Calorie restriction initiated at a young age activates the Akt/PKC ζ / λ -Glut4 pathway in rat white adipose tissue in an insulin-independent manner

Seongjoon Park · Toshimitsu Komatsu ·
Hiroko Hayashi · Haruyoshi Yamaza ·
Takuya Chiba · Yoshikazu Higami ·
Kazunao Kuramoto · Isao Shimokawa

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Abstract Calorie restriction (CR) may exert an anti-aging effect through a metabolic adaptation to limited energy intake. The present study investigated the effect of CR on insulin signaling in response to glucose load in the epididymal adipose tissue of male F344 rats at 7 and 22 months of age. Young and middle-aged rats were fed ad libitum (AL) or 30% CR diets for 4 months, underwent glucose tolerance tests and were sacrificed 15 min after an intraperitoneal glucose or saline injection to evaluate glucose-stimulated insulin response and subsequent activation of insulin signaling molecules in the adipose tissue. In the 7- and 22-month AL groups, glucose administration increased serum insulin levels and also increased phosphorylated (p) levels of

the insulin receptor (IR), v-akt murine thymoma viral oncogene homolog (Akt), protein kinase C (PKC) ζ / λ and the membrane fraction of glucose transporter 4 (mGlut4). In contrast, in the 7-month CR group, p-Akt, p-PKC ζ / λ and mGlut4 levels were upregulated without glucose stimulation; the glucose load augmented the p-IR level but there was no additional activation of the downstream molecules. In the 22-month CR group, these unexpected findings were not observed. In summary, basal levels of insulin signaling molecules such as p-Akt, p-PKC ζ / λ , and mGlut4 were significantly increased with a low insulin response in the 7-month CR group. The present results suggest the presence of an age-specific insulin-independent mechanism that is induced by CR to regulate energy metabolism in white adipose tissue.

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S. Park · T. Komatsu · H. Hayashi · H. Yamaza · T. Chiba ·
Y. Higami · I. Shimokawa (✉)
Department of Investigative Pathology,
Unit of Basic Medical Sciences,
Nagasaki University Graduate School of Biomedical Sciences,
1-12-4 Sakamoto,
Nagasaki City 852-8523, Japan
e-mail: shimo@nagasaki-u.ac.jp

K. Kuramoto
Animal center,
Tokyo Metropolitan Institute of Gerontology,
35-2 Sakae-cho, Itabashi-ku,
Tokyo 173-0015, Japan

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Introduction

Calorie restriction (CR) ameliorates the glucose intolerance and whole body insulin resistance that occurs in aged animals (Reaven et al. 1983; Arciero et al. 1999). Our previous study revealed that CR for 4 months, initiated either at a young age or in middle age, improved glucose tolerance without any significant surge of serum insulin (Park et al. 2006). Understand-

ing the mechanism by which CR modulates glucose-insulin homeostasis would give an insight into potential therapeutic approaches for diabetic patients.

Previous studies evaluating the effect of CR have emphasized the sensitization of insulin action in skeletal muscle—a major site of glucose disposal (DeFronzo et al. 1981; James et al. 1986). Insulin binding to the insulin receptor (IR) leads to autophosphorylation of IR and activation of PI3K, which in turn phosphorylates v-akt murine thymoma viral oncogene homolog (Akt), and protein kinase C (PKC) ζ/λ (Ishiki and Klip 2005). The signaling cascade promotes translocation of Glut4 into the cell membrane to increase glucose uptake in insulin-sensitive tissues (Henriksen et al. 1990). CR was hypothesized to inhibit or restore the age-related impairment in insulin signaling in response to glucose, as supported by earlier studies (Reaven et al. 1983; Arciero et al. 1999). In our previous study of rat quadriceps femoris muscle (QFM) tissue, young-aged CR rats had similar or slightly higher activation (phosphorylation) of IR and Akt with lower serum insulin levels in response to glucose administration. This confirms the sensitization of insulin action in the skeletal muscle by CR. CR in middle-aged rats, however, had no significant effect on the age-related impairments of insulin signaling in response to glucose, whereas glucose intolerance was improved. These findings indicate two possibilities: firstly, the presence of an insulin-independent mechanism in skeletal muscle for glucose metabolism, and, secondly, the involvement of non-muscle tissues in glucose disposal (Park et al. 2006).

