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Down-regulation of hepatic AMP-activated protein kinase and up-regulation of CREB coactivator CRTC2 for gluconeogenesis under calorie-restricted conditions at a young age

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AMP-activated protein kinase (AMPK) is a key molecule that controls energy homeostasis at cellular and whole body levels. Calorie restriction (CR) may exhibit the anti-aging effect through modulation of AMPK activity. We investigated the hepatic AMPK pathways for gluconeogenesis (the transducer of regulated cyclic adenosine monophosphate response element-binding protein (CREB) 2; CRTC2) and cell growth (mammalian target of rapamycin, mTOR). Male F344 rats at 2.5 months (mo) and 18 mo of age were subjected to 4-mo-long 30% CR; control rats were fed *ad libitum* (AL) throughout the experiment. Rats were killed 15 min after saline or glucose injection to evaluate activation of signal molecules under transient hyperglycemic and subsequent hyperinsulinemic conditions. Western blot analyses demonstrated a modest reduction of threonine-172-phosphorylated (p)-AMPK*α* levels and an increment of nuclear CRTC2 in the young CR group as compared with the age-matched AL group. We also confirmed the increased binding of CRTC2 and CREB and up-regulation of gluconeogenic genes (PGC-1*α* and PEPCK) in the CR group. However, there was no CR-specific alteration in total or phosphorylated mTOR levels. The results suggest down-regulation of hepatic AMPK activity by CR for metabolic adaptation that promotes gluconeogenesis. The effect of CR on mTOR remains elusive.

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Key words: Calorie restriction, AMPK, CRCT2, CREB, Gluconeogenesis, mTOR

Introduction

AMP-activated protein kinase (AMPK) is a cellular energy sensor that responds immediately to a reduction of ATP[1]. AMPK also regulates whole body energy metabolism particularly through adipokines such as adiponectin, leptin, and ghrelin on a long-term basis[2]. AMPK suppresses hepatic fatty acid and cholesterol synthesis while activating fatty acid oxidation[3]. AMPK also represses hepatic gluconeogenesis. In the skeletal muscle, AMPK enhances glucose uptake through upregulation of membrane translocation of glucose transporter 4 and also augments fatty acid oxidation[3]. In the white adipose tissue, AMPK suppresses fatty acid synthesis and lipolysis[3]. These effects of AMPK are expected to totally reduce blood glucose, serum insulin and IGF-1 levels as does CR and thus chronic activation of AMPK is considered to be a pro-longevity strategy[4].

Current studies have indicated a possible involvement of AMPK signal pathways in the regulation of aging and lifespan in organisms. Activation of AMPK signaling extends lifespan in *C. elegans*[5,6]. AMPK suppresses the target of rapamycin (TOR) signaling[3]; abrogation of TOR signal-

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ing also increases lifespan in *C. elegans*[7]. Deletion of ribosomal protein S6 kinase 1 (S6K1), a component of the mammalian TOR (mTOR) signal pathway, is also reported to extend the lifespan in mice, though only in female mice[8]. Deletion of S6K1 induced gene expression patterns similar to those seen in CR or with pharmacological activation of AMPK in the liver and skeletal muscle[8]. Therefore, one might speculate that CR affects aging and lifespan partly through activation of AMPK and thus suppression of the mTOR pathway in multiple organs.

However, the effect of CR on AMPK has been controversial even in cases where a specified organ was studied. In the liver, we reported a decrease in the threonine-172-phosphorylated (p) AMPKa level (hence the active form) in CR rats[9]. However, other laboratories have reported similar levels between CR and control groups[10] or even up-regulation of the p-AMPK levels in CR rats[11].

