0022-3565/11/3371-9–15
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
U.S. Government work not protected by U.S. copyright
JPET 337:9–15, 2011

Vol. 337, No. 1
175380/3672045
Printed in U.S.A.

Endothelial Endothelin B Receptor-Mediated Prevention of Cerebrovascular Remodeling Is Attenuated in Diabetes Because of Up-Regulation of Smooth Muscle Endothelin Receptors

Aisha I. Kelly-Cobbs, Alex K. Harris, Mostafa M. Elgebaly, Weiguo Li, Kamakshi Sachidanandam, Vera Portik-Dobos, Maribeth Johnson, and Adviye Ergul

Departments of Physiology (A.I.K.-C., W.L., V.P.-D., A.E.) and Biostatistics (M.J.), Medical College of Georgia, Augusta, Georgia; Program in Clinical and Experimental Therapeutics, University of Georgia College of Pharmacy, Augusta, Georgia (A.K.H., M.M.E., K.S.); and Charlie Norwood Veterans Administration Medical Center, Augusta, Georgia (A.E.)

Received September 21, 2010; accepted December 21, 2010

ABSTRACT

Structure and function of the cerebrovasculature is critical for ischemic stroke outcome. We showed that diabetes causes cerebrovascular remodeling by activation of the endothelin A (ET_A) receptors. The goal of this study was to test the hypotheses that vasculoprotective endothelial ET_B receptors are decreased and pharmacological inhibition of the ET_B receptor augments vascular remodeling of middle cerebral arteries (MCAs) in type 2 diabetes. MCA structure, matrix metalloprotease (MMP) activity, and matrix proteins as well as ET_A and ET_B receptor profiles were assessed in control Wistar and diabetic Goto-Kakizaki rats treated with vehicle, the ET_B receptor antagonist (2*R*,3*R*,4*S*)-4-(1,3-benzodioxol-5-yl)-1-[2-[(2,6-diethylphenyl)amino]-2-oxoethyl]-2-(4-propoxyphenyl)pyrrolidine-3-carboxylic acid (A192621) (30 mg/kg/day), or the dual ET receptor antagonist bosentan (100 mg/kg/day) for 4 weeks. Diabetes increased vascular smooth muscle (VSM) ET_A and ET_B receptors; the increase was prevented by chronic bosentan treatment. MCA wall thickness was increased in diabetes, and this was associated with increased MMP-2 activity and collagen deposition but reduced MMP-13 activity. Because of up-regulation of VSM ET receptors in diabetes, selective ET_B receptor antagonism with A192621 blunts this response, and combined ET_A and ET_B receptor blockade with bosentan completely prevents this response. On the other hand, A192621 treatment augmented remodeling in control animals, indicating a physiological protective role for this receptor subtype. Attenuation of changes in ET receptor profile with bosentan treatment suggests that ET-1 has a positive feedback on the expression of its receptors in the cerebrovasculature. These results emphasize that ET receptor antagonism may yield different results in healthy and diseased states.

Introduction

The extracellular matrix (ECM) is a dynamic scaffold with the purpose of maintaining the integrity of blood vessels as well as other tissues. Disruption of the delicate balance of ECM synthesis/degradation leads to structural and functional changes in the vasculature often associated with complications of diabetes (Kannel and McGee, 1979; Ding and Triggle, 2010). Hypertrophic vascular remodeling is associated with both human and experimental diabetes and in both the microvasculature and macrovasculature (Rumble et al., 1997; Harris et al., 2005; Calcutt et al., 2009). However, the mechanistic regulation of these events and how it is altered by diabetes is not well understood.

The potent vasoconstrictor ET-1, which is elevated in human and experimental diabetes, has both vasoactive and mitogenic properties that are carried out by two distinct receptor subtypes (Ergul et al., 2005). ET_A receptors located on VSM promote vasoconstriction and cellular proliferation in many cell types. ET_B receptors have a putative role of being primarily vasodilatory as well as vasculoprotective

This study was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grant R01-DK074385; American Recovery and Reinvestment Act Supplement R01-DK074385-05S1]; an American Heart Association Established Investigator Award [Award 0740002N]; and a Veterans Administration Merit Award (to A.E.).

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

doi:10.1124/jpet.110.175380.

ABBREVIATIONS: ECM, extracellular matrix; ET, endothelin; eET, endothelial ET receptor; VSM, vascular smooth muscle; vET, VSM ET receptor; GK, Goto-Kakizaki; MCA, middle cerebral artery; MMP, matrix metalloprotease; TIMP, tissue inhibitor of metalloprotease; W/L, wall/lumen; A192621, (2*R*,3*R*,4*S*)-4-(1,3-benzodioxol-5-yl)-1-[2-[(2,6-diethylphenyl)amino]-2-oxoethyl]-2-(4-propoxyphenyl)pyrrolidine-3-carboxylic acid.

(Murakoshi et al., 2002). However, these receptors produce heterogeneous responses depending on their localization (Mizuguchi et al., 1997). ET_B receptors, located on endothelial cells, do indeed elicit vasodilation via cGMP. However ET_B receptors, located on VSM, produce effects akin to the ET_A (Schneider et al., 2007; Pollock, 2010). Mounting evidence suggests that the balance of these receptors within the vasculature may be tipped toward the contractile ET_B phenotype under pathological circumstances (Ikeda et al., 2001; Stenman et al., 2002; Alabadi et al., 2004; Matsumoto et al., 2004; Ortmann et al., 2004). We have demonstrated previously that chronic blockade of the ET_A receptor in type 2 diabetes decreases vascular remodeling by attenuation of MMP dysregulation (Harris et al., 2005). However, whether and to what extent the ET_B receptors contribute to this process and how diabetes affects the ET receptor profile that ultimately influences the net effect of ET-1 on cerebrovascular structure remained unknown. Building on past studies by us and others that demonstrate ET_B receptors are vasculoprotective (Murakoshi et al., 2002; Sachidanandam et al., 2007), the current study tested the hypotheses that endothelial ET_B receptors are decreased in diabetes and pharmacological inhibition of the ET_B receptor augments vascular remodeling of middle cerebral arteries (MCAs). The rationale was that better understanding of receptor profile and function in disease states will determine the use of ET receptor subtype-selective or nonselective antagonists in the prevention of diabetic complications.

