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


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Running title: UCP2 and ROS on cardiovascular function in diabetic rats

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Abbreviations list

Uncoupling protein, UCP2; reactive oxygen species, ROS; Otsuka-Long Evans Tokushima Fatty, OLETF; Long Evans Tokushima Otsuka, LETO; plasminogen activator inhibitor-1, PAI-1; Transforming growth factor, TGF; endothelial NO synthase, eNOS; phosphorylated vasodilator-stimulated phosphoprotein, pVASP; cytochrome oxidase, COX; 4-hydroxy-2-nonenal, HNE; superoxide dismutase, SOD.

Sections: Endocrine and Diabetes or Cardiovascular
Abstract

UCP2 is an important regulator of intracellular ROS production. We determined the effects of calorie restriction (CR) on the dynamic aspects of mitochondrial reactive oxygen species (ROS) production, uncoupling protein 2 (UCP2) and 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 non-diabetic control (LETO) rats at 29 weeks. Mitochondrial ROS production and UCP2 protein expression significantly increased in the heart and aorta of OLETF rats compared with that in LETO rats. A fibrogenic growth factor, transforming growth factor (TGF)-β1 in the coronary vessels, endothelial NO synthase (eNOS), 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 SOD activity in the aorta was significantly diminished. The relationship between UCP2 and ROS production in the cardiovascular function of diabetic rats being fed a CR 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. Decrease of 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.
Introduction

Calorie restriction (CR) has shown to improve some diabetic abnormalities in this paper. 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 noninsulin-dependent diabetes mellitus. In the aorta from OLETF rats, endothelial NADH oxidase is enhanced after the onset of hyperglycemia, thereby resulting in increased vascular production of superoxide (Kim et al., 2003). The role of endothelial dysfunction in macro- and microvascular disease has been studied during diabetes. In this context, attenuated endothelium-dependent vascular relaxation has been reported in different vascular beds of human and animal models of diabetes (De Vriese et al., 2000). However, a single unifying mechanism to account for endothelial dysfunction in diabetes has yet to emerge. Insulin resistance or sensitivity is susceptible to changes in
whole body redox balance (Bonnefont-Rousselot et al., 2000; Hayden and Tyagi, 2003; Davi et al., 2005). Therefore, oxidative stress may be involved in the development of insulin resistance. In view of the above information, a hypothesis was formulated stating that endothelial dysfunction and insulin resistance are associated with a heightened state of oxidative stress in diabetes mellitus.

Uncoupling protein 2 (UCP2) is a new member of the 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 (RNS) 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, USA). Anti-endothelial nitric oxide synthase (eNOS/NOS-III) and anti-plasminogen activator inhibitor-I (PAI-1) antibodies were purchased from BD Biosciences (San Jose, CA, USA). Anti-UCP2 antibody was purchased from Biolegend (San Diego, CA, USA), and
phosphorylated-vasodilator-stimulated phosphoprotein (pVASP) (Ser239) and VASP antibodies were from Calbiochem (Darmstadt, Germany), respectively. 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 (Saint Louis, MO, USA), Oxford Biotechnology (Oxfordshire, UK) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), 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 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, ip). 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 12 000 g 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 (Glutest Ace®, Sanwa Kagaku Kenkyusyo, Nagoya, Japan). Total cholesterol, triglyceride and free fatty acid were determined with commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). As an index of atherosclerosis, plasma PAI-1 (ZYMUTEST Rat-PAI-1; Hyphen BioMed, Neuville-sur-Oise, France) was measured with commercially available ELISA kits.

**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 50 mM HEPES, pH 7.5, containing 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 200 mM NaCl, 10% glycerol, 0.1% Tween-20, 0.1 mM Na3VO4, 1 mM NaF, 1 mM PMSF, and a protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at 12 000 g 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 PVDF membranes (Immobilon™-PSQ; Millipore, Billerica, MA, USA). Membranes were blocked overnight in buffer containing 1×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 (33kDa) 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 (Amersham Biosciences, 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) 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 Signal™ solution (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-I activity. Applied fractions were separated by SDS-PAGE and transferred electrophoretically to PVDF 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 (Scion Image version 1.63, MD, USA). Band intensity was presented as a percentage of the mean value for each group. Activation of VASP 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, USA) that measures malondialdehyde (MDA) together with 4-hydroxyalkanals. The colorimetric assay used a chromogenic reagent which reacted with the products mentioned above, generating a stable chromophore which 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 3000 g 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 (TOYOBO).

