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Comparison of Selective versus Dual ET Receptor Antagonism on Cerebrovascular Dysfunction in Diabetes

Weiguo Li¹, Kamakshi Sachidanandam², and Advije Ergul^{1,2,3}

¹Department of Physiology, Medical College of Georgia, 1120 15th Street, Augusta, Georgia 30912

²Program in Clinical and Experimental Therapeutics, University of Georgia College of Pharmacy, 1120 15th Street, Augusta, Georgia 30912

³Charlie Norwood Veterans Affairs Medical Center, 1120 15th Street, Augusta, Georgia 30912

Abstract

Objectives—Cerebrovascular tone plays a key role in controlling cerebral blood flow. Our studies have demonstrated that the endothelin (ET) system is upregulated in type 2 diabetes leading to increased sensitivity to ET-1 and decreased relaxation in basilar artery (BA). While chronic ET_A receptor blockade restored relaxation, selective ET_B blockade caused paradoxical constriction in diabetes. Whether this effect was due to activation of ET_A receptors in the presence of ET_B receptor blockade or due to the loss of vasculoprotective effects of ET_B receptors remained unknown. The current study hypothesize that due to the antagonism of the vasculoprotective ET_B receptors, dual blockade will not be as effective as selective ET_A receptor antagonism in improving cerebrovascular dysfunction in type 2 diabetes.

Methods—These studies were done in non-obese, type 2 diabetic Goto-Kakizaki rats administered either vehicle, selective ET_A receptor antagonist Atrasentan (5 mg/kg) or dual ET antagonist Bosentan (100 mg/kg) for 4 weeks. At sacrifice, basilar arteries were collected and mounted on a wire-myograph and cumulative dose-responses to ET-1 (1-500 nM) and acetylcholine (Ach, 1 nM-5 μM) were studied.

Results—BA was highly sensitive to ET-1-mediated constriction in diabetic animals. While neither Atrasentan nor Bosentan affected endothelium-dependent vascular relaxation in control animals, both treatments improved the maximum dilatation in diabetes and Atrasentan also improved sensitivity to Ach.

Conclusion—In light of our previous data which showed that ET_B receptors are vasculoprotective and blockade of this receptor worsens relaxation, current findings suggest that when blocked simultaneously with the ET_A receptors, the ET_B receptor antagonism is protective by reducing the hyperreactivity and improving cerebrovascular function in diabetes.

Keywords

cerebrovascular function; diabetes; endothelin; ET_A receptor; ET_B receptor

INTRODUCTION

Diabetes exists as an independent risk factor for cardiovascular disease (CVD) [1]. Studies have demonstrated that there is a strong correlation between diabetes and cerebrovascular disorders such as cerebral ischemia and stroke [2, 3]. Regulation of vascular tone is important for maintenance of proper blood flow. In the cerebral circulation, changes in blood flow are buffered by the myogenic response to maintain cerebral blood flow. However, alterations to this system may be detrimental and could contribute to cerebrovascular disease. Myogenic tone is increased in experimental diabetes [4-6]. In addition to increased basal tone, cerebral arteries from diabetic animals exhibit diminished endothelium derived relaxation [4, 7, 8].

Endothelin-1 (ET-1) being a potent vasoconstrictor with profibrotic and proliferative properties that change vessel function and structure is an important mediator of these vascular pathologies [9, 10]. The effects of endothelin are mediated via two G-protein coupled receptors: ET_A and ET_B [11]. ET_A receptors reside on the smooth muscle cell (SMC) and produce vasoconstriction. ET_B receptors on endothelial cells promote vasodilation via cGMP while VSMC ET_B receptors elude ET_A-like responses. Studies have demonstrated enhanced contractile responses to ET-1 [12, 13] as well as a reduction of increased myogenic tone after ET receptor antagonism [4] in type 1 diabetes. However, the relative roles of ET-1 and its receptors in cerebrovascular dysfunction in type 2 diabetes, which is a common comorbidity in stroke patients, are poorly elucidated. Given that physiologically ET_B receptors on endothelial vs. VSM cells have opposing actions on vascular reactivity [14], we asked the question whether ET_B receptors contribute to or balance detrimental effects of ET_A receptor activation in diabetic vascular dysfunction. We reported that chronic ET_A receptor blockade restored relaxation, but selective ET_B blockade caused paradoxical constriction in diabetes. Whether this effect was due to activation of ET_A receptors in the presence of ET_B receptor blockade or due to the loss of vasculoprotective effects of ET_B receptors remained unknown. We hypothesized that due to the antagonism of the vasculoprotective ET_B receptors, bosentan treatment will not be as effective as selective ET_A receptor antagonism in improving cerebrovascular dysfunction in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals

All experiments were performed on male Wistar (Harlan, Indianapolis, IN) and diabetic Goto-Kakizaki (GK) (in-house bred, derived from the Tampa colony) rats [15, 16]. All protocols were approved by the Institutional Animal Care and Use Committee. Weight and blood glucose measurements were monitored twice a week till sacrifice. Blood glucose was measured from the tail vein using a commercially available glucometer (Freesstyle, Alameda, CA). After the spontaneous onset of diabetes, starting at 14 weeks of age, animals received either vehicle, Atrasentan (5 mg/kg/day in drinking water) or dual ET receptor antagonist Bosentan (100 mg/kg/day in diet) for 4 weeks as we previously reported [17-20]. Animals were anesthetized with sodium pentobarbital and exsanguinated via cardiac puncture. The basilar artery was then harvested for functional studies as described below.

Plasma measurements

Plasma ET-1 was measured by specific ELISA kit from ALPCO Diagnostics (Windham, NH) according manufacture's protocol.

Vascular function

Isometric tension exerted by the vessels was recorded via a force transducer using the wire-myograph technique (Danish Myo Technologies, Denmark). The myograph chambers were filled with Krebs buffer (NaCl 118.3, NaHCO₃ 25, KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 1.5 and Dextrose 11.1 mM), gassed with 95% O₂ and 5% CO₂ and maintained at 37°C. Vessel segments were mounted in the chamber using 40 µm OD wires and adjusted to a baseline tension of 0.4g. Cumulative dose response curves to ET-1 (0.1-100 nM) were generated and the force generated was expressed as % change of baseline. Endothelium-dependent relaxation to acetylcholine (ACh, 1 nM-1 µM) was assessed after vessels were constricted to 60% of the baseline tension with serotonin (5-HT) or directly after ET-1 dose response. Sensitivity (EC₅₀) and maximum response (R_{max}) values were calculated from the respective dose-response equations.

Statistical analysis

Results are given as mean ± SEM. For EC₅₀ and R_{max} values, a two-way analysis of variance (ANOVA) was done to analyze disease and treatment effects with a post-hoc Bonferroni test. A repeated measures ANOVA was used to determine group differences (Control vs. Diabetic) across the ET-1 or ACh concentrations. Post-hoc group comparisons at each concentration used a Tukey's adjustment for the multiple comparisons. Graphpad Prism 5.0 was used for all statistical tests performed.

RESULTS

Animal data

Metabolic parameters for all study groups are summarized in Table 1. Diabetic animals were significantly smaller than control and ET receptor antagonism did not affect animal weight. GK animals displayed elevated blood glucose in all treatment groups.

ET-1-mediated contractility

Basilar arteries of diabetic rats were hypersensitive to ET-1 (EC₅₀ 7.9 ± 2.0 vs 20 ± 1.5 nM in controls, p<0.05) (Fig. 1A). In control animals, Atrasentan treatment has no effect on either sensitivity (Fig 1B) or maximum response (Fig 1C). Bosentan, on the other hand, caused a rightward shift lowering the sensitivity to ET-1 with no effect on R_{max}. In diabetic rats, both Atrasentan and Bosentan treatment lowered sensitivity but did not influence the maximum response to ET-1.

Endothelium-dependent relaxation

Diabetic rats exhibited impaired endothelium relaxation following pre-constriction with 5-HT. Basilar arteries of GK rats relaxed only 31.3 ± 3.6% in response to ACh whereas the arteries of control rat relaxed 58.2 ± 8.9% (Fig. 2A). In the control group, the treatment affected neither the sensitivity nor the maximum relaxation response. In diabetic group, both treatments improved relaxation response (Fig. 2B) and Atrasentan increased sensitivity to ACh (Fig. 2C).

DISCUSSION

We previously showed that selective ET_A receptor antagonism prevents whereas selective ET_B receptor antagonisms exaggerates diabetes-mediated cerebrovascular dysfunction which suggesting a vasculoprotective effect by ET_B receptors [19]. However, it remained unknown whether this effect was due to the stimulation of unoccupied ET_A receptors when ET_B receptors are blocked or loss of vasculoprotection conferred by this receptor subtype.

To address this question, control and diabetic animals were treated with vehicle, selective ET_A receptor antagonist Atrasentan or dual ET receptor antagonist Bosentan using the same treatment paradigm employed in our previous study [19] with the basic assumption that: 1) If ET_A activation drives vascular dysfunction, a similar effect to that of selective ET_A blockade should be seen, or 2) If it is the loss of vasculoprotective effects of endothelial ET_B receptors, vascular dysfunction should have no change as ET_B antagonism negates ET_A antagonism effects. The working hypothesis was that due to the antagonism of the vasculoprotective ET_B receptors, treatment with nonselective ET receptor antagonist will not be as effective as selective ET_A receptor antagonism in improving vascular function in type 2 diabetes. Our findings demonstrate that the dual antagonism is as effective if not better than selective ET_A antagonism in decreasing hypersensitivity to ET-1 and improving vascular relaxation of basilar arteries in diabetes.