Materials and Methods

Animal Studies. The Medical College of Georgia Institutional Animal Care and Use Committee approved all protocols. Male Wistar (Harlan, Indianapolis, IN) and Goto-Kakizaki (GK; in-house bred, derived from the Tampa colony) rats were used for all studies. All animals were individually housed at the Medical College of Georgia's animal care facility, allowed access to food and water ad libitum, and maintained on a 12/12-h light/dark cycle. During housing, weight and blood glucose measurements were performed twice weekly. Glucose measurements were taken from the tail vein and measured on a commercially available glucose meter (AccuChek; Roche Diagnostics, Indianapolis, IN). Mean arterial blood pressure (mm Hg) was measured either by telemetry or the tail-cuff method, which we validated on animals with telemetric implants. Animals on telemetry had transmitters implanted at week 12 and were allowed to recover for 2 weeks. Mean arterial blood pressure was recorded from weeks 14 through 18. Tail-cuff blood pressure was measured on animals not on telemetry following the same time course (Elgebaly et al., 2007). Results are given as the average of the readings during the treatment period. The spontaneous onset of diabetes was approximately 6 weeks of age. Starting at 14 weeks of age, animals received either vehicle, the ET_B-selective antagonist (2*R*,3*R*,4*S*)-4-(1,3-benzodioxol-5-yl)-1-[2-[(2,6-diethylphenyl)amino]-2-oxoethyl]-2-(4-propoxyphenyl)pyrrolidine-3-carboxylic acid (A192621) (30 mg/kg/day daily by oral gavage), or the dual ET_A/ET_B receptor antagonist bosentan (100 mg/kg/day food admixture) for 4 weeks. Structures for these compounds were reviewed in Battistini et al. (2006). This treatment paradigm was based on our previous studies (Harris et al., 2005, 2008; Sachidanandam et al., 2007, 2008). Treatment was maintained until sacrifice at 18 weeks of age. To monitor the vascular changes over the course of the disease, additional control and diabetic animals at 10 weeks (shortly after the onset of diabetes) and 14 weeks (at the start of treatment) were included for morphometry. Animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture.

Vessel Morphometry. Upon sacrifice, the brain was removed and one MCA was perfused with Histogel (Richard Allen Scientific, Kalamazoo, MI), then excised and embedded in the same matrix. Upon gelling of the matrix, the embedded vessel was placed in 10% formalin for storage. The other MCA was excised, snap-frozen in liquid nitrogen, and stored at −80°C for protein studies. For morphometric analysis, 4-μm vessel cross-sections were stained with Masson's trichrome stain. Slides were viewed using an Axiovert microscope (Carl Zeiss Inc., Thornwood, NY), and wall thickness, lumen, and outer diameter were measured using SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI). In additional control and diabetic animals, MCAs were mounted on the pressurized arteriograph, and after equilibration vessels were fixed in formalin using the quick-transfer freezing chamber at a constant 80-mm Hg intraluminal pressure (Living Systems Instrumentation, Burlington, VT) to confirm that there were no variations in vascular structure caused by inconsistencies that might have occurred during manual perfusion of Histogel.

Tissue Homogenization and Gelatin Zymography. Snap-frozen MCAs were homogenized, and gelatin zymography was performed on them as described previously (Harris et al., 2005). Gelatinolytic activity was assessed by densitometric analysis (Gel-Pro version 3.1; Media Cybernetics, Carlsbad, CA). Tissue inhibitor of metalloproteinase-2 (TIMP-2) levels were measured by enzyme-linked immunosorbent assay (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Immunoblotting. MCAs were homogenized in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride) and sonicated at room temperature for 8- to 10-s bursts. Samples were placed on ice between sonications. Total protein was measured using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Vascular extracts (20 μg) were separated on 10% SDS gels and transferred to a nitrocellulose membrane in Tris-glycine transfer buffer supplemented with 20% methanol. The immunoblots were blocked for 1 h in 5% bovine serum albumin diluted in 0.2 M Tris-base, 1.4 M NaCl, 0.1% Tween 20, and 0.02% NaN_3. MMP-2 and MMP-13 antibodies were from Calbiochem (Cambridge, MA). Collagen type-1 levels were quantified by slot-blot analysis using an antibody from BD Biosciences Transduction Laboratories (San Jose, CA). To determine the effect of diabetes and bosentan treatment on ET receptor expression, in a separate set of vehicle- and bosentan-treated control and diabetic animals, one MCA was frozen intact, and in the other MCA endothelium was denuded by passing an air bubble after the vessel was mounted on a pressurized arteriograph (Miller et al., 2001). To identify VSM ET receptors (vETs) and endothelial ET receptors (eETs), endothelium-intact (vET_A, vET_B, and eET_B) and endothelium-denuded (vET_A and vET_B) MCAs were used in immunoblotting experiments using antibodies for ET_A and ET_B receptors as recommended by the manufacturer (Alomone Labs, Jerusalem, Israel). Specificity of the bands was confirmed by using increasing concentrations of competing peptide for each antibody. eET_B was calculated as the difference in band intensities of intact and denuded vessels. In all immunoblotting experiments, bands were visualized using ChemiGlow and images were captured using Alpha Imager from Alpha Innotech (San Leandro, CA). All blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading.

Statistical Analysis. A rank transformation was applied to the data before analysis to address issues of non-normality and heterogeneity of variance (Conover and Iman, 1981). A 2×3 analysis of variance was used to investigate the main effects of disease (Wistar versus GK) and drug (vehicle versus bosentan versus A192621) and the interaction between disease and drug. vET_A, vET_B, and eET_B data were not available on rats treated with A192621, so a 2×2 analysis of variance was used to investigate the main effects of

TABLE 1
Physiological parameters of animal groups
Results are given as mean ± S.E.M.

