

Divergent regulation of adipose tissue metabolism by calorie restriction and inhibition of growth hormone signaling

Seongjoon Park^a, Toshimitsu Komatsu^a, Hiroko Hayashi^a, Lucas Siqueira Trindade^a, Haruyoshi Yamaza^a, Takuya Chiba^a, Isao Shimokawa^{a*}

^a Department of Investigative Pathology, Unit of Basic Medical Science, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki City 852-8523, Japan

* Corresponding author:

Department of Investigative Pathology, Unit of Basic Medical Science, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki City 852-8523, Japan

Tel: +81-95-819-7051; Fax: +81-95-819-7052

E-mail address: shimo@nagasaki-u.ac.jp

Abstract

Calorie restriction (CR) and a reduced growth hormone (GH) signal affect insulin sensitivity and lifespan in mammals in a similar manner. We investigated the effects of CR and moderate inhibition of GH on glucose-stimulated activation of insulin signaling and the expression of genes related to fat metabolism in white adipose tissue (WAT) in rats. We used 10-month-old male, wild-type (W) Wistar rats, fed ad libitum (AL) or a 30% CR diet from 6 weeks of age, and transgenic (Tg) rats with moderately suppressed GH signaling. Rats were killed 15 min after an intraperitoneal injection of glucose or saline. In control W-AL rats, the levels of serum insulin, phosphorylated (p) insulin receptor (pY-IR), p-Akt, and the expression of glucose transporter (Glut) 4 in the membrane fraction were greater in the glucose-injected group than in the saline-injected group, indicating significant activation of insulin signaling in response to glucose loading. In the W-CR and Tg-AL rats, the serum insulin and pY-IR levels were lower than those in the W-AL rats. The Akt-Glut pathway was up-regulated even after saline-injection. Expression levels of adipogenic and lipogenic genes including PPAR γ , adiponectin, and its receptors, were higher in the W-CR rats than in the W-AL and Tg-AL rats. The present findings indicate adipose tissue metabolic profiles specific to CR.

Keywords: Calorie restriction, Growth hormone, Glucose uptake, Insulin signal, Adipose tissue

Introduction

Calorie restriction (CR) and reduced growth hormone (GH)-IGF-1 signaling extend the lifespan and delay the onset of age-related diseases in rodents (Bartke et al. 2002; Masoro, 2003; Katic and Kahn, 2005). Our earlier study demonstrated that the lifespan of transgenic (Tg) rats, in which the GH-IGF-1 axis was moderately suppressed by over-expression of an antisense GH gene, was 10% longer than that of wild (W)-type rats (Shimokawa et al. 2002). Tg rats, even those fed ad libitum (AL), ~~shared some phenotypes with~~ showed findings similar to those in CR rats, including food intake and body weight, and plasma parameters associated with insulin sensitivity, particularly the adipokines, adiponectin, leptin and resistin (Yamaza et al. 2007; Chiba et al. 2008). CR is known to suppress the GH-IGF-1 axis (Breeze et al., 1991; Shimokawa et al., 2003). Thus, the GH-IGF-1 pathway could, at least in part, mediate the effect of CR.

Our previous study demonstrated that W-CR and Tg-AL rats had normal or slightly improved glucose tolerance without a significant surge in the serum insulin concentration after a glucose load (Yamaza et al., 2004). In a similar experimental setting, we also showed that the activation of insulin signaling in response to glucose was minimized in the liver, while similar findings were observed in the skeletal muscle in W-CR and Tg-AL rats, in comparison with the findings in W-AL rats (Hayashi et al., 2008), suggesting a role for the GH-IGF-1 axis in the effect of CR. Although skeletal muscles are known to contribute greatly to whole-body glucose clearance, adipose tissue also play a pivotal role in glucose tolerance and insulin resistance by affecting insulin signaling and

energy metabolism in other tissues partly through the effects of adipokines. One such adipokine is adiponectin, which is reported to not only enhance insulin-stimulated glucose uptake in tissues, but can also act as an insulin-independent signal (Yamauchi et al. 2002; Wu et al. 2003). In our studies, the plasma adiponectin concentration was increased in CR and Tg rats (Yamaza et al. 2007), whereas the leptin and resistin concentrations were decreased (Chiba et al. 2008). Because overexpression of adiponectin is reported to extend the lifespan in mice (Otabe et al. 2007), CR and reduced GH signaling might increase the lifespan by modulating energy metabolism and subsequently the secretion of adipokines from WAT.

CR rodents exhibit a metabolic shift between the fed and fasted phases in a feeding cycle (Duffy et al. 1989; McCarter and Palmer, 1992). In the post-prandial phase, animals predominantly use carbohydrates as the energy source, while fatty acids and ketone bodies are also used in the pre-prandial phase. The metabolic shift predicts that CR rodents efficiently activate adipogenic or lipogenic pathways in the postprandial phase to supply fatty acids to other organs in the pre-prandial phase. Indeed, a previous study has demonstrated some unique effects of CR in epididymal adipose tissue (Park et al. 2008). These include increased baseline levels of p-Akt and p-PKC ζ/λ , and Glut4 in the membrane fraction, suggesting that CR enhances glucose uptake in WAT via an insulin-independent manner.

In the present study, we compared the effects of CR and modestly inhibited GH signaling on glucose-stimulated activation of insulin signaling molecules involved in glucose uptake and the expression of genes associated with adipogenesis and lipogenesis in

WAT to evaluate the role of GH signaling in mediating the effects of CR.

Materials and Methods

Experimental animals

Tg rats (Jcl: Wistar-TgN[ARGHGEN]1Nts) were kindly provided by the Nippon Institute for Biological Science (Oume City, Tokyo, Japan) and non-transgenic wild-type (W) Wistar rats were purchased from Japan Clea Inc (Tokyo, Japan). The Tg and W rats were maintained at the Center for Frontier Life Sciences, Nagasaki University, as previously described (Shimokawa et al. 2002). The transgene consists of four copies of the thyroid hormone responsive element, the rat GH promoter, and an antisense complementary DNA (cDNA) sequence for rat GH (Matsumoto et al. 1993). Male heterozygous rats were used in this study because these Tg rats have phenotypes similar to those of non-transgenic W rats subjected to 30% CR (Shimokawa et al. 2003).

The W-CR rats received 30% less food than the W-AL group by a modified alternate-day feeding program. The CR group received two allotments of food, i.e., 140% of the mean daily food intake in the AL group, every other day 30 min before the lights were turned off. The CR regimen was initiated at 6 weeks of age. The food intake in the AL group was monitored every 2 weeks and the allotment for the CR group was adjusted accordingly.

Overnight-fasted rats were sacrificed by decapitation 15 min after an intraperitoneal (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. Perirenal adipose tissue was immediately collected, weighed, quickly diced, and frozen in liquid nitrogen. At the same time, trunk blood was collected and divided into two aliquots for preparation of serum and plasma samples. Fifty- μ l aliquots of serum or plasma samples were kept at -80°C until assayed for insulin and glucose levels. Serum insulin concentrations were measured using enzyme-linked immunosorbent assay (ELISA) kits specific for rat insulin (Amersham Laboratories Inc., Webster, TX). Serum glucose concentrations were measured using kits based on the glucose oxidase method (Wako Pure Chemical Industries Ltd., Osaka, Japan). All samples were stored at -80°C .

The present study was conducted in accordance with the provisions of the Ethics Review Committee for Animal experimentation at Nagasaki University.

Tissue preparation for 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 (10000 g, 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).

ELISA for p-IR and p-Akt

The protein abundance of p-IR and p-Akt in the cell lysates was determined using

an insulin receptor [pYpY1162/1163] ELISA kit and an Akt [pS473] ELISA kit (both from BioSource International, Camarillo, CA), respectively. All samples were tested in duplicate. The optical density (OD) of each sample was read at 450 nm using a Labsystems Multiskan (Dainihonseiyaku, Tokyo, Japan). In preliminary experiments, we performed Western blotting of IR and Akt proteins and confirmed that the levels of IR and Akt were similar between the rat groups. p-IR and p-Akt levels were also analyzed by Western blotting and the results showed trends similar to those obtained by ELISA, as indicated in the supplemental data published elsewhere (Hayashi H et al., 2008).