The present study evaluated the effect of CR on downstream molecules of AMPK, a transducer of regulated CREB activity 2 (CRTC2; formerly TORC2) and mTOR in the liver to resolve the reported contradictions. CRTC2 is a transcriptional regulator that promotes translation of the PPAR gamma coactivator-1α (PGC-1α) gene in concert with the cAMP responsive element binding protein (CREB) for induction of gluconeogenic enzyme genes[12]. Insulin and AMPK phosphorylate CRTC2 and exclude it from the nucleus, leading to attenuation of gluconeogenesis. We focused on the liver in the present study because the liver is a critical energy converter that supplies energy to other organs in concert with the white adipose tissue. Studies suggesting the relation between up-regulation of AMPK and longevity or an anti-neoplastic effect of CR have confirmed the findings in the rodent liver[8,11,13]. The present results support the downregulation of hepatic AMPK activity by CR. We discuss the role of AMPK in the metabolic adaptation to long-term CR.

Materials and methods

Experimental animals and tissues

All experiments in the present study were performed in accordance with the provisions of the Ethics Review Committee for Animal Experimentation at Nagasaki University. The experimental animals and procedures were reported elsewhere[14,15]. Briefly, male Fischer 344 rats (F344/DuCrj), certified to be specific pathogen-free, were purchased from Charles River Laboratory Japan (Yokohama, Japan). Rats were housed separately in a barrier facility (temperature 21-24°C; 12 hour/12 hour-light/ dark cycle) at an animal facility at the Biomedical Research Center of Nagasaki University. Rats fed ad libitum (AL) until 2.5 months (young) and 18 months (middle-age) of age were randomly assigned to the control AL group or the calorie restricted (CR) group. The young- and middle-age-AL groups continued to receive food AL; the CR groups received 30% less food than the respective AL groups by a modified alternate-day feeding program. The CR groups received two lots of 30% less food every other day, 30 min before lights were turned off. Food intakes in the AL groups were monitored every 2 weeks and the amounts of food given to the CR groups were adjusted accordingly.

At 7 and 22 months of age, when rats were subjected to glucose tolerance tests, the CR groups had 24% and 28% lower body weights as compared to the age-matched AL groups[14]. The results indicating that CR improved glucose tolerance at both ages were reported elsewhere[14]. With an interval of 2 or more weeks after GTT, rats were fasted overnight and sacrificed 15 min after an intraperitoneal 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- and/ or glucose-related signal molecules. Liver tissue was collected immediately, weighed, quickly diced, and frozen in liquid nitrogen.

The blood glucose and serum insulin concentrations in this experimental setting were reported elsewhere[14]. The data indicated that glucose-stimulated serum insulin response was lower in the CR groups as compared to the AL groups. However, the phosphorylated Akt1 level, an index of activation of insulin signaling, was greater in the glucose-injected CR group than in the glucose injected AL group at 7 mo, suggesting sensitization of insulin signaling in the liver of CR group (Supplemental data).

Tissue preparation for Western blotting

For analysis of p-AMPKα (Thr-172) and p-mTOR, the cytosolic fractions were isolated from frozen liver and skeletal muscle tissues according to the polyethylene glycol (PEG) precipitation method[16]. Briefly, frozen tissues (200–250 mg) were homogenized in a homogenization buffer consisting of 50 mM Tris–HCl (pH 7.7 at 4°C), 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.25 M mannitol, 1 mM dithiothreitol (DTT) and the following protein inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 4μM soybean trypsin inhibitor and 1 mM benzamidine. After homogenization for 40 s at 4°C, the mixture was centrifuged at 14,000g for 20 min at 4°C. The resulting supernatant was

mixed with a final concentration of 2.5% PEG, vortexed for 10 min and centrifuged at 10,000g for 10 min at 4°C. The resulting supernatant was resuspended in a buffer consisting of 100 mM Tris–HCl (pH 7.5 at 4° C), 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1 mM DTT, 0.02% sodium azide and the following protein inhibitors: 1 mM PMSF, 4μM soybean trypsin inhibitor and 1 mM benzamidine.

For analysis of CREB, 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 mg/mL aprotinin, 2 mg/mL leupeptin, 1% Triton X-100]. Homogenates were centrifuged (10,000g, 20 min, 4°C) and the supernatant was collected. The protein content of the lysates was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL).