	Control			Diabetes		
	Vehicle (n = 11)	A192621 (n = 3)	Bosentan (n = 8)	Vehicle (n = 11)	A192621 (n = 8)	Bosentan (n = 8)
Body weight, g	485 ± 16	406 ± 2#	418 ± 7#	352 ± 6*	320 ± 5*#	333 ± 5*#
Systolic blood pressure, mm Hg	129 ± 5	159 ± 5#	142 ± 6	142 ± 3	157 ± 6#	157 ± 6
Diastolic blood pressure, mm Hg	92 ± 3	100 ± 4#	95 ± 5	89 ± 5	100 ± 5#	96 ± 3
Blood glucose, mg/dl	108 ± 4	104 ± 5	93 ± 9	161 ± 17*	152 ± 8*	194 ± 27*

# $P < 0.0001$ vs. vehicle; * $P < 0.0001$ vs. control.

disease (Wistar versus GK) and drug (vehicle versus bosentan) and the interaction between disease and drug. Effects were considered statistically significant at $p < 0.05$. SAS version 9.2 (SAS Institute, Cary, NC) was used for all analyses.

Results

Animal Data. Metabolic parameters for control and diabetic (GK) animals are summarized in Table 1. Diabetic animals were significantly smaller than control. Both bosentan and A192621 blockade caused a reduction of body weight in control and diabetic animals. There was no difference in food or water intake of the animals. GK animals displayed higher blood glucose that was not affected by treatments. Blood pressure was similar between control and diabetic animals. Treatment with A192621 elevated blood pressure in both groups.

Morphology of Middle Cerebral Arteries. Diabetic animals exhibited significantly increased wall thickness and wall/lumen (W/L) ratio (Fig. 1). There was a disease and treatment interaction such that both bosentan and A192621 increased wall thickness and W/L ratio in controls but decreased them in diabetic animals. To monitor the temporal development of vascular remodeling in this model, morphometry was repeated at three different time points after the onset of diabetes. Wall thickness increased over time in all groups, and there was no difference between control and diabetic rats at 10 or 14 weeks (Fig. 2). However, at 18 weeks diabetic rats had significantly thicker walls and increased W/L ratio.

MMP Protein Expression and Activity. MMP-2 protein was more abundant in diabetic animals compared with controls, and both receptor antagonists reduced MMP-2 in diabetic animals but not in the control group (Fig. 3A). MMP-2 activity was slightly greater in diabetic animals. Bosentan, but not A192621, lowered MMP-2 activity to control levels. It is noteworthy that selective ET_B blockade with A192621 increased enzyme activity in control but not diabetic rats, indicating a disease and drug interaction (Fig. 3B). TIMP-2 levels, an endogenous inhibitor of MMP-2, were assessed in MCAs by enzyme-linked immunosorbent assay. There was no disease or treatment effect (Fig. 3C).

Fibrillar collagenase MMP-13 levels were measured by immunoblotting. There was a trend for lower MMP-13 protein in diabetic animals, and bosentan but not A192621 treat-

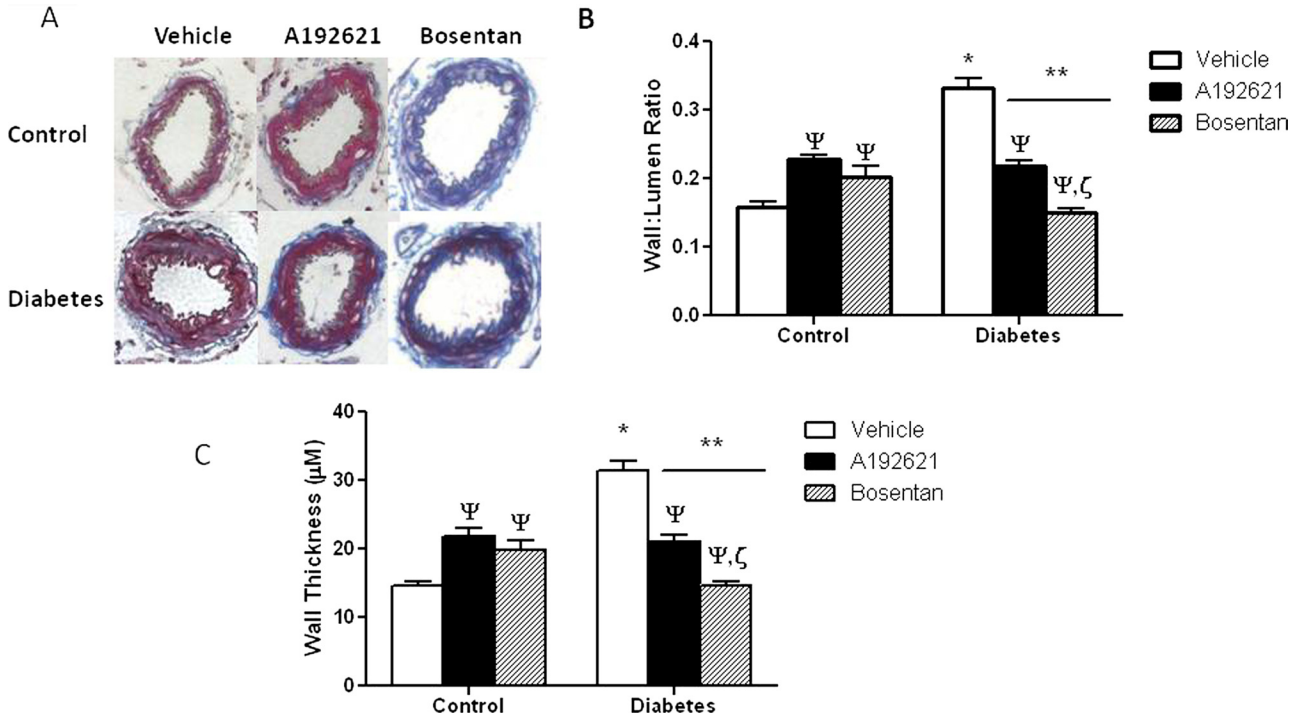

Fig. 1. A, representative cross-sections of Masson trichrome stained MCAs. B and C, summary of W/L ratios (B) and wall thickness (C) in diabetic and control MCAs with and without ET receptor antagonism. Results are given as mean ± S.E.M., n = 6–8 in all groups except control + A192621 = 3. *, $p < 0.05$ versus control; **, $p < 0.0001$ disease and treatment interaction. ξ, $p < 0.05$ versus vehicle; ψ, $p < 0.05$ versus diabetes + A192621.

