Effects of an Endothelin Receptor Antagonist on a Model of Hypertensive Retinopathy

Louise McDonald Graham R. Lee Tanyth E. deGooyer Denise McDonald
Paul Canning Elizabeth J. Kelso Barbara J. McDermott Alan W. Stitt

Centre for Vision and Vascular Science, Queen's University Belfast, Belfast, Northern Ireland

Key Words

Hypertensive retinopathy · Endothelin · Bosentan · Retina · Microvasculature

Abstract

Hypertensive retinopathy manifests itself as progressive retinal microvascular pathology in response to aberrant blood flow. The current study sought to evaluate whether dysfunction of the vasoactive endothelin-1 (ET-1) system is involved in the pathogenesis of hypertension-induced retinopathy in an animal model of systemic hypertension. The endothelin receptor antagonist, bosentan, was administered to spontaneously hypertensive rats (SHRs) and comparisons were made with untreated SHRs and normotensive Wistar Kyoto (WKY) rats. The retinal mRNA expression of ET-1, ET-converting enzyme-1, ET_A and ET_B receptors and the basement membrane proteins, laminin β 1, collagen IV and fibronectin was quantified using real-time RT-PCR. In addition, retinal arteriole and/or capillary bed damage was assessed by qualitative and quantitative microscopy. mRNA for the ET_A receptor was increased in SHRs, when compared to WKY control animals ($p < 0.001$). Treatment with bosentan in SHRs significantly reduced the expression of ET-1 ($p < 0.05$), and both the ET_A ($p < 0.0001$) and ET_B ($p < 0.05$) receptor subtypes. The laminin β 1, collagen IV and fibronectin mRNA expression was significantly higher in SHRs when compared to

WKY control animals ($p < 0.001$). Treatment with bosentan abolished these responses and also the appearance of various microvascular lesions. ET-mediated vasoregulation abnormalities in the retinal microvasculature could play an associative role in lesion formation during hypertensive retinopathy.

Copyright © 2009 S. Karger AG, Basel

Introduction

Ocular manifestations of hypertension comprise a spectrum of pathological processes encompassing hypertensive choroidopathy, optic nerve neuropathy and retinopathy [1]. Blood-pressure-related retinal abnormalities may be the initial finding in an asymptomatic patient and hypertensive retinopathy is manifest as cumulative microvascular damage arising from bouts of elevated blood pressure. Hypertensive retinopathy is characterised clinically and histologically by retinal arteriolar wall hyalinisation, vascular basement membrane (BM) thickening, markedly constricted precapillary arterioles, fibrotic necrosis of arteriolar walls, closure of capillaries, cotton wool spots, smooth-muscle degeneration and blood-retinal barrier dysfunction [2–4]. Damage to precapillary arterioles leading to the breakdown of autoregulatory processes and compromise of the blood-retinal barrier are

central to the pathophysiology of hypertensive retinopathy [5]. However, despite the well-established link between systemic hypertension and retinopathy, the precise pathogenesis of this disease remains largely unelucidated.

The control of the retinal blood flow is heavily dependent on autoregulatory mechanisms that protect against physiological changes in perfusion pressure [6]. The retinal microcirculation is mainly under the local control of vasoconstrictive and vasodilatory molecules released by the vascular endothelium, neurons and glia [6]. Amongst these regulatory agents, the endothelin (ET) family of vasoconstrictive peptides appears to have a role in the autoregulation of microvascular beds and may contribute to systemic blood pressure regulation [7, 8].

Two ET receptor subtypes have been identified that transduce ET bioactivity; the endothelin A receptor (ET_A) and the endothelin B receptor (ET_B) [9], which are classified on the basis of differing affinities for ET molecules and tissue distribution [10]. The presence of ET-1 and its receptors has been confirmed in the retinal microvasculature [11, 12] with the receptor distribution suggesting a dual vaso- and neuromodulatory role. Vasoconstriction produced by ET-1 in retinal arterial vessels is primarily ET_A-receptor-mediated by vascular smooth muscle [13]. In the retinal capillary beds, ET-1 has been identified as a putative endothelium-pericyte signal, although precise mechanisms underlying its regulation of pericyte function remain unclear [6].

