Long-Term Endothelin Receptor Blockade Improves Cardiovascular Function in Diabetes

Subodh Verma, Emi Arikawa, and John H. McNeill

To evaluate the potential contribution of endothelin-1 (ET-1) toward the cardiovascular complications of diabetes, the present study examined the effects of chronic ET receptor blockade with bosentan on heart function and vascular reactivity in streptozotocin (STZ)-induced diabetic rats. Wistar rats were divided into four groups: control, control bosentan-treated, diabetic, and diabetic bosentan-treated. After chronic bosentan treatment, cardiac function and vascular reactivity were assessed. Ex vivo working heart function was determined in terms of rate of contraction (+dP/dt), rate of relaxation (−dP/dt), and left ventricular developed pressure (LVDP). Contractile responses to ET-1 were determined in isolated superior mesenteric arteries. In addition, ET-1-like immunoreactivity was determined in ventricular and vascular tissues by immunohistochemistry. Cardiac function was depressed in the untreated-diabetic group. Bosentan treatment improved working heart function; hearts from the diabetic bosentan-treated group exhibited improved LVDP and −dP/dt. The contractile responses of mesenteric arteries to ET-1 were exaggerated in the untreated-diabetic group. Long-term bosentan treatment normalized these responses. Immunohistochemical analyses revealed increased ET-1-like immunoreactivity in ventricular and vascular tissues from untreated diabetic rats. These data show the beneficial effects of ETA/B receptor blockade on cardiovascular function in STZ-diabetic rats. An altered ET-1 system may contribute toward the pathogenesis of cardiovascular dysfunction in diabetes.

Key Words: Endothelin-1, streptozotocin-induced diabetes, endothelin receptor blocker, bosentan, cardiac function, vascular reactivity.

Cardiovascular complications of long-standing diabetes are the leading cause of morbidity and mortality among diabetic patients. A variety of mechanisms including coronary atherosclerosis, autonomic neuropathy, macroangiopathy, and diabetic cardiomyopathy have been implicated in the development of cardiovascular disease in diabetes.

A growing body of evidence suggests that the potent vasoconstrictor endothelin-1 (ET-1) may play an important role in the pathogenesis or reinforcement of cardiovascular diseases. Endothelins are a family of structurally related 21 amino-acid peptides in which ET-1 is the most predominant and important subtype. The biological actions of ET-1 are principally mediated via two major subtypes of receptors, ET\textsubscript{A} and ET\textsubscript{B}. Both ET\textsubscript{A} and ET\textsubscript{B} receptors have been shown to be present on vascular smooth muscle cells, where they mediate vasoconstriction. Most endothelial cells express only ET\textsubscript{B}, the activation of which leads to vasodilation.

Increased ET-1 production and altered vascular reactivity to ET-1 have also been demonstrated in experimental and clinical diabetes, suggesting that this peptide may mediate diabetes-induced cardiovascular dysfunction as well. To examine this proposition, we studied the effects of chronic ET receptor blockade (with bosentan) on cardiac function and vascular reactivity in streptozotocin (STZ)-induced diabetic rats. We report here, for the first time, that long-term ET receptor blockade improves cardiovascular function in experimental diabetes.

Methods

Animals and Research Design

Male Wistar rats (200 to 250 g) were obtained from the Animal Care Unit, The University of British Columbia, Vancouver, BC, and were randomly assigned to two groups. One group received a single caudal vein injection of bosentan treatment.

Canada Research Traineeship. The gift of bosentan from Actelion Ltd, Switzerland, is gratefully acknowledged.