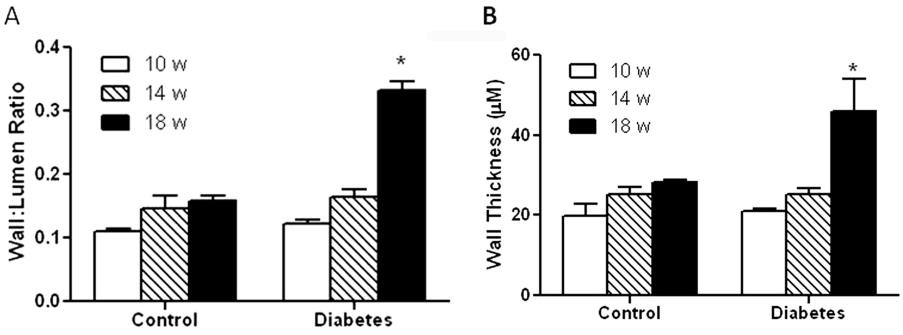

Fig. 2. MCA morphology shortly after onset of diabetes (10 weeks), at the start of treatment (14 weeks), and at the end of the treatment (18 weeks). Summary of W/L ratio (A) and wall thickness (B) indicate that MCA structure is comparable at the beginning of the treatment but by 18 weeks diabetic animals develop significant remodeling. Results are given as mean ± S.E.M., $n = 4–8$. *, $p < 0.001$ versus other groups.

ment increased enzyme levels (Fig. 4A). In the control group, on the other hand, selective ET_B blockade with A192621 decreased MMP-13, indicating that there is a disease and treatment interaction so that A192621 treatment is affecting vessels from control rats differently. In accordance with the MMP-13 levels, collagen type 1 levels were higher in diabetic animals (Fig. 4B). Dual blockade with bosentan normalized collagen deposition, whereas A192621 was not as effective. In the control group, bosentan had no effect, but A192621 treatment increased collagen levels.

ET Receptor Expression. VSM ET_A and ET_B receptors, as determined by thicker immunoreactive bands detected on endothelium-denuded samples, were increased in diabetic animals (Fig. 5, A–C). Bosentan treatment reduced receptor expression to control levels. Endothelial ET_B receptors were similar between groups, and bosentan treatment had no effect (Fig. 5D).

Discussion

Maintenance of extracellular matrix homeostasis is an important event to ensure proper structure and function of vessels. Numerous studies have demonstrated medial hypertrophy and increased media/lumen ratios in the peripheral vessels in diabetes (Cooper et al., 1997; Rumble et al., 1997; Gilbert et al., 2000; Intengan and Schiffrin, 2000). However, much less is known about the effects of diabetes on the cerebrovasculature where blood flow is tightly regulated to maintain a relatively stable cerebral perfusion. We have demonstrated previously that experimental type 2 diabetes induces vascular remodeling of MCAs and ET_A receptor blockade partially prevented this response, implicating a role for ET-1 in the regulation of this process (Harris et al., 2005). Given that the ET_B receptor subtype is a clearance receptor and may serve to balance the ET_A receptor-mediated actions