ET receptor antagonists have been crucial in defining the cellular activity and pathological effects of these vasoactive peptides. Bosentan is a recently developed, non-selective ET receptor antagonist, blocking both ET_A and ET_B receptors [14]. Studies with bosentan have demonstrated blood-pressure-lowering activity in animal models of hypertension [15], as well as in patients with essential hypertension [16] and congestive heart failure [16]. This agent also suppressed aspects of ET-mediated remodelling in senescent spontaneously hypertensive rats (SHRs) [17]. Moreover, clinical trials have established that this drug is beneficial in the treatment of pulmonary hypertension [18], although this effect is not demonstrated in the SHR [19]. The ET system has been implicated in hyperglycaemia-mediated retinal microvascular dysfunction in studies of diabetic rats with or without bosentan treatment [20, 21], and has been shown to rectify diabetes-induced increases in retinal ET-1 mRNA expression, BM thickening and activation of the transcription factors NF- κ B and activating protein [22]. Following on from these studies, the current research has investigated

retinal pathology in the SHR model and the ability of bosentan to attenuate hypertension-related lesions in the retinal microvasculature.

Materials and Methods

Experimental Model

Male SHRs and age- and sex-matched normotensive Wistar Kyoto (WKY) rats were obtained from Harlan (Blackthorn, Oxon, UK), housed at 4 per cage and given free access to rat chow and tap water. The investigation included 3 groups of rats entered into the study at 15 months of age: (i) untreated WKY controls; (ii) untreated SHRs, and (iii) SHRs treated chronically with the non-selective ET receptor antagonist, bosentan, obtained as a gift from Dr. Martine Clozel at Actelion Ltd., Switzerland. The animals received a diet of powdered chow with no addition (vehicle) or blended with bosentan to provide 100 mg/kg/day, with daily monitoring of food intake and body weight to confirm accuracy of dosage. A generally poor state of health was evident in all SHR rats, which were monitored daily to check for signs of tachypnea and laboured respiration. Animals reaching a point of significant deterioration before completion of the protocol were culled and removed from the study.

The animals in each group were terminally anaesthetised with isoflurane and their eyes were enucleated. One eye was used for trypsin digest examination of arteriole and capillary bed damage and the other for the measurement of mRNA expression of various genes using real-time RT-PCR. The entire study was performed in accordance with Home Office (UK) regulations.

RNA Isolation and Real-Time RT-PCR

Real-time RT-PCR was conducted according to previously described protocols [23]. Briefly, fresh dissected retinas were immediately submerged in RNAlater™ (Ambion Ltd., Cambridgeshire, UK) and stored at -20°C. Retinal RNA extraction was conducted using TRI Reagent™ (Sigma-Aldrich, Poole, Dorset, UK) and yield determined using a GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech, Cambridge, UK). RNA samples were reverse transcribed into cDNA and real-time RT-PCR was conducted for quantitative analysis of mRNA expression using the Lightcycler® rapid thermal cycler system (Roche Diagnostics Ltd., Lewes, UK) [24].

cDNA was amplified with sequence-specific primers for ET-1 (forward primer: 5' ACT TCT GCC ACC TGG ACA TC 3', reverse primer: 5' GGC TCG GAG TTC TTT GTC TG 3'), endothelin-converting enzyme-1 (ECE-1; forward primer: 5' CCG ACT CCA AGA ACT CCA AC 3', reverse primer: 5' ATC CAG GAT CTG CTG CAT CT 3'), ET_A (forward primer: 5' CCC TTT TTG CAG AAG TCG TC 3', reverse primer: 5' GTA CCA TGA CGA AGC CGA TT 3'), ET_B (forward primer: 5' CTG TGG GGA TCA CAG TGT TG 3', reverse primer: 5' GGG GCT TTC CTT TGT AGT CC 3'), fibronectin (forward primer: 5' ACT GAC GAA GAG CCC TTA C 3', reverse primer: 5' AGA TAA CCG CTC CCA TTC C 3'), laminin β 1 chain (forward primer: 5' AAA CCC TGA CCA ATG CCT CC 3', reverse primer: 5' TTG TAG CAG CTG CTC AGC TTT CC 3') or collagen IV (forward primer: 5' AGT TAC GCA AAT CCC TGT AAG 3', reverse primer: 5' GAG AAG AAC ATA GTG ATG CCC 3') to amplify 200-, 253- and 176-bp frag-

ments, respectively. Data were analysed with the LightCycler7 analysis software as described previously [24]. For each gene, PCR amplifications were performed in triplicate on at least 2 independent RT reactions. The housekeeping gene, 28S (forward: 5' TTG AAA ATC CCG GGG AGA G 3'; reverse 5' ACA TTG TTC CAA CAT GCC AG 3') was also examined using the same cDNAs and the mRNA expression on each of the groups was normalised to this level as has been previously described [24, 25].

