Coronary endothelial dysfunction and mitochondrial reactive oxygen species in type 2 diabetic mice

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Cho YE, Basu A, Dai A, Heldak M, Makino A. Coronary endothelial dysfunction and mitochondrial reactive oxygen species in type 2 diabetic mice. Am J Physiol Cell Physiol 305: C1033–C1040, 2013. First published August 28, 2013; doi:10.1152/ajpcell.00234.2013.—Endothelial (EC) dysfunction is implicated in cardiovascular diseases, including diabetes. The decrease in nitric oxide (NO) bioavailability is the hallmark of endothelial dysfunction, and it leads to attenuated vascular relaxation and atherosclerosis followed by a decrease in blood flow. In the heart, decreased coronary blood flow is responsible for insufficient oxygen supply to cardiomyocytes and, subsequently, increases the incidence of cardiac ischemia. In this study we investigate whether and how reactive oxygen species (ROS) in mitochondria contribute to coronary endothelial dysfunction in type 2 diabetic (T2D) mice. T2D was induced in mice by a high-fat diet combined with a single injection of low-dose streptozotocin. ACh-induced vascular relaxation was significantly attenuated in coronary arteries (CAS) from T2D mice compared with controls. The pharmacological approach reveals that NO-dependent, but not hyperpolarization- or prostacyclindependent, relaxation was decreased in CAS from T2D mice. Attenuated ACh-induced relaxation in CAS from T2D mice was restored toward control level by treatment with mitoTempol (a mitochondria-specific O2− scavenger). Coronary ECs isolated from T2D mice exhibited a significant increase in mitochondrial ROS concentration and decrease in SOD2 protein expression compared with coronary ECs isolated from control mice. Furthermore, protein ubiquitination of SOD2 was significantly increased in coronary ECs isolated from T2D mice. These results suggest that augmented SOD2 ubiquitination leads to the increase in mitochondrial ROS concentration in coronary ECs from T2D mice and attenuates coronary vascular relaxation in T2D mice.

hyperglycemia; diabetic vascular complication; posttranslational modification; ubiquitin-proteasome system

VASCULAR ENDOTHELIAL CELLS (ECs) play an important role in regulation of vascular tone, vascular permeability, blood cell coagulation, and new vascular formation. The endothelium regulates vascular tone by producing and releasing vasconstrictors (e.g., endothelin-1, angiotensin II, and thromboxane A2) and vasodilators [e.g., nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors] and 2) inducing hyperpolarization of smooth muscle cells (SMCs) via electrical propagation through the gap junctions. NO serves as a vasodilator, as well as an inhibitor of aggregation of platelets and infiltration of inflammatory cells. The decrease of NO bioavailability, as a result of endothelial dysfunction, causes increased vascular tension and atherosclerotic plaque and leads to the decrease in blood flow. In the heart, decreased coronary blood flow is responsible for insufficient oxygen supply to the cardiomyocytes and increase in the incidence of cardiac ischemia.

Ischemic heart diseases are involved in >50% of diabetes-related deaths, and the incidence of cardiac ischemia is higher in diabetic patients than control subjects. Impaired endothelium-dependent vasodilatation due to chronic elevation of blood glucose level has been reported in various diabetic animal models and in diabetic patients (5, 42, 54, 67, 80), and the reduced NO bioavailability is a common feature of EC dysfunction in diabetes (62, 67, 75).

Under physiological conditions, ECs constantly generate reactive oxygen species (ROS), including superoxide anion (O2−), and ROS participate in modulation of essential endothelial functions. The potential sources of O2− include mitochondrial electron transport chain (ETC), xanthine oxidase, uncoupled endothelial NO synthases (eNOS), cytochrome P-450 enzymes, and NADPH oxidases (NOXs). ECs express several antioxidant enzymes, such as SOD, catalase, and glutathione peroxidase. The imbalance between ROS production and elimination results in increased oxidative stress and tissue injury and leads to many cardiovascular diseases (51, 87). There is increasing evidence showing that elevated levels of O2− in ECs contribute to development of atherosclerosis (34), vascular complications of diabetes (2, 25, 31, 36, 57), and hypertension (12, 35). In this study we demonstrate that decreased endothelium-dependent vascular relaxation (EDR) in coronary arteries (CAS) from type 2 diabetic (T2D) mice is due to an increase in mitochondrial ROS production in coronary ECs from T2D mice. It is, at least in part, attributed to decreased SOD2 protein expression via augmented ubiquitination of SOD2 in coronary ECs from T2D mice.

