Biochemical and Biophysical Research Communications 382 (2009) 51–56

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Adiponectin suppresses hepatic SREBP1c expression in an AdipoR1/LKB1/AMPK dependent pathway

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ARTICLE INFO

Article history:
Received 18 February 2009
Available online 28 February 2009

Keywords:
Diabetes mellitus
Adipocytokine
Adiponectin
SREBP1c
Fatty acid synthesis
AMPK

ABSTRACT

Adiponectin, one of the insulin-sensitizing adipokines, has been shown to activate fatty acid oxidation in liver and skeletal muscle, thus maintaining insulin sensitivity. However, the precise roles of adiponectin in fatty acid synthesis are poorly understood. Here we show that adiponectin administration acutely suppresses expression of sterol regulatory element-binding protein (SREBP) 1c, the master regulator which controls and upregulates the enzymes involved in fatty acid synthesis, in the liver of $+Lepr^{db}/+Lepr^{db}$ (*db/db*) mouse as well as in cultured hepatocytes. We also show that adiponectin suppresses SREBP1c by AdipoR1, one of the functional receptors for adiponetin, and furthermore that suppressing either AMP-activated protein kinase (AMPK) via its upstream kinase LKB1 deletion cancels the negative effect of adiponectin on SREBP1c expression. These data show that adiponectin suppresses SREBP1c through the AdipoR1/LKB1/AMPK pathway, and suggest a possible role for adiponectin in the regulation of hepatic fatty acid synthesis.

In the pathogenesis of insulin resistance, fat accumulation in liver, or hepatic steatosis, is of great importance. Steatosis can develop as a result of decreased lipid oxidation and increased lipid synthesis. SREBP1c is a critical transcription factor that controls and upregulates the enzymes involved in fatty acid synthesis such as fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC)-1 in the liver [1–4]. SREBP1c is constitutively upregulated in obese insulin-resistant animal models [5,6], while deletion of SREBP1c in obese model mice ameliorates hepatic steatosis [6]. These data clearly indicate that accelerated lipid synthesis in the liver contributes to the development of hepatic steatosis at least in rodent models, and that SREBP1c could play critical roles in the pathogenesis of fatty liver and the metabolic syndrome.

Adiponectin, whose expression and plasma concentration are inversely correlated with obesity and insulin resistance and type 2 diabetes [7–10], activates AMPK and peroxisome proliferator-activated receptor (PPAR) α, thus increasing fatty acid oxidation in the liver [11–13].

It has previously been reported that adiponectin prevents the development of alcohol-induced steatosis, and also ameliorates fatty liver disease in Lep^{ob}/Lep^{ob} mice [14]. These were attributed to increasing fatty acid oxidation as well as suppressing fatty acid synthesis by adiponectin. However, given the possibilities that improved insulin sensitivity by adiponectin treatment could secondarily affect fatty acid metabolism in the liver, and that central adiponectin signaling interferes with hypothalamic leptin signaling [15,16], it remains unclear whether adiponectin directly suppresses lipid synthesis.

Therefore, the current study is designed to more precisely elucidate the role of adiponectin in hepatic fatty acid synthesis. Here, we show that adiponectin directly suppresses SREBP1c via the AdipoR1/LKB1/AMPK pathway in hepatocytes. The data presented here have revealed an unknown function of adiponectin in the regulation of SREBP1c.

Materials and methods

Generation of recombinant adiponectin. Bacterially expressed murine adiponectin was prepared as described previously [12].