While there are many models of diabetes, most are either chemically induced type 1 model or have co-morbid conditions such as hypertension, obesity and hyperlipidemia. The spontaneously diabetic GK rat is a non-obese, normotensive rat model originally developed from selective inbreeding of glucose intolerant Wistar rats [21]. Previous studies have demonstrated that GK rats retain greater than 40% of their beta cell mass and have a reduction of post-prandial glucose when given the insulin secretagogue nateglinide [22-25]. In addition, our laboratory has previously shown that these rats have impaired glucose tolerance as compared to control Wistars [17]. Therefore, the GK rat serves as an excellent model for studying the effects of hyperglycemia alone on cerebrovascular function in type 2 diabetes.

Several laboratories including ours reported elevated plasma ET-1 levels in both clinical and experimental diabetes [17, 26-28]. Contractile response to ET-1 in aorta and peripheral arteries is increased in diabetes [29-31]. A study by McIntyre et al reported increased sensitivity to ET-1 in subcutaneous resistance arteries from patients with type 1 diabetes [32]. Two independent groups have both demonstrated similar results in the rat and rabbit basilar arteries, respectively [12, 13]. However, another investigation reported no differences in contractile responses to ET-1 in isolated rat basilar arteries [33]. These discrepancies may be attributable to the differences in methodologies (e.g. in-vivo vs. in-vitro). These previous studies were all performed in chemically induced type 1 diabetes model. We have recently extended these studies to type 2 diabetes and have shown an increased sensitivity to ET-1 in the rat basilar artery in diabetes [19]. The current study also confirmed this finding. The vascular effects of ET-1 are mediated by two distinct receptor subtypes: ET_A and ET_B. In the present study, we used Atrasentan to selectively block the ET_A receptor and Bosentan to antagonize both receptors simultaneously. While neither treatment affected the maximum force generated by ET-1 in both control and diabetic groups, increased sensitivity to ET-1 in the diabetic group was ameliorated by both Atrasentan and Bosentan as indicated by increased EC₅₀ values. While it did not reach significance, Bosentan appeared to be more effective. This finding was in contrast to our hypothesis that blockade of vasculoprotective ET_B receptors would render Bosentan treatment not as effective as Atrasentan treatment. It has been suggested that ET-1 mediated contraction in the cerebrovasculature may be, in part, mediated through crosstalk of the ET receptor subtypes. Zuccarello et al previously demonstrated that ET_B mediated vasoconstriction relies on activation of the smooth muscle as well as the endothelial ET_B receptors [34]. Thus, simultaneous blockade of both receptors which is readily achieved by Bosentan appears to inhibit ET-1-mediated constriction of basilar arteries.

Vascular reactivity comprises both constriction and relaxation of blood vessels. Previous studies have demonstrated that endothelium derived relaxation is impaired in aortas and the peripheral vasculature of type 2 diabetes model [12, 35-37]. However, in the study of

cerebrovasculature, type 1 diabetes models, such as STZ rat or alloxan rabbit, were heavily used [7, 38, 39]. Only in recent years have investigators begun to study the effects of type 2 diabetes on the cerebral circulation [6, 40-43]. Schwaninger and Karagiannis both reported diminished vasodilation in response to Ach in obese Zucker rats (OZR) [42, 43]. However, the OZR is a model of type 2 diabetes which known to have co-morbid conditions such as hypertension and hyperlipidemia that may contribute to altered vascular states. More recently, diabetic *db/db* mice, characterized by hyperinsulinemia, severe hyperglycemia and obesity, were shown to exhibit similar reductions in endothelium dependent relaxation [6]. In the present study, we found that type 2 diabetes significantly impairs Ach induced relaxation in serotonin preconstricted basilar arteries. ET receptor antagonism completely restored Ach induced relaxation in diabetic basilar arteries indicating that an activated endothelin system contributes to this impairment. This data, in accordance with previous studies done in varying models of diabetes, suggests that diabetes impairs vascular relaxation without regard to the etiology of the disease or other co-morbid conditions.