Western blotting

Antibodies to Glut4 and p-AMPK (Thr172) were obtained from Cell Signaling Technology (Beverly, USA). Anti-Glut1 was obtained from Santa Cruz Biotechnology (California, USA). 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 other chemicals were obtained from Sigma Chemical Co. (Missouri, USA).

All samples were boiled for 3 min and chilled on ice. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The 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 secondary antibody for 1 h 30 min. All incubations were at room temperature. Antibody labeling was detected using an ECL kit in accordance with the manufacturers instructions. Specific signals were

quantified by Fluorchem (DE500-5T, Alphainnotech Corporation, San Leandro, CA) with associated image analysis software (AlphaEase FC, Alphainnotech Corporation). To minimize the variance in signal intensity between the blots, a standard sample prepared from tissue from a 7-month-old rat was included in each blot. Immunoblotting for β -actin was also performed to confirm the quality and quantity of loaded samples.

Subcellular fractionation

To investigate the effect of CR on the subcellular distribution of Glut4 and Glut1, 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 μ g/ml aprotinin] at 4 °C with a polytron homogenizer. Homogenates were centrifuged (1000 g, 25 min, 4 °C) and the supernatant was removed and centrifuged (100000 g, 60 min, 4 °C) to obtain the cytosolic fraction. This pellet was resuspended in buffer A, which was supplemented with 1% NP-40, and kept on ice for 20 min before a final centrifugation (100000 g, 20 min, 4 °C) to obtain the membrane fraction. The protein concentration was determined using the BCA protein assay kit, and the samples were used immediately to determine Glut4 by Western blotting.

Real-time PCR

Total RNA was extracted as previously described (Chiba et al. 2008). 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 manufacturers protocol, and real-time PCR was performed as previously described (Yamaza et al. 2007) using primers and probes for acetyl-CoA carboxylase (ACC) 1, and β -actin (Applied Biosystems, Tokyo, Japan). The sequences of the primers and TaqMan probes were as follows: ACC1: sense, 5'-ATTTTCGTCAGGATCTTTGATGAAGTAA-3'; antisense, 5'-ATAGAGTGAAGTGTGACCGGACTCT-3'; TaqMan probe, 5'-FAM-CTGCTTCTGCGACTCCCCACCC-TAMRA-3'; β -actin: sense, 5'-ACTGCCCTGGCTCCTAGCA-3'; antisense, 5'-GAGCCACCAATCCACACAGA-3'; TaqMan probe, 5'-FAM-ATCAAGATCATTGCTCCTCC TGAGCGC-TAMRA-3'.

For real-time PCR of Glut1, Glut4, PPAR γ , fatty acid synthase (FAS), adiponectin, and adiponectin R1 and R2, mixed TaqMan[®] MGB probes and primers were used (Glut1, Rn01417099_m1; Glut4, Rn00562597_m1; PPAR γ , Rn00440940_m1; FAS, Rn00569117_m1; Adiponectin, Rn00595250_m1; Adiponectin R1, Rn01114954_g1; Adiponectin R2, Rn01463177_a1; PPAR γ , Rn00440940_m1 Applied Biosystems, Tokyo, Japan). 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 (supplemental data). All samples and standard curves were tested in triplicate.

Statistical analysis

Data are expressed as means \pm SEM for four or five rats and were analyzed by

one-factor (1f) ANOVA, followed by Fischers protected least significant difference test as a post hoc test. The level of significance was set at $p < 0.05$.

Results

General data

The general data, including the body weight and fat pad weight in the W-CR and Tg-AL rats, are published elsewhere (Hayashi et al. 2008; Chiba et al., 2008) and are summarized in Table 1. The body weight was 32% and 35% lower in W-CR and Tg-AL rats respectively than in W-AL rats. The Tg-AL rats consumed 30% less food than W-AL rats; thus, the food intake in the Tg-AL rats was similar to that provided for the W-CR rats (data not shown; refer to Shimokawa et al. 2003). The peri-renal fat tissue weight normalized for body weight was 36% lower in the W-CR and 29% lower in the Tg-AL rats than in the W-AL rats.

The pituitary GH-mRNA expression level, normalized for β -actin mRNA, was 30% and 80% lower in W-CR and Tg-AL rats respectively than in the W-AL rats (Chiba et al. 2008). The level was significantly lower in Tg-AL rats than in W-CR rats. The plasma concentration of IGF-1 was approximately 25% lower in W-CR and Tg-AL rats than in the W-AL rats; there was no difference between W-CR and Tg-AL rats.

The blood glucose concentration in the glucose-injected group was greater than that in the saline-injected group in all three groups of rats (Hayashi et al. 2008). Although the blood glucose level in the saline-injected group did not differ significantly between the

groups of rats, the blood glucose levels in the glucose-injected group were 16% and 29% lower in the W-CR and Tg-AL rats than in the W-AL rats. There was no significant difference between the W-CR and Tg-AL rats. The serum insulin concentration in the glucose-injected group was also greater than that in the saline-injected group, particularly in W-AL rats. The insulin levels in the glucose-injected group were significantly lower in the W-CR and Tg-AL rats than in the W-AL rats. There was no difference between W-CR and Tg-AL rats.

Activation state of insulin signaling molecules

The pY-IR level in WAT in the saline-injected group did not differ between the groups of rats (Fig. 1). The levels of pY-IR in the glucose-injected group in W-AL and W-CR rats were significantly higher than in the respective groups of saline-injected rats, although the level was lower in the W-CR rats than in the W-AL rats. In Tg-AL rats, the pY-IR level did not differ between the saline- and the glucose-injected groups.

The levels of p-Akt (Ser473) in the saline-injected group were 1.8-fold higher in the W-CR and Tg-AL rats than in the W-AL rats (Fig. 2). In the W-AL rats, the p-Akt level in the glucose-injected group was higher than that in the saline-injected group. In the W-CR and Tg-AL rats, the p-Akt levels in the glucose-injected groups did not differ from those in the saline-injected groups. As a result, the p-Akt levels in the glucose-injected group did not differ significantly among the W-AL, W-CR and Tg-AL rats.

The expression of Glut4 in the membrane fraction in the saline-injected group did

not differ significantly between the W-CR and W-AL rats (Fig. 3A), but it was significantly greater in the W-CR rats than in the Tg-AL rats. In the W-AL rats, the expression of Glut4 in the membrane fraction in the glucose-injected group was greater than that in the saline-injected group. In the W-CR and Tg-AL rats, the expression of Glut4 in the membrane fraction in the glucose-injected group did not differ significantly from that in the saline-injected group.

The expression of Glut1 in the membrane fraction in the saline-injected group in Tg-AL rats was greater, although only marginally significant ($p = 0.0521$), than that in the W-AL rats and in W-CR rats (Fig 3B). By contrast, the expression of Glut1 in the membrane fraction in the glucose-injected group did not differ from that in the saline-injected group in each group of rats and did not differ between the groups of rats injected with glucose.

Because the membrane fraction of Glut4 or Glut1 might not represent the net amount of each Glut protein in the membrane, the mRNA level was examined as a reference. The Glut4 mRNA level in the saline-injected group was significantly higher in the W-CR rats than in the W-AL rats and Tg-AL rats (Fig. 3C); there was no difference between the W-AL and Tg-AL rats. The Glut4 mRNA level in the glucose-injected group did not differ from that in the saline-injected group in each group of rats. The Glut1 mRNA level was slightly higher in the W-CR and Tg-AL rats than in the W-AL rats, although this was not statistically significant (Fig. 3D).

