Ramipril Improves Oxidative Stress-Related Vascular Endothelial Dysfunction in db/db Mice

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Abstract: Endothelial dysfunction often precedes Type 2 diabetes–associated cardiovascular complications. One important cause of endothelial dysfunction is oxidative stress, which can lead to reduced nitric oxide (NO) bioavailability. In this study, we examined the effects of ramipril (an angiotensin-converting enzyme inhibitor, ACEI) on reactive oxygen species (ROS) production and endothelium-dependent vasodilation using a Type 2 diabetic (db/db) murine model. Plasma concentration of 8-isoprostane ([8-isoP]) was measured and used as an indication of the amount of ROS production. Six weeks of ramipril (10 mg/kg/day) treatment significantly reduced [8-isoP] and improved acetylcholine (ACh)-induced vasodilation in db/db mice without altering responses in wild-type (WT) mice. Responsiveness of smooth muscle cells to NO, assessed by sodium nitroprusside–induced vasodilation, was not different between db/db and WT mice regardless of ramipril or vehicle treatment. Our results suggest that ramipril specifically improved endothelium-dependent vasodilation in Type 2 diabetic mice, possibly by reducing ROS levels.

Key words: diabetes, oxidative stress, nitric oxide, angiotensin-converting enzyme inhibitor, vasodilation.

About 90% of patients with diabetes mellitus have Type 2 diabetes, amounting to more than 120 million people worldwide [1]. A genetic predisposition, obesity, and a sedentary lifestyle contribute to the onset of the disease [2]. Type 2 diabetes is characterized by insulin resistance and dysfunctional pancreatic β-cells, causing impaired insulin effects and/or secretion. Endothelial dysfunction is a key feature of Type 2 diabetes and is postulated to be the major cause of cardiovascular complications associated with the disease [3–5]. When stimulated with acetylcholine (ACh), a healthy endothelium secretes a range of vasoactive substances, with nitric oxide (NO) being one of the most important endogenous vasodilators [6]. In Type 2 diabetes, NO bioavailability is often diminished, leading to significantly reduced ACh-mediated vasodilation [7]. Reduced NO levels can be attributed to oxidative stress that is related to elevated levels of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and peroxynitrite. Elevated NADPH oxidase expression and activity leads to high superoxide levels [8]. Superoxide combines with NO, which is synthesized by endothelial nitric oxide synthase (eNOS) to form peroxynitrite. In turn, peroxynitrite oxidizes and destabilizes eNOS to produce more superoxide [9, 10]. The consequence is an overall increase in ROS and reduced ability of endothelium-dependent vasodilation, as observed in Type 2 diabetic patients. The overproduction of ROS can be exacerbated by angiotensin II (AngII), the secretion of which is increased by hyperglycemia [11], as exemplified in Type 2 diabetic patients. A potent vasoconstrictor itself, AngII also activates NADPH oxidase, leading to ROS production [12, 13]. Given its precipitating role in increasing ROS and endothelial damage, the ability to lower circulating AngII levels has been a focus in managing Type 2 diabetes. Two AngII-associated strategies to improve vascular function have been proposed. Antagonists specific to the AngII subtype 1 receptor (AT₁R) reduce ROS levels [14], but apparently without improving endothelium-dependent vasodilation in diabetic patients [15–17]. On the other hand, angiotensin-converting enzyme inhibitors (ACEIs), which block AngII synthesis, improve endothelium-
dependent vasodilatation in Type 2 diabetes [18]. In this study we examine the effects of the ACEI ramipril on production of ROS and restoration of endothelium-dependent vasodilatation in a mouse model of Type 2 diabetes.

METHODS

Animals. Thirteen five-week-old male diabetic db/db (BKS.Cg-m+/+Leprdb/j strain) and 15 wild-type littermates +/+db (WT) were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). The mice were housed in a temperature- (26°C) and humidity-controlled environment with a 12 h light-and-dark cycle and had free access to lab diet and water. Protocols were in accordance with the University of British Columbia Animal Care Committee Guidelines. Body weights and blood glucose levels were recorded on a weekly basis, and water consumed per cage was recorded every other day. WT and db/db mice were both divided randomly into two groups of 5 mice each, and each group received either vehicle or ramipril (10 mg/kg/day) via the drinking water. Doses were adjusted according to body weight and age-related changes in water consumption.