MATERIALS AND METHODS

Biological Materials and Reagents

N-nitro-l-arginine methyl ester (l-NAME) was purchased from Cayman Chemical (Ann Arbor, MI); apamin (Apa) and charybdotoxin (ChTx) from AnaSpec (Fremont, CA); streptozotocin (STZ) and anti-SOD1 and anti-SOD2 antibodies from Enzo Life Science (Plymouth Meeting, PA); M199, antibiotic reagents, dispase II, MitoTracker Green FM, and MitoSOX Red from Invitrogen (Carlsbad, CA); anti-ubiquitin (Ub) and anti-actin antibodies and ImmunoCruz from Santa Cruz Biotechnology (Santa Cruz, CA); anti-CD31 and EC growth supplement from BD Biosciences (San Jose, CA); and collagenase II from Worthington Biochemical (Lakewood, NJ). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animal Preparation

All investigations conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985). This study was conducted in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the University of Illinois at Chicago. Our protocols were approved by the Office of Animal Care and Institu-
Figure 1. Plasma insulin levels, oral glucose tolerance test (OGTT), and insulin tolerance test (ITT) results. A: insulin concentration in plasma samples from non-fasting type 2 diabetic (T2D, n = 5) and control (Cont, n = 5) mice. B: percent glucose in control (n = 10) and T2D (n = 13) mice fasted for 6 h before the OGTT. C: percent glucose in control (n = 10) and T2D (n = 13) mice fasted for 4 h before the ITT. Values are means ± SEM. *P < 0.05 vs. Cont.
mmol/l). In a control group of cells, equimolar mannitol was added to exclude the potential effect of changes in osmolarity (normal glucose, 5 mmol/l). For free fatty acid (FFA) treatment, 300 μmol/l palmitic acid was added to the medium. Control cells were treated with vehicle (BSA) only. Cells were cultured for 48 h and used for the immunoprecipitation (IP) experiment.

**Immunoprecipitation**

- **IP-Ub/immunoblotting-SOD2.** Mouse coronary ECs (from 2–3 hearts/sample) were lysed, and 25 μg of proteins were incubated with ExactaCruz C IP matrix (Santa Cruz Biotechnology), which was prepared as follows: IP matrix (40 μl) was incubated with anti-Ub (2 μg) at 4°C for 2 h and then washed with binding buffer. Lysate with IP matrix was incubated overnight at 4°C. The matrix-bound proteins were collected, and samples were used for immunoblotting.
- **IP-SOD2/immunoblotting-Ub.** Human coronary ECs were lysed, and 50 μg of proteins were incubated with ExactaCruz B IP matrix, which was prepared as follows: IP matrix (20 μl) was incubated with anti-SOD2 (1 μg/ml) at 4°C for 2 h and then washed with PBS.

**Statistical Analysis**

Values are means ± SE. Statistical comparison between dose-response curves was made by two-way ANOVA with Bonferroni’s correction. Student’s t-test for unpaired samples was used to identify significant differences. Differences were considered to be statistically significant when *P* < 0.05.

**RESULTS**

**Metabolic Characteristics**

Blood samples were collected for measurement of metabolic characteristics cholesterol, Body weight and plasma levels of glucose and total cholesterol, HDL, and LDL were significantly higher in T2D than control mice (Table 1). Plasma insulin level was significantly higher in T2D than control mice (Table 1). Plasma glucose and total cholesterol, HDL, and LDL were significantly higher in T2D than control mice (Table 1). Plasma glucose and total cholesterol, HDL, and LDL were significantly higher in T2D than control mice (Table 1).