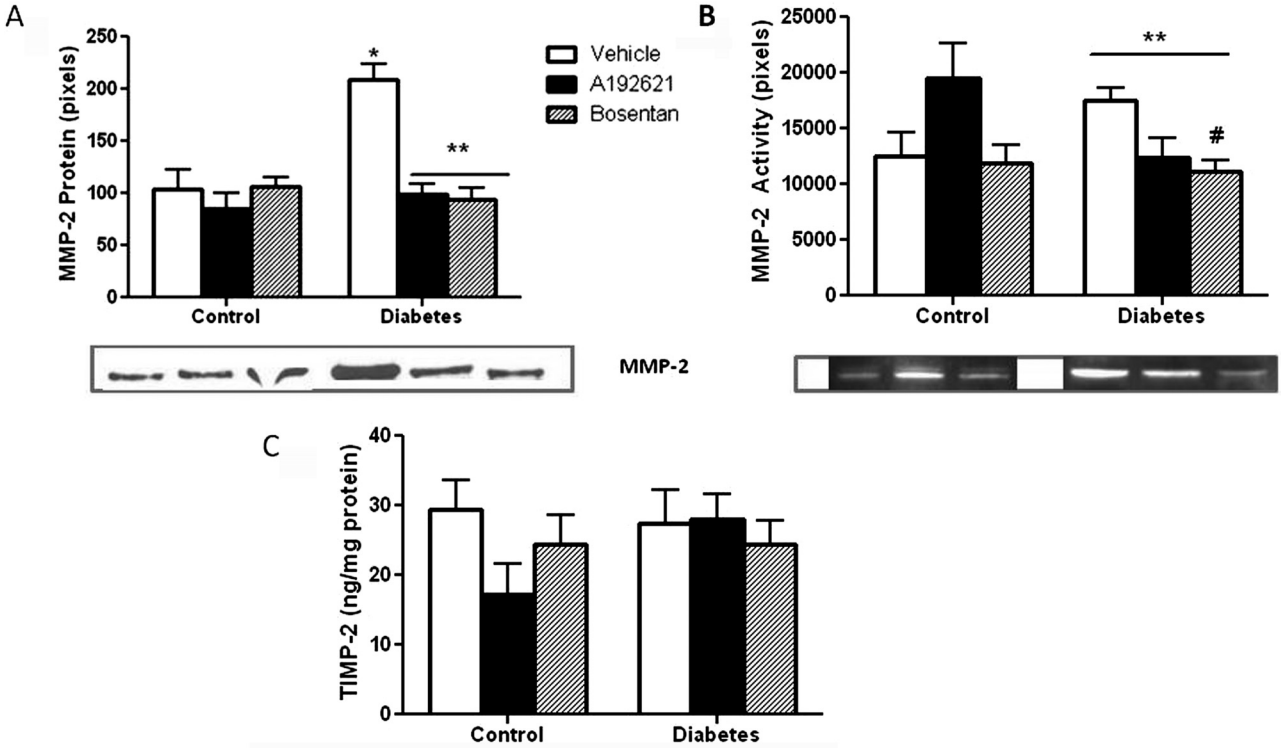

Fig. 3. MMP-2 protein and activity levels in MCAs from diabetic and control MCAs. A, MMP-2 protein was elevated in diabetes and was restored to control levels by ET receptor blockade. Representative immunoblot is shown below the histogram. B, MMP-2 activity was also greater in diabetes and reduced to control levels by either treatment. It is noteworthy that A192621 increased MMP-2 activity in control animals without a change in protein levels. A representative zymogram is shown below the histogram. C, Levels of TIMP-2 protein, endogenous MMP-2 inhibitor, were not different among groups. Results are given as mean ± S.E.M., $n = 6–8$ in all groups except control + A192621 = 3. *, $p < 0.05$ versus control. **, $p = 0.0005$ disease and treatment interaction. #, $p < 0.05$ versus diabetes.

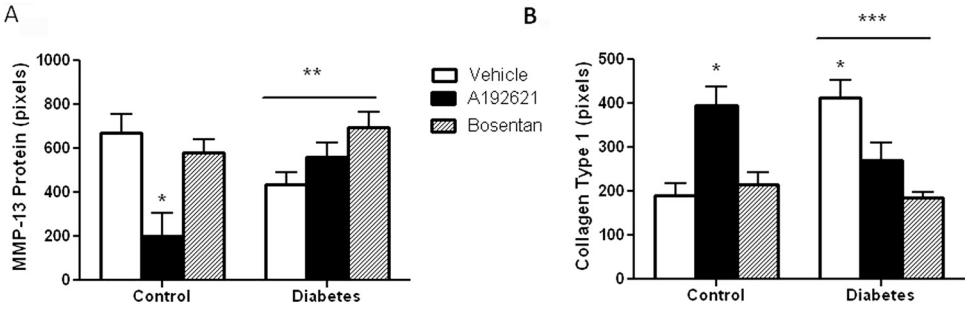

Fig. 4. Differential effect of ET receptor antagonism on MMP-13 and collagen levels in control and diabetic animals. A, MMP-13 protein was decreased in diabetes and was restored to control levels by either ET receptor blockade. Selective ETB receptor blockade with A192621 but not bosentan reduced MMP-13. B, ET receptor antagonism prevented the increase in collagen type 1 in diabetes, but selective ETB blockade caused an increase in control animals. Results are given as mean ± S.E.M., $n = 6–8$ in all groups except control + A192621 = 3. *, $p < 0.05$ versus control vehicle. **, $p = 0.0039$ disease and treatment interaction. ***, $p = 0.0001$ disease and treatment interaction.