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of STZ (Sigma, St Louis, MO) at a dose of 55 to 60 mg/kg under halothane anesthesia and served as the diabetic group. The other group was injected with saline (0.9% sodium chloride) and served as the control group. The rats injected with STZ were checked for hyperglycemia at 48 and 72 h using Glucostix (Miles Canada, Etobicoke, ON) reagent strips read by a Glucometer II (Ames, Miles Laboratories, Elkhart, IN). The control and diabetic groups were then further divided into untreated control (C), control bosentan-treated (CB), diabetic (D), and diabetic bosentan-treated (DB). The rats were housed two per cage on a 12-h light–dark cycle and received food (Purina rat Chow; Ralston Purina, St Louis, MO) and water ad libitum. The CB and DB groups received bosentan (100 mg/kg/day) via a single daily oral gavage. The therapeutic efficacy of this dose has been previously demonstrated.9 Rats were treated for 7 and 10 weeks for the isolated working heart function and vascular reactivity studies, respectively, as described below.

**Isolated Working Heart Function**

After 7 weeks of bosentan treatment, isolated working heart function was assessed as described previously.10 Briefly, the rats were anesthetized with sodium pentobarbital (60 mg/kg), and the hearts were excised and mounted on the working heart apparatus. Perfusion was first initiated in the retrograde manner through the aorta. After cannulation of the left pulmonary vein, cardiac work was initiated by switching from the retrograde mode to the working heart mode such that buffer entered the left ventricle via the left atrium and exited through the aorta. The aortic outflow was subjected to a constant afterload of 600 g/mm², maintained at 37°C, and oxygenated with 95% O2 and 5% CO2. Each ring was subjected to a resting tension of 1.0 g to allow maximum force generation. After an initial equilibration period (60 min), isometric dose–response curves (DRC) to cumulative addition of ET-1 (10⁻¹⁰ – 3 × 10⁻⁸ mol/L) were recorded. For each concentration, a plateau was obtained before the subsequent dose was added. After each DRC, buffer was replaced several times to wash the tissues until the resting tension of each tissue was reached. At the end of the experiment, the tissues were removed, blotted dry, and the cross-sectional area of each vascular ring was calculated as follows: cross-sectional area (mm²) = weight (mg)/[length (mm) × density (mg/mm²)]. The density of the vascular smooth muscle was assumed to be 1.05 mg/mm². The absolute tension generated was corrected for cross-sectional area (expressed as g/mm²). Agonist pD₂ values (−log ED₅₀) were calculated by nonlinear regression analysis of the DRC and used as an index of sensitivity.

**Measurement of ET-1 Levels**

Following 10 weeks of bosentan treatment, plasma samples as well as ventricular and vascular tissues were obtained from all of the four experimental rat groups for the measurement of ET-1 levels as described below.

**Plasma ET-1 Levels** A quantity of 2 mL of plasma was acidified with 0.5 mL HCl (2 mol/L) and centrifuged at 8000 g for 10 mins. Extraction of ET-1 from plasma was performed using Amprep 500 mg C2 mini-columns (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom). The supernatant was passed through the column, washed initially with 5 mL of water containing 0.1% TFA, and then washed with 2 mL of 80% acetonitrile containing 0.1% TFA to elute ET-1. The eluent was then dried in a Speed-Vac (Savant Instruments, Farmingdale, NY) overnight and reconstituted in the assay buffer. Immunoreactive ET-1 was measured by a radioimmunoassay (RIA) using a specific antiserum to ET-1 obtained from Peninsula Laboratories (Belmont, CA).