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doi:10.1016/j.bbrc.2009.02.131

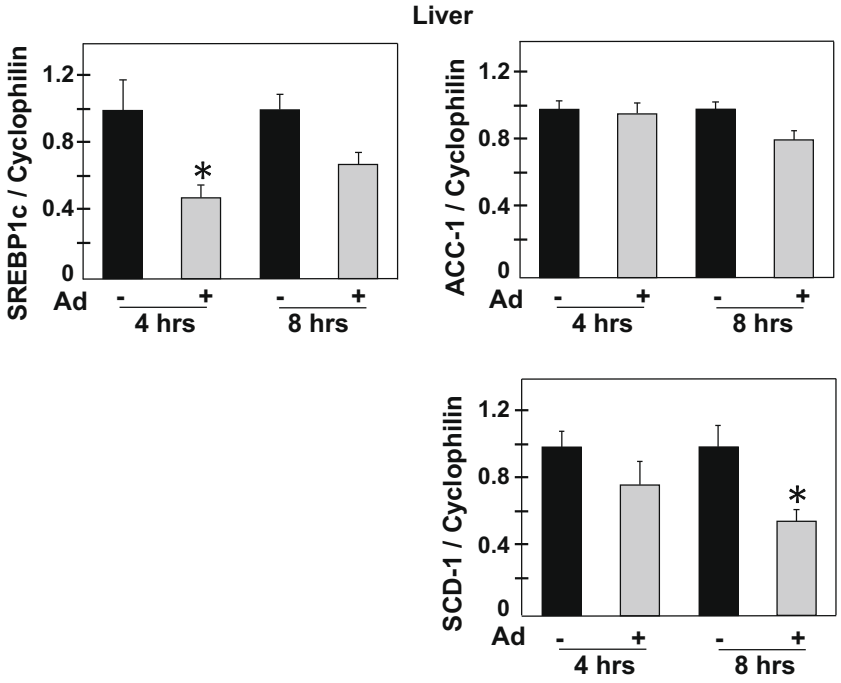

Fig. 1. Intraperitoneal administration of adiponectin acutely suppresses SREBP1c and its downstream molecules. The total RNA was extracted from the liver of *db/db* mice 4 or 8 h after adiponectin administration. Each bar represents the mean ± SE ($n = 9$, $^{*}P < 0.05$).

Animals. Female +*Lepr*^{db}/+*Lepr*^{db} (*db/db*) mice were purchased from Japan CLEA. For the immunoblot and gene expression analysis, the mice at the age of 7 weeks were fasted overnight and then injected with 3 μg/g body weight (BW) of recombinant adiponectin intraperitoneally. At 4 or 8 h after injection, their livers were removed. The mice homozygous for a conditional floxed allele of LKB1 (hereafter *LKB1*$^{lox/lox}$ mice) were generated as described previously [17]. The female *LKB1*$^{lox/lox}$ mice were subjected to experiments at the age of 7 weeks. The Animal Care Committee of the University of Tokyo approved the animal care and experimental procedures.

Quantitative real-time PCR. The total RNA was extracted from the liver or cultured cells by using an RNeasy kit (QIAGEN). cDNA was prepared by Taqman Reverse Transcription Reagents (Applied Biosystems). Quantitative real-time PCR was performed with ABI Prism by using PCR Master Mix Reagent (Applied Biosystems). Levels of mRNA were normalized to those of cyclophilin mRNA. The sequences of the probes and primers used are as follows; SREBP1c fwd:AAGCTGTCGGGGTAGCGTC, rev: GAGCTGGAGCATGTCTTCAA, probe:ACCACGGAGCCATGGATTGCACATT;cyclophilin fwd: GGTCC TGGCATCTTGTCCAT,rev: CAGTCTTGGCAGTGCAGATAAAA, probe: CTGGACCAAACACAAACGGTTCCCA. The primers and probes of the other genes examined were purchased from Applied Biosystems.

Cells and cell culture. Fao cells were cultured in 6 cm dishes (CORNING) with RPMI1640 medium containing 10% (vol./vol.) fetal bovine serum (GIBCO) and 50 units/ml of penicillin/streptomycin. After equilibration with serum free medium overnight, the cells were incubated with 25 μg/ml of adiponectin.

