Calorie Restriction Improves Cardiovascular Risk Factors via Reduction of Mitochondrial Reactive Oxygen Species in Type II Diabetic Rats

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ABSTRACT

Uncoupling protein 2 (UCP2) is an important regulator of intracellular reactive oxygen species (ROS) production. We determined the effects of calorie restriction (CR) on the dynamic aspects of mitochondrial ROS production, UCP2, and the nitric oxide (NO)-cGMP pathway in the cardiovascular tissues of type II diabetic Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Some rats were on restricted diets (30% reduction from free intake) from age 29 to 42 weeks. Blood glucose, hemoglobin A1c, plasma levels of free fatty acid, triacylglycerol, and plasminogen activator inhibitor-1 in OLETF rats were significantly higher than those in nondiabetic control [Long-Evans Tokushima Otsuka (LETO)] rats at 29 weeks. Mitochondrial ROS production and UCP2 expression significantly increased in the heart and aorta of OLETF rats compared with those in LETO rats. A fibrogenic growth factor, transforming growth factor (TGF)-β1 in the coronary vessels, endothelial nitric-oxide synthase, and aortic nitrotyrosine were increased in OLETF rats at 42 weeks. In contrast, an index of the NO-cGMP pathway, phosphorylated vasodilator-stimulated phosphoprotein, and superoxide dismutase activity in the aorta were significantly diminished. The relationship between UCP2 and ROS production in the cardiovascular function of diabetic rats being fed a calorie-restricted diet is unknown. These abnormalities in OLETF rats were reversed to normal levels by CR. CR significantly improved the NO-cGMP pathway via normalizing ROS generation in OLETF rats. A decrease in UCP2 expression by CR may be a compensatory mechanism to counteract decreased intracellular oxidative stress. The data suggest that CR may prevent cardiovascular tissues from oxidative stress provoked by diabetes mellitus.

Calorie restriction (CR) is shown to improve some diabetic abnormalities in this article. Cardiovascular disease is prevalent in patients with diabetes mellitus. High blood glucose levels, altered insulin signaling, reactive oxygen species (ROS), inflammation, and protein kinase C activation may lead to a decrease in nitric oxide (NO) bioavailability (Endemann and Schiffrin, 2004). It is well known that diabetes mellitus provokes an increase in oxidative stress in vascular cells and cardiomyocytes. High glucose concentrations, which contribute to the pathogenesis of atherosclerosis in patients with diabetes, lead to intracellular oxidative stress (Baynes, 1991; Baynes and Thorpe, 1999).

The Otsuka Long-Evans Tokushima Fatty (OLETF) rat, a model of spontaneous type II diabetes, develops hyperglycemic obesity with hyperinsulinemia and insulin resistance after the age of 25 weeks, similar to patients with non-insulin-dependent diabetes mellitus. In the aorta from OLETF rats, this work was supported by grants from Osaka City University and Okayama University Medical Research Fund for Medical Research and from the Special Coordination Funds of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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ABBREVIATIONS: CR, calorie restriction; ROS, reactive oxygen species; NO, nitric oxide; OLETF, Otsuka-Long Evans Tokushima Fatty; UCP2, uncoupling protein; MTR, MitoTracker Red; MTG, MitoTracker Green; COX, cytochrome oxidase; eNOS, endothelial nitric-oxide synthase; PAI-1, plasminogen activator inhibitor-1; pVASP, phosphorylated vasodilator-stimulated phosphoprotein; VASP, phosphorylated vasodilator-stimulated phosphoprotein; HNE, 4-hydroxy-2-nonenal; TGF, transforming growth factor; GLUT4, glucose transporter 4; LETO, Long-Evans Tokushima Otsuka; TBS, Tris-buffered saline; TBS-T, TBS-Tween 20; PAGE, polyacrylamide gel electrophoresis; SOD, superoxide dismutase; AP-1, activator protein-1; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)diene.
and mitochondrial anion carrier family and an important regulator of intracellular ROS production (Arsenijevic et al., 2000; Duval et al., 2002). However, the pathophysiological contribution of cardiovascular UCP2 in diabetes is still ambiguous. The aim of this study was to investigate the possible pathological role of UCP2-regulated ROS production and the NO-cGMP pathway in the cardiac vasculature in OLETF rats. In addition, because CR suppresses ROS/reactive nitrogen species in plasma and aorta (Zou et al., 2004), we studied the effects of CR after the development of diabetes.