Adiponectin-AMPK pathway as an insulin-independent mechanism

In our previous study, the plasma adiponectin level was reported to be increased in W-CR and Tg-AL rats compared that in W-AL rats (Yamaza et al. 2008). In the present study, the adiponectin mRNA level in WAT was significantly higher in the W-CR rats than in the W-AL and Tg-AL rats (Fig. 4A); there was no difference between the W-AL and Tg-AL rats. The level in the saline-injected group did not differ from that in the glucose-injected group.

The mRNA levels of adiponectin R1 and R2 did not differ between the saline- and the glucose-injected groups in each group of rats (Fig. 4B and 4C). The level of R1 was higher in the W-CR rats than in the W-AL or Tg-AL rats in both the saline- and the glucose-injected groups. There was no difference between W-AL and Tg-AL rats. The mRNA level of R2 showed similar results.

The level of p-AMPK (Thr172) in WAT in the saline group did not differ from that in the glucose-injected group in any of the groups of rats (Fig. 4D). The level of p-AMPK (Thr172) in the saline-injected group in W-CR rats was marginally higher ($p = 0.0521$) than that in W-AL, and was significantly higher than that in Tg-AL rats.

Gene expression levels of PPAR γ and lipogenic genes

There was no significant difference in the mRNA level of PPAR γ between the saline- and glucose-injected groups of rats (Fig 5A). The mRNA level of PPAR γ was 2-fold higher in the W-CR rats than in the W-AL and Tg-AL rats; there was no difference between

the W-AL and Tg-AL rats.

The mRNA level of ACC1 did not differ between the saline- and glucose-injected groups (Fig. 5B). The level was significantly greater in the W-CR rats than in the W-AL and Tg-AL rats. The level of ACC1 in the W-AL rats did not differ from that in the Tg-AL rats. The mRNA level of FAS did not differ between the saline- and glucose-injected groups (Fig 5C), but was significantly greater in the W-CR rats than in the W-AL and Tg-AL rats. There was no difference between the W-AL and Tg-AL rats.

Discussion

Low levels of blood glucose and insulin are common findings in most animal models of longevity (Katic and Kahn, 2005). In our previous study, we demonstrated that long-lived W-CR and Tg-AL rats exhibited normal or slightly improved glucose tolerance with lower serum insulin responses (Yamaza et al. 2004). Subsequent analyses have shown that CR modulates the activation of insulin signaling in response to glucose loading in a tissue-specific manner. CR minimized the glucose-stimulated serum insulin response and the activation of insulin signaling in the liver, while CR sensitized the insulin pathway in skeletal muscle (Hayashi et al. 2008). Tg rats also showed findings similar to those in the CR rat liver and skeletal muscle, suggesting that the GH axis plays an important role the effect of CR on energy metabolism and, thus, aging or lifespan (Hayashi et al. 2008).

The present study showed up-regulation of p-Akt and Glut expression in WAT of CR and Tg rats under unstimulated conditions, although the up-regulated subtype of Glut

differed between the two groups of rats. In W-CR rats, the expression of Glut4 in the membrane fraction was not significantly increased, but the mRNA level was 2-fold greater, suggesting an increment in the net amount of Glut4 or accelerated turnover of Glut4 in the membrane by CR. By contrast, in Tg-AL rats, the present findings indicate an increment in Glut1 expression. It should be noted that the mGlut4 level was increased in W-AL rats in response to glucose loading and, thus, insulin action. However, CR rats did not show a Glut4 response to glucose loading, suggesting an insulin-independent mechanism for the upregulation of Glut4 by CR. Our findings are consistent with a previous study demonstrating that glucose uptake is significantly increased in WAT and in the preprandial and postprandial phases in CR rats (Wetter et al. 1999).

In the present study, the peri-renal fat pad was used for the analysis. CR and GH suppression may affect physiological functions as well as adipogenesis or lipogenesis differently in different fat pads. Indeed, Li and colleagues (Li et al 2003) have reported that the responsiveness of lipid metabolism-related genes to fasting is more sensitive in visceral fat (including peri-renal and epididymal fat pads), and is almost unchanged in subcutaneous fat in rats. In male Wistar rats subjected to alternate-day feeding for CR, it was reported that the peri-renal fat and the epididymal fat pads, but not the inguinal fat pad, show similar age- and CR-related changes in fat cell size and number (Bailey et al., 1993). In our previous study using the epididymal fat pad in male F344 rats (Park et al. 2008), the p-Akt/PKC ζ / λ -Glut4 pathway was upregulated at baseline in CR rats. Another research group also reported augmented FAS-mRNA expression levels and the protein levels of adiponectin in the epididymal fat pad in CR rats (Zhu et al 2007). Thus, the present findings

showing activation of insulin signaling and gene expression in the peri-renal fat pad represent some of the unique effects of CR in WAT, although the responses to the suppression of GH in the other fat pads need to be elucidated.

Reduced plasma GH levels are reported to increase the protein levels of Glut1 but not Glut4 (Kilgour et al. 1995). The extent of the GH axis suppression was greater in the Tg rats than in the W-CR rats. The mGlut1 and mRNA levels in the W-CR and Tg-AL rats seem to be correlated with the extent of GH suppression. Although Tg-AL rats showed an increment in WAT Glut1 expression, glucose uptake may not be increased as much as in CR rats, because the glucose-transport capacity of Glut1 is 3-fold lower than that of Glut4 (Palfreyman et al. 1992). Thus, the augmented glucose uptake in WAT via up-regulation of Glut4 may characterize the metabolic effect of CR.

The mechanisms involved in insulin-independent upregulation of Glut4 and promotion of glucose uptake in WAT by CR should be investigated, because the insulin independent, Akt-dependent characteristics ~~is~~ seem to be inconsistent with the Daf2-Age-1-Daf16 pathway, an extensively studied signal for longevity in nematodes (Kenyon et al. 1993; Dorman et al. 1995). The present study focused on a potential role of the adiponectin-AMPK pathway, because in vitro studies indicate that adiponectin promotes glucose uptake via AMPK in cultured adipocytes in an insulin-independent manner (Wu et al. 2003; Yamaguchi et al. 2005). In accordance with the in vitro studies, the present in vivo study also indicates that the mRNA levels of adiponectin and its specific receptors (Adiponectin R1 and R2) in WAT were elevated in that W-CR rats but not in the Tg-AL rats. The p-AMPK level also seemed to be increased, but only in the W-CR rats.

Although our previous study reported an increment in the plasma adiponectin concentrations in Tg-AL rats (Yamaza et al. 2007), the present study did not confirm an increase in adiponectin gene expression in WAT. Ames dwarf mice have also been reported to show similar characteristics with increased plasma adiponectin levels without increase of adiponectin mRNA levels in WAT (Wang et al. 2006). Thus, CR and GH differentially upregulate plasma adiponectin levels. Recent studies have demonstrated that adiponectin is expressed in the skeletal muscle and liver (Krause et al. 2008; Wolf et al. 2006). Accordingly, these tissues might also be involved in the regulation of plasma adiponectin by GH.

Another possible mediator of the insulin-independent mechanism is ghrelin, a hormone secreted from the stomach (Kojima et al. 1999). Ghrelin signaling may be involved in the activation of Akt at baseline under CR and GH-suppressed conditions. The specific receptor for ghrelin is expressed abundantly in WAT (Petersenn 2004) and the plasma level of ghrelin was reported to increase after CR (Komatsu 2006). Ghrelin can activate the insulin receptor substrate-associated phosphatidylinositol 3-kinase/Akt pathway in 3T3-L1 preadipocytes and adipocytes to stimulate cellular proliferation and differentiation to mature adipocytes in addition to enhancing basal glucose uptake (Kim et al., 2004).