White adipose tissue (WAT) also plays an important role in glucose disposal, although the glucose metabolic index is lower than in skeletal muscle (Wetter et al. 1999). Adipose tissue-specific Glut4 knockout mice develop insulin resistance and have an increased risk of diabetes (Abel et al. 2001). In contrast, adipose tissue-specific overexpression of Glut4 reverses insulin resistance and diabetes in muscle-specific Glut4 knockout mice (Carvalho et al. 2005). An *in vivo* study has indicated that postprandial glucose uptake in WAT is greater in CR rats than in control *ad libitum* (AL) rats (Wetter et al. 1999). Therefore, in the present study, we addressed the issue of whether WAT is involved in glucose-insulin homeostasis modulated by CR. For this purpose, we analyzed the insulin signaling molecules in WAT in response to a glucose load. The adiponectin-AMPK pathway was also analyzed, be-

cause this pathway is involved in an insulin-independent mechanism for glucose metabolism. As noted in our previous paper (Park et al. 2006), the effect of CR initiated in middle age is also important, because many clinical programs that aim to reduce calorie intake, and thus decrease body fat, are provided to prediabetic obese patients. Therefore, in the present study, we compared the effects of CR initiated in young or middle-aged rats.

Materials and methods

Experimental animals and tissues

Four-week-old male Fischer 344 rats (F344/DuCrj) were purchased from Charles River Laboratory Japan (Yokohama, Japan). Seventeen-month-old male F344 rats (F344/DuCrj) were transferred from the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan) to the Biomedical Research Center at the Center for Frontier Life Sciences, Nagasaki University. All rats were certified to be specific pathogen-free. Rats were housed separately and maintained in a barrier facility (temperature 21–24°C; 12 h/12 h-light/dark cycle). Young ($n=20$) and middle-aged ($n=15$) rats received food (CRF-1 diet, Oriental Yeast, Tokyo) *ad libitum* (AL) until 2.5 or 18 months of age to measure the amount of food intake and for acclimatization to the facility. Rats were divided into two diet groups: AL and CR. In the AL group, rats continued to receive food AL, whereas in the CR group, rats received 30% less food than the AL groups by a modified alternate-day feeding program. The CR groups received two lots of food, i.e., 140% of the mean daily food intake of the respective AL groups, every other day, 30 min before lights were turned off. Food intake in each AL group was monitored every 2 weeks and the amount of food given to each CR group was adjusted accordingly.

At 7 and 22 months, with at least an interval of 2 weeks after glucose tolerance tests (GTT), rats were fasted overnight and sacrificed by decapitation 15 min after an *ip* injection of D-glucose (1.0 g/kg body weight; 50% solution) or saline to evaluate the glucose-stimulated insulin response and subsequent activation of insulin signaling molecules. Epididymal adipose tissue was collected immediately, weighed, quickly diced, and frozen in liquid nitrogen. Serum samples were prepared from trunk blood for measure-

ment of adiponectin concentrations. All samples were stored at -80°C .

All experiments in this study were performed in accordance with the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University.

Serum insulin and adiponectin determination

Serum insulin and adiponectin concentrations were determined using commercially available ELISA kits (LINCO Research, St. Louis, MO, and B-Bridge International, Tokyo, Japan, respectively).

Tissue preparation for immunoprecipitation and western blotting

Total tissue lysates were prepared using lysis buffer [50 mM Tris HCl at pH 7.4, 50 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin, 1% Triton X-100]. Homogenates were centrifuged (10,000 g, 20 min, 4°C) and the supernatant was collected. The protein content of lysates was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL).

Immunoprecipitation

Insulin receptor and phosphor-tyrosine antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody to beta actin was purchased from Abcam (Cambridge, UK).

Four milligrams of protein from each sample was rotated overnight at 4°C in the presence of 10 μg IR antibody before 50 μl protein G agarose beads was added and rotation at 4°C continued for a further 3 h. Immune complexes were pelleted in a micro-centrifuge for 2 min at 4,000 rpm at 4°C , followed by four washes with IP buffer (40 mM Tris-HCl, pH 7.6, 5 mM EDTA, 120 mM NaCl, 0.1% NP-40, 1 mM PMSF, 2 $\mu\text{g}/\text{ml}$ Leupeptin, 1 $\mu\text{g}/\text{ml}$ Aprotinin, 20 mM NaF, and 2 mM Na_3VO_4). The final pellet was resuspended in 30 μl sample buffer (100 mM Tris HCl, pH 6.8, 4% SDS, 1.2% β -mercaptoethanol, 2% glycerol, 0.2 mg/ml bromophenol blue). All samples were boiled for 3 min and chilled on ice. The supernatant was then resolved on an 8% SDS-polyacrylamide gel and

transferred to nitrocellulose paper. Immunoblots for tyrosine-phosphorylated IR followed the method described for western blotting, in which the membrane was incubated with anti-phospho IR at (1:1,000) in Tris-buffered saline with 0.1% Tween 20 (TBS-T) overnight.