Preparation of hepatic nuclear fraction

Livers (0.2 g) were homogenized (buffer) for 30 sec in 1.2 mL of 10 mM HEPES-NaOH buffer (pH 7.8) containing 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 2 μg of leupeptin per mL, 1 μg of pepstatin per mL, 5 μg of aprotinin per mL, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4. Incubated on ice for 20 min, then homogenized with 150 μL of homogenized buffer with 10% NP-40 for 15 sec. The homogenate was carefully layered onto 5 mL of the same buffer in a centrifuge tube, followed by centrifugation at 12,000 rpm and 4°C for 2 min. The precipitate was washed twice with homogenized buffer with 1% NP-40 followed by centrifugation at 12,000 rpm and 4°C for 30 sec. The precipitate was resuspended in 100 μL (50 mM HEPES-NaOH buffer [pH 7.8] containing 50 mM KCl, 3 mM NaCl, 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, 2 μg of leupeptin per mL, 1 μg of pepstatin per mL, 5 μg of aprotinin per mL, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4). The homogenate was incubated at 4°C for 30 min with gentle shaking each 5 min. The supernatant of the centrifugation at 12,000 rpm for 10 min was stored at –80°C. The protein content of the lysates was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, Rockford, IL).

Western blotting

The following antibodies were used in this study; anti-p (Thr172)-AMPKα anti-CREB, anti-mTOR, and anti-p (Ser 2448)-mTOR (Cell Signaling Technology, Beverly, USA), anti-CRCT2 (Santa Cruz Biotechnology, California, USA), and anti-pan AMPK (Upstate Biotechnology Inc., Lake Placid, NY); Enhanced chemiluminescence (ECL) Western blotting detection reagents and ECL-anti-rabbit or mouse IgG, horseradish peroxidase-linked species-specific antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). All the dilutions were performed according to the manufacturer's protocol. All other chemicals were obtained from Sigma Chemical Co. (Missouri, USA). All samples were boiled for 3 min and chilled on ice. Samples were diluted in protein sample buffer, and 40 μg of protein from the liver was loaded onto 7.5% SDS–polyacrylamide mini-gels. Samples were resolved by electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were immediately placed in blocking solution (containing 0.1% Tween, 5% BSA in TBS-T buffer) for 30 min and incubated with the primary antibody (2.5% BSA in TBS-T buffer) 4°C overnight. The membranes were washed and followed by the secondary antibody recommended by the manufacturer for 1 h at room temperature. Antibody labeling was detected using an ECL kit in accordance with the manufacturer's instructions. Specific signals were quantified by Fluorchem (DE500-5T, Alpha Innotech Corporation, San Leandro, CA) with associated image analysis software (AlphaEase FC, Alpha Innotech Corporation). To minimize the variance in signal intensity between the blots, a standard sample prepared from a mix of all tissues was included in each blot. Immunoblotting for β-actin was also performed to confirm the quality and quantity of loaded samples (data not shown).

Immunoprecipitation (IP)

Nuclear extracts (4 mg) were diluted in 1 mL IP buffer (40 mM Tris-HCl – pH 7.6, 5mM EDTA, 120 mM NaCl, 0.1% NP-40, 1 mM PMSF, 2 μg/mL leupeptin, 1 μg/mL aprotinin, 20 mM NaF, 2 mM Na_3VO_4), centrifuged at 15,000 rpm at 4°C for 30 min and transferred to a new tube. One milligram of anti-CREB was incubated overnight at 4°C with rotation followed by the addition of protein G-agarose beads (20 μl of 50% slurry) (Upstate, California, USA) and rotating at 4°C overnight. The immunoprecipitates were washed three times sequentially with IP buffer and a Western blot for CRCT2 was performed.[17]

