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Molecular Mechanism of Moderate Insulin Resistance in Adiponectin-Knockout Mice

WATARU YANO*, NAOTO KUBOTA*,**,***, SHINSUKE ITOH*, TETSUYA KUBOTA*,***,****, MOTOHARU AWAZAWA*, MASAO MOROI****, KAORU SUGI****, ISEKI TAKAMOTO*,**,***, HITOMI OGATA#, KUMPEI TOKUYAMA#, TETSUO NODA##, YASUO TERAUCHI###, KOHJIRO UEKI* AND TAKASHI KADOWAKI*,**,***

**Department of Diabetes and Metabolic Disease, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
***Translational Systems Biology and Medicine Initiative, University of Tokyo, Tokyo, Japan*
****Division of Applied Nutrition, National Institute of Health and Nutrition, Tokyo, Japan*
*****Division of Cardiovascular Medicine, Toho University, Ohashi Hospital, Tokyo, Japan*
#Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan
##Department of Cell Biology, Japanese Foundation for Cancer Research-Cancer Institute, Tokyo, Japan
###Department of Endocrinology and Metabolism, Yokohama City University Graduate School of Medicine, Yokohama, Japan

Abstract. Adiponectin has been proposed to act as an antidiabetic adipokine, suppressing gluconeogenesis and stimulating fatty acid oxidation in the liver and skeletal muscle. Although adiponectin-knockout (adipo(–/–)) mice are known to exhibit insulin resistance, the degrees of insulin resistance and glucose intolerance are unexpectedly only moderate. In this study, the adipo(–/–) mice showed hepatic, but not muscle, insulin resistance. Insulin-stimulated phosphorylation of IRS-1 and IRS-2 was impaired, the IRS-2 protein level was decreased, and insulin-stimulated phosphorylation of Akt was decreased in the liver of the adipo(–/–) mice. However, the triglyceride content in the liver was not increased in these mice, despite the decrease in the PPARalpha expression involved in lipid combustion, since the expressions of lipogenic genes such as SREBP-1 and SCD-1 were decreased in association with the increased leptin sensitivity. Consistent with this, the down-regulation SREBP-1 and SCD-1 observed in the adipo(–/–) mice was no longer observed, and the hepatic triglyceride content was significantly increased in the adiponectin leptin double-knockout (adipo(–/–)ob/ob) mice. On the other hand, the triglyceride content in the skeletal muscle was significantly decreased in the adipo(–/–) mice, probably due to up-regulated AMPK activity associated with the increased leptin sensitivity. In fact, these phenotypes in the skeletal muscle of these mice were no longer observed in the adipo(–/–)ob/ob mice. In conclusion, adipo(–/–) mice showed impaired insulin signaling in the liver to cause hepatic insulin resistance, however, no increase in the triglyceride content was observed in either the liver or the skeletal muscle, presumably on account of the increased leptin sensitivity.

Key words: Adiponectin, Insulin resistance, Adiponectin-knockout mice, Euglycemic-hyperinsulinemic clamp.
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ADIPONECTIN (also known as Acrp30) [1–4] is a hormone secreted by adipocytes that acts as a major antidiabetic adipokine. Plasma adiponectin levels are decreased in obesity, insulin resistance and type 2 diabetes mellitus [1–4]. Decreased adiponectin has been implicated in the development of insulin resistance in obesity, which has been shown to be reversed by replenishment of adiponectin [5–7]. This insulin-sensitizing effect of adiponectin seems to be mediated by the inhibition of gluconeogenesis in the liver and stimulation of fatty acid oxidation via activation of AMP-activated protein kinase (AMPK) and peroxi-

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Correspondence to: Takashi KADOWAKI, M.D., Ph.D. Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan.
Abbreviations: EGP, Endogenous Glucose Production; Rd Rate of disappearance

some proliferator-activated receptor (PPAR) alpha in the liver and skeletal muscle [8–12]. Thus, adiponectin ameliorates insulin resistance in both the liver and skeletal muscle.