Western blotting

Antibodies to Phospho (p)-Akt (Ser473), p-PKC ζ/λ and p-AMPK α (Thr172) were obtained from Cell Signaling Technology (Beverly, MA). Anti-AMPK α antibody was purchased from Upstate (New York, NY) and anti-Glut4 antibody was obtained from Chemicon (Tamaulaca, CA). Enhanced chemiluminescence (ECL) western blotting detection reagents and ECL-anti-rabbit or mouse IgG, horseradish peroxidase-linked species-specific whole antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). All other chemicals were obtained from Sigma (St. Louis, MO).

All samples were boiled for 3 min and chilled on ice. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were immediately placed in blocking solution (5% non-fat dry milk in TBS-T buffer) for 1 h and incubated with the primary antibody for 2 h 30 min followed by the second antibody for 1 h 30 min. All incubations were at room temperature. Antibody labeling was detected using ECL as per the manufacturer's instructions. Specific signals were quantified by Fluorchem (DE500-5T, Alpha Innotech, San Leandro, CA) with associated image analysis software (AlphaEase FC, Alpha Innotech). To minimize the variance in signal intensity between the blots, a standard sample, prepared from a 7-month-old rat tissue was included in each blot. Immunoblotting of β -actin was also performed to confirm the quality and quantity of the loaded samples.

Subcellular fractionation

To investigate the effect of CR on the subcellular distribution of Glut4, a subcellular fractionation protocol was used as previously described (Oliveira et al. 2004). The total crude membrane fraction, but not the purified membrane fraction, was prepared as follows. Tissues were homogenized in five volumes of buffer A [0.32 M sucrose, 20 mM Tris-HCl (pH 7.4),

2 mM EDTA, 1 mM DTT, 100 mM sodium fluoride, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 1 mM PMSF, and 5 µg/ml aprotinin] at 4°C with a polytron homogenizer. Homogenates were centrifuged (1,000 g, 25 min, 4°C) and the supernatant was removed and centrifuged (100,000 g, 60 min, 4°C) to obtain the cytosol fraction. This pellet was resuspended in buffer A, which was added to 1% NP-40 and kept on ice for 20 min before a final centrifugation (100,000 g, 20 min, 4°C) to obtain the membrane fraction. Protein content was determined using the BCA protein assay kit, and used immediately for determination of Glut4 by Western blotting.

Statistical analysis

Data are expressed as means ±SEM and were analyzed by one-factor ANOVA with post hoc Fischer's protected LSD test for multiple comparisons. The level of significance was set at $P < 0.05$.

Results

Effect of CR on body weight and epididymal fat pad weight

The body weight of AL rats increased significantly with age (Table 1). At 7 and 22 months of age, CR rats had a 24% and 28% reduction in body weight, respectively. The normalized fat pad weight of AL rats increased by 24% with age (Table 1). Although the

Table 1 Body and relative epididymal fat pad weights. Values represent mean ± SD. The number of rats is in parenthesis. AL Ad libitum, CR calorie restriction

	Age (months)	
	7	22
Body weight (BW, g)		
AL	340.4±19.3 (10)	408.3±20.1* (9)
CR	258.4±7.4 *** ^a (10)	292.3±18.5 */*** ^a (6)
Epididymal fat pad (g/100 g BW)		
AL	1.7±0.2 (10)	2.1±0.2 * (9)
CR	1.5±0.4 (10)	1.0±0.4 */*** ^a (6)

* $P < 0.01$ versus 7 months in each diet group, ** $P < 0.01$ versus AL at each age

^a Data published elsewhere (Park et al. 2006)

normalized fat pad weight of the 7-month CR group did not differ from that of the 7-month AL group, by 22 months of age, the fat pad weight of the CR group was reduced to approximately 50% of that of the AL group.