Materials and Methods

Chemicals. Reduced-type MitoTracker Red (MTR) CM-H2XRos (M-7513), MitoTracker Green (MTG) FM (M-7514), and anti-cytochrome oxidase (COX) II antibody were purchased from Molecular Probes (Eugene, OR). Anti-endothelial nitric-oxide synthase (eNOS/NOS-III) and anti-plasminogen activator inhibitor-1 (PAI-1) antibodies were purchased from BD Biosciences (San Jose, CA). Anti-UCP2 antibody was purchased from Biologend (San Diego, CA), and phosphorylated-vasodilator-stimulated phosphoprotein (pVASP) (Ser219) and VASP antibodies were from Calbiochem (Darmstadt, Germany). Anti-4-hydroxy-2-nonenal (HNE) antibody was from the Japan Institute for the Control of Aging (Shizuoka, Japan). Anti-nitrotyrosine antibody, anti-transforming growth factor (TGF)-β1 antibody, and anti-glucose transporter 4 (GLUT4) antibody (C-20) were purchased from Sigma-Aldrich (St. Louis, MO), Oxford Biotechnology (Oxfordshire, UK), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

Animals. Male OLETF (aged 4 weeks) and age-matched Long–Evans Tokushima Otsuka (LETO) rats were obtained from the Animal Center of Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and maintained until they reached an appropriate age for the experiment. All rats had free access to standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and tap water, and were taken care of under the specifications outlined in the Guiding Principles for the Care and Use of Laboratory Animals—Approved by the Authorities of the Local Committee on Experimental Animal Research. Blood, urine, and tissue samples were taken at 29 and 42 weeks of age. Some OLETF rats were subjected to 70% CR between 29 and 42 weeks of age.

Animals were sacrificed under anesthesia with urethane (5 g/kg i.p.). Blood was collected with heparinized syringes, and heart, thoracic aorta, and other tissues were dissected out and frozen in liquid nitrogen. Plasma samples were obtained by centrifugation at 12,000g for 5 min. A small piece of the heart and aorta was removed for morphometric, immunohistochemical, or biochemical analysis.

Biochemical Measurements. Blood glucose levels were measured immediately after sampling with a glucose test meter (Gluestest; Sanwa Kagaku Kenkyusho, Nagoya, Japan). Total cholesterol, triglyceride, and free fatty acid were determined with commercially available kits (Wako Pure Chemicals, Osaka, Japan). As an index of atherosclerosis, plasma PAI-1 was measured with a commercially available enzyme-linked immunosorbent assay kit (ZYMUTEST Rat-PAI-1; Hyphen BioMed, Neuville-sur-Oise, France).

Immunoblot Analysis for UCP2/COXII, eNOS, pVASP and VASP, PAI-1, and TGF-β1. The heart and aortic tissues (100 mg) were homogenized and sonicated in 0.3 ml of 50 mM HEPES, pH 7.5, containing 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiotreitol, 200 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.1 mM Na3VO4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 12,000g for 20 min. The supernatants (30 μg) were used for detection of UCP2 and COXII proteins and were separated on 15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon-PSQ; Millipore Corpor
racion, Billerica, MA). Membranes were blocked overnight in buffer containing 1X Tris-buffered saline (TBS), 0.1% Tween 20, and 5% (w/v) powdered milk. Membranes were cut at the 50 kDa marker and incubated with anti-UCP2 (33 kDa) or anti-COXII (70 kDa) overnight at 4°C. The membranes were washed in TBS-Tween 20 (TBS-T) and incubated with each secondary antibody conjugated with horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1.5 h at room temperature. After being washed again in TBS-T, the membranes were exposed to enhanced chemiluminescence (Amersham Biosciences UK, Ltd.) on the film. Protein bands were quantified by densitometry and expressed as a relative proportion of the average value for control blots. COXII is one of the mitochondrial respiratory chain enzymes; therefore, UCP2 levels in the mitochondrion were expressed as the ratio of the intensity of UCP2/COXII. Antibodies against UCP2 and COXII were diluted by Can Get Signalsolution (Toyobo, Osaka, Japan).