Adiponectin-knockout (adipo(–/–)) mice have been described mainly by four groups. We reported that adipo(–/–) mice showed insulin resistance [13], indicating that adiponectin acts as an insulin-sensitizing hormone *in vivo*. Maeda *et al*. reported that adipo(–/–) mice fed a normal diet failed to show insulin resistance and glucose intolerance [14]. Ma *et al*. described the absence of insulin resistance and unexpectedly, increased fatty acid oxidation, in the skeletal muscle of adipo(–/–) mice [15]. Nawrocki *et al*. described that adipo(–/–) mice exhibited hepatic, but not muscle, insulin resistance, and increased endogenous glucose production (EGP), with absence of any change in the rate of disappearance (Rd) of glucose during the euglycemic-hyperinsulinemic clamp study [16]. However, the degrees of insulin resistance and glucose intolerance were unexpectedly moderate in these adipo(–/–) mice [13, 15, 16]. We recently reported increased leptin sensitivity in adipo(–/–) mice [17]. Leptin is known to decrease the expressions of lipogenic genes and also the triglyceride content in the liver [18, 19] and, it has also been shown to activate AMPK in the skeletal muscle [20]. In fact, muscle AMPK activity was shown to be increased in the adipo(–/–) mice [17]. In this study, we investigated the molecular mechanisms of the insulin resistance observed in the adipo(–/–) and adiponectin/leptin double-knockout (adipo(–/–)ob/ob) mice.

Materials and methods

Animals

Mice lacking adiponectin were generated as described previously [13, 21]. Adiponectin/leptin double-knockout mice and ob/ob mice were generated by intercrossing of adipo(+/–)ob/+ mice [21]. The mice were allowed free access to water and ordinary laboratory diet. All experiments in this study were conducted on littermate male mice. The animal care and procedures of the experiments were approved by the Animal Care Committee of the University of Tokyo.

Euglycemic-hyperinsulinemic clamp study

A clamp study was carried out as described previously [21]. A catheter was inserted into the jugular vein 2-3 days prior to the clamp study. After overnight food deprivation, insulin was injected constitutively by intravenous infusion at 4 mU/kg/min, and 50% glucose solution enriched to 20% with 6,6-d_2 glucose as tracer was injected to maintain the blood glucose at about 120 mg/dl under conscious and unstressed conditions. Blood was sampled via tail tip bleeds at 90, 105 and 120 min to determine the rate of glucose disappearance (Rd) and endogenous glucose production (EGP).

RNA preparation and analysis

Tissue samples were homogenized to isolate RNA with ISOGEN reagent (Wako, Japan) and analyzed by northern blotting and real-time quantitive PCR. Northern blotting for PPARalpha and SREBP-1 mRNA was carried out as described previously [6, 19]. For real-time quantitive PCR, the ABI 7900 sequence detection system (Applied Biosystems, CA, USA) was used. The RNA sample was processed with TURBO DNase (Ambion, TX, USA) before reverse transcription to synthesize cDNA. 36B4 mRNA was used as the internal control. The primer sets for PEPCK and G6Pase were purchased from Applied Biosystems. The sequences of the primer sets for SREBP-1c, PPARalpha and 36B4 were as follows [22]; SREBP-1c: forward primer, ATCGGCGCGGAAGCTGTCGGGGTAG CGTC; reverse primer, TGAGCTGGAGCATGTCT TCAA; probe, FAM-ACCACGGAGCCATGGATT GCACATT-TAMRA. PPARalpha: forward primer, CAACGGCGTCGAAGACAAA; reverse primer, GACGGTCTCCACGGACATG; probe, FAM-CAGAGGTCCGATTCTTCCACTGCTGC-TAMRA. 36B4: forward primer, TGCCACACTCCATCAT CAATG; reverse primer, CCGCAAATGCAGATG GATC; probe, FAM-CCCACTTACTGAAAAGGT CAAGGCCTTCCTG-TAMRA.

