Caloric Restriction Stimulates Revascularization in Response to Ischemia via Adiponectin-mediated Activation of Endothelial Nitric-oxide Synthase*

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Caloric restriction (CR) can extend longevity and modulate the features of obesity-related metabolic and vascular diseases. However, the functional roles of CR in regulation of revascularization in response to ischemia have not been examined. Here we investigated whether CR modulates vascular response by employing a murine hindlimb ischemia model. Wild-type (WT) mice were randomly divided into two groups that were fed either ad libitum (AL) or CR (65% of the diet consumption of AL). Four weeks later, mice were subjected to unilateral hindlimb ischemic surgery. Body weight of WT mice fed CR (CR-WT) was decreased by 26% compared with WT mice fed AL (AL-WT). Revascularization of ischemic hindlimb relative to the contralateral limb was accelerated in CR-WT compared with AL-WT as evaluated by laser Doppler blood flow and capillary density analyses. CR-WT mice had significantly higher plasma levels of the fat-derived hormone adiponectin compared with AL-WT mice. In contrast to WT mice, CR did not affect the revascularization of ischemic limbs of adiponectin-deficient (APN-KO) mice. CR stimulated the phosphorylation of endothelial nitric-oxide synthase (eNOS) in the ischemic limbs of WT mice. CR increased plasma adiponectin levels in eNOS-KO mice but did not stimulate limb perfusion in this strain. CR-WT mice showed enhanced phosphorylation of AMP-activated protein kinase (AMPK) in ischemic muscle, and administration of AMPK inhibitor compound C abolished CR-induced increase in limb perfusion and eNOS phosphorylation in WT mice. Our observations indicate that CR can promote revascularization in response to tissue ischemia via an AMPK-eNOS-dependent mechanism that is mediated by adiponectin.

Obesity is closely associated with the development of metabolic syndrome and type 2 diabetes (1), which contribute to microvascular rarefaction and impaired collateral vessel growth under ischemic conditions (2–4). These conditions lead to increased vulnerability to ischemic injury and impaired wound healing and promote the progression of cardiovascular diseases. Thus, therapeutic approaches that enhance revascularization could be beneficial for ischemic vascular disease.

Caloric restriction (CR) has been shown to extend the life span of multiple species by retarding the aging process (5). In obese subjects CR has been shown to reduce visceral fat accumulation and also decrease body weight (6). CR have also been reported to lead to a reduction of hyperglycemia and hyperlipidemia that are major risk factors for ischemic heart diseases (7–12). A number of experimental studies have shown that CR attenuates atherosclerotic lesion formation (11), pathological cardiac hypertrophy (13), and ischemia-induced myocardial damage (14). These findings suggest that CR counteracts the unfavorable features of obese complications. However, the consequences of CR on vascular responses to tissue ischemia have not been examined.

Adipose tissue secretes a variety of bioactive molecules, referred to as adipokines, that directly affect obesity-linked disorders in remote organs (15). Adiponectin is an adipokine that is down-regulated in obesity-linked diseases, including ischemic heart disease and peripheral arterial disease (16, 17). Adiponectin exerts protective actions on a variety of metabolic and cardiovascular disorders, including insulin resistance, atherosclerosis, vascular dysfunction, and cardiac injury (18). CR has been shown to markedly increase circulating levels of adiponectin (14, 19). Thus, it is plausible that adiponectin mediates the salutary actions of CR in the setting of obesity-linked vascular complications. Here we investigated whether CR modulates the process of ischemia-induced revascularization in vivo. We first examined the effect of CR on revascularization using a mouse model of vascular insufficiency. We also examined the potential contribution of adiponectin to CR-mediated revascularization process in ischemic muscles. Our observations indicate that CR

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4 The abbreviations used are: CR, caloric restriction; AL, ad libitum; WT, wild-type; KO, knockout; eNOS, endothelial nitric-oxide synthase; HR, heart rate; LDBF, laser Doppler blood flow; AMO PK, AMP-activated protein kinase; pfu, plaque-forming unit; NOS, nitric-oxide synthase; Ad-APN, adenovirus expressing adiponectin.
promotes revascularization in response to tissue ischemia via modulation of adiponectin production.