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 non-fluorescent. 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 $\psi$ 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 37ºC for 15 min with or without 500 µM NADH, and addition of 200 nM MTG. After incubation with Mito Tracker, sections were washed twice with Mito Tracker-free PBS. 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, 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. Theoretical xy- and z-axis resolution was 0.45 and 0.9 µm, respectively. Stack size of the image was 512×512 µm with an 8-bit color depth.

For co-localization 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 derivative 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 (NaCl 99.01 mmol/l, KCl 4.69 mmol/l, CaCl₂ 1.87 mmol/l, MgSO₄ 1.20 mmol/l, Na HEPES 20.0 mmol/l, K₂HPO₄ 1.03 mmol/l, NaHCO₃ 25.0 mmol/l and D-(+)-glucose 11.1 mmol/l, 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. Chemiluminescence was then 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 protein) to inhibit the L-012-chemiluminescence induced by the addition of 200 µM L-012, 100 µM hypoxanthine, 0.0025 U/ml xanthine oxidase (Grade III, Sigma) 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.

Immunohistochemistry was conducted using rabbit or mouse streptavidin–biotin peroxidase kits (Vectastain Universal Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA). Antibodies for immunohistochemical staining were nitrotyrosine, PAI-1 and GLUT4. Negative control sections were processed in an identical manner by substitution of primary antibody with normal mouse immunoglobulin 1.

Statistical analysis

Unless otherwise stated, data are presented as means±SE. Results were considered significant at $P<0.05$. Statistical analysis was performed by analysis of variance (ANOVA).

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). Left ventricle/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 Mito Tracker fluorescence of heart (Fig. 2) and aorta (Fig. 3A) tissue sections (n=4 per 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 co-localized 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. Mitochondrial ROS generation of CR group was inhibited. Fig. 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 twofold 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 nitration 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 nitration 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 about twofold 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 to that in LETO rats. In the CR-treated OLETF rats, the decreases of SOD activity was suppressed (Fig. 7).

**PAI-1 in the aorta and plasma**

Fig. 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 wall 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).

Discussion

This study revealed that diabetes-induced excess mitochondrial ROS generation resulted in vascular NO/cGMP dysfunction in spite of UCP2 overexpression in OLETF rats. CR significantly improved NO-cGMP pathway and other cardiovascular risk factors via normalizing 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-kappaB (NF-kB) 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 induces 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 NF-kB, AP-1 and HIF-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-β1 expression by CR may be due to suppress above 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 (Ser473) 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-following heart diseases. The decrease of free fatty acids after CR may be due to the improvement of β-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, more than twofold 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 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 NO-cGMP pathway (VASP phospholyration) is decreased yet increase eNOS levels? The bioavailability of NO is diminished through NO is quenched by superoxide to form peroxynitrite resulted in nitrotyrosine formation. In fact, it has been reported that blood pressure in OLETF rats is 10~20 mmHg higher than LETO rats (Yagi et al., 1997; Fukui et al., 2000). Because we could not measure blood pressure in this study, it has been unknown 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 CR diet is contributing or controlling factors in cardiovascular response in OLETF rats remains to be determined.

Recent reports suggest that UCP2 overexpression (considering possibly completely 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; Park et al., 2005b). 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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Footnotes

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Figure legends

**Fig. 1. Expression of UCP2 in the heart and aorta**

Representative images and quantitative results of immunoblots from the heart (A) and aorta (B). Whole cell lysates were subjected to Western blotting. Representative images of UCP2 and COXII are shown for LETO (L), OLETF (O) and CR rats. To examine the ratio of UCP2 levels to the number of mitochondria, COXII, a constitutive mitochondrial protein, was reflected on levels of mitochondrial fractions in equal loading of protein in each lane. Open column, LETO rats; closed column, OLETF rats; oblique column, calorie-restricted OLETF rats. Data are presented as means±SEM (n=5). *P<0.05 compared with age-matched LETO rats, #P<0.05 compared with age-matched OLETF rats.