cDNA was amplified using sequence-specific primers for ET-1 (forward primer: 5' ACT TCT GCC ACC TGG ACA TC 3'; reverse primer: 5' GGC TCG GAG TTC TTT GTC TG 3'), ECE-1 (forward primer: 5' CCG ACT CCA AGA ACT CCA AC 3'; reverse primer: 5' ATC CAG GAT CTG CTG CAT CT 3'), ET_A (forward primer: 5' CCC TTT TTG CAG AAG TCG TC 3'; reverse primer: 5' GTA CCA TGA CGA AGC CGA TT 3'), ET_B (forward primer: 5' CTG TGG GGA TCA CAG TGT TG 3'; reverse primer: 5' GGG GCT TTC CTT TGT AGT CC 3'), fibronectin (forward primer: 5' ACT GAC GAA GAG CCC TTA C 3'; reverse primer: 5' AGA TAA CCG CTC CCA TTC C 3'), laminin β 1 chain (forward primer: 5' AAA CCC TGA CCA ATG CCT CC 3'; reverse primer: 5' TTG TAG CAG CTG CTC AGC TTT CC 3') or collagen IV (forward primer: 5' AGT TAC GCA AAT CCC TGT AAG 3'; reverse primer: 5' GAG AAG AAC ATA GTG ATG CCC 3') to amplify 200-, 253- and 176-bp fragments, respectively. Data were analysed with the LightCycler analysis software as described previously [24]. For each gene, PCR amplifications were performed in triplicate on at least 2 independent RT reactions. The housekeeping gene, 28S (forward: 5' TTG AAA ATC CCG GGG AGA G 3'; reverse 5' ACA TTG TTC CAA CAT GCC AG 3') was also examined using the same cDNAs and the mRNA expression on each of the groups was normalised to this level as has been previously described [24, 25].

Vascular Digest Preparation

Following enucleation, whole eyes were fixed in 2% Carson's fixative for at least 24 h, after which they were placed in PBS. For retinal isolation, the eye was cut at the equator and the retina was dissected away from the optic cup, washed in 0.2 M Tris buffer and then placed in 3% trypsin solution (in 0.2 M Tris) at 37.4°C for 45 min using a previously presented protocol [26]. The entire retinal vascular tree was mounted onto a silane-treated slide as described [26]. The slides were then histologically stained with haematoxylin and light green.

The gross pathology was noted and the density of acellular capillaries was analyzed by an experienced investigator (in a blinded fashion) counting the number of acellular strands per field in the central retinal capillary beds (mean of 12 fields of view/specimen at $\times 250$ magnification). The fields were counted in a clockwise fashion at a fixed distance from the optic disc and expressed as acellular strands/square millimeter retina. The capillary density was determined using a Lucia G Image Analysis Software Program (Nikon UK Ltd.) which provided an area fraction of capillaries. Arteries/arterioles and veins/venules were not included in the fraction which was multiplied by total area measured to give the vascular density. The arterial and arteriolar diameter was quantified with at least 20 diameters measured for each vessel type in the central retina of each vascular tree. Vessels were chosen at random in fields collected in a clockwise fashion and diameters were measured at areas that were at least 50 μ m away from a branching point.

Statistical Analysis

Statistical analyses were performed using Instat (Graphpad software, San Diego, Calif., USA). Statistical significance was assessed by ANOVA with either Bonferroni or Tukey-Kramer used for post-hoc analysis. All data were expressed as the mean $\pm$ standard error of the mean (SEM), and $p < 0.05$ was considered statistically significant.

Results

General Features of Pressure-Overload-Induced Disease

As reported elsewhere, the systolic blood pressure (SBP) in SHR rats reaches a plateau before 20 weeks of age [27], and in an acute study, SBP was 85% higher in these SHR rats at 20 weeks (247.8 ± 9.7 mm Hg, mean $\pm$ SEM) than in age-matched WKY rats (129.5 ± 4.4 mm Hg, mean $\pm$ SEM). Prior treatment with bosentan for 10 weeks at 100 mg/kg/day did not reduce the systemic blood pressure in SHR rats (236.4 ± 7.0 mm Hg, mean $\pm$ SEM), and so, based on these data, it was considered unnecessary to measure SBP in this chronic study performed in the same population. Treatment was initiated during the phase of compensatory left ventricular hypertrophy in the SHR and prior to any evidence of decompensation, which occurs between the ages of 18 and 24 months [28]. In the absence of blood-pressure-lowering effects of bosentan, it was reasoned that contribution of the direct effects of ET-1 on late events in the vasculature could be prevented by treatment with bosentan initiated well before the decompensation phase and maintained until there was evidence of severe morbidity. Starting at 65 weeks of age, the average duration of treatment of SHR rats was 18 weeks, sampling being performed at 83 weeks (range, 81–87; $n = 9$). Untreated SHR rats were sampled at an average of 80 weeks (range, 72–88; $n = 6$) and WKY rats at 82 weeks ($n = 4$).