Measurement of the tissue triglyceride content

Tissue homogenate was extracted with 2:1 (vol/vol) chloroform/methanol, and the triglyceride content was determined as described previously [22]. In brief, chloroform/methanol was added to the homogenate and shaken for 15 min. After centrifugation at 14,000

rpm for 10 min, the organic layer was collected. This extraction step was repeated three times. The collected sample was dried and resuspended in 1% Triton X-100/ethanol, and measured using L-type Wako (Wako, Japan).

Immunoprecipitation and western blotting

Immunoprecipitation and western blot analyses were carried out as described previously [23]. Tissue lysate was immunoprecipitated with anti-IRS-1 antibody or anti-IRS-2 antibody (Upstate, VA, USA) and blotted with an anti-phospho-tyrosine (anti-pY) antibody (Upstate, VA, USA) to assess the degree of phosphorylation of IRS-1 or IRS-2. For the western blot analyses, antibodies against Akt, phospho-Akt, AMPK and phospho-AMPK (Cell Signaling Technology, MA, USA) were used.

Statistics

All values were expressed as means ± SEM. The statistical significances of differences were calculated using the *t*-test.

Results

The euglycemic-hyperinsulinemic clamp study revealed hepatic insulin resistance in the adipo(–/–) mice

We carried out the euglycemic-hyperinsulinemic clamp study using the tracer technique in the wild-type and adipo(–/–) mice. Significant decrease of the GIR was observed in the adipo(–/–) mice as compared with that in the wild-type mice (Fig. 1A), indicating that the adipo(–/–) mice indeed exhibited insulin resistance, as previously reported [13]. Adipo(–/–) mice showed similar Rd to the wild-type mice, but significantly increased EGP. (Fig. 1B, C). The expressions of PEPCK (Fig. 1D) and G6Pase (Fig. 1E), which are involved in gluconeogenesis, were up-regulated during the euglycemic-hyperinsulinemic clamp study in the adipo(–/–) mice, indicating the hepatic insulin resistance in the adipo(–/–) mice.

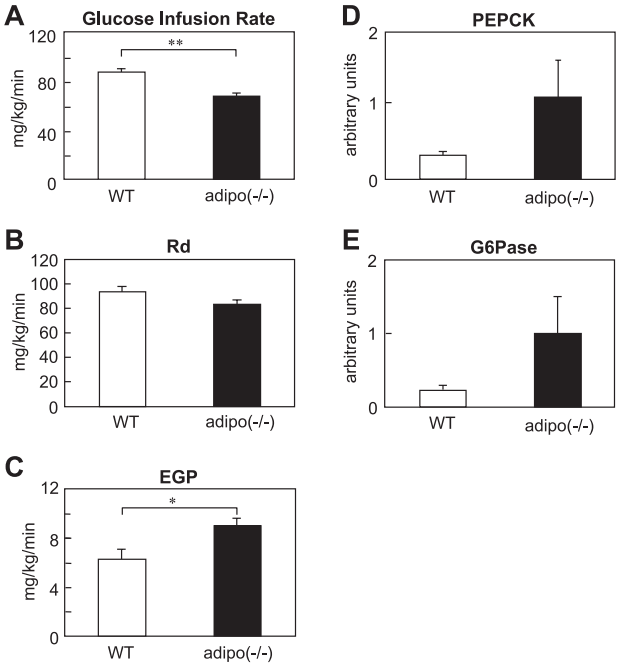

Fig. 1. The euglycemic-hyperinsulinemic clamp study revealed hepatic insulin resistance in the adipo(–/–) mice. (A-E) Glucose infusion rate (GIR) (A), rate of glucose disappearance (Rd) (B), endogenous glucose production (EGP) (C), PEPCK (D) and G6Pase (E) mRNA levels in the liver during the euglycemic-hyperinsulinemic clamp study. All values are expressed as means ± SEM of data (n = 7) obtained from the analysis of wild-type (*open bars*) and adipo(–/–) mice (*closed bars*). **p*<0.05, ***p*<0.01.