RT-PCR

Total RNA was extracted as previously described[18]. The

quality of extracted RNA was evaluated as the densitometric ratio of 28S and 16S ribosomal RNA. The extracted RNA was reverse-transcribed using a Bio-Rad reverse transcriptase reagent kit (Hercules, USA) according to the manufacturer's protocol, and real-time PCR was performed as previously described[19] using primers and probes for PEPCK, PGC-1 and β-actin (Applied Biosystems, Tokyo, Japan). The sequences of the primers and TaqMan probes were as follows: PEPCK: sense, 50-TCCGGGCACCTCAGTGAA-30; antisense, 50-ACGTTGGTGAAGATGGTGTTTTT-30; TaqMan probe, 50-FAM-CGCCATTAAGACCATC-TA MRA-30; β-actin: sense, 50-ACTGCCCTGGCTCCTAGCA-30; antisense, 50-GAGCCACCAATCCACACAGA-30; TaqMan probe,50-FAM-ATCAAGATCATTGCTCCTCCTGAGCGC-TAMRA-30. The results were normalized for β-actin expression levels in all samples; the expression levels of β-actin were confirmed to be equivalent among the groups examined (data not shown). All samples and standard curves were tested in triplicate.

Statistical analysis

Data are expressed as means ± standard deviation (SD). Data were analyzed by three-factor ANOVA with the main effects of age, diet, and glucose injection. Data were also analyzed by one-factor ANOVA with a post hoc test (Fisher's protected LSD test) for multiple comparisons). The level of significance was set at $p < 0.05$.

Results

AMPK levels

The protein abundance of AMPK did not differ between the age groups or between the diet groups (Figure 1A). The levels in the glucose-injected groups were also not different from those in the saline groups. The Thr172-phosphorylated (p-) AMPK levels were lower in the middle-aged groups than in the young groups, particularly in the AL groups (Age, $p = 0.0051$; Age x Diet, $p = 0.0870$; Figure 1B). The p-AMPK levels did not differ between the glucose- and saline-injected groups. When the data were decomposed by age, the p-AMPK levels were lower in the CR groups at the young age (Diet, $p = 0.002$) and there was no difference at the middle age.

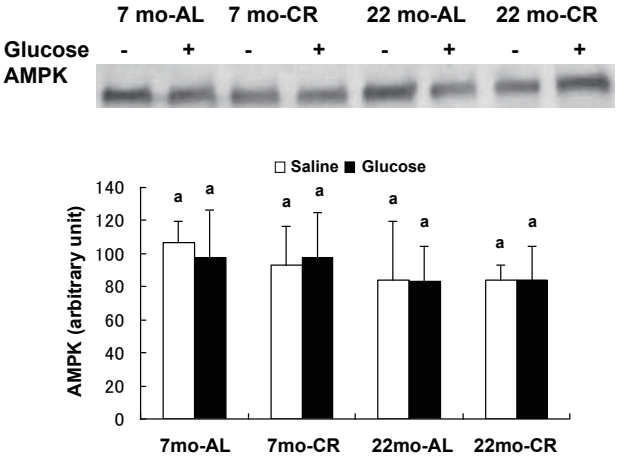

Figure 1A

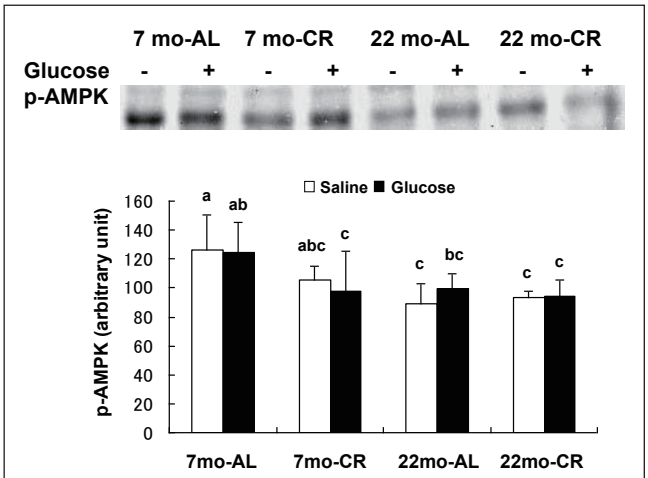

Figure 1B

Figure 1. Effects of calorie restriction (CR) on the level of abundance of AMPKα protein (A) and threonine-172-phosphorylated (p)-AMPKα (B) in the liver of 7- and 22-month-old (mo) rats. Representative immunoblots are shown. Data are means ± SD (n = 5 in each group, except n = 3 for the 22 mo CR group). Within a panel, different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

CRTC2 and CREB levels and their interaction for gluconeogenesis

The CRTC 2 protein abundance in the hepatic nuclear fraction was significantly greater in the CR groups (Diet, $p = 0.0007$; Figure 2), particularly at the young age (Diet ×Age, $p = 0.0730$; Age = 0.0752; see also the results for multiple comparisons). The levels of CRTC2 in the glucose groups did not differ from those in the saline groups.