Transfections and luciferase assays of SREBP1c promoter activity. Luciferase reporter plasmid harboring 2.6 Kbp of 5′-flanking region of mouse SREBP1c exon 1 subcloned to pGL2 basic vector was kindly provided by H. Shimano, Tsukuba University, Tsukuba, Japan. Fao cells placed onto a 24-well plate (CORNING) were transfected with 1 μg of the luciferase reporter plasmid and 0.02 μg of Renilla luciferase plasmid with HSV-TK promoter (phRL-TK, Promega) by using Lipofectamine 2000 (Invitrogen). On the 4th day after overnight starvation, the cells were stimulated with reagents. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacture's protocol.

Generation and infection of adenoviruses. The adenoviruses encoding siAdipoRs were generated according to the manufacture's protocol (TaKaRa Biotechnology). The sequences of target genes were; siAdipoR1, GACGATGCTGAGACCAAAT; siAdipoR2, CCCGACT CTTCTCTAAATTG. An adenovirus encoding shRNA sequence for GFP was used for their control. The adenovirus encoding a dominant negative mutant of AMPKα1 subunit, Cre recombinase and

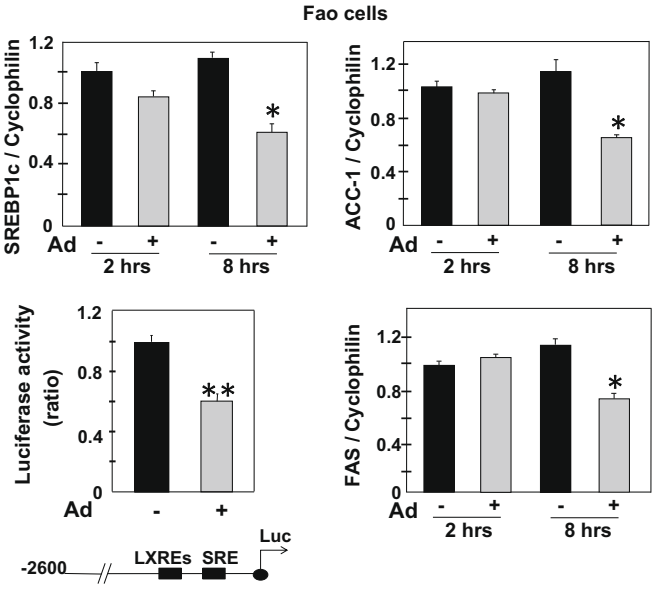

Fig. 2. Adiponectin stimulation acutely suppresses the mRNA expressions involved in fatty acid synthesis in Fao cells. Fao cells are incubated with adiponectin for 2 or 8 h. Each bar represents the mean ± S.E. ($n = 9$, $^{*}P < 0.05$). For luciferase assay, Fao cells transfeced with the reporter plasmid were incubated with adiponectin and 40 pM of insulin. Each bar represents the mean ± SE ($n = 3$, $^{**}P < 0.005$).

LacZ were prepared as previously described [12,17]. The mice were injected with the adenoviruses at a dose of 4×10^9 PFU/gBW for AdipoR knockdown, or at a dose of 0.9×10^{12} PFU/body for LKB1 gene deletion. On the 7th day, the mice were subjected to the experiments.

Immunoprecipitation and immunoblotting. The liver lysates were extracted with the homogenization buffer [18]. A total of 7 mg of liver protein for adipoR1 and 15 mg of liver protein for AdipoR2 were immunoprecipitated with 0.5 μg of the respective antibodies (prepared by IBL Japan). The samples were prepared with Laemmli buffer without boiling. Fao cells were lysed with buffer A [18]. The lysates or the precipitates were subjected to Western blotting by using antibodies for AdipoRs, AMPK (Cell Signaling Technology) and phosphoAMPK (Cell Signaling Technology). The blot was detected by using a chemiluminescence (ECL) system (Roche Molecular Biochemicals).

Statistical analysis. Statistical analysis was performed by 2-sample *t*-test assuming unequal variances or paired 2-sample *t*-test for means. Statistical significance was accepted at $P < 0.05$ unless otherwise indicated.