Impaired insulin signaling in the liver of the adipo(–/–) mice

Insulin signaling was investigated in the liver and the skeletal muscle of the adipo(–/–) mice. Insulin-stimulated tyrosine phosphorylation of IRS-1 was significantly decreased and that of IRS-2 was markedly decreased in the liver of the adipo(–/–) mice as compared with that in the liver of the wild-type mice (Fig. 2A). The protein level of IRS-2 was significantly decreased, while that of IRS-1 was not altered (Fig. 2A). Insulin-stimulated phosphorylation of Akt was also significantly reduced in the adipo(–/–) mice (Fig. 2B). In the skeletal muscle, the insulin-stimulated tyrosine phosphorylation level of IRS-1 was similar in the wild-type and adipo(–/–) mice (Fig. 2C). The insulin-stimulated phosphorylation level of Akt was also similar in the two genotypes (Fig. 2D). These data indicate impairment of hepatic insulin signaling in the adipo(–/–) mice.

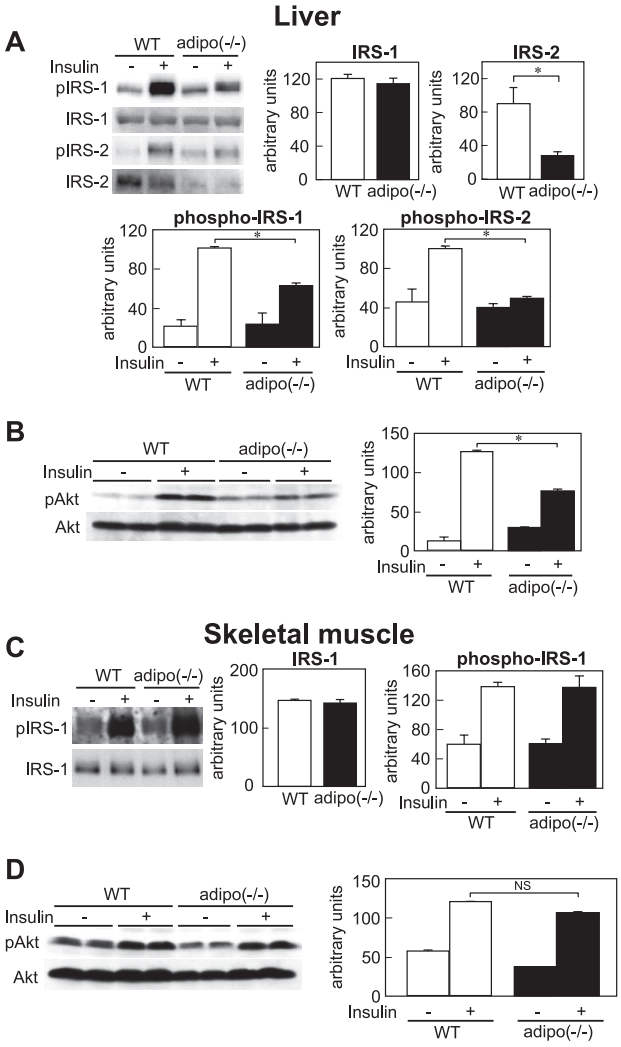

Fig. 2. Impaired insulin signaling in the liver of the adipo(–/–) mice.
(A) Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 in the liver. Quantification of the protein levels (*upper, right*) and phosphorylation levels of IRS-1 and IRS-2 (*lower*). (B) Insulin-stimulated phosphorylation of Akt in the liver. (C) Insulin-stimulated tyrosine phosphorylation of IRS-1 in the skeletal muscle. Quantification of the protein level (*middle*) and phosphorylation level of IRS-1 (*right*). (D) Insulin-stimulated phosphorylation of Akt in the skeletal muscle. All values are expressed as means ± SEM of data (n = 4) obtained from the analysis of wild-type (*open bars*) and adipo(–/–) mice (*closed bars*). **p*<0.05.