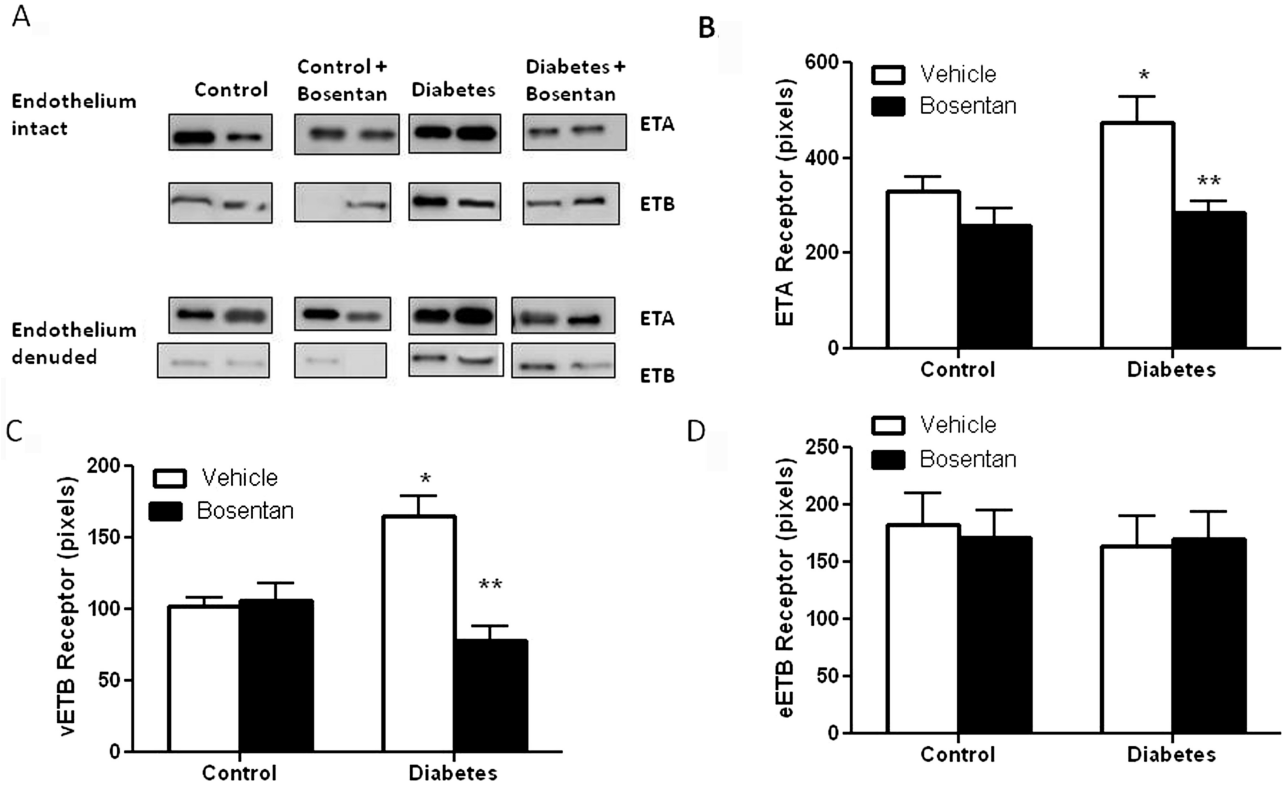

Fig. 5. Effect of diabetes and bosentan treatment on VSM and endothelial cell ET receptors. Endothelium-intact (vETA, vETB, and eETB) and endothelium-denuded (vETA and vETB) MCAs were used in immunoblotting experiments, and eETB was calculated as the difference in band intensities of intact and denuded vessels. A, representative immunoblots are shown. B and C, ETA (B) and vETB (C) receptor density measured on endothelium-denuded vessels was increased in diabetes and was restored to control levels by bosentan treatment. D, there was no disease or treatment effect on eETB protein levels. Results are given as mean ± S.E.M. *, $p < 0.001$ versus control. **, $p < 0.001$ versus vehicle.

of ET-1, the goals of the current study were to determine 1) the effect of diabetes on cerebrovascular ET receptor subtype expression and 2) the relative roles of ET receptors in cerebrovascular remodeling by taking advantage of selective and dual ET receptor antagonists. Important findings are 1) endothelial ET_B receptors that counterbalance ET-1-mediated vascular contraction and remodeling are not changed but rather the contractile and proliferative VSM ET_A and ET_B receptors are increased in diabetic animals, 2) dual ET receptor antagonism completely prevents vascular remodeling in diabetic animals, 3) because of changes in ET_B receptor expression in diabetic animals, selective ET_B receptor blockade also prevents remodeling response in diabetic animals but worsens it in control animals, indicating a protective role for this receptor subtype under physiological conditions, and 4) chronic treatment with bosentan alters ET receptor levels. Collectively, these findings strongly suggest that ET receptor antagonism may result in different responses under control and disease conditions.

The vascular effects of ET-1 are mediated by two receptor subtypes: ET_A and ET_B. ET_A receptors are located on smooth muscle cells and mediate vasoconstriction and proliferation, whereas ET_B receptor locations/actions vary. ET_B receptors located on endothelial cells mediate relaxation via cGMP, whereas ET_B receptors are located on smooth muscle cells and behave as ET_A receptors. It has been demonstrated that diabetes up-regulates vascular ET_B receptor expression. Mumtaz et al. (1999) found increased ET_B receptor density in

the diabetic rabbit urinary bladder, whereas Ikeda et al. (2001) showed a significantly increased ET_B gene expression in streptozotocin diabetic rat adrenal glands. Most notably, contractile ET_B receptors are up-regulated in rat MCAs after focal cerebral ischemia (Henriksson et al., 2003). This apparent change of receptor balance has been noted in functional studies as well. Diabetes-mediated ET-1 hyper-reactivity is attenuated in cerebral arteries after ET_B receptor blockade (Alabadi et al., 2004; Matsumoto et al., 2004), producing effects similar to those observed with ET_A antagonism. In the current study, we found increased ET_A and ET_B receptors on VSM. This explains our past and current findings. Selective ET_A blockade (Harris et al., 2005) or ET_B receptor antagonism as in the current study partially prevents cerebrovascular remodeling, suggesting that ET-1 through the unoccupied receptors still promotes restructuring of vessels, and when dual antagonism is applied remodeling is completely prevented. Another factor contributing to this response may be the fact that dual antagonism with bosentan not only prevents receptor activation but also reduces VSM ET receptors, indicating that ET-1 has a positive feedback on the expression of its receptors in the cerebrovasculature.

Previous studies have demonstrated that ET-1 influences ECM dynamics in diabetes. In diabetic animals, ET-1 receptor antagonism prevents ECM deposition in the retina as well as in renal arteries (Evans et al., 2000; Gilbert et al., 2000). Amiri et al. (2004) have demonstrated that endothelium-restricted overexpression of ET-1 causes marked hypertrophic remodeling and endothelial dysfunction in mice. Similar to our previous results, Spiers et al. (2005) reported attenuation of increased vascular MMP-2 activity after ET antagonism, whereas others have shown that treatment with the ET_A-selective antagonist sitaxsentan reduces postmyocardial infarction left ventricular dilation as well as MMP activity in cardiac tissue (Podesser et al., 2001). These observations indicate a putative role for ET in the regulation of MMPs, which are important for the regulation of ECM dynamics. We showed that MMP-2 activity is increased in the cerebrovasculature in diabetes, which may contribute to the activation of various growth-promoting signals mediating vascular remodeling (Harris et al., 2005; Sachidanandam et al., 2007). Growing evidence suggests that MMPs not only degrade the matrix but also stimulate formation of matrix. In the current study, MMP-2 protein and activity were increased in diabetes, which was reduced by ET antagonism. MMP activity may be regulated by endogenous inhibitors such as TIMP-2, but in the current study we did not detect any significant changes in TIMP-2 levels by disease or treatment. However, it has to be noted that there was a trend for increased MMP-2 activity and a corresponding decrease in TIMP-2 levels in control animals treated with A192621. We also found that another MMP class enzyme, collagenase MMP-13, is significantly decreased in diabetes, which may explain increased collagen deposition. Whereas dual ET_B receptor blockade completely restored MMP-13 levels in diabetic animals, selective blockade impaired collagenase activity in control animals and mediated collagen deposition. ET-1 has been shown to stimulate collagen synthesis and fibrosis (Iglarz and Clozel, 2010). In our study we did not investigate collagen expression; therefore, we cannot differentiate whether prevention of collagen deposition is caused by improvement of MMP-13 activity or inhibition of collagen synthesis. However, our findings provide strong evidence that ET-1 modulates MMP proteins.