Results and discussion

Adiponectin suppressed the expression of SREBP1c in the liver of db/db mice

We used *db/db* mice to evaluate the effects of adiponectin on hepatic lipid metabolism. These mice not only show obesity and

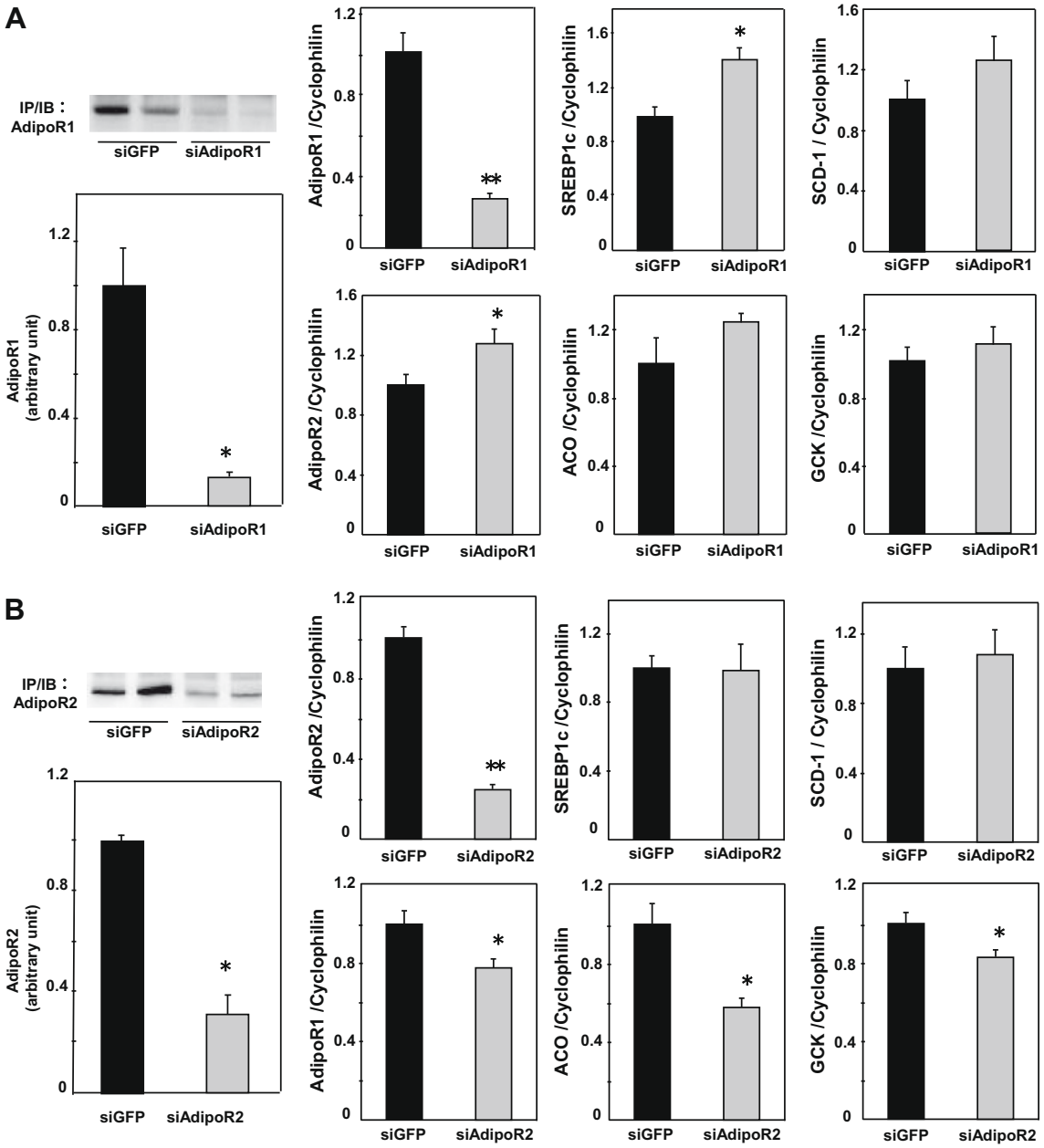

Fig. 3. Adenovirus-mediated gene transfer of siAdipoR1, not siAdipoR2, upregulates SREBP1c expression. The livers were removed from the *db/db* mice on the 7th day after injection of an adenovirus encoding (A) siAdipoR1 or (B) siAdipoR2. The left panels show the representative blot and the results of the quantificational analysis of AdipoRs. Each bar represents the mean ± SE ($n = 4$, $^{*}P < 0.005$). The right panels show the changes in gene expressions in the liver. Each bar represents the mean ± SE (subjects $n = 13$, except for the control group in siAdipoR1 experiment, where $n = 12$, $^{*}P < 0.005$, $^{**}P < 0.0001$).

several characteristics of the metabolic syndrome [19], but also possess some additional advantages; the hyperinsulinemic and hyperglycemic phenotypes make it feasible to observe the direct effects of adiponectin on fatty acid metabolism, apart from the possible secondary changes in the metabolic parameters [1–3,20]. Furthermore, adiponectin could interfere with the central leptin signaling [16], which substantially affects fatty acid metabolism in the liver [21,22]. Thus, *db/db* mice, which lack the leptin receptor, are appropriate for us to rule out the possible involvement of the interactions between these two adipokines. In the first place, we administered recombinant adiponectin to *db/db* mice intraperitoneally. The administration of adiponectin caused significant suppression of SREBP1c mRNA at 4 h in the liver (*left panel*, Fig. 1). At 8 h, the mRNA expressions of ACC-1 and SCD-1, the genes involved in fatty acid synthesis and regulated by SREBP1c expression, were also reduced (*right panel*, Fig. 1). The suppression of these genes was not attributed to the changes in the plasma insulin or glucose concentrations, both of which remained unchanged during the entire time course as expected (data not shown).

Adiponectin suppressed the expression of SREBP1c and lipogenic enzymes in Fao cells

Next we stimulated Fao cells, a well differentiated hepatoma cell line [23] with adiponectin. Adiponectin suppressed the expressions of SREBP1c in a time-dependent manner (*upper left panel*,