**Immunohistochemistry** Immunohistochemical analysis of ET-1–like immunoreactivity (ET-1-ir) was performed in ventricular and vascular tissues from the four experimental groups. Ventricles and superior mesenteric arteries were immersed in 10% formalin for 8 h and embedded in paraffin. Sections of paraffin-embedded tissue were dewaxed in xylene, preincubated with 0.6% hydrogen peroxide in methanol for 1.5 h to block endogenous peroxidase activity and blocked with 5% normal goat serum for 1 h at room temperature. The slides were subsequently incubated with a rabbit polyclonal anti-ET-1 antiserum (Peninsula Laboratories) at a 1:1000 dilution for 48 h at
**Table 1.** General characteristics of the rats after 10 weeks of bosentan treatment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>C (n = 8)</th>
<th>CB (n = 8)</th>
<th>D (n = 9)</th>
<th>DB (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>497 ± 14</td>
<td>509 ± 9</td>
<td>377 ± 16*†</td>
<td>383 ± 16*†</td>
</tr>
<tr>
<td>Food intake (g/day)</td>
<td>29 ± 1</td>
<td>28 ± 1</td>
<td>57 ± 2*†</td>
<td>52 ± 1*†</td>
</tr>
<tr>
<td>Fluid intake (mL/day)</td>
<td>57 ± 3</td>
<td>62 ± 4</td>
<td>281 ± 12†</td>
<td>257 ± 13†</td>
</tr>
<tr>
<td>Plasma insulin (ng/mL)</td>
<td>2.65 ± 0.33</td>
<td>1.91 ± 0.29</td>
<td>0.33 ± 0.04†</td>
<td>0.44 ± 0.1*†</td>
</tr>
<tr>
<td>Plasma glucose (mmol/L)</td>
<td>7.93 ± 0.26</td>
<td>7.13 ± 0.22</td>
<td>25.24 ± 0.5†</td>
<td>22.72 ± 1.0*†</td>
</tr>
<tr>
<td>Plasma cholesterol (mmol/L)</td>
<td>2.05 ± 0.08</td>
<td>1.77 ± 0.04</td>
<td>2.43 ± 0.25</td>
<td>2.27 ± 0.23</td>
</tr>
<tr>
<td>Plasma triglycerides (mmol/L)</td>
<td>1.34 ± 0.25</td>
<td>1.51 ± 0.1</td>
<td>3.71 ± 1.38</td>
<td>5.81 ± 0.87†</td>
</tr>
<tr>
<td>Plasma endothealin (pg/mL)</td>
<td>5.40 ± 0.56</td>
<td>3.80 ± 0.67‡</td>
<td>5.97 ± 0.22</td>
<td>9.96 ± 1.07*†</td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>129 ± 4</td>
<td>123 ± 8</td>
<td>119 ± 7</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>Heart: Body weight ratio (mg/g)</td>
<td>2.53 ± 0.04</td>
<td>2.37 ± 0.05</td>
<td>3.01 ± 0.13†</td>
<td>3.00 ± 0.11†</td>
</tr>
</tbody>
</table>

C = control rats; CB = control bosentan-treated rats; D = diabetic rats; DB = diabetic bosentan-treated rats.

Values expressed as means ± SEM.

* P < .05, different from C.
† P < .05, different from CB.
‡ P < .05, different from D.

4°C. The slides were then washed in phosphate buffered saline (PBS) for 10 min and immunostained with an avidin-biotin-peroxidase system (Vectastain Elite kit, Vector Laboratories, Burlingame, CA) using dianinobenzidine as the chromagen. Slides incubated with nonimmune normal rabbit serum (versus anti-ET-1 antiserum) served as the negative control. A digital imaging system was used for assessment of ET-1-ir. Slides were viewed through a Nikon Diaphot TMD inverted microscope. A video camera connected to an IBM compatible computer with Northern Eclipse Software (Empix Imaging, Mississauga, ON) converted the data to a digital image, each with a gray value ranging from 0 to 255. For each tissue, four different images were taken. Any areas darker than the threshold would be recognized as “objects”. Percent object area (total object area/selected area × 100%) from each image was obtained. Values from four images were averaged for each rat tissue.

**Plasma Analyses**

Five-hour fasted plasma glucose, insulin, triglyceride, and cholesterol levels were measured. Glucose, triglyceride and cholesterol levels were determined using enzymatic colorimetric assay kits (Boehringer Mannheim, Laval, Quebec). Plasma insulin levels were determined using a double antibody RIA using a kit from Linco Research Diagnostics, St. Charles, MO.

**Statistical Analyses**

Values are expressed as mean ± SEM. Statistical analyses were performed using a one-way analysis of variance (ANOVA) or a repeated measures ANOVA, followed by a Newman-Keuls test. The level of significance was set at P < .05.

**Chemicals**

Unless otherwise indicated, all chemicals were reagent grade and obtained from Sigma Chemicals, St. Louis, MO. Bosentan was provided by Actelion, Allschwil, Switzerland.