The interaction of CRTC2 and CREB was confirmed by coimmunoprecipitation and immunoblotting using samples from young saline-injected animals (Figure 3). To confirm

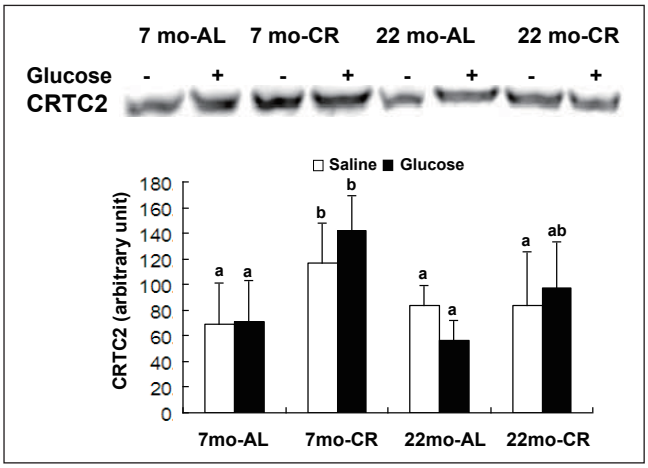

Figure 2. Effects of calorie restriction (CR) on the level of abundance of CRTC2 protein in the hepatic nuclear fractions of 7- and 22-month-old (mo) rats. Representative immunoblots are shown. Data are means ± SD (n = 5 in each group, except n = 3 for the 22 mo CR group). Within a panel, different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

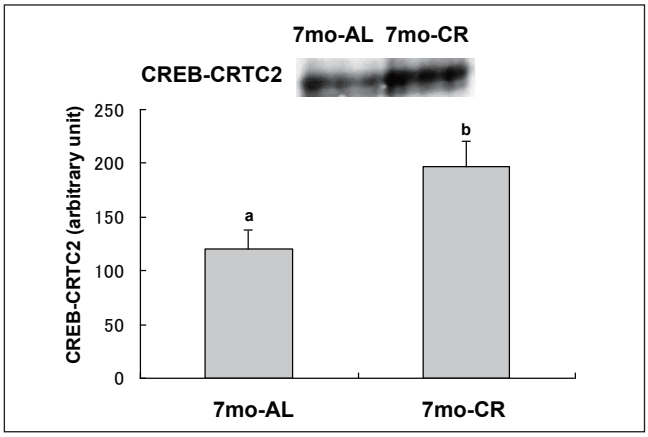

Figure 3. Effects of calorie restriction (CR) on binding of CREB and CRTC2 in the hepatic nuclear fraction of 7-month-old (mo) rats. Representative coimmunoprecipitations are shown. Data are means ± SD (n = 5 in each group). Different letters indicate statistical differences at $p < 0.01$, as determined by one-factor ANOVA with a post hoc test.

the role for the transcriptional complex in gluconeogenesis, we measured the expression levels of PGC-1α and PEPCK mRNA. In parallel with the findings of CRTC2 and CREB, the expression levels of PGC-1α and PEPCK mRNA were marginally or significantly greater in the CR groups than in the AL groups (Diet, $p = 0.087$ and $p = 0.010$ respectively; Figure 4A, B).