The present findings as well as those published from other laboratories (Wetter et al., 1999; Zhu et al., 2007) suggest that adipogenesis and/or lipogenesis are upregulated in WAT in CR rats if the total energy intake is limited. The present results of the gene expression of PPAR γ , ACC1, and Fas support the upregulation of both adipogenic and

lipogenic metabolism in WAT by CR but not suppression of GH. The promotion of lipid metabolism is consistent with the evolutionary view of CR (Holliday, 1989). It is reasonable to conclude that organisms retain mechanisms to efficiently store energy sources as fat to avoid death as a result of famine.

We emphasize the potential importance of PPAR γ in WAT in the effect of CR, because PPARs are believed to play a role in the effect of CR by regulating diverse physiological functions such as energy metabolism, insulin action and inflammation in multiple tissues (Masternak 2007; Chung et al 2008). In WAT, PPAR γ is known to upregulate the gene expression of adiponectin, which has anti-diabetic and anti-atherosclerotic effects (Yamauchi et al, 2003a,b) and reduces oxidative stress in peripheral tissues (Li et al, 2007; Tao et al., 2007). Overexpression of adiponectin gene was also reported to extend the lifespan of mice (Otabe et al. 2007). Thus, upregulation of adiponectin in WAT through the activation of a PPAR γ -associated transcriptional mechanism may comprise part of the machinery for CR.

In conclusion, the present study has revealed a unique effect of CR in WAT, which is potentially associated with the anti-aging effect of CR. A modest reduction in GH activity partially mimicked the effect of CR on activation of insulin signaling in response to glucose loading, but not for fat metabolism. Modification of fat metabolism could be associated with the CR-specific mechanism for longevity.

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Figure Legends

Figure 1. Levels of tyrosine-phosphorylated insulin receptor (pY-IR) in white adipose tissue: effects of CR and GH suppression. Data represent means + SEM (n = 5 for each treatment in the W-AL, W-CR, and Tg-AL groups, except for n = 4 for the saline-injected Tg-AL group). W-AL, wild-type rats fed ad libitum. W-CR, wild-type rats fed a calorie-restricted diet. Tg-AL, GH-suppressed transgenic rats fed ad libitum. Different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

Figure 2. Levels of phosphorylated (p)-Akt (Ser473) in the white adipose tissue: effects of CR and GH suppression. Data represent means + SEM (n = 5 for each treatment in the W-AL, W-CR, and Tg-AL groups, except for n = 4 for the saline-injected Tg-AL group). W-AL, wild-type rats fed ad libitum. W-CR, wild-type rats fed a calorie-restricted diet. Tg-AL, GH-suppressed transgenic rats fed ad libitum. Different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test.

Figure 3. Levels of glucose transporter (Glut)4 and Glut1 proteins in the membrane fraction of the white adipose tissue: effects of CR and GH suppression. Upper panel: Representative immunoblots. Data represent means + SEM (n = 5 for each treatment in the W-AL, W-CR, and Tg-AL groups, except for n = 4 for the saline-injected Tg-AL group). W-AL, wild-type rats fed ad libitum. W-CR, wild-type rats fed a calorie-restricted diet. Tg-AL, GH-suppressed transgenic rats fed ad libitum. Different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test. The expression of Glut4 (A) in the membrane fraction ($m(\text{membrane})/(m+c(\text{cytosol}))$) and Glut1($m/(m+c)$) (B). mRNA levels of Glut4 (C) and Glut1 (D). mRNA levels were normalized for β -actin mRNA levels.

Figure 4. Activation of the adiponectin-AMPK pathway in white adipose tissue: effects of CR and GH suppression. Data represent means + SEM (n = 5 for each treatment in the W-AL, W-CR, and Tg-AL groups, except for n = 4 for the saline-injected Tg-AL group).

W-AL, wild-type rats fed ad libitum. W-CR, wild-type rats fed a calorie-restricted diet.

Tg-AL, GH-suppressed transgenic rats fed ad libitum. Different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test. mRNA levels of adiponectin (A), adiponectin R1 (B) and R2 (C). mRNA levels were normalized for β -actin mRNA levels. (D) The protein abundance of phosphorylated (p)-AMPK (Thr172).

Figure 5. mRNA expression levels of PPAR γ and lipogenic genes in white adipose tissue: effects of CR and GH suppression. Data represent means + SEM (n = 5 for each treatment in the W-AL, W-CR, and Tg-AL groups, except for n = 4 for the saline-injected Tg-AL group). W-AL, wild-type rats fed ad libitum. W-CR, wild-type rats fed a calorie-restricted diet. Tg-AL, GH-suppressed transgenic rats fed ad libitum. Different letters indicate statistical differences at $p < 0.05$, as determined by one-factor ANOVA with a post hoc test. mRNA levels of PPAR γ (A), acetyl-CoA carboxylase (ACC)1 (B), and fatty acid synthase (FAS) (C). mRNA levels were normalized for β -actin mRNA levels.