**EXPERIMENTAL PROCEDURES**

**Materials**—Endothelial nitric-oxide synthase (eNOS) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-eNOS (Ser-1177), phospho-AMPK (Thr-172), and pan-α-AMPK antibody were purchased from Cell Signaling Technology (Beverly, MA). Tubulin antibody was from Oncogene (Cambridge, MA). Adenovirus vectors containing the gene for β-galactosidase (Ad-βgal) and full-length mouse adiponectin (Ad-APN) were described previously (20). Compound C was purchased from Calbiochem.

**CR Protocols**—Studies using wild-type (WT), eNOS-deficient (eNOS-KO), and adiponectin-deficient (APN-KO) mice in a C57/BL6 background were approved by the Institutional Animal Care and Use Committee at Nagoya University. Male mice at the ages of 6 weeks were housed in individual cages and fed ad libitum (AL) on a normal chow for 2 weeks. Food was provided at the same time (3:00 p.m.), and food intake of individual mice was measured daily. The average value of caloric intake was calculated from daily food intake for 2 weeks. After that, mice were randomly divided into two groups. The AL group was fed ad libitum for an additional 4 weeks. CR mice were fed with 65% of the average caloric intake of control AL diet for the next 4 weeks. At 4 weeks after the CR or control AL diet, mice were subjected to unilateral hindlimb surgery. All mice were weighed at weekly intervals. Heart rate (HR) and systolic blood pressure were determined using a tail-cuff pressure analysis system in the conscious state.

**Mouse Model of Revascularization**—Mice were subjected to unilateral hindlimb surgery under anesthesia with sodium pentobarbital (50 mg/kg intraperitoneally). In this model, the entire left femoral artery and vein were removed surgically as described previously (21). In some experiments, 2 × 10^8 plaque-forming units (pfu) of Ad-APN or Ad-βgal were systemically injected into the jugular vein of mice 3 days before the ischemic hindlimb surgery (22). In some experiments, we intraperitoneally injected NOS inhibitor L-NAME (20 mg/kg/day) in phosphate-buffered saline or vehicle (phosphate-buffered saline) into WT mice 1 day prior to surgery and daily until sacrifice. In some experiments, AMPK inhibitor compound C (20 mg/kg/3 times per week) dissolved in dimethyl sulfoxide (DMSO) or vehicle (DMSO) was intraperitoneally injected into the abdomen of WT mice 1 day before the operation until sacrifice (23, 24).

**Analysis of Hindlimb Blood Flow**—Hindlimb blood flow was determined using a laser Doppler blood flow (LDBF) analyzer (Moor LDI; Moor Instruments, Devon, UK). LDBF analyses were performed on legs and feet before surgery and on postoperative days 3, 7, and 14. Blood flow was shown as changes in the laser frequency using different color pixels. Quantitative analysis of blood flow was expressed as the ratio of left (ischemic) to right (nonischemic) LDBF to avoid data variations because of ambient light and temperature (22).

**Measurement of Capillary Density**—Capillary density in adductor muscle was assessed by immunohistochemical analysis. Muscle samples were embedded in OCT compound (Miles Scientific, Elkhart, IN) and snap-frozen in liquid nitrogen. Tissue slices (7 μm in thickness) were prepared and stained with CD31 (PECAM-1, BD Biosciences) followed by treatment with fluorescein isothiocyanate-conjugated secondary antibody to detect CD31. The signals were detected and analyzed by fluorescence microscopy. Capillary endothelial cells were quantified by measuring the number of CD31-positive cells per high power field (×400), and the number of capillaries per muscle fiber in 15 randomly chosen microscopic fields from three different sections in each tissue block (22).