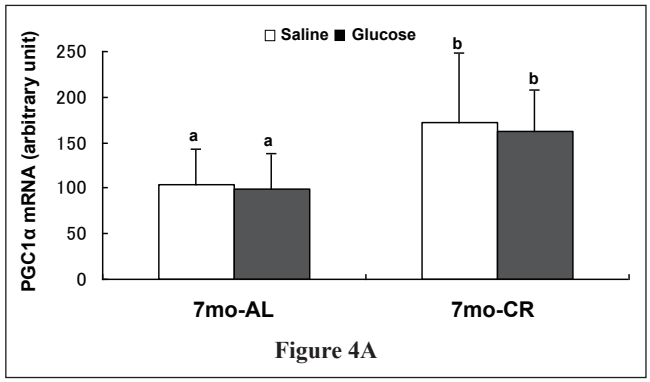

Figure 4A

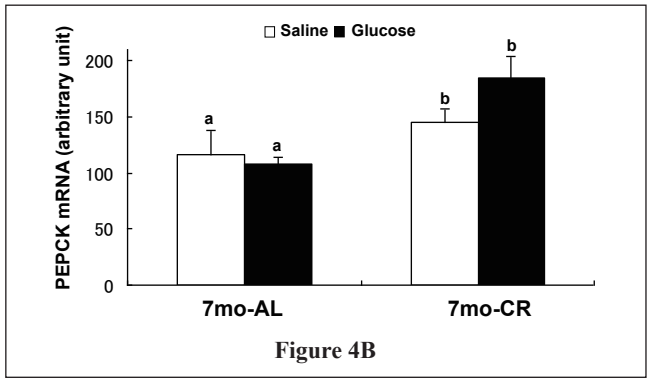

Figure 4B

Figure 4. Effects of calorie restriction (CR) on mRNA expression levels of PGC-1α (A) and PEPCK (B) in the liver of 7-month-old (mo) rats. Data are means ± SD (n = 5 in each group). Within a panel, different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

mTOR levels

The protein abundance of mTOR did not differ between the groups by age, diet, and glucose or saline-injection (Figure 5A). The p-mTOR levels were greater in the glucose-injected groups than in the saline groups (Glucose, $p < 0.0001$; Figure 5B). There was no significant difference in p-mTOR levels between the age groups or between the diet groups.

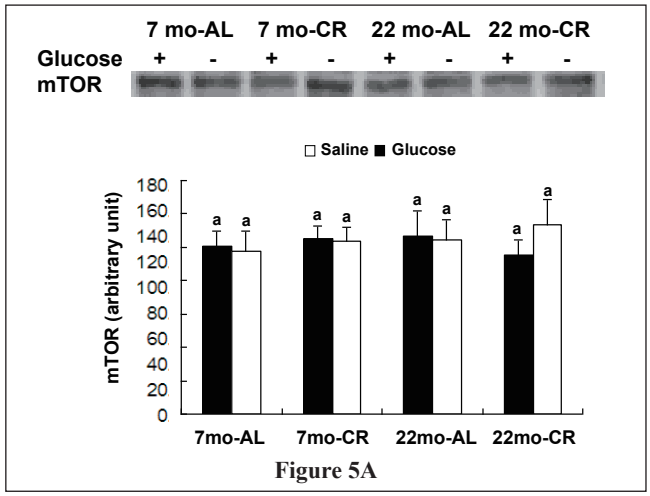

Figure 5A

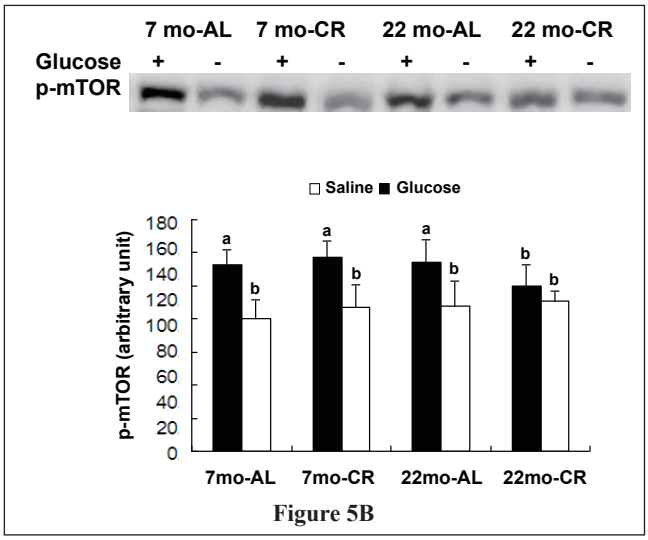

Figure 5. Effects of calorie restriction (CR) on the level of abundance of mTOR (A) and phosphorylated (p)-mTOR (B) in the liver of 7- and 22-month-old rats. Representative immunoblots are shown. Data are means ± SD (n = 5 in each group, except for n = 3 for the 22 mo CR group). Within a panel, different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