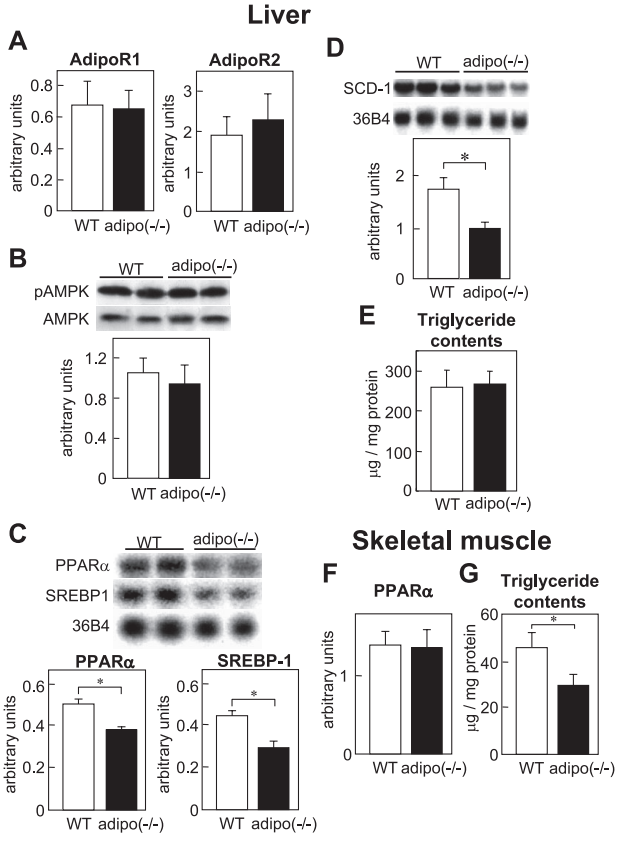

Fig. 3. Hepatic triglyceride content was not elevated and the expressions of lipogenic genes were down-regulated in the adipo(–/–) mice.
(A) AdipoR1 and AdipoR2 mRNA expression levels in the liver (n = 3). (B) AMPK phosphorylation in the liver (n = 8–10). (C-D) The mRNA levels of PPARalpha, SREBP-1 (C) and SCD-1 (D) in the liver (n = 4–5). (E) Hepatic triglyceride content (n = 11–14). (F) PPARalpha expression level in the skeletal muscle (n = 5). Triglyceride content (G) in the skeletal muscle (n = 5). All values are expressed as means ± SEM of data obtained from the analysis of wild-type (*open bars*) and adipo(–/–) mice (*closed bars*). **p*<0.05.

Hepatic triglyceride content was not elevated and the expressions of lipogenic genes were down-regulated in the adipo(–/–) mice

We next investigated the lipid metabolism in the liver of these mice. No significant differences in the expression levels of the adiponectin receptors AdipoR1 and AdipoR2 [24] were observed between the wild-type and adipo(–/–) mice (Fig. 3A). While the degree of AMPK phosphorylation remained unchanged (Fig. 3B), significant down-regulation of PPARalpha