Blood glucose and ROS measurements. For measurements of blood glucose levels, blood was collected from mouse tails using a handheld blood glucose monitoring system (Ascensia® Contour®, Bayer HealthCare, Toronto, Canada) on a weekly basis. At 11 weeks of age (following 6 weeks of treatment), the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and sacrificed. Blood was drawn and placed in an ice-cooled EDTA-coated MicroTainer (Fisher Scientific, Pittsburgh, Pennsylvania, USA), and immediately centrifuged at 2,000 to 3,000 rpm for 15 min at 4°C. The plasma and blood clots were carefully separated and the plasma frozen at –80°C in the presence of 0.005% butylated hydroxy toluene (BHT) (10 µl of 5 mg/ml solution in ethanol per 1 ml sample) for ROS measurements.

Determination of 8-isoprostane levels using enzyme immunoassay. The isoprostanes are a family of eicosanoids of nonenzymatic origin produced by the random oxidation of tissue phospholipids by oxygen radicals. At least one family of isoprostanes, 8-isoprostanes (8-isoP), has been implicated as a causative mediator and specific marker of free radical–induced damage in a variety of diseases related to oxidative stress [19]. Plasma levels of 8-isoP are indicative of ROS production [14, 20] and were determined by an enzyme immunoassay kit using the manufacturer’s protocol (Cayman Chemical, Ann Arbor, Michigan, USA). Absorbance readings at 450 nm with a Tecan Spectra Rainbow plate reader (Tecan, Männedorf, Switzerland). A standard curve was plotted using known 8-isoP concentrations (8-isoP). The following equation, derived from fitting the standard curve, was used in converting values of absorbance readings to absolute 8-isoP concentrations (in pg/ml): \[ [8-isoP] = e^{-(% \text{ binding} - 90.91)/13.5} \], where percent binding has been corrected for nonspecific binding.

Contractility studies. The thoracic aortas were cleaned of surrounding tissues carefully and cut into 2 mm rings. The aortic rings were mounted on tungsten wires (diameter 40 µm) and placed in separate 5 ml wire myograph chambers (Multi Myograph Model 610 M, Danish Myotech, Aarhus, Denmark). The chambers were filled with physiological salt solution (PSS) and maintained at 37°C that was aerated with carbogen (95% oxygen and 5% carbon dioxide). After we mounted the aortic rings, the baseline tension was gradually increased to 5 mN over 60 min as previously described [21]. Bath solution was changed at 15 min intervals. After the equilibration period, the aortic rings were challenged twice at 15 min intervals with 80 mM K+-containing PSS (80K-PSS) to ensure tissue viability.

Concentration-response curves (CRCs) of ACh and sodium nitroprusside (SNP). The presence of functional endothelial cells in aortic rings was first tested with ACh. A single concentration of phenylephrine (PE, 10−6 M) was added to establish a submaximal contraction, followed by the addition of ACh (10−6 M) to induce endothelium-dependent vasodilatation. After refreshing the PSS several times to reestablish baseline tension, the tissues were left to equilibrate for 20 min. A single concentration of PE (10−6 M) was added again. Upon reaching steady-state contraction, ACh (10−9 M to 10−5 M) was added in a cumulative manner to construct a CRC. After repeated washouts with PSS, endothelium-independent vasodilatation induced by SNP was conducted to examine the viability of smooth muscle cells (SMCs). Tissues were precontracted with PE (10−6 M) until reaching a steady state, when SNP (10−9 M to 10−5 M) was added in a cumulative manner.

Drugs and chemicals. The composition of PSS was (in mM) NaCl (119), KCl (4.7), KH2PO4 (1.18), NaHCO3 (24), MgSO4.7H2O (1.17), CaCl2 (1.6), glucose (5.5), and EDTA (0.026) at pH 7.4. The composition of 80K-PSS was (in mM) NaCl (43.7), KCl (80), KH2PO4 (1.18), NaHCO3 (24), MgSO4.7H2O (1.17), CaCl2 (1.6), glucose (5.5), and EDTA (0.026) at pH 7.4.

Statistical analysis. The results are expressed as the mean ± SEM. GraphPad Prism (version 4.03-2005) was used for one- and two-way ANOVA with a Bonferroni post hoc test wherever appropriate. P values of less than 0.05 (P < 0.05) were considered to be statistically significant.

RESULTS

Ramipril had no effect on body weights or blood glucose levels

Body weights and blood glucose levels of WT (vehicle or ramipril) and db/db (vehicle or ramipril) mice were...
measured at weekly intervals during the treatment period, as shown in Fig. 1. At the start of the experiment, the five-week-old WT and diabetic mice did not differ significantly in either their body weights or plasma glucose levels, suggesting that the phenotypic characteristics of Type 2 diabetes had not emerged yet. By 6 weeks of age, db/db mice had significantly increased their body weights and had higher blood glucose levels. Over the remaining course of ramipril (or vehicle treatment), all mice continued to gain body weight so that by 11 weeks of age, the db/db mice weighed nearly as much as the WT mice. Ramipril treatment did not affect body weight changes in either the WT or the db/db mice. Hyperglycemia was evident in db/db mice by 6 weeks of age. Blood glucose levels continued to increase until the db/db mice were 8 weeks old, at which point blood glucose levels of db/db mice were maintained at about 4 times higher than their WT counterparts, whose blood glucose remained relatively constant throughout the six-week period. Ramipril treatment did not noticeably affect blood glucose levels in either WT or db/db mice.