Discussion

The present study demonstrated that CR initiated at the young age in rats modestly reduced the hepatic p-AMPK level thus suggesting the decreased activity of AMPK by CR. CR initiated at the middle age did not further decrease the p-AMPK level, although the AL group showed an aging-related reduction of p-AMPK. Concomitantly, the nuclear CRTC2 level was greater in the CR group, particularly at the young age. The present study also confirmed the binding between CRTC2 and CREB and the up-regulation of gluconeogenic genes. Because AMPK represses hepatic gluconeogenesis partly by excluding CRTC2 from the nucleus by phosphorylation[12], the present data suggest that CR induces a physiological milieu where the gluconeogenic program is immediately induced in response to fasting and thus reduced blood glucose. From an evolutional viewpoint, the enhanced gluconeogenic response is also likely to cope with famine.

The present study showed that CR did not affect the total or p-mTOR level, even though the hepatic p-AMPK level was reduced. The p-mTOR level acutely responded to glucose load and thus activation of insulin signaling as indicated by an increment of pAkt1 levels in both AL and CR groups (Supplemental data). Thus, at least in the liver, CR did not seem to regulate mTOR on a long-term basis. mTOR forms two complexes in cells, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1, which is composed of mTOR, Raptor, and mLST8 proteins, regulates cell growth and energy metabolism[20]. AMPK negatively regulates mTORC1. Thus, further analyses will be necessary for understanding an implication of the mTORC1 pathway on the effect of CR, because recent studies have indicated the importance of mTOR signaling in the regulation of lifespan as well as tumorigenesis[8,11,21,22].

Long-term activation of hepatic AMPK by overexpression of a constitutively active form of AMPK-α1 represses Srebp-1c and its target-genes, lipogenic genes including fatty acid synthesis, which in turn decreases fat storage in the white adipose tissue[23]. By contrast, deletion of hepatic LKB1 and thus attenuated AMPK activity upregulates lipogenic genes more significantly under feeding conditions[24]. Thus, hepatic AMPK is also considered as a repressor for lipogenesis. Our previous study has demonstrated that one of the lipogenic genes, acetyl-CoA carboxylase 1 mRNA, was greatly upregulated in the fed phase of CR as compared to the AL phase[9]. Therefore, the down-regulated hepatic AMPK by CR could also contribute to rapid induction of lipogenic enzyme genes in response to energy influx.

Our previous study in the white adipose tissue in the same experimental setting demonstrated that CR promotes lipogenesis even in the fasted condition when lipolysis is activated15. In this condition, the p-AMPK level was consistently up-regulated in the CR group, although the level was only increased after glucose load and thus activation of insulin signaling in the AL group. AMPK is reported to repress lipolysis by a negative feedback loop mechanism when lipolysis is activated[25]. AMPK inhibits hormone-sensitive lipase (HSL) by phosphorylation[26]. Indeed, our preliminary data suggest a modest reduction of the p-HSL level at the young age although statistically insignificant[15] (supplemental data). Thus, under CR conditions, AMPK seems to act as a repressor for lipolysis to limit excess loss of fat storage in the white adipose tissue.

In summary, our studies suggest that CR sensitizes hepatic gluconeogenic and lipogenic responses in the fasted-and-fed cycle of food availability through consistent down-regulation of AMPK. By contrast, in the white adipose tissue, AMPK seems to act as a repressor for excess energy loss under CR conditions. Thus, our data implies that AMPK coordinates energy flux between the liver and adipose tissues under CR conditions in order to ensure animal survival.