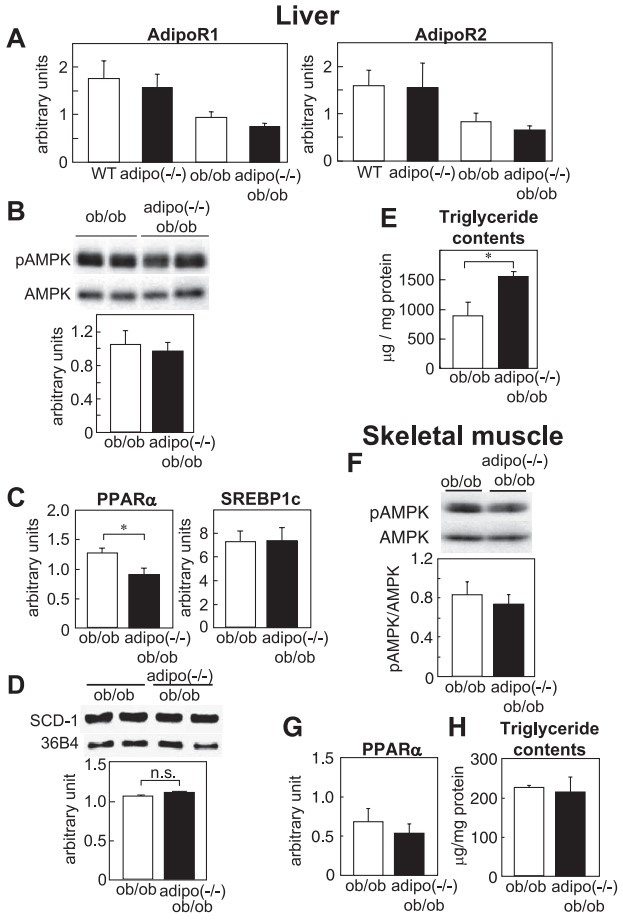

Fig. 4. The reduced expressions of the lipogenic genes in the adipo(–/–) mice were no longer seen in the adipo(–/–) mice with a leptin-deficient background.
(A) AdipoR1 and AdipoR2 mRNA expression levels in the liver (n = 3–6). (B) AMPK phosphorylation in the liver (n = 5). (C-D) mRNA levels of PPARalpha, SREBP-1c (C) and SCD-1 (D) in the liver (n = 4–5). (E) Hepatic triglyceride content (n = 5). (F) AMPK phosphorylation in the skeletal muscle (n = 5). (G) PPARalpha expression level in the skeletal muscle (n = 5). (H) Triglyceride content in the skeletal muscle (n = 5). All values are expressed as means ± SEM of data obtained from the analysis of ob/ob (*open bars*) and adipo(–/–)ob/ob mice (*closed bars*). **p*<0.05.

was observed in the adipo(–/–) mice as compared with that in the wild-type mice (Fig. 3C), suggesting that fatty acid oxidation may be reduced in the liver of the adipo(–/–) mice. The expressions of lipogenic genes such as SREBP-1 (Fig. 3C) and SCD-1 (Fig. 3D) were also significantly down-regulated in the adipo(–/–) mice, and the hepatic triglyceride content was not elevated in the adipo(–/–) mice (Fig. 3E). This unexpected down-regulation of lipogenic genes, which may be explained by the increased leptin sensitivity seen in the adipo(–/–) mice [17], might have prevented the elevation of the triglyceride content in the liver of the adipo(–/–) mice. In the skeletal muscle, we previously reported that the phosphorylation of AMPK was increased in adipo(–/–) mice, presumably due to increased leptin sensitivity [17]. Consistent with this, although the expression of PPARalpha was similar in the wild-type and adipo(–/–) mice (Fig. 3F), the muscle triglyceride content was significantly decreased in the adipo(–/–) mice (Fig. 3G).

The reduced expressions of the lipogenic genes observed in the adipo(–/–) mice no longer seen in the adipo(–/–) mice with a leptin-deficient background

To evaluate the existence of the aforementioned compensatory mechanism in the adipo(–/–) mice, we generated adipo(–/–)ob/ob mice. The expression levels of AdipoR1 and AdipoR2 were similar in the liver of the ob/ob and adipo(–/–)ob/ob mice. Comparison of the wild-type with ob/ob mice, and of the adipo(–/–) with adipo(–/–)ob/ob mice demonstrated a tendency towards reduced expression levels of the adiponectin receptors in the leptin-deficient background (Fig. 4A). Significant decrease of PPARalpha expression was observed in the liver of the adipo(–/–)ob/ob mice (Fig. 4C), as in the liver of the adipo(–/–) mice (Fig. 3C). The expression levels of SREBP-1c (Fig. 4C) and SCD-1 (Fig. 4D) were not altered in the liver of the adipo(–/–)ob/ob mice, unlike in the liver of the adipo(–/–) mice, indicating that the compensatory mechanism in the adipo(–/–) mice was no longer operative in the adipo(–/–)ob/ob mice. In fact, the hepatic triglyceride content was significantly increased in the adipo(–/–)ob/ob mice (Fig. 4E). These data suggest that the leptin pathway might have contributed to the reduced expressions of the lipogenic genes and absence of elevation of the hepatic triglyceride content in the adipo(–/–) mice. In the skeletal muscle, increased phosphorylation of AMPK in the adipo(–/–) was no longer observed in the adipo(–/–)ob/ob mice (Fig. 4F). The expression level of PPARalpha (Fig. 4G) and the triglyceride content (Fig. 4H) were also unaltered in the skeletal muscle of adipo(–/–)ob/ob mice.