Effect of CR on blood glucose and glucose-stimulated serum insulin response

The results of GTT for the present animals are published elsewhere (Park et al. 2006). Briefly, blood glucose concentrations increased to a peak at 15 min after ip glucose injection and gradually decreased thereafter. When compared with the 7-month AL group, the 22-month AL group had higher blood glucose levels during the GTT. Blood glucose levels were lower in the 7- and 22-month CR groups than in age-matched AL groups. When compared with the 7-month CR group, blood glucose levels were slightly higher in the 22-month CR group.

Serum insulin concentrations were significantly augmented in the AL groups but not in the CR groups at 15 min after glucose administration (Table 2). There was a small, age-related increase in serum insulin in both diet groups. Blood glucose concentrations were significantly increased after ip glucose injection in both the AL and CR groups (Table 2).

Effect of CR on p-IR levels in adipose tissue

Administration of glucose significantly elevated levels of p-IR in adipose tissue in the 7-month AL and CR groups (Fig. 1) but there was no significant effect of diet. There was a small, age-related decrease in the glucose-stimulated level of p-IR in the CR group. Protein levels of IR were not significantly changed by age, diet, or glucose administration (Fig. 1).

Effect of CR on p-Akt and p-PKCζ/λ levels in adipose tissue

As a whole, glucose administration tended to elevate levels of p-Akt in the AL groups (Fig. 2a); there was no age-related change in basal or glucose-stimulated p-Akt levels between AL groups. The basal level of p-Akt in the 7-month CR group was significantly higher than that of the 7-month AL group but was similar to the glucose-stimulated levels in the AL group. The basal level of p-Akt was significantly reduced in the

Table 2 Effects of CR on blood glucose and glucose-stimulated serum insulin response. Samples were prepared from tail vein blood at 0 and 15 min after glucose administration. Data represent mean (insulin, ng/ml; glucose, mg/dl) $\pm$ SD

			Time (after glucose injection)	
			0 min	15 min
Glucose	7 months	AL	99.1 $\pm$ 8.5 (12)	183.5 $\pm$ 41.4** (12)
		CR	96.0 $\pm$ 9.2 (14)	173.4 $\pm$ 21.0** (14)
	22 months	AL	100.0 $\pm$ 8.9 (9)	226.8 $\pm$ 29.7** (9)
		CR	103.1 $\pm$ 5.9 (8)	196.4 $\pm$ 49.5** (8)
Insulin	7 months	AL	1.5 $\pm$ 0.4 (10)	2.3 $\pm$ 0.5* (10)
		CR	1.3 $\pm$ 0.3 (10)	1.4 $\pm$ 0.7 ***** 10 ^a
	22 months	AL	2.1 $\pm$ 0.5 (9)	3.8 $\pm$ 1.3*/***** (9) ^a
		CR	2.1 $\pm$ 0.6 *** (8) ^a	1.8 $\pm$ 0.9 ***** (8) ^a

* P <0.01 vs 0-min at each age in AL, ** P <0.001 vs 0-min at each age, *** P <0.05 versus 7 month-CR at 0-min, **** P <0.01 versus 7 month-AL at 15 min, ***** P <0.01, versus AL at 15 min of each age

^aData published elsewhere (Park et al. 2006)

22-month CR group as compared with the 7-month CR group but levels tended to increase ($P=0.0987$) in the 22-month CR group after glucose injection.

Glucose injection also resulted in a significant increase in p-PKC ζ/λ levels in the 7-month AL group (Fig. 2b). The basal p-PKC ζ/λ level in the 7-month CR group was greater than that of the 7-month AL group and did not differ from the glucose-stimulated level. There was no age-related difference in basal or glucose-stimulated p-PKC ζ/λ levels between the AL groups. The basal level was decreased at 22 months in the CR group compared with the 7-month CR group.

Effect of CR on mGlut4 protein levels in adipose tissue

Glucose injection led to an increase in mGlut4 protein levels in the 7-month AL group (Fig. 3a). There was no significant age-related change between the AL groups. The basal mGlut4 level in the 7-month CR group was significantly greater than that of the 7-month AL group, but there were no further changes in Glut4 levels following glucose injection. Basal mGlut4 levels were significantly decreased in the 22-month CR group compared with the 7-month CR group. There was a small but significant increase in mGlut4 levels after glucose injection in the 22-month CR group. The total protein levels of Glut4 did not differ between the groups (see [Electronic supplementary material](#)), although the number of rats in the 22-month CR group was limited.