**Fig. 2. Effects of CR on cardiovascular ROS generation**

Confocal microscopy of frozen sections of rat heart aged 42 weeks. Co-localization of MTG and MTR fluorescence is represented as shades of orange in the overlay image shown on the right (Merge). Upper panel, LETO rats; middle panel, OLETF rats; lower panel, calorie-restricted OLETF rats.

**Fig. 3. Effects of CR on aorta ROS generation**

(A) Confocal microscopy of frozen sections of rat aorta aged 42 weeks. Co-localization of MTG and MTR fluorescence is represented as shades of orange in the overlay image shown on the right (Merge). Upper panel, LETO rats; middle panel, OLETF rats; lower panel, CR 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 \((n=4–5\) per group). Reaction mixtures contained in a final volume of 500 \(\mu\)l Krebs buffer, 5 mm aorta ring sample, and 400 \(\mu\)M L-012. Data show KCPM; \(10^3\) counts per min (maximum intensity of chemiluminescence counts after 1 mM NADPH) - (minimum intensity after 100 U/ml SOD) (mean ±SE). *\(P<0.05\) as compared with LETO rats, #\(P<0.05\) as compared with OLETF rats.

**Fig. 4. Effects of CR on heart lipid peroxidation**

Markers of oxidative stress, including 4-HNE-modified proteins (66 and 42 kD), were measured in cardiac homogenates. Values are means±SEM \((n=5)\). *\(P<0.05\), **\(P<0.01\) vs. age-matched LETO rats, #\(P<0.05\), ##\(P<0.01\) vs. age-matched OLETF rats.

**Fig. 5. Effects of CR on aortic eNOS and nitrotyrosine in the aorta**

Aortic expression of immunoreactive eNOS (A) and nitrotyrosine (B). Aortic lysates were resolved by SDS-PAGE. Densitometry data are presented as means±SEM \((n=5)\). *\(P<0.05\) vs. age-matched LETO rats, #\(P<0.05\) vs. age-matched OLETF rats. (B) shows isolated aorta sections stained for nitrotyrosine from age-matched LETO, satiated OLETF, and calorie-restricted (CR) OLETF rats aged 42 weeks (Original magnification \(\times40\)).

**Fig. 6. Effects of CR on aortic pVASP expression**

To examine NO/cGMP signal activation, total VASP and phosphorylated (Ser239)-VASP expression were assessed. Aortic lysates were resolved by SDS-PAGE.
Densitometry data are presented as means±SEM (n=5). (A) total VASP, (B) pVASP/total VASP ratio. *P<0.05 vs. age-matched LETO rats, #P<0.05 vs. age-matched OLETF rats.

**Fig. 7. Effects of CR on total activity in aorta**

Aortas were homogenized as described in Materials and Methods. SOD activity was determined by measuring the ability of the samples (20 µg protein) to inhibit hypoxanthine/xanthine oxidase-induced superoxide production. Values are means±SEM (n=4-5). *P<0.05 vs. age-matched LETO rats, #P<0.05 vs. age-matched OLETF rats.

**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, satiated OLETF, and calorie-restricted OLETF (CR) rats at age 42 weeks. Original magnification ×40. Open column, LETO rats; closed column, OLETF rats; oblique column, calorie-restricted OLETF rats. Values are means±SEM (n=5). *P<0.05 vs. age-matched LETO rats, #P<0.05 vs. age-matched OLETF rats.