The supernatants (2–10 µg) were analyzed by immunoblotting for pVASP and VASP using 7.5% gels as described above. Commercial VASP (20 ng) was used as an internal standard for Western blotting. Anti-VASP phosphoserine 239 antibody was used for the analysis of the phosphorylation state of VASP at Ser239 (pVASP), which is a reliable biochemical marker of vascular cGMP-dependent protein kinase-1 activity. Applied fractions were separated by SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membranes. The supernatants (5 µg) were also analyzed for eNOS, PAI-1, and TGF-β using 7.5, 7.5, and 15% gels, respectively, as described above. Western blots were probed with a specific antibody and quantified using Scion Image software (version 1.63; Scion Corporation, Fredrick, MD). Band intensity was presented as a percentage of the mean value for each group. Activation of pVASP was indicated by the intensity ratio pVASP/VASP.

**Lipid Peroxidation of Plasma and Heart Tissues.** Lipid peroxidation in the plasma was assayed using a Bioxytech LPO-586 kit (Oxis International, Portland, OR) that measures malondialdehyde together with 4-hydroxyalkanal. The colorimetric assay used a chromogenic reagent that reacted with the products mentioned above, generating a stable chromophore that was measured spectrophotometrically at 586 nm. This technique requires sample incubation at 45°C to avoid undesirable artifacts.

Heart tissues were homogenized with ice-cold 20 mM phosphate buffer (pH 7.4) and centrifuged at 3000g for 10 min. The collected supernatants (20 µg) were analyzed by immunoblotting for HNE-J2 using 12.5% gels, as described above. Antibodies were diluted by Can Get Signal solution.

**In Situ Determination of Mitochondrial ROS Generation.** Loading of sections with MTG or MTR, mitochondrial fluorescence probes, showed that mitochondria were predominantly situated in the perigranular, subplasmalemmal, and perinuclear regions. MTR (CM-H2XRos, reduced form of the probe) is nonfluorescent. When the probe is oxidized by ROS, it becomes fluorescent. The oxidized product binds to thiol groups and proteins within mitochondria. MTR is excited at 579 nm with an emission spectrum of 599 nm (Johnson et al., 2003). MTG (excitation, 490 nm; emission, 516 nm) is a mitochondrion-selective fluorescent label (Presley et al., 2003). MTG covalently binds to the inner mitochondrial membrane and fluoresces independently of membrane potential ψ and [Ca^{2+}]_{mt}. Unfixed frozen tissues were cut into 10-µm-thick sections and placed on glass slides. MTR (2 µM) was applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 15 min with or without 500 µM NADH and addition of 200 nM MTG. After incubation with MitoTracker, sections were washed twice with MitoTracker-free phosphate-buffered saline. Images were obtained with an LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) coupled to an upright microscope (Zeiss) with a PlanFluor objective (63×, N.A. 1.4) at Central Research Laboratory, Okayama University Medical School, Okayama, Japan. Laser settings were identical to those used for the acquisition of images. The refraction index of the immersion media (Zeiss 518F) was 1.518.

![Fig. 1. Expression of UCP2 in the heart and aorta.](https://example.com/fig1.jpg)

Theoretical xy- and z-axis resolutions were 0.45 and 0.9 µm, respectively. Stack size of the image was 512 × 512 µm with an 8-bit color depth.