**Western Blot Analysis**—Tissue samples obtained on postoperative day 7 were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor mixture (Sigma). Protein content was determined by the Bradford method. The same amounts of protein (50 μg) were separated with denaturing SDS-10% polyacrylamide gels. The membranes were immunobotted with the primary antibodies at a 1:1000 dilution followed by the secondary antibody conjugated with horseradish peroxidase at a 1:5000 dilution. Bands were visualized using ECL Western blotting detection kit (Amersham Biosciences).

**Measurement of Plasma Parameters**—Total cholesterol, high density lipoprotein cholesterol, and glucose levels were measured with enzymatic kits (Wako Chemicals, Richmond, VA). Insulin levels were measured with electroimmunoassay kits (Wako Chemicals). Adiponectin levels were determined using enzyme-linked immunosorbent assay kits (Otsuka Pharmaceutical Co Ltd., Tokyo, Japan). Blood was collected by heart puncture from mice on 4 weeks of CR.

**Statistical Analysis**—Data are presented as mean ± S.E. Statistical analysis was performed by analysis of variance followed by Scheffe’s F test. A value of p < 0.05 was accepted as statistically significant.

**RESULTS**

**Effect of CR on Body Weight in WT Mice**—A linear reduction in body weight occurred during the first 2 weeks of CR, and the reduction in body weight was maintained between 2 and 6 weeks (Fig. 1). The WT mice fed AL (AL-WT mice) showed a moderate increase in body weight of 7.3% over this time course. At the end of the 6-week time course, the difference in body weight between AL-WT mice and WT mice fed CR diet (CR-WT mice) was 26% (Fig. 1). HR and systolic blood pressure did not differ between the two groups (Table 1). Consistent with previous reports (12, 25), there was a significant reduction in total cholesterol, triglyceride, and plasma glucose levels in the CR-WT mice compared with AL-WT mice.

**CR Promotes Revascularization in Response to Tissue Ischemia in WT Mice**—AL-WT and CR-WT mice underwent surgical induction of unilateral hindlimb ischemia 4 weeks after the initiation of diets. All mice survived and appeared healthy during the follow-up period. Fig. 2A shows representative LDBF images of hindlimb blood flow before surgery and at different time points after surgery in the AL- and CR-WT mice. In AL-WT mice, hindlimb perfusion fell precipitously after surgery, increased to 20–30% of the nonischemic limb by day 7, and ultimately returned to 50% of the nonischemic limb by day
CR-WT mice showed a significant increase in limb flow at 7 and 14 days after hindlimb surgery compared with AL-WT mice (Fig. 2B) (p < 0.05; n = 6).

To investigate the extent of revascularization at the microcirculatory level, capillary density was measured in histological sections harvested from the ischemic tissues. Representative photomicrographs of CD31-stained ischemic muscles are shown in Fig. 3A. Quantitative analysis revealed that the capillary density was significantly increased in CR-WT mice compared with AL-WT mice at 14 days after surgery (Fig. 3B).

Role of Adiponectin in CR-mediated Revascularization—CR has been shown to increase serum levels of adiponectin (14, 19), and we have shown that adiponectin promotes blood flow recovery in response to ischemia (22). These findings led us to hypothesize that the increase in circulating adiponectin levels contributes to the improved vascular response in the CR-treated animals. We assessed plasma adiponectin levels in WT mice at 4 weeks (n = 6). Each value is mean ± S.E. HR indicates heart rate (beats/min); sBP, systolic blood pressure (mm Hg); TC, total cholesterol (mg/dl); HDL-C, high density lipoprotein cholesterol (mg/dl); PG, plasma glucose (mg/dl); TG, triglyceride.