**Fig. 9. Effects of CR on aortic TGF-β1 expression**

Aortic levels of TGF-β1 expression as shown by Western blotting. Open column, LETO rats; closed column, OLETF rats; oblique column, calorie-restricted OLETF rats. Values are means±SEM (n=5).
Fig. 10. GLUT4 expression in the aorta

Immunohistochemistry of GLUT4 in the aorta. Age-matched LETO, satiated OLETF, and calorie-restricted OLETF (CR) rats aged 42 weeks. Original magnification ×20 (left), ×40 (right).
Table 1. Body weight and heart weight in satiated and calorie-restricted rats

<table>
<thead>
<tr>
<th>Age</th>
<th>Body Weight (g)</th>
<th>LV /Heart weight (%)</th>
<th>Food intake (g/day)</th>
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<td>Age 29 weeks</td>
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<td>LETO</td>
<td>506±6</td>
<td>0.722±0.008</td>
<td>18.5±1.5</td>
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<tr>
<td>OLETF</td>
<td>643±10**</td>
<td>0.722±0.010</td>
<td>26.6±2.4*</td>
</tr>
<tr>
<td>Age 42 weeks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>537±13</td>
<td>0.695±0.011</td>
<td></td>
</tr>
<tr>
<td>OLETF</td>
<td>691±10**</td>
<td>0.699±0.012</td>
<td></td>
</tr>
<tr>
<td>CR</td>
<td>553±12</td>
<td>0.706±0.010</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means±SE; n=5–8 for each group. LV, Left ventricle

*P<0.05, **P<0.01 vs. LETO rats
### Table 2. Changes in blood chemistry

<table>
<thead>
<tr>
<th></th>
<th>HbA1c (%)</th>
<th>BS (mg/dl)</th>
<th>T-chol (mg/dl)</th>
<th>TG (mg/dl)</th>
<th>FFA (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age 29 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>2.71±0.05</td>
<td>160±7</td>
<td>97±6</td>
<td>47±4</td>
<td>303±53</td>
</tr>
<tr>
<td>OLETF</td>
<td>3.92±0.23*</td>
<td>259±9*</td>
<td>131±16</td>
<td>112±10**</td>
<td>469±16*</td>
</tr>
<tr>
<td><strong>Age 42 weeks</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>2.62±0.04</td>
<td>193±12</td>
<td>115±3</td>
<td>47±23</td>
<td>397±23</td>
</tr>
<tr>
<td>OLETF</td>
<td>4.10±0.50*</td>
<td>319±30*</td>
<td>160±11*</td>
<td>110±21*</td>
<td>473±51*</td>
</tr>
<tr>
<td>CR</td>
<td>2.64±0.45†</td>
<td>206±27†</td>
<td>104±6†</td>
<td>53±11†</td>
<td>244±95†</td>
</tr>
</tbody>
</table>

Data are expressed as means±SE. $n=5–8$. *P<0.05, **P<0.01 vs. LETO rats. †P<0.05 vs. OLETF rats. HbA1c, hemoglobin A1c; BS, blood sugar; T-chol, total cholesterol; TG, triglyceride; FFA, free fatty acid.
Fig. 1

A

UCP2/COXII ratio

L      O        L       O          CR

29W  42W

B

UCP2/COXII ratio

L      O        L       O          CR

29W  42W

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Fig. 3B

![Graph showing KCPM values for different conditions: NADPH (-), LETO, OLETF, and CR. Significant differences are indicated by asterisks (*) and hash marks (#).]
Fig. 4

![Graph showing relative intensity for 66kD and 42kD proteins across different groups (LETO, OLET, CR) at 29W and 42W, with statistical significance indicated by asterisks (*) and double asterisks (**) for 66kD and # for 42kD.](image)
Fig. 6

A

Total VASP

VASP (µg/mg protein)

2.5

5.0

0

29W

42W

L

O

L

O

CR

B

pVASP

pVASP/VASP ratio

0

1

2

29W

42W

LETO

OLET

LETO

OLET

CR

* #
Fig. 7

[Graph showing Total SOD Activity (U/mg protein) for LETO, OLETF, and CR.]

- LETO
- OLETF
- CR

42W

Total SOD Activity (U/mg protein)
Fig. 9

The figure shows a bar graph comparing relative intensity at different time points (29W and 42W) for different conditions (LETO, OLET, and CR). The y-axis represents relative intensity, while the x-axis lists the conditions. The 12.5 kDa protein band is indicated at the bottom of the graph. Significant differences are marked with asterisks (*) and a hash (#).