Discussion

In the present study, we investigated the molecular mechanisms of the insulin resistance in adipo(–/–) mice. Adipo(–/–) mice showed hepatic, but not muscle, insulin resistance. Insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 was impaired in the liver of the adipo(–/–) mice, despite the absence of any change in the hepatic triglyceride content. One of the underlying mechanisms responsible for this may be the increased phosphorylation of the serine/threonine residue of IRS-1 in the liver of these mice, which is currently under investigation. Moreover, the IRS-2 protein level was also significantly decreased in the adipo(–/–) mice; as a result, insulin-stimulated phosphorylation of Akt was significantly decreased in these mice. In the skeletal muscle, on the other hand, insulin-stimulated phosphorylation of IRS-1 and Akt was similar in degree between the wild-type and adipo(–/–) mice. In fact, while the EGP and expression levels of PEPCK and G6Pase were increased, the Rd was not found to be significantly changed in the adipo(–/–) mice during the euglycemic-hyperinsulinemic clamp study.

Triglyceride content in the liver was not increased in the adipo(–/–) mice, despite the decrease in PPARalpha expression involved in lipid combustion, since the expressions of lipogenic genes such as SREBP-1 and SCD-1 were decreased in association with the increased leptin sensitivity. Consistent with this, down-regulation of SREBP-1 and SCD-1 observed in the adipo(–/–) mice was no longer observed in the adipo(–/–)ob/ob mice, and the hepatic triglyceride content was significantly increased in the adipo(–/–)ob/ob mice as compared with that in the ob/ob mice. On the other hand, the triglyceride content in the skeletal muscle was significantly decreased in the adipo(–/–) mice, probably due to the up-regulated muscle AMPK activity associated with the increased leptin sensitivity in these mice [17]. In fact, the increase in AMPK activity [17] and decrease triglyceride content in the skeletal muscle were no longer observed in the adipo(–/–)ob/ob mice. Therefore, increase in the leptin actions appears to compensate for the adiponectin deficiency in both the liver and the skeletal muscle of the adipo(–/–) mice, accounting for the unexpected absence of a increase in the hepatic triglyceride content and rather decreased muscle triglyceride content in the adipo(–/–) mice [13].

Why was the degree of insulin resistance different between the liver and skeletal muscle of the adipo(–/–) mice? We recently demonstrated that adiponectin induces the expression of IRS-2 in the liver (Awazawa M, Ueki K and Kadowaki T, manuscript in preparation). IRS-2 is a major IRS in the liver, but not in the skeletal muscle [25], suggesting that the reduction of IRS-2 due to adiponectin deficiency may have little effect on the insulin signaling in the skeletal muscle of adipo(–/–) mice.

A similar degree of phosphorylation of AMPK was seen in the liver of the wild-type and adipo(–/–) mice, even though adiponectin is known to activate AMPK [9, 10]. SCD-1 expression was down-regulated in the adipo(–/–) mice. Increased phosphorylation of AMPK has been reported in the liver of SCD-1-knockout mice [26]. It is suggested that the down-regulation of AMPK resulting from adiponectin deficiency may be balanced by the up-regulation of AMPK occurring as a result of the decreased SCD-1 expression, resulting in the absence of any net change in the phosphorylation level of AMPK in the liver of the adipo(–/–) mice. In Nawrocki's study, there appeared to be no differences in the phosphorylation level and activity of AMPK between the wild-type and adipo(–/–) mice [16].

In conclusion, adipo(–/–) mice showed impaired insulin signaling in the liver to cause hepatic insulin resistance, however, no increase in the triglyceride content was observed in either the liver or the skeletal muscle, presumably on account of the increased leptin sensitivity.

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