TABLE 1

| Diet | HR  | sBP  | TC  | HDL-C | TG  | PG  |
|------|-----|------|-----|-------|-----|-----|
| AL   | 640 | 104  | 77.6| 54.9  | 112.4| 95.3|
| CR   | 605 | 121  | 77.9| 52.6  | 113.8| 95.6|

*p < 0.05 versus AL-WT mice.

14 (Fig. 2B). CR-WT mice showed a significant increase in limb flow at 7 and 14 days after hindlimb surgery compared with AL-WT mice (Fig. 2B) (p < 0.05; n = 6).

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induced revascularization is mediated by the up-regulation of adiponectin.

**eNOS Activation Is Essential for CR-induced Revascularization** — eNOS plays an important role in revascularization following hindlimb ischemia (21). To analyze the potential involvement of eNOS in CR-induced revascularization, the expression and phosphorylation of eNOS in ischemic adductor muscle were assessed by Western blot analysis. The expression of total eNOS protein in ischemic muscles did not differ between AL and CR-mice. However, phosphorylation of eNOS at Ser-1177 in ischemic muscle was significantly greater in CR-WT mice than in AL-WT mice (Fig. 5, A and B). To test the possible contribution of adiponectin to regulation of eNOS activation by CR, we assessed the phosphorylation of eNOS in ischemic tissue of APN-KO mice treated with AL or CR. In contrast to the stimulatory effects of CR on eNOS phosphorylation in WT mice, CR did not influence eNOS phosphorylation in APN-KO mice (Fig. 5, A and B).

To test whether increased expression of adiponectin affects phosphorylation of eNOS in ischemic muscle, Ad-βgal or Ad-APN was intravenously delivered to APN-KO and WT mice. Circulating adiponectin levels were 9.9 ± 2.1 μg/ml in wild-type/Ad-βgal, 18.1 ± 3.6 μg/ml in wild-type/Ad-APN, <0.05 μg/ml in APN-KO/Ad-βgal, and 9.1 ± 2.2 μg/ml in APN-KO/Ad-APN on postoperative day 7. Treatment with Ad-APN significantly increased eNOS phosphorylation in ischemic muscle of WT and APN-KO mice at day 7 after surgery (Fig. 5, A and B).

To further analyze the involvement of eNOS signaling in revascularization by CR, we examined the impact of CR on blood flow of ischemic muscles in eNOS-KO mice. CR-eNOS-KO mice had increased plasma adiponectin levels compared with AL-eNOS-KO mice (8.1 ± 1.4 in AL-eNOS-KO mice and 15.1 ± 2.3 μg/ml in CR-eNOS-KO mice, respectively, p < 0.05). LDBF analysis revealed that no significant differences in limb perfusion were observed between AL-eNOS-KO and CR-eNOS-KO mice on postoperative day 0, 3, 7, and 14 (Fig. 5C). Because eNOS-KO mice exhibit severe ischemia-induced vascular insufficiency, which is accompanied by amputation, we assessed lower limb function and tissue salvage post-surgery using a clinical scoring system (27). Similarly, the index of severity of tissue ischemia after hindlimb surgery did not differ between AL-eNOS-KO and CR-eNOS-KO mice (Fig. 5D). We also analyzed the effect of CR on blood flow recovery of ischemic muscles in WT mice receiving NOS inhibitor L-NAME or vehicle. Although vehicle-treated CR-WT mice showed increased recovery of blood flow compared with vehicle-treated AL-WT mice, treatment with L-NAME abrogated the increase in limb perfusion recovery in CR-WT mice (Fig. 5E). Collectively, these data suggest that CR-stimulated revascularization is attributed to eNOS activation that is involved in adiponectin production.
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Role of AMPK in CR-induced Revascularization—To examine the possible participation of AMPK in CR-induced revascularization, the expression and phosphorylation of AMPK in ischemic adductor muscle were assessed by Western blot analysis. The expression of total AMPK protein in ischemic muscles did not differ between AL and CR mice. However, phosphorylation of AMPK in ischemic muscle was significantly greater in CR-WT mice than in AL-WT mice at day 7 after the operation (Fig. 6, A and B). To further analyze the involvement of AMPK in CR-induced revascularization, we examined the effect of CR on blood flow recovery of ischemic muscles in WT mice receiving AMPK inhibitor compound C or vehicle. CR-WT mice receiving compound C or vehicle had increased plasma adiponectin levels compared with AL-WT mice receiving vehicle (8.3 ± 1.6 in vehicle-treated AL-WT mice, 16.1 ± 2.4 in vehicle-treated CR-WT mice, and 15.4 ± 2.4 μg/ml in compound C-treated CR-WT mice, respectively). Treatment of CR-WT mice with compound C blocked increased limb perfusion caused by CR compared with vehicle-treated CR-WT mice (Fig. 6C). Furthermore, treatment with compound C significantly diminished CR-induced increase in eNOS phosphorylation in ischemic muscle tissue (Fig. 6, D and E). These data suggest that AMPK is involved in CR-induced eNOS activation and revascularization.