**Attenuated ACh-Induced Relaxation in CAs From T2D Mice**

To investigate endothelial function, ACh-induced vascular relaxation was examined using isolated CA rings from control and T2D mice. As shown in Fig. 2A, endothelium-dependent relaxation (EDR) was significantly decreased in CAs from T2D mice compared with controls, while there was no difference in sodium nitroprusside (SNP)-dependent relaxation between the two groups (Fig. 2B). To identify the molecular mechanism involved in attenuated EDR in diabetic CAs, a series of pharmacological experiments were conducted using the various inhibitors. Pretreatment with l-NAME (a NOS inhibitor, 100 μmol/l) and indomethacin (Indo, a cyclooxygenase inhibitor, 10 μmol/l) attenuated EDR in CAs from control and T2D mice, and the magnitude of the relaxation was not different between control and T2D CAs (Fig. 2C). However, in the presence of the Ca2+-activated K+ channel blockers, Apa and ChTX (100 nmol/l), and Indo, EDR was significantly decreased in CAs from T2D mice compared with controls (Fig. 2D). Pretreatment with all inhibitors (l-NAME, Indo, Apa, and ChTX) diminished EDR in both groups (Fig. 2E). Pretreatment with Indo alone did not affect ACh-induced relaxation in CAs from control and T2D mice (data not shown). These data suggest that an attenuated NO-mediated relaxation contributes to the decrease in ACh-induced relaxation in CAs from T2D mice, while prostacyclin- and endothelium-derived hyperpolarization-dependent relaxation was not altered in CAs from T2D mice.

**Increased Mitochondrial ROS Concentration in Coronary ECs From T2D Mice**

We examined whether mitochondrial ROS concentration was altered in coronary ECs from T2D mice. Coronary ECs were isolated from control and T2D mice and cultured on coverslips. Figure 3, A and B, demonstrates a significant increase of mitochondrial ROS in coronary ECs from T2D mice.
controls. These data suggest that increased O$_2^-$ augmented EDR in CAs from T2D mice toward the level in control mice by pretreatment with 10 μmol/l mitoTempol (a mitochondria-specific O$_2^-$ scavenger). Scale bars, 10 μm. B: summarized data showing average of mitochondrial ROS intensity in primary cultured coronary ECs from control (n = 203), T2D (n = 175), and mitoT-treated T2D (n = 177) mice. C: ACh-induced vascular relaxation in control (n = 12), T2D (n = 13), and mitoT-treated (100 μmol/l) T2D (n = 3) mice. Values are means ± SE. *p < 0.05 vs. Cont. #p < 0.05 vs. T2D.

Fig. 3. Effect of mitoTempol on mitochondrial reactive oxygen species (ROS) concentration in mouse coronary endothelial cells (ECs) and endothelium-dependent vascular relaxation in diabetes. A: representative images showing mitochondrial ROS concentration in primary cultured coronary ECs isolated from control and T2D mice and ECs from T2D mice treated with mitoTempol (mitoT, a mitochondrial O$_2^-$ scavenger, 10 μmol/l). Compared with control mice by pretreatment with 10 μmol/l mitoTempol (a mitochondria-specific O$_2^-$ scavenger).

mitoTempol Treatment Restores ACh-Induced Relaxation in Diabetic CA

To determine the role of O$_2^-$ in decreased EDR in CAs from T2D mice, CAs were pretreated with mitoTempol (100 μmol/l) for 20 min. As shown in Fig. 3C, mitoTempol treatment augmented EDR in CAs from T2D mice toward the level in controls. These data suggest that increased O$_2^-$ production leads to decreased EDR in CAs from T2D mice.

Decreased SOD2 Protein Expression in Coronary ECs from T2D Mice

To determine which subtypes of SOD protein expression were altered, we used coronary ECs freshly isolated from control and T2D mice. As shown in Fig. 4, SOD2 protein expression was significantly decreased in coronary ECs from T2D mice compared with controls [1.03 ± 0.07 (n = 8) and 0.66 ± 0.12 (n = 9) in control and T2D, respectively, \( p = 0.02 \)], whereas SOD1 protein expression level was not changed in coronary ECs from T2D mice.

Protein Ubiquitination of SOD2

To identify the mechanism whereby SOD2 protein expression was decreased in coronary ECs from T2D mice, we first measured and compared SOD2 mRNA level in coronary ECs isolated from control and T2D mice. Figure 5A demonstrates no difference of SOD2 mRNA expression between coronary ECs from control and T2D mice. Next, we determined the level of SOD2 protein ubiquitination in coronary ECs isolated from control and T2D mice. Coronary ECs from T2D mice exhibit significant increase in the level of ubiquitinated SOD2 protein compared with coronary ECs from controls (Fig. 5B). These data suggest that increased mitochondrial O$_2^-$ in coronary ECs from T2D mice may be due to decreased SOD2 protein expression via the increase in ubiquitination of SOD2 protein in coronary ECs from T2D mice.