**Ramipril treatment reduced plasma ROS levels to a greater extent in db/db mice**

Prior to isolating the aortas for evaluation of endothelial function, blood was collected from all mice for measurements of circulating ROS levels. Using an enzyme immunoassay kit and computational methods as described before, we determined the absolute concentrations of 8-isoprostanes (8-isoP), an ROS marker, from the blood samples, as reported in Fig. 2. ROS production was significantly higher (by 55.9 ± 23.4%, *P* < 0.05) in vehicle-treated db/db than in WT mice. In ramipril-treated db/db mice, 8-isoP levels were 238.8 ± 22.6 pg/ml, compared to 486.4 ± 68.8 pg/ml in vehicle-treated db/db mice. Ramipril treatment resulted in a 50.9 ± 23.6% (*P* < 0.05) lower ROS production in db/db mice. In WT mice, ramipril had a statistically insignificant effect on ROS production. In ramipril-treated WT mice, [8-isoP] was 206.6 ± 12.7 pg/ml, compared to 312.0 ± 28.9 pg/ml in vehicle-treated WT mice. Levels of 8-isoP were comparable between ramipril-treated db/db and WT mice (*P* > 0.05).

**ACh-induced vasodilatation is primarily due to NO release in both WT and db/db murine aortas**

Oxidative stress-related endothelial dysfunction could be a consequence of an imbalance in the production of NO and other ROS. Thus we first examined ACh-induced vasodilatation in PE-precontracted WT aorta to determine if NO was the sole endothelium-derived vasorelaxant factor in normal, healthy endothelium. We compared...
The results above suggested that ramipril decreased ROS production to improve ACh-stimulated NO release and the resultant vasodilation. To test this hypothesis, we compared ACh-induced vasodilation in aortas from ramipril-treated (or vehicle-treated) WT and db/db mice (Fig. 4). Complete vasodilation was observed in aortas from WT mice (n = 10) regardless of treatments. Vehicle-treated db/db aortas (n = 5) showed marked suppression in ACh-induced vasodilation when compared to aortas from WT mice (P < 0.05). With ramipril treatment, db/db aortas (n = 4) showed significant greater vasodilating response to ACh, compared to aortas from db/db mice receiving vehicle treatment (P < 0.05). After incubating with L-NAME, aorta from ramipril-treated mice showed no ACh-induced vasodilatation, complementing the data in Fig. 3 where L-NAME completely abolished the ACh response in WT aortas.

Table 1 compares the maximal efficacies, expressed as $E_{\text{max}}$, of the ACh-induced vasodilatation in different conditions. Wild-type mice with and without ramipril treatment displayed equal maximal efficacy to ACh. Untreated db/db mice showed only 19.08 ± 1.54% of vasodilation, which was significantly smaller than that of the ramipril-treated db/db mice (85.00 ± 2.48%, P < 0.05). Maximal vasodilation in ramipril-treated db/db mice was marginally but not significantly smaller than that in WT mice. This indicated ramipril treatment might not completely restore ACh-induced endothelium-dependent vasodilation, which was further supported by the ACh potency data. In Table 1, the potencies expressed as negative logarithms of the effective concentrations at half-maximal response ($pEC_{50}$) of ACh among the 4 groups of aortas were also compared. The $pEC_{50}$ of vehicle-treated WT murine aortas was $-7.45 ± 0.13$ and was not different from that of the ramipril-treated WT murine aortas ($-7.30 ± 0.07$). The potency of ACh of ramipril-treated db/db murine aortas ($-7.05 ± 0.06$) was significantly higher than that of the vehicle-treated db/db mice ($-6.77 ± 0.09$, P < 0.05). The vehicle-treated db/db mice also displayed significantly lower ACh potency than both groups of WT mice.
Smooth muscle cell function was not different between WT and db/db mice

We examined the vasodilation produced by SNP, an exogenous donor of NO, to determine if ramipril treatment modified SMC responsiveness to NO (Fig. 5). In aortas from both WT (n = 10) and db/db (n = 9) mice, SNP elicited maximal vasodilation regardless of ramipril or vehicle treatment. Potencies of SNP in db/db murine aortas were lower than those in WT, but not statistically different (Table 1). Also shown in Table 1 was the equal maximal efficacy to SNP across all 4 groups of mice.