We hypothesized that ET_B receptor blockade would block the vasculoprotective effects of endothelial ET_B receptors and exacerbate vascular remodeling in diabetes. Conversely, we found that ET_B blockade significantly reduced medial hypertrophy and decreased W/L ratio. This was completely unexpected based on previous reports of enhanced neointimal hyperplasia and medial thickening (Murakoshi et al., 2002) after genetic deletion or pharmacological inhibition of ET_B receptors and our studies that showed enhancement of mesenteric resistance vessel remodeling after ET_B receptor blockade (Sachidanandam et al., 2007). Intriguingly, in the mesenteric circulation, we did not find any changes in ET_B receptor expression in diabetes (Sachidanandam et al., 2008), which may explain the differences with the results of the current study. Our findings provide strong evidence that there is a delicate balance of vascular and endothelial ET receptors. When this balance is optimal, blockade of ET_B receptors either directly via inhibition of ET_B receptor activation or indirectly through the activation of ET_A receptors leads to remodeling. Because recent studies suggest a rather complex and antihypertensive effects of ET_B receptors, it is also possible that elevated blood pressure with A192621 may contribute to cerebrovascular remodeling in control animals (Pollock, 2010). However, the fact that both treatments prevent remodeling in diabetic animals despite similar increases in blood pressure argues against this possibility. These results point to a contrasting effect of ET_B blockade in control and diabetic animals. Although concomitant ET_A blockade does not add or subtract from effects mediated by selective ET_B blockade in controls, additional ET_A blockade improves beneficial effects of ET_B antagonism in diabetes. When the vascular/endothelial ET receptor balance is compromised as we found in the diabetic animals in the current study, vascular ET_A and ET_B receptors override protection conferred by the endothelial ET_B receptors and therefore dual ET_A/ET_B receptor blockade completely restored indices of remodeling to control values.

There are several technical limitations of the current study. First, because of the limited availability of the selective ET_B antagonist A192621 we had a relatively small number of animals in the control group, and the receptor density studies were performed only in the bosentan treatment group. Second, we measured ET receptor densities by immunoblotting because receptor binding studies require significantly more MCA samples and additional animals treated with these antagonists are needed. Along the same lines, eET_B receptor density was determined by an indirect method. Although antibodies yielded specific bands in immunoblots, immunohistochemical studies to determine endothelial versus VSM ET_B receptors were not clear (data not shown). We have also observed a 10 to 15% weight loss in both control and diabetic animals treated with antagonists. Our finding that treated control animals show increased remodeling despite weight loss argues against the fact weight loss may be contributing to the beneficial effects observed in diabetic animals. However, the cause of weight loss and effect on remodeling endpoints need to be studied further. Despite these shortcomings, our results have demonstrated that selective ET_B receptor antagonism exerts different effects under physiological or diabetic conditions and

dual ET receptor blockade completely prevents cerebrovascular remodeling in diabetes. These findings emphasize that the relative ET_A and ET_B receptor density is an important determinant of response to ET receptor antagonist treatment especially in disease states.

Acknowledgments

We thank staff from Actelion Pharmaceutical (Basel, Switzerland) and Abbott Laboratories (Chicago, IL) for providing bosentan and A192621, respectively.

Authorship Contributions

Participated in research design: Ergul.

Conducted experiments: Kelly-Cobbs, Harris, Elgebaly, Sachidanandam, and Portik-Dobos.

Performed data analysis: Kelly-Cobbs, Johnson, and Ergul.

Wrote or contributed to the writing of the manuscript: Kelly-Cobbs, Harris, and Ergul.

Other: Ergul acquired funding for the project.

References

Alabadí JA, Miranda FJ, Lloréns S, Centeno JM, Marrachelli VG, and Alborch E (2004) Mechanisms underlying diabetes enhancement of endothelin-1-induced contraction in rabbit basilar artery. *Eur J Pharmacol* **486:**289–296.

Amiri F, Virdis A, Neves MF, Iglarz M, Seidah NG, Touyz RM, Reudelhuber TL, and Schiffrin EL (2004) Endothelium-restricted overexpression of human endothelin-1 causes vascular remodeling and endothelial dysfunction. *Circulation* **110:**2233–2240.

Battistini B, Berthiaume N, Kelland NF, Webb DJ, and Kohan DE (2006) Profile of past and current clinical trials involving endothelin receptor antagonists: the novel "-sentan" class of drug. *Exp Biol Med (Maywood)* **231:**653–695.

Calcutt NA, Cooper ME, Kern TS, and Schmidt AM (2009) Therapies for hyperglycaemia-induced diabetic complications: from animal models to clinical trials. *Nat Rev Drug Discov* **8:**417–429.

Conover W and Iman R (1981) Rank transformations as a bridge between parametric and nonparametric statistics. *Am Stat* **35:**124–133.

Cooper ME, Gilbert RE, and Jerums G (1997) Diabetic vascular complications. *Clin Exp Pharmacol Physiol* **24:**770–775.

Ding H and Triggle CR (2010) Endothelial dysfunction in diabetes: multiple targets for treatment. *Pflugers Arch* **459:**977–994.

Elgebaly MM, Portik-Dobos V, Sachidanandam K, Rychly D, Malcom D, Johnson MH, and Ergul A (2007) Differential effects of ET(A) and ET(B) receptor antagonism on oxidative stress in type 2 diabetes. *Vascul Pharmacol* **47:**125–130.

Ergul A, Johansen JS, Strømhaug C, Harris AK, Hutchinson J, Tawfik A, Rahimi A, Rhim E, Wells B, Caldwell RW, et al. (2005) Vascular dysfunction of venous bypass conduits is mediated by reactive oxygen species in diabetes: role of endothelin-1. *J Pharmacol Exp Ther* **313:**70–77.

Evans T, Deng DX, Chen S, and Chakrabarti S (2000) 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* **49:**662–666.