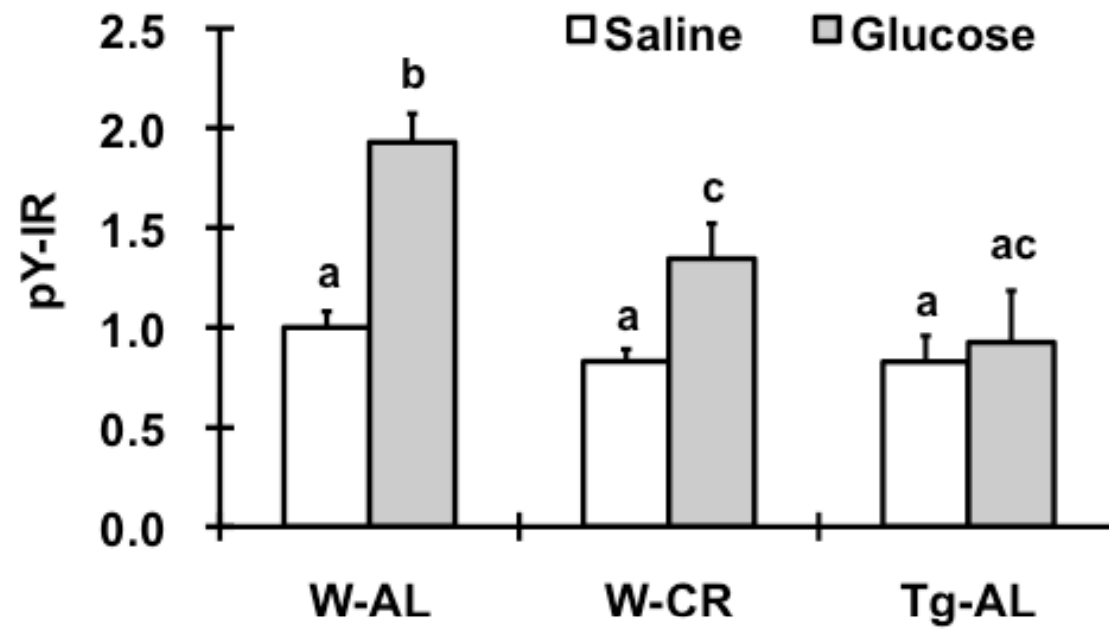


Fig. 1

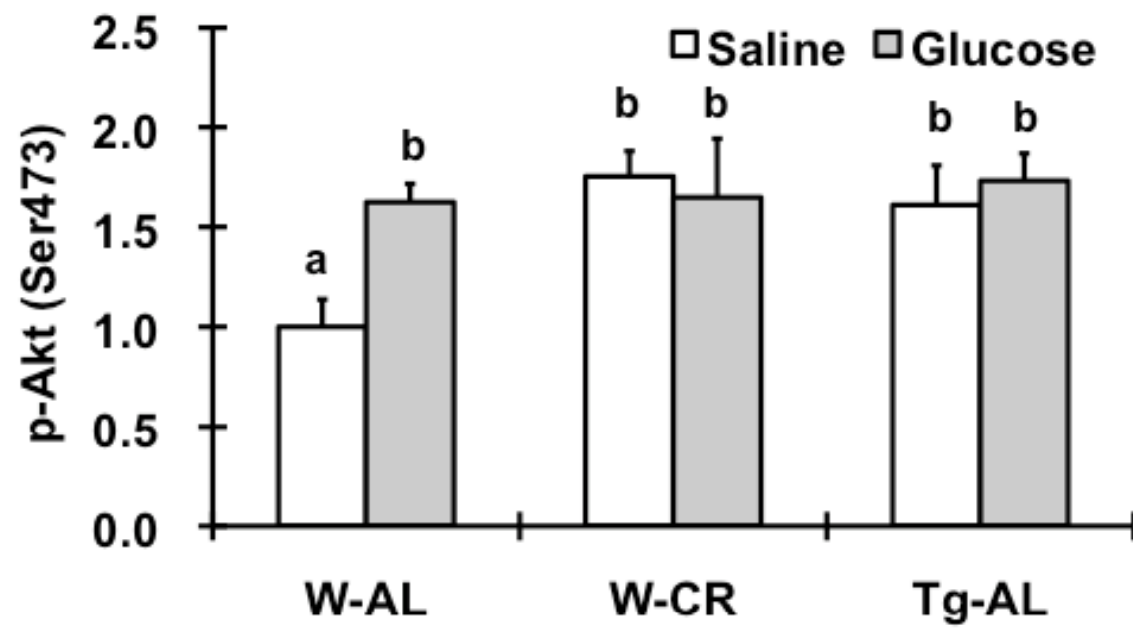


Fig. 2

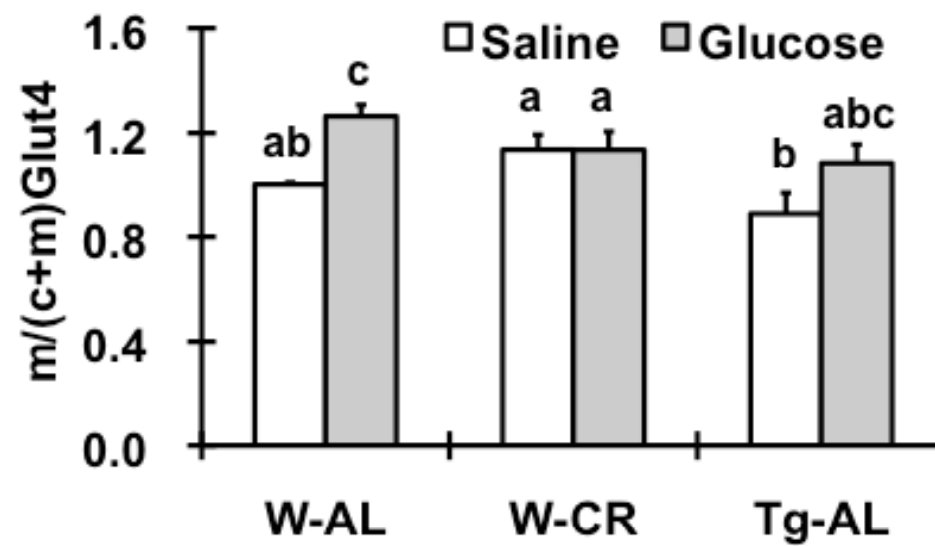
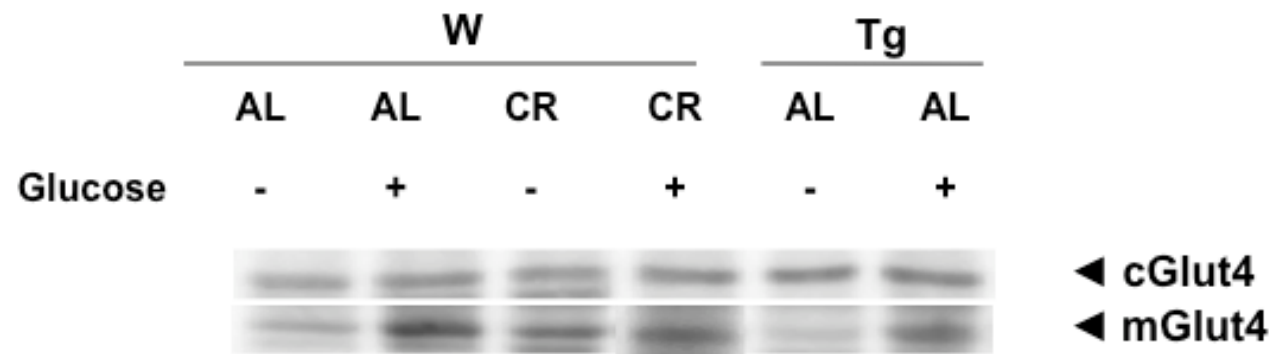