Effect of CR on plasma adiponectin and p-AMPK α

The adiponectin-AMPK pathway is reported to activate glucose uptake in primary rat adipocytes (Wu et al. 2003). Therefore, in the present study we analyzed serum adiponectin concentrations and p-AMPK α levels. Glucose injection did not affect the serum adiponectin concentration significantly (Fig. 4a). There was an age-related decrease in serum adiponectin concen-

Fig. 1 Effects of age and calorie restriction (CR) on phosphotyrosine insulin receptor (pY-IR) levels in whole tissue extracts of rat epididymal fat tissue. *Upper panel* Representative immunoblotting, *lower panel* relative pY-IR levels. Data represent mean $\pm$ SEM [7 month ad libitum (AL)/CR, $n=5$; 22 month AL, $n=4$ (saline) or 5 (glucose), CR $n=3$]. * P <0.05 vs 7-month AL/saline, or 7-month CR/saline

Fig. 2a,b Effects of age and CR on phosphorylated Akt (p-Akt) and protein kinase C (p-PKCζ/λ) in whole tissue extracts of rat epididymal fat tissue. *Upper panels* Representative immunoblotting, *lower panels* relative levels of p-Akt (**a**) or p-PKCζ/λ (**b**). Data represent mean + SEM [7-month AL/CR, $n=5$; 22-month AL, $n=4$ (saline) or 5 (glucose), CR $n=3$]. **a** p-Akt levels, * $P<0.05$ vs 7-month AL/saline; ^{§§} $P<0.01$ vs 7-month AL/saline; ^{††} $P<0.01$ vs 7-month CR/saline. **b** p-PKCζ/λ levels, * $P<0.05$ vs 7-month AL/saline or 22-month AL/saline; [§] $P<0.05$ vs 7-month AL/saline; [†] $P<0.05$ vs 7-month CR/saline

trations in the AL group, but not in the CR group. CR resulted in a significant increase in serum adiponectin.

The p-AMPKα level was significantly increased by glucose administration in the 7-month and 22-month AL groups (Fig. 4b) but there were no age-related changes. The basal p-AMPKα level was significantly greater in the 7-month CR group than in the 7-month AL group; however, levels of p-AMPKα were lower

in the 22-month CR group compared with the 7-month CR group. There were no significant increases in p-AMPKα levels after glucose injection in the 7-month or 22-month CR group.

Discussion

Glucose administration increased serum insulin levels, subsequent activation of the IR-Akt/PKCζ/λ pathway, and translocation of Glut4 to the plasma membrane in young rats fed AL. A similar level of activation of the IR-Akt/PKCζ/λ-Glut4 pathway required higher serum insulin levels in middle-aged rats fed AL, reflecting an age-related desensitization of insulin signaling. However, in young CR rats, the Akt/PKCζ/λ-Glut4 pathway was activated under basal conditions and no further activation was observed even when the IR was phosphorylated after glucose administration.

In the young rats, CR appears to activate the insulin signaling pathway in an insulin-independent manner. In support of this finding, an *in vivo* study of 12-month-old male rats showed that WAT exhibited greater glucose metabolic indices. Glucose utilization in the preprandial phase, when blood glucose and

Fig. 3 Effects of age and CR on Glut4 levels in membrane extracts from rat epididymal fat tissue. *Upper panel* Representative immunoblotting, *lower panel* mGlut4 levels. Data represent mean + SEM [7-month AL/CR, $n=5$; 22-month AL, $n=4$ (saline) or 5 (glucose), CR $n=3$]. * $P<0.05$ vs 7-month AL/saline; ** $P<0.01$ vs 22-month CR/saline; [§] $P<0.05$ vs 7-month AL/saline; [†] $P<0.05$ vs 7-month CR/saline

Fig. 4a,b Effects of age and CR on serum adiponectin concentration and phosphorylated AMPK α (p-AMPK) levels in whole extracts of rat epididymal fat tissue. *Upper panels* Representative immunoblotting, *lower panels* serum level of adiponectin (**a**) or p-AMPK (**b**). Data represent means $\pm$ SD $\pm$ SEM [7-month AL/CR, $n=5$; 22-month AL, $n=4$ (saline) or 5 (glucose), CR $n=3$]. **a** Adiponectin serum concentration, * $P<0.05$ vs 7-month AL/saline; ** $P<0.01$ vs 22-month AL/glucose; *** $P<0.001$ vs 7-month AL/glucose or 22-month AL/saline; $\dagger$ $P<0.05$ vs 7-month AL/glucose; $\dagger\dagger$ $P<0.01$ vs 7-month AL/saline. **b** Phosphorylated AMPK α levels * $P<0.05$ vs 7-month AL/saline; ** $P<0.01$ vs 22-month AL/saline; $\S$ $P<0.05$ vs 7 month AL/saline