Gilbert RE, Rumble JR, Cao Z, Cox AJ, van Eeden P, Allen TJ, Kelly DJ, and Cooper ME (2000) Endothelin receptor antagonism ameliorates mast cell infiltration, vascular hypertrophy, and epidermal growth factor expression in experimental diabetes. *Circ Res* **86:**158–165.

Harris AK, Elgebaly MM, Li W, Sachidanandam K, and Ergul A (2008) Effect of chronic endothelin receptor antagonism on cerebrovascular function in type 2 diabetes. *Am J Physiol Regul Integr Comp Physiol* **294:**R1213–R1219.

Harris AK, Hutchinson JR, Sachidanandam K, Johnson MH, Dorrance AM, Stepp DW, Fagan SC, and Ergul A (2005) Type 2 diabetes causes remodeling of cerebrovasculature via differential regulation of matrix metalloproteinases and collagen synthesis: role of endothelin-1. *Diabetes* **54:**2638–2644.

Henriksson M, Stenman E, and Edvinsson L (2003) Intracellular pathways involved in upregulation of vascular endothelin type B receptors in cerebral arteries of the rat. *Stroke* **34:**1479–1483.

Iglarz M and Clozel M (2010) At the heart of tissue: endothelin system and end-organ damage. *Clin Sci (Lond)* **119:**453–463.

Ikeda K, Wada Y, Sanematsu H, Foster HE Jr, Shin D, Weiss RM, and Latifpour J (2001) Regulatory effect of experimental diabetes on the expression of endothelin receptor subtypes and their gene transcripts in the rat adrenal gland. *J Endocrinol* **168:**163–175.

Intengan HD and Schiffrin EL (2000) Structure and mechanical properties of resistance arteries in hypertension: role of adhesion molecules and extracellular matrix determinants. *Hypertension* **36:**312–318.

Kannel WB and McGee DL (1979) Diabetes and cardiovascular disease. The Framingham study. *Jama* **241:**2035–2038.

Matsumoto T, Yoshiyama S, Kobayashi T, and Kamata K (2004) Mechanisms underlying enhanced contractile response to endothelin-1 in diabetic rat basilar artery. *Peptides* **25:**1985–1994.

Miller AW, Dimitropoulou C, Han G, White RE, Busija DW, and Carrier GO (2001) Epoxyeicosatrienoic acid-induced relaxation is impaired in insulin resistance. *Am J Physiol Heart Circ Physiol* **281:**H1524–H1531.

Mizuguchi T, Nishiyama M, Moroi K, Tanaka H, Saito T, Masuda Y, Masaki T, de Wit D, Yanagisawa M, and Kimura S (1997) Analysis of two pharmacologically predicted endothelin B receptor subtypes by using the endothelin B receptor gene knockout mouse. *Br J Pharmacol* **120:**1427–1430.

Mumtaz FH, Dashwood MR, Thompson CS, Sullivan ME, Mikhailidis DP, and Morgan RJ (1999) Increased expression of endothelin B receptors in the diabetic rabbit urinary bladder: functional relevance. *BJU Int* **83:**113–122.

Murakoshi N, Miyauchi T, Kakinuma Y, Ohuchi T, Goto K, Yanagisawa M, and Yamaguchi I (2002) Vascular endothelin-B receptor system in vivo plays a favorable inhibitory role in vascular remodeling after injury revealed by endothelin-B receptor-knockout mice. *Circulation* **106:**1991–1998.

Ortmann J, Traupe T, Nett P, Celeiro J, Hofmann-Lehmann R, Lange M, Vetter W, and Barton M (2004) Upregulation of vascular ET(B) receptor gene expression after chronic ET(A) receptor blockade in prediabetic NOD mice. *J Cardiovasc Pharmacol* **44:**S105–S108.

Podesser BK, Siwik DA, Eberli FR, Sam F, Ngoy S, Lambert J, Ngo K, Apstein CS, and Colucci WS (2001) ET(A)-receptor blockade prevents matrix metalloproteinase activation late postmyocardial infarction in the rat. *Am J Physiol Heart Circ Physiol* **280:**H984–H991.

Pollock DM (2010) Dissecting the complex physiology of endothelin: new lessons from genetic models. *Hypertension* **56:**31–33.

Rumble JR, Cooper ME, Soulis T, Cox A, Wu L, Youssef S, Jasik M, Jerums G, and Gilbert RE (1997) Vascular hypertrophy in experimental diabetes. Role of advanced glycation end products. *J Clin Invest* **99:**1016–1027.

Sachidanandam K, Elgebaly MM, Harris AK, Hutchinson JR, Mezzetti EM, Portik-Dobos V, and Ergul A (2008) Effect of chronic and selective endothelin receptor antagonism on microvascular function in type 2 diabetes. *Am J Physiol Heart Circ Physiol* **294:**H2743–H2749.

Sachidanandam K, Portik-Dobos V, Harris AK, Hutchinson JR, Muller E, Johnson MH, and Ergul A (2007) Evidence for vasculoprotective effects of ETB receptors in resistance artery remodeling in diabetes. *Diabetes* **56:**2753–2758.

Schneider MP, Boesen EI, and Pollock DM (2007) Contrasting actions of endothelin ET(A) and ET(B) receptors in cardiovascular disease. *Annu Rev Pharmacol Toxicol* **47:**731–759.

Spiers JP, Kelso EJ, Siah WF, Edge G, Song G, McDermott BJ, and Hennessy M (2005) Alterations in vascular matrix metalloproteinase due to ageing and chronic hypertension: effects of endothelin receptor blockade. *J Hypertens* **23:**1717–1724.

Stenman E, Malmsjö M, Uddman E, Gidö G, Wieloch T, and Edvinsson L (2002) Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. *Stroke* **33:**2311–2316.

Address correspondence to: Dr. Adviye Ergul, Medical College of Georgia, 1120 15th Street CA2094, Augusta, GA 30912. E-mail: aergul@mcg.edu