**Results**

**General Characteristics**

The general characteristics of the four rat groups are given in Table 1. Induction of diabetes resulted in characteristic symptoms of diabetes including hyperglycemia, hypoinsulinemia, decreased body weight gain, and increased food and fluid intake when compared to age-matched controls. These values remained unchanged after bosentan treatment. Systolic blood pressure was similar among all groups (Table 1). Plasma ET-1 levels were similar in the C and D groups (Table 1). Bosentan treatment resulted in an increase in plasma ET-1 levels in both CB and DB groups consistent with previous reports in human studies. Immunohistochemical analysis revealed a marked increase in ET-1-ir in left and right ventricular tissue as well as superior mesenteric arteries from diabetic rats (Figs. 1–3), which remained unchanged by bosentan treatment. Sections incubated with nonimmune rabbit serum did not demonstrate immunostaining, indicating the specificity of the ET-1 antiserum (not shown).

**Effect of Bosentan Treatment on Myocardial Performance**

Functional cardiac performance was assessed by measuring left ventricular responses to left atrial filling pressures in terms of LVDP, −dP/dt, and +dP/dt. In control hearts, there was a progressive increase in these indices in response to increases in left atrial filling pressure (Fig. 4; data on +dP/dt not shown in graphs). Cardiac dysfunction
was apparent in untreated diabetic rats hearts, which exhibited an inability to respond to increases in left atrial filling pressure when compared to those of controls (Fig. 4; $+\frac{dP}{dt}$ at 10 mm Hg: C $4400 \pm 340$ mm Hg/sec vs D $2700 \pm 560$ mm Hg/sec, $P < .05$). Treatment of diabetic rats with bosentan (DB group) improved LVDP and $-\frac{dP}{dt}$ without affecting these indices in the CB group. Bosentan treatment did not affect the $+\frac{dP}{dt}$ in any group ($+\frac{dP}{dt}$ at 10 mm Hg: C $4400 \pm 340$ mm Hg/sec vs CB $3600 \pm 700$ mm Hg/sec, $P > .05$; D $2700 \pm 560$ mm Hg/sec, $P > .05$).
Calculation of the time to half relaxation \( t_{1/2R} \) revealed that bosentan treatment restored the \( t_{1/2R} \) in diabetic rats even at lower pressures \( t_{1/2R} = 6.3 \text{ mm Hg·sec} \) at 6.3 mm Hg: C 28 ± 6* msec, D 43.5 ± 6 msec, DB 35 ± 3* msec, *P < .05 different from C.

Effect of Bosentan Treatment on Vascular Reactivity

The maximum contractile response of superior mesenteric arteries to ET-1 was increased in diabetic rats when compared to controls (Fig. 5). This exaggerated response was corrected after bosentan treatment. Bosentan did not affect the contractile responses to ET-1 in control rat arteries. No change in agonist sensitivity between groups was noted (Table 2).

Discussion

The primary observations from this study are that chronic ET receptor blockade with bosentan improves functional cardiac performance and corrects vascular hyperreactivity to ET-1 in diabetes. These data are important, as they uncover, presumably for the first time, the beneficial effects of ET antagonism on cardiac and vascular function in experimental diabetes and suggest that exaggerated ET-1 production or action may play a role in the development of cardiovascular dysfunction in chronic experimental diabetes.

Elevated glucose levels are a potent stimulus for the gene expression and production of ET-1. However, data on plasma ET-1 levels in diabetes are conflicting; studies have demonstrated increased, decreased, or no change in ET-1 levels in diabetes. Because ET-1 is released primarily toward the vascular smooth muscle, plasma levels are neither indicative nor useful in assessing the contribution of this peptide toward local vascular homeostasis. Tissue levels are a better indicator of local ET-1 production and regulation. Given this preamble, the lack of difference in plasma ET-1 levels between the control and diabetic rat groups is not surprising, and is consistent with reports by Hopfner et al indicating the marked variation in ET-1 plasma levels at different stages of STZ-diabetes. Clearly, plasma ET-1 is a poor index of local production and we have observed that plasma levels do not correlate with tissue levels in STZ-diabetes (not shown). An increase in plasma ET-1 levels observed after bosentan treatment is a well known phenomenon related to dissociation of ET-1 from ET receptors after ET receptor blockade.