For colocalization studies, both fluorophores were separated through careful selection of emission beam splitters and barrier...
filters. Signal bleed-through of either probe was imaged using identical settings (gain, iris, and black level).

Images from MTG (green) and CM-H2XRos (red) fluorescence patterns of the cells were processed as one-color images or two-color overlays as indicated. Neither signal showed significant photobleaching during the time frame required for analysis. The findings reported here were replicated in three independent experiments. An examination of the intracellular distribution of ROS generation was made by comparison with the staining pattern of the mitochondrion-specific probe.

Assay for ROS Generation in Aortic Segments. Superoxide release in aortic segments was determined by L-012 (Minamiyama et al., 2006). L-012 is a luminol derivate with a high sensitivity for ROS, which does not exert redox cycling itself. Fresh aortas were carefully excised and placed in chilled, modified Krebs-HEPES buffer (99.01 mM NaCl, 4.69 mM KCl, 1.87 mM CaCl₂, 1.20 mM MgSO₄, 20.0 mM Na HEPES, 1.03 mM K₂HPO₄, 25.0 mM NaHCO₃, and 11.1 mM D-(+)-glucose, pH 7.4). The buffer was gassed with 95% O₂ and 5% CO₂ and was temperature controlled at 37°C in a water bath until assay. Connective tissue was removed, and aortas were cut into 5-mm segments. The aortic segments were transferred into scintillation vials containing Krebs-HEPES buffer and incubated for 5 min at 37°C, and chemiluminescence was assessed with 400 μM L-012 in a luminescence reader (BLR-201; Aloka, Tokyo, Japan) at 1-min intervals. The aortic segments were then added to 1 mM NADPH. Superoxide release from the aorta segment was expressed as the relative chemiluminescence of the difference between the peak levels of NADPH and the levels after the addition of superoxide dismutase (SOD) (100 U/ml).

Aorta SOD Activity. Excised thoracic aorta was prepared as described in immunoblot analysis. SOD activity was determined by the ability of the supernatant (10 μg of protein) to inhibit the L-012-cheliuminescence induced by the addition of 200 μM L-012, 100 μM hypoxanthine, and 0.0025 U/ml xanthine oxidase (grade III; Sigma-Aldrich) in 500 μl of HEPES-NaOH (pH 7.8). The SOD activity was calibrated with known units of recombinant SOD.

Histology and Immunohistochemistry. Resected tissue samples were fixed immediately in 10% buffered formalin. All tissues were embedded in paraffin within 48 h of formalin fixation and were cut to a thickness of 4 μm just before staining. Histological staining was performed using hematoxylin and eosin, anti-PAI-1, or anti-nitrotyrosine. Other parts of the tissues were frozen in OCT compound and sectioned to a thickness of 10 μm for GLUT4 staining. Immunohistochemical analysis was performed using rabbit or mouse streptavidin-biotin peroxidase kits (Vectastain Universal Elite ABC Kit; Vector Laboratories, Burlingame, CA). Antibodies for immunohistochemical staining were nitrotyrosine, PAI-1, and GLUT4. Negative control sections were processed in an identical manner by substitution of the primary antibody with normal mouse immunoglobulin 1.

Statistical Analysis. Unless otherwise stated, data are presented as means ± S.E. Results were considered significant at P < 0.05. Statistical analysis was performed by analysis of variance.
Results

Body Weight, Heart Weight, and Blood Chemistry. Body weight increased in both the satiated LETO and OLETF rats. CR suppressed the body weight gains in OLETF rats (Table 1). The left ventricle weight/body weight ratio showed no difference between the two groups.

Table 2 summarizes the blood chemistry results. Hemoglobin A1c, blood glucose, plasma triglyceride, total cholesterol, and free fatty acid in both ages of OLETF rats were significantly higher than in the age-matched LETO rats. CR significantly decreased all these parameters in OLETF rats.