Acknowledgments

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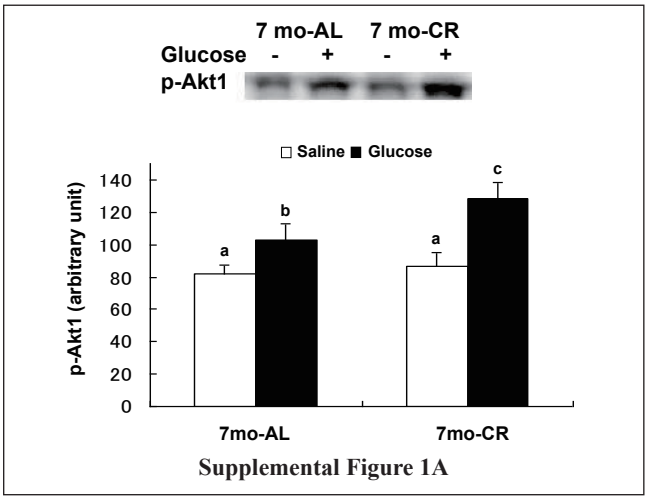
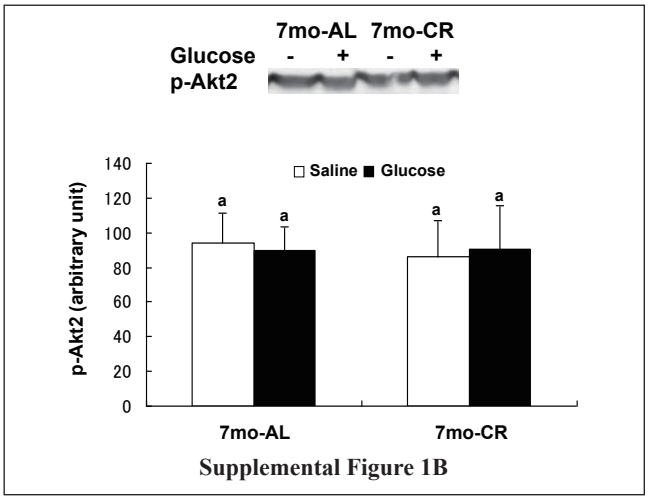
Table 1. Glucose-stimulated serum insulin response

	7 mo AL	7 mo CR	22 mo AL	22 mo CR
Body weight (g)	340.4 ± 19.3[b]	258.4 ± 7.4[d]	408.3 ± 20.1[a]	292.3 ± 18.5[c]
Glucose (mg/dL)				
Saline-injected group	99.1 ± 8.5[c]	96.0 ± 9.2[c]	100.0 ± 8.9[c]	103.1 ± 5.9[c]
Glucose-injected group	183.5 ± 41.4[b]	173.4 ± 21.0[b]	226.8 ± 29.7[a]	196.4 ± 49.5[b]
Insulin (ng/mL)				
Saline-injected group	4.94 ± 0.89[b]	2.79 ± 0.75[c]	2.03 ± 0.47[c]	1.24 ± 0.10[c]
Glucose-injected group	12.50 ± 3.21[a]	8.58 ± 4.49[a]	8.72 ± 4.65[a]	4.24 ± 3.10[b]

Blood glucose concentrations and serum insulin concentrations were measured at 15 min after saline or glucose injection. Within a panel, different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test. The data are previously published elsewhere[14].



Supplemental Figure 1A



Supplemental Figure 1B

Supplemental Figure 1. Effects of calorie restriction (CR) on the level of abundance of p-AKT1 (A), p-AKT2 (B), in the liver of 7-month-old (mo) rats.. Representative immunoblots are shown. Data are means + SD (n = 5 in each group). Within a panel, bars with common letters above them do not differ significantly, $P > 0.05$. as determined by one-factor ANOVA with a post hoc test. The antibodies to p-AKT1 and p-AKT2 were purchased from Cell Signaling Technology (Beverlym MA). Western Blots were performed following the procedure described in the Material and Methods Section.