To circumvent the problems associated with the interpretation of plasma ET-1, we assessed ET-1-ir (by immunohistochemistry) in ventricles and vascular tissue from diabetic and control rats. Our data reveal an increased production of ET-1 in these tissues. Wu and Tang have demonstrated similar increases in vascular ET-1 levels in aortae and mesenteric arteries from STZ rats, without concomitant increases in plasma ET-1 levels. Because ET-1 levels are increased in the heart and vasculature from diabetic rats, it would be reasonable to ascribe the beneficial effects of bosentan to antagonism of ET-1. Bosentan is a potent inhibitor of ET\(_{AB}\) receptors and its pharmacological profile is well documented. Bosentan works to antagonize the actions of ET-1 on ET receptors and does not affect the production of ET-1 from endothelial cells. Furthermore, bosentan did not alter glucose levels in any group. Hence, the primary stimulus for ET-1 release (ie, elevated glucose levels) was intact. The observation that ET-1 levels were increased in the heart and vasculature in untreated diabetic rats is sufficient to suggest that the beneficial effects of bosentan were medi-
ated, at least in part, by antagonism of ET receptors and ET-1 action.

It is well known that arteries from STZ-induced diabetic rats exhibit enhanced contractile responses to a variety of vasoconstrictor agents. This effect is not related to decreased endothelium-derived nitric oxide production and may be related to alterations in G protein function, increased phosphoinositide turnover, or altered intracellular calcium flux. The observations that the contractile responses of mesenteric arteries to ET-1 are exaggerated are consistent with previous data in STZ-diabetic rats. In the face of increased mesenteric ET-1 levels, an increased ET-1 responsiveness is difficult to construe, as down-regulation of ET receptors would be expected. One explanation may be that the observed increase in ET-1 responses may be due to a nonspecific elevation in vascular smooth muscle contractility as a result of hyperglycemia-induced protein kinase C activation; this would explain the consistent enhancement of contractile responses to a variety of vasoactive agents in the presence of diabetes. Similar effects have been noted in rats with congestive heart failure, in which increased ET-1 levels are associated with parallel and paradoxical increases in ET-1 binding sites. However, from a functional standpoint, what perhaps is more important is the observed normalization of these vascular responses in diabetic rats after chronic bosentan treatment.

Cardiac dysfunction in diabetes is well documented, and the reader is referred to excellent reviews on the subject. In the present study, long-term administration of bosentan improved $-dP/dt$, $t_{1/2}$, and LVDP in the diabetic group without affecting $+dP/dt$. These parameters $-dP/dt$ and $t_{1/2}$, are very sensitive indices of diabetic diastolic dysfunction that appear to manifest first in STZ-induced diabetes. Improvement in the $t_{1/2}$ was observed in the diabetic-treated group even at low preload values, suggesting that bosentan treatment had a marked effect on ventricular relaxation. In our experience, $+dP/dt$ is the last factor to be corrected, possibly after improvement in diastolic function. Prolonging the treatment protocol may have uncovered beneficial effects on this parameter as well; however, this remains to be determined. The mechanisms of the beneficial effects of bosentan on diabetic heart function are unknown but seem to be unrelated to glycemic status or lipid metabolism; no changes in glucose, triglyceride, or cholesterol levels were noted after bosentan treatment in this study. Endothelin has a direct toxic effect on cardiac myocytes, and bosentan may serve to counter this effect.

