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.—Coronary endothelial cell (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 prostacyclin-dependent, 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 1) producing and releasing vasoconstrictors (e.g., endothelin-1, angiotensin II, and thromboxane A2) and vasodilators [e.g., nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors] (28, 29, 44, 64, 68) and 2) inducing hyperpolarization of smooth muscle cells (SMCs) via electrical propagation through the gap junctions (10, 26). NO serves as a vasodilator, as well as an inhibitor of aggregation of platelets and infiltration of inflammatory cells (22). 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 (5, 20).

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 endothelial-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 (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 (9, 78). 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, 55, 70), and hypertension (12, 35). In this study we demonstrate that decreased endothelium-dependent vascular relaxation (EDR) in coronary arteries (CAs) from 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 (Lakeview, 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-
Isometric Tension Measurement of CA Rings

Isometric tension was measured as previously described (58). The heart was isolated and placed in Krebs-Henseleit solution for dissection. Third-order small CAs were cleaned of any adherent connective tissue and cardiomycocytes and cut into 1- to 1.5-mm segments. Rings were mounted in a wire myograph (DMT-USA) with 20-m wires and wash in Krebs-Henseleit solution for dissection. Isometric tension was measured at time 0 after glucose administration and 15, 30, and 60 min after glucose administration. An insulin tolerance test was performed as follows: mice were fasted for 4 h; then insulin was injected (0.2 U/kg body wt), and plasma glucose concentration was measured at time 0 (before insulin injection) and 15, 30, 60, and 120 min after insulin injection. Data were normalized by the glucose level at time 0 and shown as percentage.

Isolation of Coronary Vascular ECs

Mouse coronary ECs were isolated as previously described (56, 57). Briefly, dissected heart tissues were minced and incubated with a kit from Wako Chemicals USA (Richmond, VA). Plasma insulin level was measured using a kit from ALPCO Diagnostics (Salem, NH). An oral glucose tolerance test was performed as follows: mice were fasted for 6 h; then glucose (2 g/kg body wt) was administrated orally, and plasma glucose concentration was measured at time 0 (before glucose administration) and 15, 30, and 60 min after glucose administration. An insulin tolerance test was performed as follows: mice were fasted for 4 h; then insulin was injected (0.2 U/kg body wt), and plasma glucose concentration was measured at time 0 (before insulin injection) and 15, 30, 60, and 120 min after insulin injection. Data were normalized by the glucose level at time 0 and shown as percentage.

Assay of SOD2 mRNA

SOD2 mRNA in coronary ECs from control and T2D mice was measured by real-time PCR. mRNA from mouse coronary ECs was isolated using the RNase-free RNA templates using random hexamers and SuperScript III (Invitrogen). The primers for SOD2 and the endogenous reference gene 18S rRNA (18S) are as follows: forward = 5'-GACCTGCCT-GACCTTGCTCCTTATTG-3' and reverse = 5'-GACCTGCTT-GACCTTGCTTCCTATTTG-AAGC-3'. (reverse) for SOD2 and 5'-GTACCCTGGTCATAGG-AGC-3' (reverse) for 18S. Measurements were made in triplicate, using 1 ng of template per well, with a Bio-Rad Real Time PCR System. The efficiency-correlated cycle threshold (ΔCt) method was used to determine the level, in arbitrary units, of SOD2 mRNA relative to 18S.
pharmacological experiments were conducted using the vari-
involved in attenuated EDR in diabetic CAs, a series of
two groups (Fig. 2
relaxation (EDR) was significantly decreased in CAs from T2D
Pretreatment with all inhibitors (L-NAME, Indo, Apa, and
presence of the Ca2+
lysis was incubated with anti-Ub (2 µg/ml) at 4°C for 2 h and then washed with binding buffer. Lysate with
IP-SOD2/immunoblotting-Ub. 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/ml) at 4°C for 2 h and then washed with binding buffer. Lysate with
IP 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 signifi-
cantly higher in T2D than control mice (Table 1). Plasma
insulin level was significantly higher in T2D than control mice
(Fig. 1A). In addition, T2D mice showed a significant decrease in
insulin sensitivity (Fig. 1, B and C).

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 vari-
ous 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

mouse, while prostacyclin- and endothelium-derived hyperpo-
larization-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 in-
crease 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 (mitoT, a mitochondrial O$_2^-$ scavenger, 10 μmol/l). 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.

compared with controls. In addition, the increased ROS in coronary ECs from T2D mice was decreased to the level in the 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

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**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). Scale bars, 10 μm. B: summarized data showing average of mitochondrial ROS intensity in primary cultured coronary ECs isolated 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. 4.** SOD2 protein expression is significantly decreased in coronary ECs isolated from T2D mice compared with controls. (A) Western blots showing SOD1 (A) and SOD2 (B) and actin protein levels. Actin was used as a loading control. (B) Western blots showing SOD2 protein expression via the increase in ubiquitination of SOD2 protein in coronary ECs from T2D mice.
Increased ROS (including \( \text{O}_2^- \)) 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 \( \text{O}_2^- \) in coronary ECs, but not SMCs, which is why SNP-induced vascular relaxation was not altered in diabetic CAs.

There are various sources of \( \text{O}_2^- \) generation in ECs, including NOX, the mitochondrial ETC, xanthine oxidase, uncoupled eNOS, and cytochrome P-450 (51). High-glucose treatment enhances mitochondrial \( \text{O}_2^- \) 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 \( \text{O}_2^- \) 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 \( \text{O}_2^- \) toward the control in coronary SMCs of T2D mice (30). Excess mitochondrial \( \text{O}_2^- \) production and impaired mitochondrial antioxidant defense result in increased mitochondrial \( \text{O}_2^- \). Mitochondrial \( \text{O}_2^- \) 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 \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \). \( \text{H}_2\text{O}_2 \) is detoxified to \( \text{H}_2\text{O} \) 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
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.

AUTHOR CONTRIBUTIONS
Y.-E.C., A.B., A.D., M.H., and A.M. performed the experiments; Y.-E.C., A.B., A.D., and A.M. analyzed the data; Y.-E.C., A.B., A.D., and A.M. prepared the figures; Y.-E.C. drafted the manuscript; Y.-E.C., A.B., A.D., M.H., and A.M. approved the final version of the manuscript; A.M. is responsible for conception and design of the research; A.M. interpreted the results of the experiments; A.M. edited and revised the manuscript.

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