ET System mRNAs

In untreated SHR rats, ET-1, ECE and ET_B receptor mRNAs were decreased (from $p < 0.001$ to $p < 0.0001$) and ET_A receptor mRNA was increased ($p < 0.001$), when compared to that found in WKY rats (fig. 1). Bosentan treatment in SHR rats significantly decreased the expression of mRNA for ET-1 ($p < 0.05$), as well as the ET_A ($p < 0.0001$) and ET_B ($p < 0.05$) receptor subtypes (fig. 1).

BM Component mRNAs

Laminin β 1, fibronectin and collagen IV mRNAs were significantly increased in untreated SHR rats when com-

Fig. 1. mRNA expression of the ET system: ET-1 (a), ECE-1 (b), ET_A (c) and ET_B (d) in bosentan-treated SHR, untreated SHR and WKY rat retinas at ~21 months of age. In comparison to WKY rats, SHRs showed significantly higher values in ET_A receptor ex-

pression only. Bosentan treatment resulted in a significant reduction in the expression levels of ET-1, ET_A and ET_B. All data are presented as mean values $\pm$ SEM. ^a $p < 0.001$, ^b $p < 0.0001$: SHR vs. WKY; ^c $p < 0.05$, ^d $p < 0.0001$: treated vs. untreated SHR.

pared to WKY controls (from $p < 0.01$ to $p < 0.001$; fig. 2). Bosentan treatment in SHRs abolished the hypertension-associated increased expression of all 3 component mRNAs (fig. 2), such that the levels were comparable to those in WKY controls.

Trypsin Digest Analysis

WKY rats demonstrated normal retinal microvascular patterns with intact arterial systems and capillaries (fig. 3a). By contrast, some untreated SHRs showed focal areas of pathology, including focal sectors of acellular capillary formation (fig. 3b–e) especially downstream of arteriolar lesions (fig. 3b, c). Microvascular lesions such as acellular capillaries (fig. 3b), microaneurysm-like structures (fig. 3e) and smooth-muscle cell loss associat-

ed with vessel ballooning (fig. 3c, d) were also observed exclusively in nontreated SHRs. These lesions were never observed in the bosentan-treated SHRs (fig. 3f).

Quantitative pathology of the entire retinal microvascular tree was performed in each animal. Microaneurysms and regions of artery/arteriolar damage were observed in the untreated SHRs, but a quantitative assessment of this pathology revealed no significant difference between the groups (data not shown). This reflects the highly focal nature of these lesions. In comparisons of mean acellular capillaries, microvascular density and arteriolar diameter performed between untreated and bosentan-treated SHRs, no significant difference between any of these groups was observed (data not shown). However, a significant difference was found between un-

Fig. 2. mRNA expression of BM components: laminin- β (a), fibronectin (b) and collagen IV (c) in bosentan-treated SHR, untreated SHR and WKY rat retinas at ~21 months of age. In comparison to WKY rats, SHRs show significantly higher mRNA expression of all 3 BM components. Bosentan treatment resulted in a marked reduction in expression levels so that there was no significant difference between bosentan-treated SHRs and WKY controls. All data are presented as mean values $\pm$ SEM. * $p < 0.01$; ** $p < 0.001$; *** $p < 0.0001$.

treated SHRs and WKY rats, with fewer acellular capillaries and lower microvascular density in the SHR group (fig. 4a, b; $p < 0.001$). Bosentan did not affect the hypertension-associated increase in capillary death or reduction in vascular density in the SHR groups (fig. 4a, b). No statistical difference was detected in arterial or arteriolar diameter between the groups when considered in relation to the hypertensive state or effect of treatment with bosentan (data not shown).

Discussion

This study sought to evaluate retinopathy in the SHR and investigate the role of the vasoactive factor ET-1 in the pathogenesis of hypertension-induced retinal microvascular disease. It has provided a quantitative and qualitative morphological analysis of the retinal microvasculature of rats suffering systemic hypertension, augmenting pre-existing histological data of hypertensive retinopathy, and has extended beyond histological characterisation to examine pathological processes occurring at the level of mRNA expression. Data reported in a separate study on the same animal groups showed that SBP of the SHRs was

consistently higher than in the WKY rat controls [27], in keeping with previous studies [29–31]. Since cross-talk between receptors may allow one receptor to compensate for the other [32, 33], it was considered important to use a mixed ET_A/ET_B receptor antagonist. Bosentan, applied chronically at a dose which did not correct systemic hypertension, has provided information regarding the direct role of ET-1 in the autoregulated retinal microvasculature in the hypertensive state, without the confounding influence of a depressor effect.