insulin are low, as well as in the postprandial phase (blood glucose and insulin are high), was greater in CR rats compared with AL-fed rats (Wetter et al. 1999). Moreover, the glucose metabolic index did not differ significantly between the pre- and post-prandial phases in CR rats; a finding that supports the insulin independency of glucose uptake in WAT of CR rats. In contrast, in the 22-month CR rats, intracytoplasmic insulin signaling was not activated at baseline but it was activated in a manner similar to that in the 22-month AL group in response to glucose loading. Because the glucose-stimulated serum insulin con-

centration was lower in the 22-month CR rats than in the 22-month AL rats, the middle-aged CR rats were able to show the improved insulin resistance that occurs in middle-aged control AL rats. However, CR initiated in middle age might not induce insulin independency in WAT. To understand the mechanisms underlying the effect of CR on glucose uptake, not only insulin dependency but also insulin independency should be considered.

A growth factor other than insulin may activate the Akt/PKC ζ / λ pathway and initiate membrane translocation of Glut4 in young CR rats, because several growth factors are known to activate the Akt and/or PKC ζ / λ pathway. One such candidate is ghrelin, a hormone that is secreted mainly from the stomach (Kojima et al. 1999) and levels of which are reported to increase in the plasma following CR (Komatsu et al. 2006). Moreover, the specific receptor for ghrelin is known to be expressed abundantly in WAT (Petersenn 2002). An in vitro study using 3T3-L1 adipocytes has indicated that ghrelin promotes glucose uptake via activation of the PI3K-Akt pathway (Kim et al. 2004). Thus, the potential role for ghrelin and other growth factors in activation of the Akt/PKC ζ / λ -Glut4 pathway in WAT of CR rats under basal conditions should be further investigated.

In young CR rats, the IR was phosphorylated after glucose-administration but no further activation of the Akt/PKC ζ / λ -Glut4 signaling pathway was observed. Activation of IR is known to activate Shc-MAPK signaling, which has mitogenic effects (Rhodes and White 2002). ERK, p38, and JNK are reported to be involved in various steps of adipogenesis in WAT (Bost et al. 2005). In support of this, a recent study suggests that CR promotes proliferation but suppresses terminal differentiation of preadipocytes through a reduction in the ratio of CCAAT/enhancer binding protein β (C/EBP β) isoforms and liver-enriched activator protein (LAP)/liver-enriched inhibitory protein (LIP) (Zhu et al. 2007). A study of isolated adipocytes from 8-month-old CR and AL rats indicated that the rate of glucose incorporation into triglyceride and fatty acids is greatly increased in the adipocytes of CR rats under both basal and insulin-stimulated conditions (Escriva et al. 2007). These findings suggest that CR stimulates adipogenesis. Our preliminary data also indicate that mRNA levels of fatty acid synthase (FAS) and acetyl CoA carboxylase 1 (ACC1) and ACC1 protein abundance were greater

in the young CR group, suggesting promoted lipogenesis (see [Electronic supplementary material](#)). Gene expression of carnitine-palmitoyl-transferase 1 β (CPT1 β), which elevates fatty acid beta oxidation during fasting, was also enhanced in young CR rats (see [Electronic supplementary material](#)). Collectively, our data imply that, in adipose tissue, CR promotes glucose uptake for lipogenesis and preferentially uses fatty acids as an energy source for differentiation and proliferation of adipocytes.

