014).

Components of the ET system are expressed in the retina (Chakravarthy et al., 1997; McDonald et al., 2010) including in patients with proliferative DR (Adamiec-Mroczek et al., 2010; Roldan-Pallares et al., 2005). However, whether individual antagonism of the ETRA and ETRB influence the development of DR as well as hypertensive DR has not been extensively explored. To determine if antagonism of the ET receptors improved DR independently of any anti-hypertensive effect, we used an intraocular route of administration. Spontaneously hypertensive rats (SHR) and their genetic controls, Wistar Kyoto rats (WKY), were made diabetic with streptozotocin, and we determined that ET transcripts and particularly ET-2 are increased in retina as well as RPE/choroid. Our findings demonstrate the ability of ETRA or ETRB antagonism to attenuate retinal vascular leakage and associated events in the presence of hypertension and diabetes.

2. Methods

2.1. Animals

All studies were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee. SHR and WKY were purchased from the Animal Resources Centre (Perth, Western Australia) and housed in AMREP Animal Services (Melbourne, VIC, Australia). All rodents received normal chow (Certified Rodent Diet #5002, LabDiet, USA) and drinking water *ad libitum,* and were housed at 22 ± 1 °C with a 12-hour light/dark cycle.

Following an overnight fast, 6 to 8-week-old SHR and WKY were randomized to become diabetic or non-diabetic according to established protocols (Miller et al., 2010). Diabetes was induced by a single tail vein injection of 55 mg/kg streptozotocin (Merck, Darmstadt, Germany) dissolved in vehicle (0.1 M citrate buffer, pH 4.5). Non-diabetic rats received a single tail vein injection of vehicle only. Rats were then randomized to receive into each eye, a single intravitreal injection (1 μl) of the ETRA inhibitor, BQ123 (100 μM dissolved in 0.01% dimethyl sulfoxide, DMSO; Merck), the ETRB inhibitor, BQ788 (100 μM, Merck) or vehicle (0.01% DMSO). Treatments were administered 5 days after the administration of streptozotocin or citrate buffer and then again 4 weeks later and rats were humanely euthanized 8 weeks after the initial intravitreal injection. Intravitreal injections were performed as previously reported following anaesthesia with an intraperitoneal injection of ketamine (40 mg/kg) and xyalzine (10 mg/kg) (Alrashdi et al., 2017). A Hamilton syringe attached to a 31-gauge needle was inserted 1 to 2 mm into the eye and 2 mm behind the limbus at a 45° downward angle to avoid the lens (Alrashdi et al., 2017). The doses of BQ123 and BQ788 were based on previous reports (Patel et al., 2014). Each week, rats were weighed and their blood glucose levels measured (Accu-check Advantage II Blood Glucose Monitor, Roche Diagnostics, USA). Only rats with blood glucose levels > 15 mmol/L were considered diabetic and studied. Insulin was administered three times per week to diabetic rats to reduce mortality and promote weight gain (2–4 units by intraperitoneal injection, Humulin NPH, Eli Lilly and Co., Indianapolis, IN, USA). At the end of the studies, rats received an anaesthetic overdose of pentobarbitone sodium (Lethabarb, 60 mg/kg, Virbac, NSW, Australia).

2.2. Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously (Alrashdi et al., 2017; Deliyanti et al., 2017) on separate preparations of retina and RPE/choroid. The primer sequences for ET-1, ET-2, ETRA,

Table 1
Rat primer sequences. FP, forward primer. RP, reverse primer.

Gene	Accession Number	Primer Sequences	PCR Efficiency (%)
ET-1	NM_012548.2	FP: 5′-TGGACATCATCTGGGTCAACA-3′ RP: 5′-GCTTAGACCTAGAAGGGCTTCC-3	97.2
ET-2	NM_012549	FP: 5′-GACTGCTGCGGGAGACCTT-3′ RP: 5′-GGGATGGCCTCTCTTGTCAA-3′	99.8
ETRA	NM_012550	FP: 5′-CTCAGCGAACACCTCAAGCA-3′ RP: 5′-GGCTTAAGTGAAGAGGGAACCA-3′	98.6
ETRB	NM_017333	FP: 5′-GTTTTGATGTGATTACGTCGGACTA-3′ RP: 5′-ACCAGTCTTTGGCTGTCTTGTAAA-3′	98.1
GFAP	NM_017009	FP: 5′-CCTTGACCTGCGACCTTGAG-3′ RP: 5′-CCTGTTCGCGCATTTGC-3′	95.2
ICAM-1	NM_012967.1	FP: 5′-AGTGGTGTACCATGATCAGAA-3′ RP: 5′-TAAATGGACGCCACGATCAC-3′	100.09
TNFα	NM_012675.3	FP: 5′-TGATCGGTCCCAACAAGGA-3′ RP: 5′-TGGGCTACGGGCTTGTCA-3′	96.4
VEGF	NM_001287113	FP: 5′-AGCAGAAGTCCCATGAAGTGATC-3′ RP: 5′-TCAATCGGACGGCAGTA-3′	97.7
18.S	NR_046237.1	FP: 5′-CCGCAGCTAGGAATAATGGAAT-3′ RP: 5′-CGGCGCAATACGAATGC-3′	99.8