High-Glucose Treatment Increases Protein Ubiquitination of SOD2

We used human coronary ECs to examine the effect of high-glucose or high-FFA treatment on ubiquitination of SOD2.
Increased ROS (including O$_2^-$) and its maladaptive effects on vascular function have been demonstrated in patients with T2D (11, 18, 24). Augmented ROS production in the vessels has also been shown in T2D mice (8, 21, 38, 74, 82) and rats (32, 70–72), but none of these studies examined coronary ECs. We demonstrated, for the first time, that mitochondrial ROS concentration was significantly increased in coronary ECs from T2D mice compared with controls and that the increased ROS concentration in coronary ECs from T2D mice was restored toward the level in controls by treatment with mitoTempol (Fig. 3, A and B). This direct evidence suggests that attenuated ACh-induced relaxation in diabetic CAs is caused by the increased O$_2^-$ in coronary ECs, but not SMCs, which is why SNP-induced vascular relaxation was not altered in diabetic CAs.

There are various sources of O$_2^-$ generation in ECs, including NOX, the mitochondrial ETC, xanthine oxidase, uncoupled eNOS, and cytochrome P-450 (51). High-glucose treatment enhances mitochondrial O$_2^-$ production by alteration of mitochondrial complex III activity in rat renal proximal tubular cells (65) or by phosphorylation of p47phox (interaction with NOX1) in rat aortic vascular SMCs (52). Treatment with oxidized LDL increases the activity of complex I in mitochondria of human umbilical vein ECs (7). In femoral arteries of type 1 diabetic rats, an increase in gp91phox (also known as NOX2) is followed by excess O$_2^-$ production (23). NOX1 knockout mice do not develop endothelial dysfunction in the aorta in type 1 diabetic mice (86). NOX inhibitor restores the level of O$_2^-$ toward the control in coronary SMCs of T2D mice (30). Excess mitochondrial O$_2^-$ production and impaired mitochondrial antioxidant defense result in increased mitochondrial O$_2^-$ production. Mitochondrial O$_2^-$ generated by the ETC is largely released to the matrix at complex I and the intermembrane space at complex III. SOD2 (also known as MnSOD) in the matrix and SOD1 (also known as Cu/ZnSOD) in the intermembrane space catalyze the dismutation of O$_2^-$ to H$_2$O$_2$. H$_2$O$_2$ is detoxified to H$_2$O in the matrix by catalase, the thioredoxin-thioredoxin peroxidase system, or the glutathione-glutathione peroxidase system. Figure 4 demonstrates that SOD2, but not SOD1, was significantly decreased in coronary ECs isolated from T2D mice compared with controls, suggesting that the

![Fig. 5. Ubiquitination of SOD2 protein is significantly increased in coronary ECs from T2D mice. A: SOD2 mRNA expression level in coronary ECs isolated from control and T2D mice detected by real-time PCR. B: immunoprecipitation (IP) with anti-ubiquitin (Ub) antibody and immunoblotting (IB) with anti-SOD2 antibody in coronary ECs from control and T2D mice. Values are means ± SE; n = 5 Cont and 5 T2D. *P < 0.05 vs. Cont.](image)

![Fig. 6. High-glucose treatment significantly increases ubiquitination of SOD2 protein. A: immunoprecipitation (IP) with anti-SOD2 antibody and immunoblotting (IB) with anti-ubiquitin antibody (Ub) or anti-SOD2 antibodies in human coronary ECs treated with normal glucose (NG, n = 8) or high glucose (HG, n = 9). Values are means ± SE. *P < 0.05 vs. NG. B: IP with anti-SOD2 antibody and IB with anti-Ub or anti-SOD2 antibodies in human coronary ECs treated with 300 μM palmitic acid (free fatty acid, n = 5) or vehicle (BSA, n = 5). Values are means ± SE.](image)