Fig. 3A

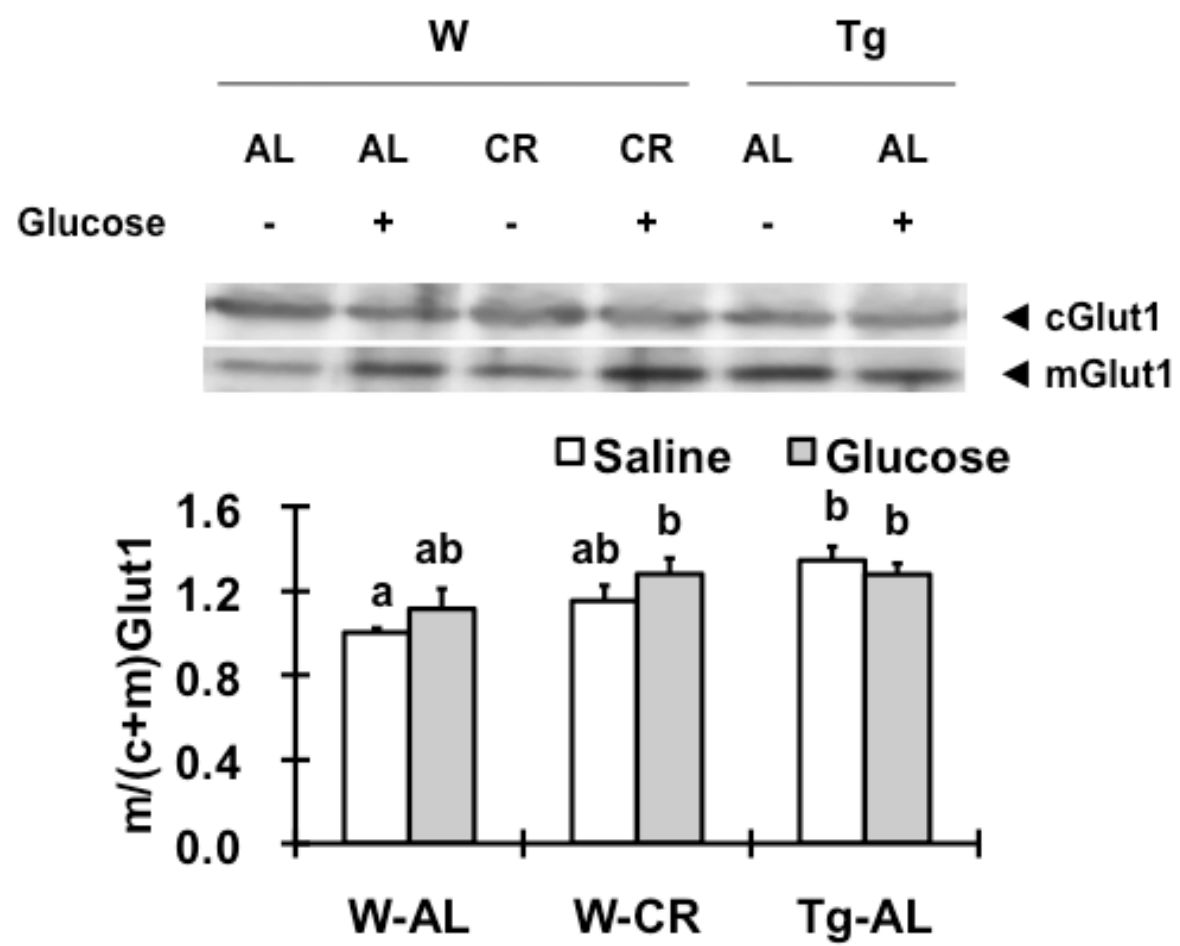


Fig. 3B

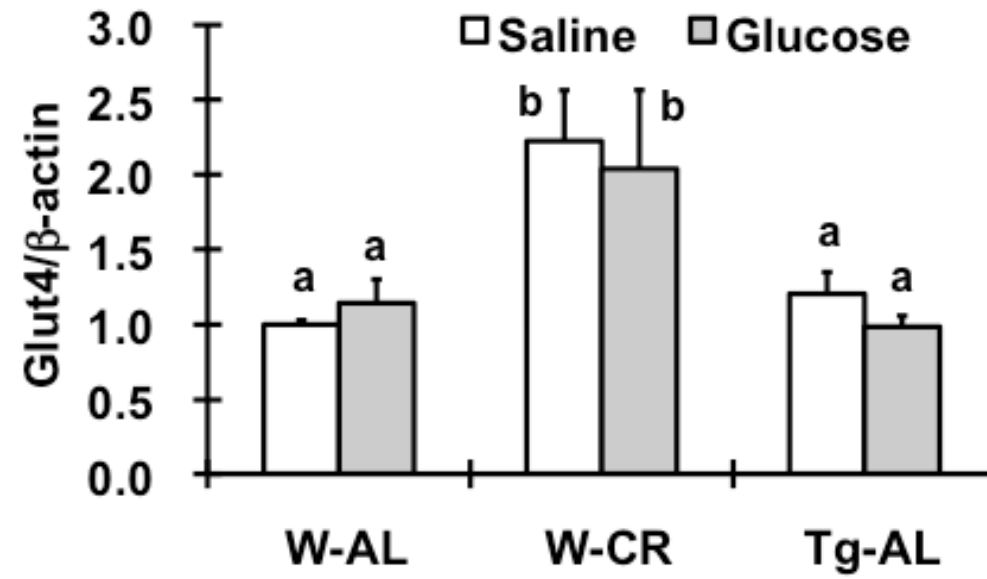


Fig. 3C

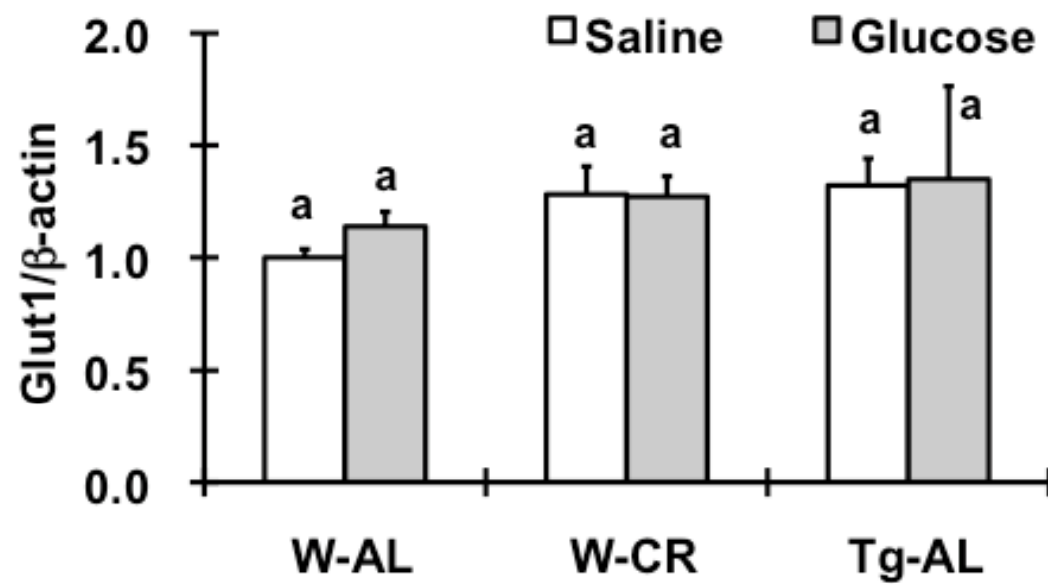


Fig. 3D

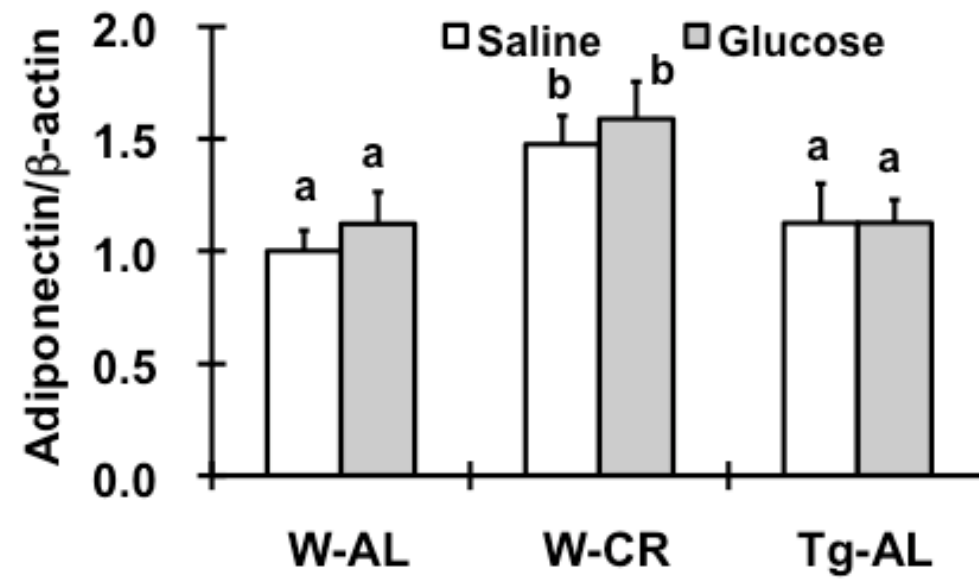


Fig. 4A

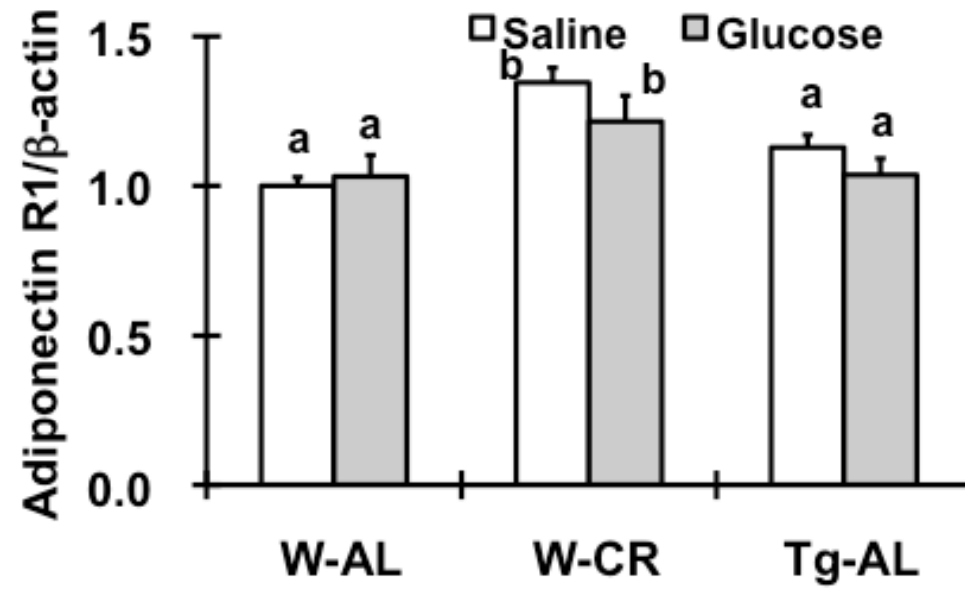


Fig. 4B

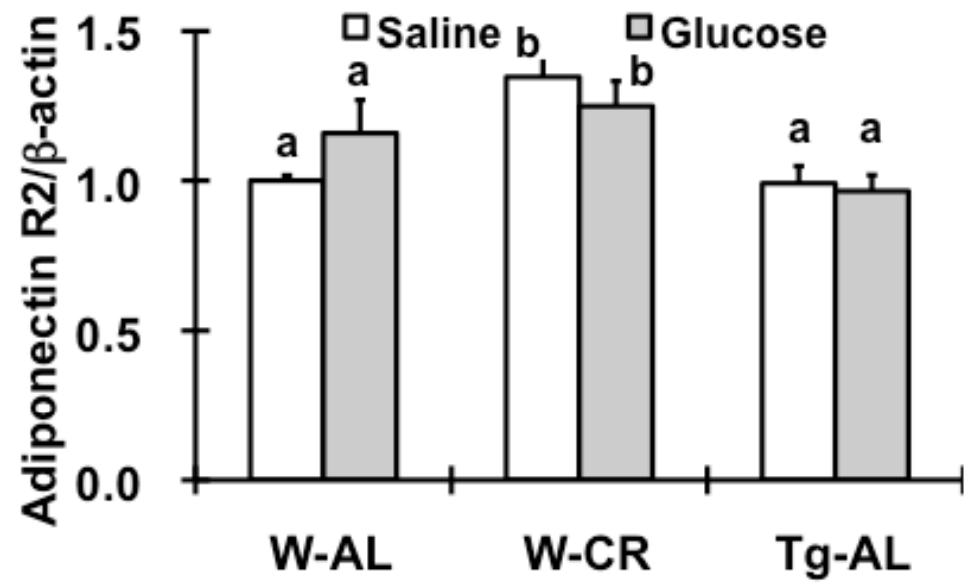


Fig. 4C

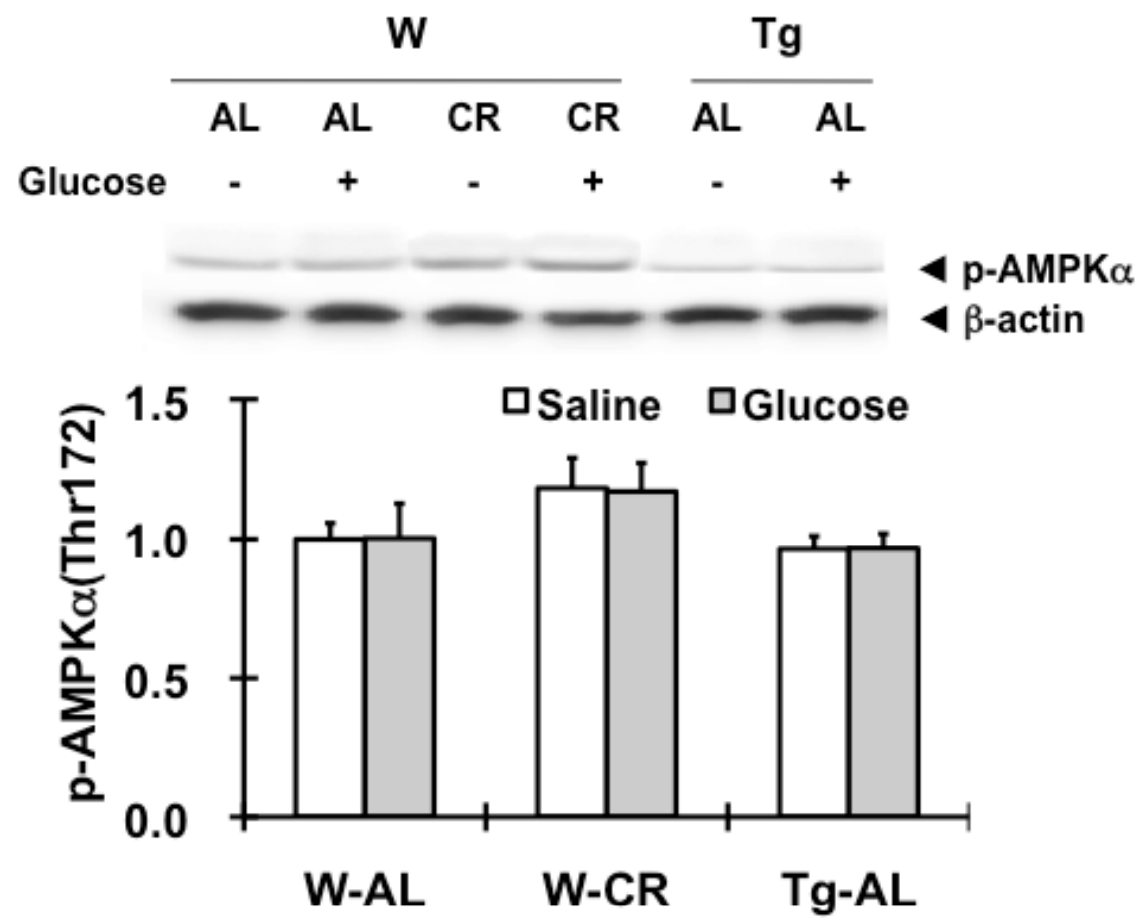


Fig. 4D

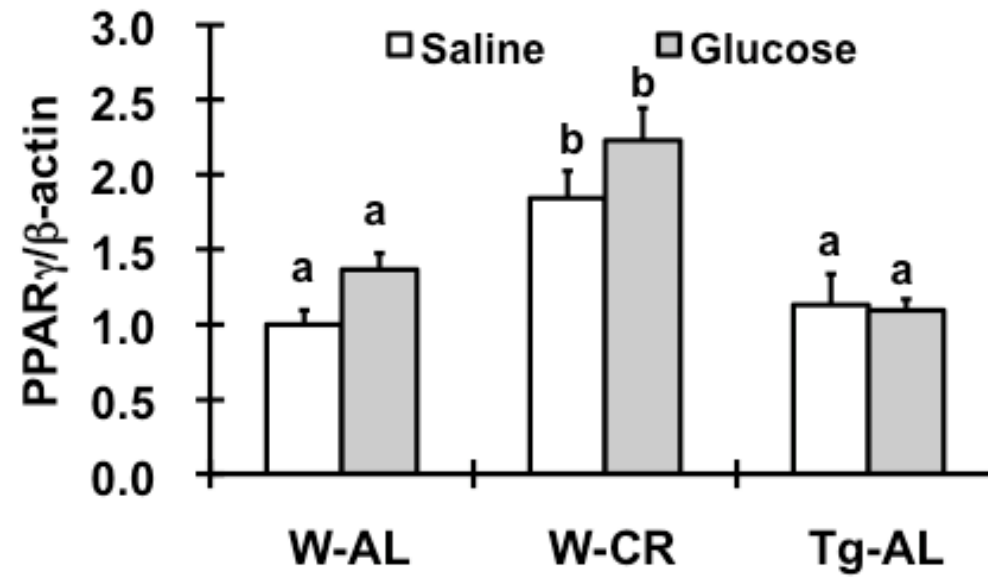


Fig. 5A

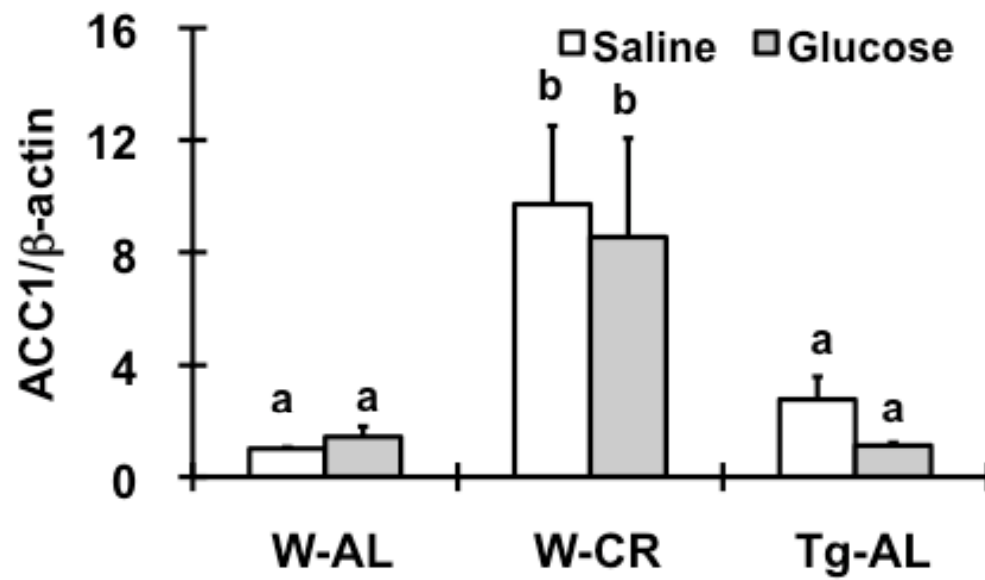


Fig. 5B

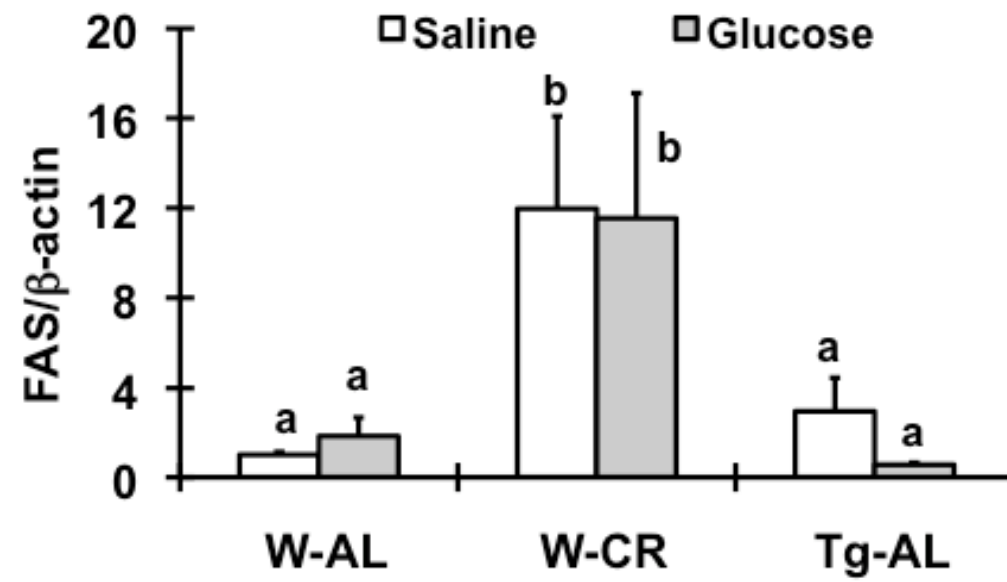
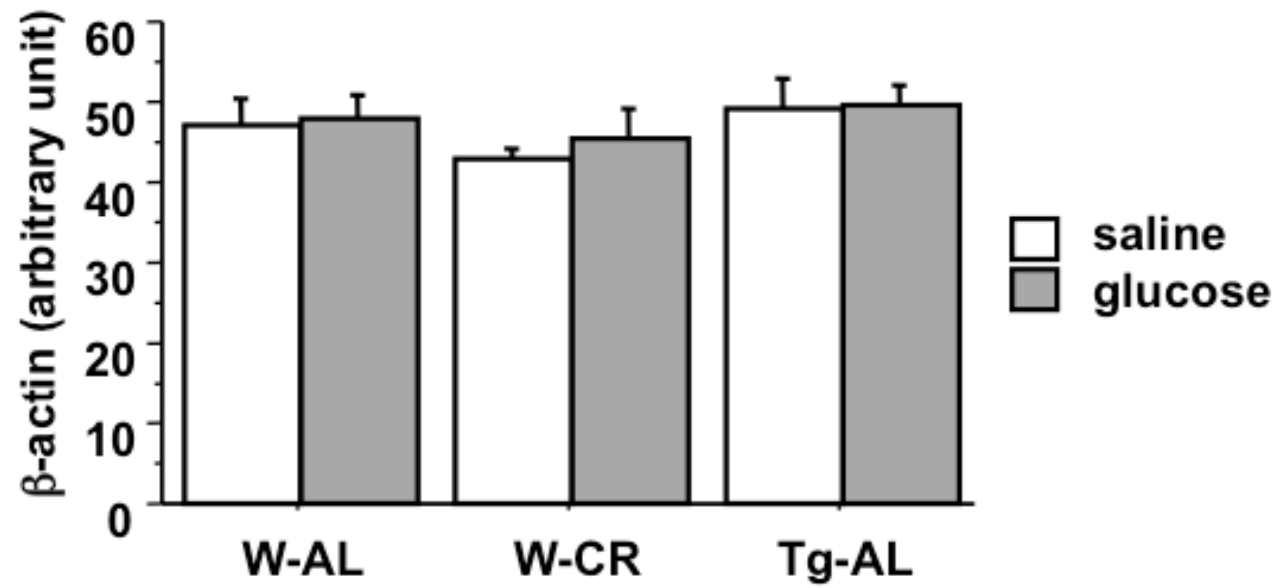


Fig. 5C



Supplemental figure. Supplementary figure.

Table 1. General data for wild type and transgenic rats

	W-AL		W-CR		Tg-AL	
	Saline	Glucose	Saline	Glucose	Saline	Glucose