Under normal circumstances, ET-1 has positive inotropic and chronotropic actions on heart muscle cells. However, under conditions of β-adrenergic stimulation, ET-1 has been shown to exert a negative modulatory effect on isolated myocytes through a $G_{q}$-mediated pathway. Endothelin-1 has been reported to hyperpolarize the membrane potential and to shorten the duration of the action.
potential in mammalian atrial myocytes, leading to suppression of electrical excitability of the heart and a decrease in heart rate. Endothelin-1 also exerts a potent inhibitory effect against isoproterenol-enhanced L-type Ca$^{2+}$ current in both atrial and ventricular myocytes; this ET-1 induced effect has been shown to be mediated via a pertussis toxin (PTX)–sensitive G-protein. In agreement with the above observations, ET-1 is reported to inhibit cAMP formation in response to isoproterenol and forskolin in adult cardiomyocytes, an effect that is PTX-sensitive and appears to be mediated via Gi. Thus, increased ET-1 levels in the diabetic heart may therefore attenuate cardiac responses to β-adrenergic agents and lead to cardiac dysfunction. Whether bosentan improves β-adrenoceptor mediated contractility is speculative at the present time.

Cardiac dysfunction in diabetes has been ascribed to alterations in myocardial energy use and the exclusive reliance of the heart on free fatty acids as a source of ATP. Increased free fatty acids exert adverse electrophysiological, biochemical, and mechanical effects on the heart with consequent changes in intracellular calcium handling, membrane permeability, and eventual cell death and cardiac dysfunction. It is possible that the effects of bosentan are mediated through improvement in myocardial energetics, and we are currently examining this proposition. Endothelin-1 is also a potent arrhythmogen. Ventricular arrhythmias after ET-1 are well documented. Inhibition of ET-1 induced arrhythmias by bosentan may exert myocardial protective effects in diabetes. Cardiac hypertrophy occurs secondary to a variety of stimuli and ET-1 exhibits potent hypertrophic effects on cardiac myocytes. Antagonism of cardiac hypertrophy by ET receptor blockade has been demonstrated in experimental congestive heart failure. Our data in diabetic rat hearts does not unmask a similar effect; heart weight to body weight ratios remained unchanged after bosentan treatment. Whether these effects

Table 2. Sensitivities of superior mesenteric arteries to ET-1

<table>
<thead>
<tr>
<th>Group</th>
<th>Sensitivity ($\text{pD}_2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>$8.18 \pm 0.19$</td>
</tr>
<tr>
<td>CB</td>
<td>$8.03 \pm 0.25$</td>
</tr>
<tr>
<td>D</td>
<td>$8.08 \pm 0.19$</td>
</tr>
<tr>
<td>DB</td>
<td>$8.14 \pm 0.15$</td>
</tr>
</tbody>
</table>

C = control rats; CB = control bosentan-treated rats; D = diabetic rats; DB = diabetic bosentan-treated rats. Values are as means ± SEM. $\text{pD}_2$ represents $-\log \text{ED}_{50}$. No significant difference was found among different rat groups.
would have become apparent if the treatment had been prolonged is an important question that needs to be addressed.

In the present study, the nonspecific receptor antagonist bosentan was used. The use of a mixed ET\textsubscript{A} and ET\textsubscript{B} receptor blocker enabled us to determine the role of endogenous ET-1 in diabetic complications. On the other hand, the nonspecific nature of bosentan prevented us from determining which specific receptor subtypes may be responsible for mediating the pathogenic role of ET-1. It has been suggested that both ET\textsubscript{A} and ET\textsubscript{B} receptors may contribute to the deleterious effects of ET-1.\textsuperscript{13} In addition, different studies including this one have demonstrated that blockade of both ET\textsubscript{A} and ET\textsubscript{B} receptors effectively ameliorated cardiac, vascular, retinal, and renal abnormalities in diabetes.\textsuperscript{33–36} However, the important question as to whether a nonselective ET\textsubscript{A} and ET\textsubscript{B} receptor blockade would provide more or fewer benefits than a selective ET\textsubscript{A} receptor blockade still remains to be solved.

In summary, the present study demonstrates the effects of long-term ET receptor blockade on cardiovascular function in STZ-diabetic rats. Chronic bosentan treatment improved functional cardiac performance and restored exaggerated vascular reactivity to ET-1. These data support a role of ET-1 as a mediator of cardiovascular dysfunction in diabetes.

Acknowledgments

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References