**DISCUSSION**

EC dysfunction is the leading cause of cardiovascular complications in diabetes (5, 19, 73, 80, 88); however, the mechanisms that cause EC dysfunction were varied between the different organs and different animal models that were used in the experiments. Our T2D mice exhibit hyperglycemia, hyperinsulinemia, and hyperlipidemia with significant increase in their body weight (Table 1, Fig. 1); this model is very close to human T2D induced by a Western diet (27, 39, 49, 53). Coronary heart disease is the common cause of death in T2D patients, and it is mainly induced by coronary atherosclerosis, cardiac ischemia, and, in some cases, by coronary vasospasm. Although coronary EC dysfunction is implicated in these vascular complications, only one report demonstrated attenuation of coronary EC function in patients with T2D and used human samples to show detailed molecular mechanisms (4). Several reports demonstrate coronary EC dysfunction in db/db (genetically derived T2D) mice (6, 13, 30, 66) and in Otsuka Long-Evans Tokushima Fatty rats (a spontaneous model of T2D) (40). EDR was attenuated in db/db mice mainly due to the decrease in NO production (6, 13), while there was no change in NO production and NO-mediated vascular relaxation between Otsuka Long-Evans Tokushima Fatty and control rats (40). In our T2D mice, we found that NO-dependent relaxation was significantly attenuated compared with controls (Fig. 2). Furthermore, scavenging mitochondrial O$_2^-$ by mitoTempol restored attenuated ACh-induced relaxation in CAs of T2D mice (Fig. 3C). O$_2^-$ is a ROS that reacts quickly with NO in the cells and forms peroxynitrite, which no longer stimulates cGMP production in smooth muscle cells. Our data imply that NO bioavailability was decreased by excess O$_2^-$ production in coronary ECs from T2D mice.

protein. At 48 h after treatment, cells were lysed and used for the IP study. High-glucose treatment significantly increased the level of ubiquitinated SOD2 protein compared with control (1.03 ± 0.06 and 2.00 ± 0.52 with normal and high glucose, respectively, *P < 0.05; Fig. 6A), whereas FFA treatment did not affect the level of SOD2 protein ubiquitination (Fig. 6B). These data suggest that increased mitochondrial O$_2^-$ in coronary ECs from T2D mice may be due to decreased SOD2 protein expression via the increase in ubiquitination of SOD2 protein by hyperglycemia.
decrease in SOD2 may contribute to the increase of mitochondrial ROS in coronary ECs from T2D mice. Our observation is in line with other investigators’ data using other tissue samples in T2D (3, 46, 61, 63), while some data show the increase in SOD2 protein expression (17, 33, 59) or no change (69, 77). Possible explanations for these different outcomes might be that their model may not increase and/or change the level of mitochondrial $O_2^-$ that was not directly measured in their study.

Contrary to our expectation, SOD2 mRNA expression was not changed in coronary ECs from T2D mice compared with controls (Fig. 5A). The level of protein expression is regulated by translational modification (e.g., RNA stability) and post-translational modification (e.g., protein stability). The Ub-proteasome system is the major intracellular proteolytic system responsible for degradation of large amounts of proteins. Dysregulation of the Ub-proteasome system has been implicated in the development of cardiovascular diseases (76, 81), and T2D is no exception (15, 16, 43, 60). Although eNOS is not ubiquitinated (47), Stangl and Stangl (76) reported that the inhibition of proteasome increases eNOS protein expression via regulation of its transcription factors, and subsequently augments EDR (84). SOD1 and SOD2 protein expression levels are regulated by ubiquitination (45, 85), and SOD2 protein ubiquitination is enhanced by oxidized LDL treatment in human aortic ECs (79). We demonstrated that coronary ECs from T2D mice significantly increased the ubiquitination of SOD2 protein (Fig. 5B). In addition, high-glucose, but not high-fat, treatment ex vivo significantly increased SOD2 ubiquitination (Fig. 6).

What is the upstream mechanism in which the ubiquitination of SOD2 protein is increased in T2D mice? The potential mechanism might be, at least in part, regulation of the ubiquitination-deubiquitination system by ROS. There is increasing evidence that the activity of deubiquitination enzymes, which dissociate Ub from the substrate protein and prevent protein degradation, is negatively regulated by ROS (14, 48, 50). Excess ROS production also inhibits the assembly of 26S proteasomes, which results in a decrease of protein ubiquitination (1, 37, 83). Further experiments are required to identify how mitochondrial ROS affects mitochondrial protein ubiquitination.

Taken together, these data suggest that increased mitochondrial ROS generation in coronary ECs from T2D mice is due to decreased SOD2 protein expression as a result of ubiquitination. The increased mitochondrial ROS generation is partially responsible for impaired EDR in diabetic CAs by decreasing NO bioavailability.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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