Body weight (g)	544.4 ± 3.7 ^a		372.4 ± 8.3 ^b		335.6 ± 8.3 ^c	
Peri-renal fat (g/100g body weight)	3.12 ± 0.12 ^a		2.01 ± 0.11 ^b		2.22 ± 0.12 ^b	
Pituitary GH-mRNA	1.00 ± 0.41 ^a		0.72 ± 0.05 ^b		0.21 ± 0.05 ^c	
IGF-1 (ng/ml)	1690 ± 112 ^a		1288 ± 276 ^b		1268 ± 210 ^b	
Glucose (mg/ml)	110.5 ± 5.2 ^a	322.2 ± 9.3 ^c	103.7 ± 1.7 ^a	272.6 ± 14.5 ^b	121.0 ± 4.9 ^a	257.9 ± 29.1 ^b
Insulin (ng/ml)	80.7 ± 15.6 ^{bc}	1214.1 ± 787.8 ^d	37.9 ± 5.8 ^{ab}	299.6 ± 168.6 ^c	20.8 ± 5.1 ^a	301.0 ± 228.7 ^{bc}

The general data have been published in more detail elsewhere. The body weight, peri-renal fat weight, blood glucose, and insulin data were published in Hayashi et al. (2008) and the same animals were used in the present study. The pituitary GH-mRNA and plasma IGF-1 levels, which were originally published in Chiba T et al. (2008), were determined in rats at the

same age as the experimental groups used here. The values of GH-mRNA were normalized relative to those in W-AL rats. Data represent the means \pm SEM (n = 10 for the body weight and peri-renal fat pad weight in the W-AL and W-CR groups; n = 8 in the Tg-AL group; n = 5 for each treatment in W-AL and W-CR rats; n = 4 for each treatment in Tg-AL rats; n = 4 or 5 for the pituitary GH-mRNA and plasma IGF-1 levels). Different letters indicate statistical differences at $p < 0.05$. Glucose and insulin levels were statistically analyzed after log transformation. W-AL, wild-type rats fed ad libitum; W-CR, wild-type 30% calorie-restricted rats; Tg-AL, transgenic rats fed ad libitum.