In this study, ET receptor blockade with Atrasentan or Bosentan did not affect endothelium-dependent dilatation in control animals but improved relaxation in diabetic rats suggesting a greater involvement of ET-1 in the regulation of vascular relaxation in diabetes. In our previous study [19], we found that selective ET_B receptor antagonism with A-192621 resulted in completely opposite results in control vs. diabetic rats, i.e., improving relaxation in control animals and causing paradoxical constriction in diabetic animals. Complete reversal of the relaxation response in A-192621-treated diabetic rats suggested that endothelial ET_B receptors are upregulated as a compensatory mechanism to offset impaired relaxation in diabetes and that blockade of these receptors ultimately results in decreased relaxation. Another possibility was that in the presence of ET_B receptor blockade, ET_A receptors are activated and worsen relaxation in diabetic rats although we did not see this response in control group. To test this possibility, in the current study we compared selective and dual blockade of ET receptors. We assumed that if it is ET_A activation driving this response, Bosentan treatment will produce similar results to Atrasentan. If it is the loss of vasculoprotective ET_B receptors, then Bosentan treatment will not be effective in improving relaxation. Both treatments were equally effective in increasing the maximum dilatation of basilar arteries in diabetes. Atrasentan, but not Bosentan, also improved the sensitivity to Ach, suggesting a greater involvement of ET_A receptors in the regulation of vascular relaxation in diabetes. These studies also suggest involvement of VSMC ET_B receptors in the regulation of vascular function in diabetes. Several studies demonstrated that diabetes upregulates vascular ET_B receptor expression. One study found increased ET_B receptor density in the diabetic rabbit urinary bladder [44] and another showed a significantly increased ET_B gene expression in STZ diabetic rat adrenal glands [45]. In 10 week old NOD mice, a type 1 diabetes model, aortic ET_B gene expression was significantly increased while ET_A expression was unchanged [46]. It is highly possible that blockade of ET_B receptors in a system which is shifted more toward a contractile phenotype, would produce effects similar to ET_A blockade. It is also possible that ET_A-ET_B heterodimerization can affect the reactivity studies. Both homo and heterodimerization of the two ET receptors have been reported by functional as well as fluorescence resonance studies [47-49]. It is suggested that when heterodimerized, the ET_A receptor overrides ET_B receptor activation and thus ET_B receptor antagonism provides an ET_A blockade-like effect. We have shown that ET_A, but not ET_B, receptor protein is increased in the mesenteric arteries in diabetes [18]. It is possible that when there is an alteration in the balance of ET_A and ET_B receptors as occurs in disease states, dimerization patterns can change ultimately affecting the responses mediated by each receptor subtype.

In conclusion, diabetes induces vascular dysfunction in isolated rat basilar arteries in the form of increased sensitivity to ET-1 and diminished relaxation capacity to acetylcholine.

Selective and dual blockade of ET receptors completely prevents vascular dysfunction. Inasmuch, these effects appear to be, at least in part, due to an increased contribution of the ET_A and smooth muscle ET_B receptor activation. These findings suggest that the relative roles of ET receptors in the regulation of vascular function may differ in states and that alterations of cerebrovascular function may potentially underlie the increased propensity for cerebrovascular disease in diabetes.

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FIG. 1.

Effects of chronic ET receptor antagonism on ET-1-mediated contractility in control (C) and diabetic (D) rats. A. Dose-response curves to ET-1 demonstrated enhanced constriction as compared to controls. The magnitude of constriction did not differ (B) but diabetic rats displayed hypersensitivity (EC_{50}) to ET-1 which was reduced by both atrasentan (+A) and bosentan (+B) treatments (C). Results are shown as mean $\pm$ SEM, $n=4-7$ /group, * $p<0.01$ vs. control, ** $p<0.05$ vs. diabetes.

FIG. 2.

Effects of chronic ET receptor antagonism on endothelium-dependent relaxation in control (C) and diabetic (D) rats. A. Dose-response curves to ACh in 5-HT-precontracted vessels demonstrated impaired relaxation in diabetes. The magnitude of dilatation was less in diabetic rats and both Atrasentan (+A) and Bosentan (+B) treatments improved relaxation (B). Atrasentan also improved sensitivity (EC_{50}) to ACh (C). Results are shown as mean $\pm$ SEM, $n=4-7$ /group, * $p<0.01$ vs. control, ** $p<0.05$ vs. all other groups.

Table 1

Physiological metabolic parameters of control and diabetes animals in each group.

	C	C+A	C+B	D	D+A	D+B
BW (g)	540 ± 10	500 ± 29	411 ± 12	389 ± 5 *	390 ± 10 *	330 ± 4 *
BG (mg/dL)	100 ± 3	96 ± 4	95 ± 8	171 ± 26 *	167 ± 11 *	179 ± 18 *
BP (mmHg)	102 ± 3	102 ± 1	133 ± 3	112 ± 2	107 ± 3	129 ± 2
ET-1 (fmol/mL)	0.7 ± 0.1	0.4 ± 0.1	N/A	1.5 ± 0.3 *	0.9 ± 0.1 *	N/A

C: contrl; D: diabetes; A: Atrasentan; B: Bosentan. N/A: not measured. Numbers are mean ± SEM.

*
p< 0.0001 vs. control.