DISCUSSION

Numerous studies confirm an association between Type 2 diabetes and vascular complications [23–25]. One common feature of diabetes in animals and humans alike is reduced endothelium-mediated vasodilation [18, 21]. Endothelial cells can be rendered dysfunctional during insulin resistance, hyperglycemia, and vascular inflammation, all of which are common features in Type 2 diabetes [24]. Insulin resistance, often characterized by low expression of the insulin receptor substrate 1 protein, slightly reduces NO production [24], and its physiological importance in vasodilation remains uncertain. Much evidence suggests that oxidative stress, which may be precipitated by hyperglycemia and vascular inflammation, is important in endothelial dysfunction. Consequently, reducing oxidative stress by lowering ROS production is crucial in the management of Type 2 diabetes. Thus this study investigated the effects of ramipril, an ACE inhibitor, on ROS production and endothelium-dependent vasodilation in a murine model of Type 2 diabetes.

Of the vasoactive substances implicated in endothelial dysfunction, AngII is most likely clinically important. Increased AngII secretion from endothelial cells has both vasoconstricting and pro-oxidative effects [25, 26]. The roles of AngII receptors vary. The blockade of AT1 receptors enhances endothelium-dependent vasodilation via reduced ROS production [14], but does not necessarily lower blood pressure in Type 2 diabetic patients [14, 17]. The activation of AT2 receptors may protect against oxidative stress, however [27]. The beneficial roles of ACEIs on endothelium-dependent vasodilation have been reported by others [18, 28, 29], but the possible link between lower ROS production and ACEI is less clear. Although some reports suggest that AngII increases ROS production, no studies have examined if ACEI treatment decreases oxidative stress in Type 2 diabetes. Our data showed that ramipril reduces plasma ROS levels to a significantly greater extent in db/db than in WT mice (Fig. 2).

The overproduction of ROS in db/db mice is very likely a result of elevated AngII synthesis, which consequently destabilizes eNOS and decreases NO bioavailability [9, 10, 26]. This is supported by the recent finding that endogenous NO levels were much lower in db/db than in WT mice [30]. In the present study, we treated mice with ramipril, which inhibits AngII production, using a dose similar to that used by others [31, 32]. Vasodilation induced by ACh was greater in ramipril-treated db/db aortas than in untreated ones (Fig. 4). The enhanced vasodilation could be attributed to increased NO production, since L-NAME (a NO synthase inhibitor) abolished the

### Table 1. Negative logarithms of potency (pEC50) and maximal efficacies (E_{max}) of acetylcholine (ACh) and sodium nitroprusside (SNP) in vasodilation of isolated aortas from vehicle- or ramipril-treated wild-type (WT) and Type 2 diabetic (db) mice (* denotes statistical significance vs. all groups; # denotes statistical significance vs. WT mice).

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<th>Wild-type (WT) mice</th>
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<td>Vehicle-treated</td>
<td>Ramipril-treated</td>
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<td>ACh</td>
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<tr>
<td>pEC50</td>
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<td>E_{max}</td>
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<tr>
<td>pEC50</td>
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<tr>
<td>E_{max}</td>
<td>90.04 ± 4.22</td>
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**Fig. 5.** Endothelium-independent vasodilation was not different between wild-type (WT) and db/db (db) mice undergoing ramipril or vehicle treatment. Smooth muscle relaxant responses were measured by sodium nitroprusside(SNP)-induced vasodilation in aortas. Concentration-response curves were constructed using data from 4 groups of 5 mice each.
The ADVANCE trial reported that intensive glycemic control, the latter resulting in significant lower blood glucose levels than the normal control group. These risks and mortality rate between normal and intensive glycemic control, the latter resulting in significant lower blood glucose levels than the normal control group. The ADVANCE trial reported that intensive glycemic control did not reduce the incidence of macrovascular events or cardiovascular-related deaths [38]. The ACCORD trial, however, reported an increase in cardiovascular-related deaths, although the incidence of nonfatal myocardial infarction was significantly lower [39]. Findings from these two trials indicate that the causal effects of glycemic control in cardiovascular protection of Type 2 diabetes patients remains to be elucidated. Further investigations on ramipril and amlodipine are warranted to determine if drugs with no known glucose-lowering effects have extra benefits in the management of Type 2 diabetes.

In summary, this study investigated the effect of ramipril on vascular endothelial function using a Type 2 diabetic mouse model. Ramipril, an ACE inhibitor, significantly decreased ROS production and enhanced ACh-induced endothelium-dependent vasodilation in aorta from db/db mice. Neither smooth muscle function nor blood glucose levels were affected by ramipril. Thus the beneficial effect of ramipril on improved vascular function is very likely because of the inhibition of angiotensin-mediated increases in free radicals associated with oxidative stress.

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