In long-term maintenance, it is clear that systemic hypertension had significant pathophysiological effects on the retinal microvasculature. As revealed by quantitative RT-PCR, there was a significant increase in BM component mRNA expression in SHR when compared to normotensive WKY controls. Capillary BM thickening has been previously noted in hypertensive retinopathy [34], and this approach has been used as a surrogate marker for retinal capillary BM thickening in the context of diabetic retinopathy [35]. In the current study, upregulation of laminin β 1, fibronectin and collagen IV would appear to be hypertension-related phenomena. Indeed, these findings are in keeping with previous reports of increased BM component expression in the mesenteric small arter-

3

Fig. 4. Morphometric analysis of the retinal microvasculature by untreated SHRs, bosentan-treated SHRs and WKY controls. The number of acellular capillaries was analyzed (**a**) and revealed that WKY rats had significantly more acellular capillaries than their

SHR counterparts, irrespective of bosentan treatment. Quantification of the capillary density in retinal capillary beds between the animal groups (**b**) demonstrated higher density in the WKY when compared to SHR. Data are the mean $\pm$ SEM. * $p < 0.001$.

ies and cerebral microvessels of the SHR [36, 37] and subcutaneous resistance arteries from hypertensive humans [36, 38]. Increased production of these proteins and subsequent BM thickening may occur as an initial response to hypertension in an adaptive process to limit vessel dilatation occurring with progressive hypertension. Hypertension-related increases in BM component mRNA expression were prevented by treatment with bosentan, implicating a role for ET-1 in this pathology during hypertensive retinopathy which draws parallels with findings in diabetic rat retina [39].

Fig. 3. Trypsin digest evaluation of retinal microvasculature from bosentan SHRs, untreated SHRs and WKY rats. **a** Normal appearance of WKY rat retinal vessels showing few apparent abnormalities in the capillary beds (CB) or on the arterial side of the circulation (A = artery). Original magnification $\times 100$. **b, c** Retinas from untreated SHR rats showing areas of extensive pathology, including sectors of acellular capillary strands (small arrows) and microaneurysm-like structures (M) always adjacent to arterioles and areas of arterial/arteriolar ballooning indicating smooth-muscle loss (large arrows). Original magnification $\times 100$. Inset shows high magnification of acellular capillary strands (original magnification $\times 200$). **d** Smooth-muscle loss in some untreated SHR rats, evidenced by lack of haematoxylin-positive nuclei (arrows) and resultant ballooning of arterioles with abundant acellular capillaries downstream. Original magnification $\times 200$. **e** Large numbers of microaneurysm-like structures (arrows) in some untreated SHR rats. Original magnification $\times 100$. Inset shows a microaneurysm-like structure at higher magnification (original magnification $\times 200$). **f** Bosentan-treated SHRs demonstrated none of the lesions observed in untreated SHRs (A = artery).

Bosentan treatment altered mRNA expression for ET-1 and both ET receptor subtypes in SHRs. Effects of bosentan on the ET system and hypertension-induced pathology are not completely in agreement [40], showing attenuation [41, 42], no change and even increases [43] in ET system mRNA expression. Tissue-specific effects of bosentan have also been observed [43]. The reported increases in circulating levels of ET-1 following chronic treatment with bosentan may be due to attenuated clearance: persistently high ET-1 levels are reported to down-regulate the number of both ET_A and ET_B receptors [14]. Also, protein kinase C (PKC) inhibitors attenuate ET_B receptor mRNA expression [44] and ET-1 is reported to regulate its own transcription through ET_B-mediated PKC [45]; if ET-1 expression were inhibited by bosentan, the activation of PKC would be reduced, which may impact on the transcription of ET receptors. Bosentan also reduces the expression of ECE-1, and ECE-1 mRNA is reported to be regulated by specific kinase-mediated signalling systems, particularly through PKC [46].

In the current study, although several parameters of the ET system were attenuated in long-term hypertensive SHRs, ET_A receptor expression was higher when compared to WKY rat controls. ET_B in addition to ET_A receptor blockade by bosentan has been shown to mediate ET-1-induced collagen type IV and fibronectin synthesis in retinas from diabetic and galactose-fed rats [47]. However, specific blockade of the ET_A receptor in coronary vasculature has been shown to be equally effective in this capacity [48] and may dictate a dominant role for

this receptor subtype in modulating ET-induced expression of BM components. Due to the hypertrophic effects mediated by ET_B receptors on vascular cells [19] and the apparent tissue-specific involvement of the ET system, this does not advocate the use of ET_A selective over ET_A/ET_B mixed receptor antagonists to normalise BM thickening in retinal microvasculature. Significantly, however, treatment with bosentan has potential to ameliorate retinal microangiopathy in diabetic rats [20, 22], which shares pathologic similarities to hypertensive retinopathy in terms of BM thickening and capillary fallout [35]. Although hypertension-induced retinopathy and diabetic retinopathy represent distinct disease entities, they share similar manifestations in terms of structural change, and so a theory of time-dependent ET action may also apply to this study. When ET-1 is intravitreally injected into control and 5- to 7-day bosentan-treated rats that had been diabetic for 3 months, there is an ET-1-mediated reduction in retinal arterial diameter and this response is prevented by bosentan [49]. However, there is no effect on retinal oxygenation as determined by MRI [49].