Effects of CR on UCP2 Expression in Heart and Aorta. Cardiac and aortic expression of UCP2 was significantly increased in the OLETF rats compared with that in the LETO rats (Fig. 1). CR significantly decreased UCP2 expression in OLETF rats.

Effects of CR on Cardiovascular ROS Generation. Cardiovascular superoxide production was assessed by MitoTracker fluorescence of heart (Fig. 2) and aorta (Fig. 3A) tissue sections (n = 4/group). MTR accumulating in response to the membrane potential formed a covalent complex with mitochondrial constituents, which prevented subsequent release of the fluorochrome. MTR almost completely colocalized with MTG (Figs. 2 and 3A). Superoxide production (MTR positive) increased in the cardiac muscle, coronary endothelial cells, and aortic vessel walls in OLETF rats. Mitochon-

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**Fig. 3.** Effects of CR on aorta ROS generation. A, confocal microscopy of frozen sections of rat aorta aged 42 weeks. Colocalization of MTG and MTR fluorescence is represented as shades of orange in the overlay image shown on the right (Merge). Top, LETO rats; middle, OLETF rats; bottom, calorie-restricted OLETF rats. B, effects of CR on ROS generation in aortic segments. Vascular superoxide production was assessed by L-012 chemiluminescence of aorta tissue sections (n = 4–5/group). Reaction mixtures contained, in a final volume of 500 μl of Krebs' buffer, a 5-mm aorta ring sample and 400 μM L-012. Data show kilocounts per minute (KCPM); 10^3 counts per min (maximum intensity of chemiluminescence counts after 1 mM NADPH) – (minimum intensity after 100 U/ml SOD) (mean ± S.E.). *, P < 0.05 compared with LETO rats; #, P < 0.05 compared with OLETF rats.
drial ROS generation of CR group was inhibited. Figure 3B shows the effects of CR on ROS generation in aortic segments using L-012 chemiluminescence. NADPH-dependent superoxide production was increased in the aortic segments from OLETF rats. Chemiluminescence of the aorta segments decreased significantly after CR.

**Lipid Peroxidation.** Further experimentation was conducted to support the premise that oxidative stress is heightened in the diabetic heart (Fig. 4). HNE-modified proteins were elevated in OLETF rats. CR returned HNE-modified protein levels to the normal level seen in LETO rats.

**eNOS Expression and Deposition of 3-Nitrotyrosine in the Aorta.** The expression of aortic eNOS in OLETF rats was elevated 2-fold compared with that in LETO rats (Fig. 5A). CR returned eNOS expression to the levels seen in LETO rats. Elevated superoxide is a prerequisite for increased peroxynitrite formation. Accordingly, we investigated whether the increase in eNOS expression, together with enhanced superoxide production in the aortic tissue in OLETF rats, was associated with peroxynitrite formation and nitrination of tyrosine residues. Immunoreactive nitrotyrosine in the aortic vessel walls of 42-week-old OLETF rats (Fig. 5B) was much greater than that in LETO rats. CR in OLETF rats decreased this immunoreactivity. The data are consistent with the concept that overproduction of superoxide during diabetes results in the formation of peroxynitrite at the expense of NO. The latter oxidant is merely responsible for the nitrination of aortic smooth muscle cells.

**pVASP and Total VASP in the Aorta.** To establish NO bioavailability, we measured pVASP and total VASP in the aorta. Total VASP in OLETF rats was elevated ~2-fold compared with that in LETO rats (Fig. 6A). CR returned total VASP in the OLETF rats to normal. In contrast, pVASP (the ratio of pVASP/VASP) was decreased in the OLETF rats and CR increased pVASP (Fig. 6B).

**Effects of CR on Aorta Total SOD Activity at 42 Weeks.** Total SOD activity in thoracic aorta of OLETF rats was significantly decreased compared with that in LETO
rats. In the CR-treated OLETF rats, the decrease in SOD activity was suppressed (Fig. 7).