Previous studies have indicated an involvement of the adiponectin-AMPK pathway in the promotion of glucose uptake, not only in skeletal muscle (Kola et al. 2006) but also in WAT (Wu et al. 2003). Adiponectin promoted glucose uptake into primary rat adipocytes via activation of AMPK (Wu et al. 2003) through a pathway independent of IR and Akt activation. In parallel with published data, plasma adiponectin and p-AMPK α levels were augmented in the young CR rats under basal conditions in the present study. However, it is unclear whether or not the activation of AMPK α contributes to the increased mGlut4 levels in young CR rats because the classic IR-Akt/PKC ζ / λ pathway was also activated at baseline. Wu et al (2003) also reported that adiponectin enhances insulin-stimulated glucose uptake in adipocytes at a submaximal concentration. In tissue from young CR rats, glucose loading increased the p-IR level at lower serum insulin concentrations, without further activation of Akt or PKC ζ / λ , or increment of mGlut4. As already mentioned, the glucose metabolic index of WAT in CR rats is not increased in the post-prandial phase when blood glucose and insulin concentrations are greater, as compared with the pre-prandial phase (Wetter et al. 1999). Thus, it is unlikely that the increased plasma adiponectin by CR contributes to insulin-stimulated glucose uptake, and thus enhanced insulin sensitivity, in the adipose tissue in young rats. However, adipose tissue from old CR rats responded to the glucose load in a similar manner to old AL rat tissue in terms of IR, Akt, PKC ζ / λ and mGlut4 activity, although the glucose-stimulated insulin response was lower. These findings suggest that insulin resistance improved in the old AL tissue. Because adiponectin has been reported to reverse the inhibitory effect of tumor necrosis factor α on insulin-stimulated glucose uptake in adipocytes (Wu et al. 2003), the elevated adiponectin by CR in middle age could contribute to the improved or enhanced insulin sensitivity.

AMPK may have a role in lipid metabolism in adipose tissue under CR conditions. CR rodents go many hours without food because they consume their provided food within a few hours of receiving it. Respiratory quotient data indicate that CR rodents start using fat as an energy source soon after finishing the food provided (Duffy et al. 1989; McCarter and Palmer 1992). Lipolysis under fasting conditions is promoted through activation of a hormone-sensitive lipase (HSL) by catecholamine. Activation of AMPK α is known to inhibit lipolysis by phosphorylation and thus inactivation of HSL (Garton et al. 1989). As already discussed, CR appears to stimulate adipogenesis in WAT, at least in young rats; thus, it is reasonable to assume that CR rodents suppress lipolysis, even in the fasting (preprandial) phase, by activating AMPK α to limit excess loss of fat components. Our preliminary data demonstrate that the protein level of pHSL in the fat tissue of young CR rats did not differ from that in the young AL rats (see Supplementary Fig. 2). Thus, the level of pHSL was not in accord with that of pAMPK α . The counter-regulatory role of AMPK in CR remains to be elucidated.

The metabolic traits observed in young CR rats had disappeared in middle-aged CR rats, which responded to glucose administration in a manner similar to that of rats fed AL. When normalized for body weight, fat pad weight did not differ between the AL and CR groups in young rats; however, it was significantly reduced in middle-aged CR rats. These findings suggest that lipolysis is facilitated and/or lipogenesis is reduced by CR in middle-aged rats. An *in vitro* study of isolated adipocytes indicated that the lipogenic effect of CR was attenuated in old compared with young rats, particularly under insulin-stimulated conditions (Zhu et al. 2007). As discussed earlier, lipolysis could also be facilitated by CR during middle age given that AMPK α has a role in suppression of lipolysis. In the middle-aged CR group, the p-AMPK α level was as low as that in the AL group under basal conditions, reflecting an attenuation of the inhibitory effect promoted by AMPK α , particularly in the fasting phase.

The present study suggests differential effects of CR on glucose and/or lipid metabolism of WAT in young and middle-aged rats. Regardless of these differences, plasma adiponectin was increased by CR in both young and middle-aged rats. An increase in plasma adiponectin has been linked to improved glucose tolerance and insulin resistance of aged animals,

especially in skeletal muscle (Yamauchi et al. 2001). In our previous study, we showed that CR during middle age improved glucose tolerance without affecting the impaired insulin signaling in skeletal muscle of rats (Park et al. 2006), suggesting the presence of an insulin-independent mechanism for glucose metabolism. Activation of the adiponectin-AMPK α pathway in skeletal muscle might occur via an insulin-independent mechanism for improved glucose tolerance and whole body insulin resistance following CR. However, we and others have been unable to confirm the upregulation of p-AMPK α in skeletal muscle following CR (Gonzalez et al. 2004; To et al. 2007).

In summary, we suggest the presence of a CR-specific, insulin- and adiponectin-AMPK α -independent mechanism that regulates glucose and thus energy metabolism. A considerable fraction (40–75%) of glucose uptake is facilitated in an insulin-independent manner, termed glucose effectiveness (Lang 1992; Pacini et al. 2001). Therefore, elucidating the potential insulin-independent pathway that is activated by CR will provide a better insight into therapeutic approaches for diabetes.

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