DISCUSSION

This study provides evidence that CR promotes ischemia-induced revascularization in a well-established hindlimb model. CR enhanced blood flow recovery and capillary formation in ischemic muscle of wild-type mice, which was accompanied by increased levels of eNOS phosphorylation and plasma adiponectin. The stimulatory actions of CR on reperfusion and eNOS phosphorylation of ischemic limbs were abolished in APN-KO mice. Furthermore, CR did not improve flow recovery in ischemic limbs of eNOS-KO mice.

The ability of CR to increase adiponectin levels is likely to contribute to the stimulation of revascularization under our experimental conditions. We have shown that adiponectin overexpression will accelerate revascularization of ischemic limbs in wild-type mice (22). Our observations here show that CR increased plasma adiponectin levels 1.8-fold in wild-type mice, in agreement with previous reports (14, 19). CR promoted ischemia-induced revascularization in wild-type mice but not APN-KO mice. Because adiponectin promotes vascular cell function and survival under conditions of stress (22, 28), we propose that adiponectin functions as an important mediator of CR-stimulated vascular responses. Recently, it was reported that CR confers resistance to myocardial ischemia-reperfusion injury by increasing adiponectin levels (14). CR also improves the survival and myocardial damage in obese mice with viral myocarditis, which is accompanied by increased adiponectin levels in plasma and myocardium (29). Thus, the up-regulation of adiponectin by CR could represent a common mechanism in the protection of cardiovascular tissues to stress.

It is well established that eNOS is beneficial for various types of vascular and metabolic diseases (21, 30, 31). In this study, CR increased the activating phosphorylation of eNOS in ischemic muscles, and the ability of CR to enhance revascularization was abrogated in eNOS-KO mice or WT mice receiving NOS inhibitor. Thus, CR appears to promote revascularization in ischemic states through its ability to activate eNOS. It is reported that CR induces mitochondrial biogenesis in various tissues of mice, and these effects are diminished in eNOS-KO mice (32). Collectively, these observations suggest that eNOS acts as a key mediator of the protective actions of CR.

It has been shown that adiponectin exerts vascular protection through modulation of eNOS signaling. We have shown