**PAI-1 in the Aorta and Plasma.** Figure 8A compares PAI-1 protein expression in the aorta, as examined by Western blotting. Aortic PAI-1 increased age-dependently in both LETO and OLETF rats and was higher in OLETF rats. CR returned PAI-1 in OLETF rats to the level in age-matched LETO rats. Plasma levels of PAI-1 were consistent with the results of aortic PAI-1 expression (Fig. 8B). However, Western blotting did not reveal any significant differences in cardiac PAI-1 protein between any of the groups (data not shown).

**Aortic TGF-β1 Levels.** TGF-β1 levels increased in the aorta of OLETF rats, but not in LETO rats (Fig. 9). CR reduced TGF-β1 in 42-week-old OLETF rats.

**GLUT4 Expression in the Aorta.** GLUT4 protein staining was positive in the aortic endothelial cells and vessel walls of LETO rats. In age-matched OLETF rats, aortic endothelial cells became thickened, and GLUT4 staining was abolished. However, in the CR group, GLUT4 proteins were seen in the endothelial cells (Fig. 10).

![Fig. 7. Effects of CR on total activity in aorta. Aortas were homogenized as described under Materials and Methods. SOD activity was determined by measuring the ability of the samples (20 μg of protein) to inhibit hypoxanthine/xanthine oxidase-induced superoxide production. Values are means ± S.E.M. (n = 4–5). *P < 0.05 versus age-matched LETO rats; #, P < 0.05 versus age-matched OLETF rats.](image)

![Fig. 8. Effects of CR on aorta and plasma PAI-1 level. PAI-1 levels in the aorta (A) and plasma (B). Hearts of age-matched LETO (L), satiated OLETF (O), and calorie-restricted OLETF (CR) rats at age 42 weeks. Original magnification, 40×. L, LETO rats; O, OLETF rats; CR, calorie-restricted OLETF rats. Values are means ± S.E.M. (n = 5). *P < 0.05 versus age-matched LETO rats; #, P < 0.05 versus age-matched OLETF rats.](image)
Discussion

This study revealed that diabetes-induced excess mitochondrial ROS generation resulted in vascular NO/cGMP dysfunction despite UCP2 overexpression in OLETF rats. CR significantly improved the NO-cGMP pathway and other cardiovascular risk factors via normalization of ROS generation in OLETF rats.

It has been reported that the expression of UCP2 is increased in the liver and skeletal muscle in OLETF rats (Mori et al., 2004). In the present study, we found that UCP2 was elevated in the heart and aorta of OLETF rats. UCP2 overexpression inhibits apoptosis and prevents mitochondrial Ca\(^{2+}\) overload in cardiomyocytes (Teshima et al., 2003) and vascular endothelial cells (Lee et al., 2005). Recently, it has been reported that UCP2 gene expression reverses high glucose-induced ROS production, NADPH oxidase activity, mitochondrial membrane potential, AP-1 activity, PAI-1 mRNA expression, and proliferation and migration of human vascular smooth muscle cells (Sakai et al., 2003; Uchida et al., 2004; Park et al., 2005a). Therefore, UCP2-regulated ROS production is thought to play a major role in the pathogenesis of atherosclerosis in patients with diabetes and hypertension.