A third of the untreated SHR displayed extensive arteriolar damage, with areas of arteriolar ballooning and acellularity in capillary beds, probably occurring as a direct result of vascular smooth-muscle cell loss. Some of these animals also displayed microaneurysm-like structures and areas of hypercellularity suggestive of proliferation of vascular endothelial cells, lesions which are extremely rare in adult rats. The occurrence of such features in the untreated SHR group and not in the bosentan-treated rats would support potential ET-1 involvement in

their pathogenesis. Vascular smooth-muscle loss observed in long-term hypertension is consistent with previous findings of increased apoptosis in the hypertensive rat heart, brain, kidney and arteries, where vascular myocyte death modulates remodelling [50, 51]. ET is noted to be a candidate modulator of apoptosis [52].

Quantification of microvascular abnormalities failed to demonstrate significant changes between treated and untreated SHRs. By comparison, the WKY groups showed greater vascular density and, surprisingly, greater numbers of acellular capillaries, although the arterial and arteriolar diameters were not significantly different between the groups. This may reflect an innate difference between the WKY and SHR strains. Reduced capillary density in the SHR group is in keeping with previous studies which found reduced microvascular density in retina of hypertensive patients [53]. From the results of the current investigation, it is evident that hypertensive changes are localised in relatively small sectors of a microvascular bed, almost exclusively downstream from a damaged arteriole. Overall quantification of the microvascular tree has failed to take this localised pathology into account, although such areas of focal retinal ischaemia are likely to have a significant impact on neovascularisation and overall retinal function.

Acknowledgements

The authors would like to acknowledge the support of the British Heart Foundation, The Wellcome Trust, Diabetes UK and Action Research.

References

- Wong TY, Hubbard LD, Klein R, Marino EK, Kronmal R, Sharrett AR, Siscovick DS, Burke G, Tielsch JM: Retinal microvascular abnormalities and blood pressure in older people: The Cardiovascular Health Study. *Br J Ophthalmol* 2002;86:1007–1013.
- Ashton N, Peltier S, Garner A: Experimental hypertensive retinopathy in the monkey. *Trans Ophthalmol Soc UK* 1969;88:167–186.
- Garner A, Ashton N, Tripathi R, Kohner EM, Bulpitt CJ, Dollery CT: Pathogenesis of hypertensive retinopathy: an experimental study in the monkey. *Br J Ophthalmol* 1975; 59:3–44.
- Crook ED: The role of hypertension, obesity, and diabetes in causing renal vascular disease. *Am J Med Sci* 1999;317:183–188.
- Klein R, Klein BE, Moss SE: The relation of systemic hypertension to changes in the retinal vasculature: The Beaver Dam Eye Study. *Trans Am Ophthalmol Soc* 1997;95:329–348; discussion 348–350.
- Kawamura H, Oku H, Li Q, Sakagami K, Puro DG: Endothelin-induced changes in the physiology of retinal pericytes. *Invest Ophthalmol Vis Sci* 2002;43:882–888.
- Webb DJ, Strachan FE: Clinical experience with endothelin antagonists. *Am J Hypertens* 1998;11:71S–79S.
- Persson PB: Renal blood flow autoregulation in blood pressure control. *Curr Opin Nephrol Hypertens* 2002;11:67–72.
- Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S: Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 1990;348:730–732.
- Rich S, McLaughlin VV: Endothelin receptor blockers in cardiovascular disease. *Circulation* 2003;108:2184–2190.
- McDonald D, Bailie J, Archer D, Chakravarthy U: Molecular characterization of endothelin receptors and the effect of insulin on their expression in retinal microvascular pericytes. *J Cardiovasc Pharmacol* 1995; 26(suppl 3):S287–S289.
- 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.
- Meyer P, Flammer J, Luscher TF: Endothelin-dependent regulation of the ophthalmic microcirculation in the perfused porcine eye: role of nitric oxide and endothelins. *Invest Ophthalmol Vis Sci* 1993;34:3614–3621.