FIGURE 5. CR promotes eNOS phosphorylation in response to tissue ischemia. A, representative immunoblots for phosphorylated eNOS (P-eNOS) and eNOS in WT and APN-KO mice fed AL or CR, and AL-WT or AL-APN-KO mice treated with Ad-APN or Ad-βgal. Western immunoblots with the indicated antibodies were performed on the ischemic adductor muscle at 7 days after surgery. Ad-APN or Ad-βgal at 2 × 10⁸ pfu total was intravenously injected into AL-WT and AL-APN-KO mice 3 days before the induction of hindlimb ischemia. B, quantitative analysis of relative changes in phosphorylation of eNOS in WT and APN-KO mice fed AL or CR, and AL-WT or AL-APN-KO mice treated with Ad-APN or Ad-βgal. Phosphorylation of eNOS was normalized to the tubulin signal and expressed as percentage of the signal intensity of AL-WT mice (n = 4). C, quantitative analysis of ischemic/nonischemic LDBF ratio in AL-eNOS-KO or CR-eNOS-KO mice on post-operative day 0, 3, 7, and 14 (p < 0.01 versus AL-eNOS-KO mice or CR-eNOS-KO mice). D, clinical score in AL-eNOS-KO or CR-eNOS-KO mice as determined by an index of severity of limb ischemia. (0 = normal, 1 = pale foot or gait abnormalities, 2 = less than half of foot is necrotic, 3 = more than half of foot is necrotic without lower leg necrosis, 4 = more than half of foot is necrotic with some lower leg necrosis, 5 = necrosis or auto amputation of entire lower limb). E, quantitative analysis of ischemic/nonischemic LDBF ratio in AL or CR-WT mice in the presence of L-NAME or vehicle on post-operative day 0, 3, 7, and 14 (*, p < 0.05; **, p < 0.01 versus CR + vehicle; n = 5).
that adiponectin stimulates phosphorylation of eNOS, which is associated with enhanced endothelial cell migration and differentiation into capillary-like structures (28, 33, 34). Adiponectin stimulates nitric oxide production by endothelial cells via the activating phosphorylation of eNOS (33, 35, 36). Recently, we have shown that APN-KO mice develop increased cerebral ischemia-reperfusion injury compared with WT mice, which is accompanied by reduced eNOS activation in ischemic cerebral tissue (37). Conversely, administration of adiponectin reduces cerebral infarct size and stimulates eNOS phosphorylation in ischemic brain. Of importance, the cerebroprotective actions of adiponectin were diminished in eNOS-KO mice, suggesting that eNOS is required for salutary vascular responses to adiponectin in ischemic brain. Consistent with these observations, this study shows that APN-KO mice have reduced phosphorylation of eNOS in ischemic muscles. The CR-induced increase in eNOS phosphorylation during ischemia was abolished in APN-KO mice. Furthermore, despite the increased plasma adiponectin in eNOS-KO mice following CR, CR had no effects on perfusion recovery of ischemic limbs in eNOS-KO mice. Taken together, these data provide evidence that thevasculo-protective actions of CR are mediated, at least in part, through the adiponectin-eNOS regulatory axis.

Our data show that AMPK is required for CR-stimulated revascularization in ischemic tissue. CR stimulated the phosphorylation of AMPK in the ischemic tissue, and administration of an AMPK inhibitor abrogated CR-induced increase in ischemia-induced revascularization and eNOS phosphorylation. AMPK is reported to directly phosphorylate eNOS at Ser-1177 (38). It has also been shown that adiponectin stimulates phosphorylation of eNOS in endothelial cells through its ability to activate AMPK (33, 36). AMPK activation has also been shown to stimulate vascular endothelial growth factor production in ischemic muscle (39). However, vascular endothelial growth factor levels in ischemic muscles did not differ between AL and CR-WT mice (data not shown), suggesting that the beneficial effect of CR on revascularization is not mediated by the angiogenic properties of this cytokine. Although other pro-angiogenic cytokines may be involved in CR-induced revascularization, we favor the hypothesis that CR-mediated increase in adiponectin has a salutary effect on the vasculature under conditions of ischemic stress by promoting AMPK-eNOS signaling and thereby promotes the revascularization response.

Obesity-linked diseases, including type 2 diabetes, cause increased mortality and morbidity of coronary and peripheral artery diseases because of microvascular rarefaction and impaired collateral vessel growth under ischemic conditions (2, 40, 41). The findings reported here suggest that CR could favor revascularization in response to tissue ischemia. Thus, nutritional approaches aimed at limiting caloric intake could be useful for treatment of ischemic heart and limb diseases.

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