Hyperglycemia is thought to play a major role in the pathogenesis of atherosclerosis in patients with diabetes and hypertension. Hyperglycemia in diabetes stimulates ROS generation in cells and activates the redox-sensitive transcription factors nuclear factor-\(\kappa\)B and AP-1 in the blood vessels (Napoli et al., 2001; De Oliveira et al., 2005). The promoter region of human UCP2 appears GC-rich, resulting in the presence of several Sp-1 motifs and AP-1/-2 binding sites near the transcription initiation site (Tu et al., 1999). ROS generation and UCP2 overexpression induce eNOS expression (Lee et al., 2005; Ho et al., 2006). Increased UCP2 and eNOS expression may contribute to prevention of atherosclerosis in diabetes. It has been reported that CR modulates redox-sensitive nuclear factor-\(\kappa\)B, AP-1, and hypoxia-inducible factor-1 binding activities by suppressing oxidative stress (Kim et al., 2002; Castello et al., 2005). Furthermore, CR induces a reduction in both mitochondrial proton leak and ROS generation (Hagopian et al., 2005). These reports collectively suggest that a reduction of eNOS, UCP2, and TGF-\(\beta\)1 expression by CR may be due to suppression of the redox-sensitive activation by ROS.

Recently, it has been reported that CR corrects insulin resistance by normalizing GLUT4 expression and/or translocation in peripheral tissue such as adipose tissue and muscle in OLETF rats (Park et al., 2005b). We also demonstrated that GLUT4 was expressed in the vascular cells in the CR group. This finding may be the first report that CR induces or restores GLUT4 in the aorta of diabetic rats. However, the GLUT4 increase in the aorta induced by CR may prove negligible in the amelioration of peripheral insulin resistance/insulin signaling, because CR did not improve the diabetes-induced decrease of AKT (Ser\(^{473}\)) phosphorylation (data not shown).

We demonstrated that CR decreased mitochondrial ROS production and PAI-1 expression, along with normalizing glucose and fatty acids levels, whereas the free radical scavenger edaravone exhibits modest cardioprotection in diabetes, independent of blood sugar level (Hayashi et al., 2003). Therefore, ROS scavenging may be important for preventing diabetes after heart disease. The decrease of free fatty acids after CR may be due to the improvement of \(\beta\)-oxidation, and/or normalization of lipid levels by the decrease of nutrient load. Thus, changes may lower the burden of mitochondrial oxidation. In OLETF rats, a \(>2\)-fold increase in expression of UCP2 compared with that in LETO rats may act as a negative feedback system for excess ROS generation because the superoxide generation pathway is overdriven. However, its expression level might not be sufficient to regulate ROS production completely. In fact, ROS generation was incompletely suppressed (Figs. 2, 3, and 4). Furthermore, in diabetic rats, oxidative stress was increased and the cardiac...
ATP level was decreased, which may have involved increased uncoupling of oxidative phosphorylation. CR prevented cardiovascular remodeling in the spontaneous diabetic model by retarding the progression of glucose intolerance, overexpressing eNOS, and inhibiting overexpression of fibrogenic/proinflammatory cytokines, including PAI-1 and TGF-β1.

In diabetes, why is the NO-cGMP pathway (VASP phosphorylation) decreased yet eNOS levels are increased? The bioavailability of NO is diminished through NO being quenched by superoxide to form peroxynitrite, resulting in nitrosyrosine formation. In fact, it has been reported that blood pressure in OLETF rats is 10 to 20 mm Hg higher than that in LETO rats (Yagi et al., 1997; Fukui et al., 2000). Because we do not measure blood pressure in this study, we do not know whether CR alters blood pressure in OLETF rats. Recently, it has been reported that 40% CR during at least 1 month reduced diastolic blood pressure in rats, although the mechanism linking CR to the changes is unclear (Mager et al., 2006). On the basis of these findings, whether a calorie-restricted diet is a contributing or a controlling factor in the cardiovascular response in OLETF rats remains to be determined.

Recent reports suggest that UCP2 overexpression (considering possibly the complete suppression of ROS generation) protects against development of endothelial dysfunction and atherosclerosis (Blanc et al., 2003; Ryu et al., 2004; Jeong et al., 2005; Kang et al., 2005; Lee et al., 2005; Mustata et al., 2005; Park et al., 2005a,b). Therefore, development of drugs that induce UCP2 in the diabetic heart will result in a new therapeutic target, which should help reduce cardiovascular risk in these patients.

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