- 14 Clozel M, Breu V, Gray GA, Kalina B, Loffler BM, Burri K, Cassal JM, Hirth G, Muller M, Neidhart W, et al: Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist. *J Pharmacol Exp Ther* 1994;270:228–235.
- 15 Li JS, Lariviere R, Schiffrin EL: Effect of a nonselective endothelin antagonist on vascular remodeling in deoxycorticosterone acetate-salt hypertensive rats. Evidence for a role of endothelin in vascular hypertrophy. *Hypertension* 1994;24:183–188.
- 16 Krum H, Liew D: Current status of endothelin blockade for the treatment of cardiovascular and pulmonary vascular disease. *Curr Opin Investig Drugs* 2003;4:298–302.
- 17 Spiers JP, Kelso EJ, Siah WF, Edge G, Song G, McDermott BJ, Hennessy M: Alterations in vascular matrix metalloproteinase due to ageing and chronic hypertension: effects of endothelin receptor blockade. *J Hypertens* 2005;23:1717–1724.
- 18 Doggrel SA: The therapeutic potential of endothelin-1 receptor antagonists and endothelin-converting enzyme inhibitors on the cardiovascular system. *Exp Opin Investig Drugs* 2002;11:1537–1552.
- 19 Schiffrin EL, Lariviere R, Li JS, Sventek P, Touyz RM: Endothelin-1 gene expression and vascular hypertrophy in DOCA-salt hypertension compared to spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol Suppl* 1995;22:S188–S190.
- 20 Deng D, Evans T, Mukherjee K, Downey D, Chakrabarti S: Diabetes-induced vascular dysfunction in the retina: role of endothelins. *Diabetologia* 1999;42:1228–1234.
- 21 Evans T, Deng DX, Chen S, Chakrabarti S: Endothelin receptor blockade prevents augmented extracellular matrix component mRNA expression and capillary basement membrane thickening in the retina of diabetic and galactose-fed rats. *Diabetes* 2000;49:662–666.
- 22 Chen S, Khan ZA, Cukiernik M, Chakrabarti S: Differential activation of NF- κ B and AP-1 in increased fibronectin synthesis in target organs of diabetic complications. *Am J Physiol Endocrinol Metab* 2003;284:E1089–E1097.
- 23 Feeney SA, Simpson DA, Gardiner TA, Boyle C, Jamison P, Stitt AW: Role of vascular endothelial growth factor and placental growth factors during retinal vascular development and hyaloid regression. *Invest Ophthalmol Vis Sci* 2003;44:839–847.
- 24 Simpson DA, Feeney S, Boyle C, Stitt AW: Retinal VEGF mRNA measured by SYBR green I fluorescence: a versatile approach to quantitative PCR. *Mol Vis* 2000;6:178–183.
- 25 Malik KJ, Chen CD, Olsen TW: Stability of RNA from the retina and retinal pigment epithelium in a porcine model simulating human eye bank conditions. *Invest Ophthalmol Vis Sci* 2003;44:2730–2735.
- 26 Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H: Advanced glycation end products (AGEs) co-localize with age receptors in the retinal vasculature of diabetic and of age-infused rats. *Am J Pathol* 1997;150:523–531.
- 27 Bell D, Kelso EJ, Argent CC, Lee GR, Allen AR, McDermott BJ: Temporal characteristics of cardiomyocyte hypertrophy in the spontaneously hypertensive rat. *Cardiovasc Pathol* 2004;13:71–78.
- 28 Bing OH, Conrad CH, Boluyt MO, Robinson KG, Brooks WW: Studies of prevention, treatment and mechanisms of heart failure in the aging spontaneously hypertensive rat. *Heart Fail Rev* 2002;7:71–88.
- 29 Adams MA, Bobik A, Korner PI: Differential development of vascular and cardiac hypertrophy in genetic hypertension: relation to sympathetic function. *Hypertension* 1989;14:191–202.
- 30 Li JS, Schiffrin EL: Effect of chronic treatment of adult spontaneously hypertensive rats with an endothelin receptor antagonist. *Hypertension* 1995;25:495–500.
- 31 Tsotetsi OJ, Woodiwiss AJ, Netjhardt M, Qubu D, Brooksbank R, Norton GR: Attenuation of cardiac failure, dilatation, damage, and detrimental interstitial remodeling without regression of hypertrophy in hypertensive rats. *Hypertension* 2001;38:846–851.
- 32 Clozel M: Endothelin receptor antagonists: current status and perspectives. *J Cardiovasc Pharmacol* 2000;35:S65–S68.
- 33 Roux S, Qiu C, Sprecher U, Osterwalder R, Clozel JP: Protective effects of endothelin receptor antagonists in dogs with aortic cross-clamping. *J Cardiovasc Pharmacol* 1999;34:199–205.
- 34 Fluckiger W, Perrin IV, Rossi GL: Morphometric studies on retinal microangiopathy and myocardiopathy in hypertensive rats (SHR) with induced diabetes. *Virchows Arch B Cell Pathol Incl Mol Pathol* 1984;47:79–94.
- 35 Stitt A, Gardiner TA, Alderson NL, Canning P, Frizzell N, Duffy N, Boyle C, Januszewski AS, Chachich M, Baynes JW, Thorpe SR, Anderson NL: The age inhibitor pyridoxamine inhibits development of retinopathy in experimental diabetes. *Diabetes* 2002;51:2826–2832.
- 36 Intengan HD, Thibault G, Li JS, Schiffrin EL: Resistance artery mechanics, structure, and extracellular components in spontaneously hypertensive rats: effects of angiotensin receptor antagonism and converting enzyme inhibition. *Circulation* 1999;100:2267–2275.
- 37 Saouaf R, Takasaki I, Eastman E, Chobanian AV, Brecher P: Fibronectin biosynthesis in the rat aorta in vitro: changes due to experimental hypertension. *J Clin Invest* 1991;88:1182–1189.
- 38 Intengan HD, Schiffrin EL: Vascular remodeling in hypertension: roles of apoptosis, inflammation, and fibrosis. *Hypertension* 2001;38:581–587.
- 39 Chen S, Khan ZA, Karmazyn M, Chakrabarti S: Role of endothelin-1, sodium hydrogen exchanger-1 and mitogen-activated protein kinase (MAPK) activation in glucose-induced cardiomyocyte hypertrophy. *Diabetes Metab Res Rev* 2007;23:356–367.
- 40 Schiffrin EL: State-of-the-art lecture: role of endothelin-1 in hypertension. *Hypertension* 1999;34:876–881.
- 41 Gomez-Garre D, Largo R, Liu XH, Gutierrez S, Lopez-Armada MJ, Palacios I, Egido J: An orally active ETA/ETB receptor antagonist ameliorates proteinuria and glomerular lesions in rats with proliferative nephritis. *Kidney Int* 1996;50:962–972.
- 42 Sanchez R, MacKenzie A, Farhat N, Nguyen TD, Stewart DJ, Mercier I, Calderone A, Thorin E: Endothelin B receptor-mediated regulation of endothelin-1 content and release in cultured porcine aorta endothelial cell. *J Cardiovasc Pharmacol* 2002;39:652–659.
- 43 Chen S, Evans T, Mukherjee K, Karmazyn M, Chakrabarti S: Diabetes-induced myocardial structural changes: role of endothelin-1 and its receptors. *J Mol Cell Cardiol* 2000;32:1621–1629.
- 44 Uddman E, Adner M, Edvinsson L: Protein kinase C inhibitors decrease endothelin ET_B receptor mRNA expression and contraction during organ culture of rat mesenteric artery. *Eur J Pharmacol* 2002;452:215–222.
- 45 Saito S, Hirata Y, Imai T, Marumo F: Auto-crine regulation of the endothelin-1 gene in rat endothelial cells. *J Cardiovasc Pharmacol* 1995;26(suppl 3):S84–S87.
- 46 Uchida K, Uchida S, Nitta K, Yumura W, Nihei H: Regulated expression of endothelin-converting enzymes in glomerular endothelial cells. *J Am Soc Nephrol* 1997;8:580–585.
- 47 Rizvi MA, Katwa L, Spadone DP, Myers PR: The effects of endothelin-1 on collagen type I and type III synthesis in cultured porcine coronary artery vascular smooth muscle cells. *J Mol Cell Cardiol* 1996;28:243–252.
- 48 De Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, Vane JR: Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. *Proc Natl Acad Sci USA* 1988;85:9797–9800.
- 49 Roberts R, Luan H, Berkowitz BA: Blocking ET-1 receptors does not correct subnormal retinal oxygenation response in experimental diabetic retinopathy. *Invest Ophthalmol Vis Sci* 2006;47:3550–3555.
- 50 Hamet P, Richard L, Dam TV, Teiger E, Orlov SN, Gaboury L, Gossard F, Tremblay J: Apoptosis in target organs of hypertension. *Hypertension* 1995;26:642–648.
- 51 Dickhout JG, Lee RM: Apoptosis in the muscular arteries from young spontaneously hypertensive rats. *J Hypertens* 1999;17:1413–1419.
- 52 Cattaruzza M, Dimigen C, Ehrenreich H, Hecker M: Stretch-induced endothelin B receptor-mediated apoptosis in vascular smooth muscle cells. *FASEB J* 2000;14:991–998.
- 53 Houben AJ, Canoy MC, Paling HA, Derhaag PJ, de Leeuw PW: Quantitative analysis of retinal vascular changes in essential and renovascular hypertension. *J Hypertens* 1995;13:1729–1733.