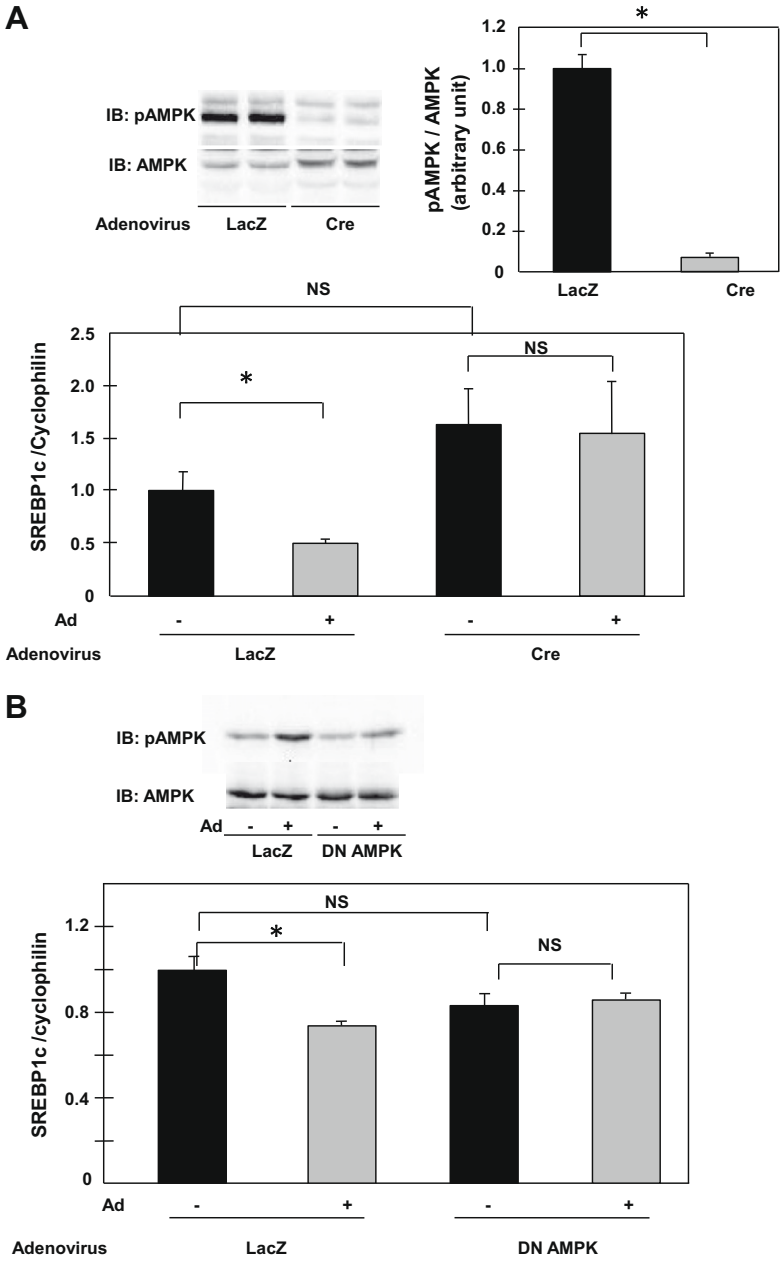

Fig. 4. Inhibition of AMPK phosphorylation abolishes SREBP1c suppression by adiponectin. (A, *upper panel*) Gene deletion of LKB1 was performed by adenovirus-mediated Cre expression in *LKB1*$^{lox/lox}$ mice. The panels show the representative blot and quantification of the phosphoAMPK/AMPK intensity of immunoblot analysis. Each bar represents the mean ± SE (subjects, $n = 5$; control, $n = 4$; $^{*}P < 0.05$). (A, *lower panel*) The mRNA expression of SREBP1c was measured 4 h after adiponectin treatment under LKB1 gene deletion. Each bar represents the mean ± SE ($n = 5$ except for the Cre-treated control mice, where the number of mice is 4, $^{*}P < 0.05$). (B, *upper panel*) The Fao cells were incubated with adiponectin under adenovirus-mediated overexpression of LacZ or DN-AMPK. The panels show the blot of the phosphoAMPK/AMPK. (B, *lower panel*) The mRNA expression of SREBP1c was measured in the Fao cells after 8 h incubation with adiponectin. Each bar represents the mean ± SE ($n = 3$, $^{*}P < 0.05$).

Fig. 2), and of other lipogenic genes such as ACC-1 and FAS at 8 h (*right panel*, Fig. 2). Next we conducted the reporter assay by using the plasmid harboring putative promoter region of SREBP1c (−2600-0 bp). In Fao cells, adiponectin significantly suppressed the SREBP1c promoter activity at 4 h (*lower left panel*, Fig. 2), consistent with the decrease in its mRNA expression. These *in vitro* assays strongly suggest that adiponectin directly suppresses the expressions of SREBP1c and its downstream enzymes in hepatocytes.

Adenovirus-mediated gene transfer of siAdipoR1 upregulated SREBP1c expression in the liver of db/db mice

Next we examined whether adiponectin suppressed SREBP1c by its functional receptors: AdipoR1 and AdipoR2. AdipoR1 has been reported to be ubiquitously expressed in various tissues, while AdipoR2 relatively abundant in the liver [13]. We constructed adenoviruses encoding siRNA for AdipoR1 and AdipoR2, and examined which receptor was responsible for the suppression of SREBP1c. Adenovirus-mediated gene transfer of siAdipoR1 and siAdipoR2 caused robust suppression of the respective receptor in the liver of *db/db* mice (*left panels*, Fig. 3A and B). With AdipoR1 suppressed, the expression of SREBP1c mRNA was significantly upregulated, and the SCD-1 mRNA expression also tended to be increased (*right panel*, Fig. 3A). There were no differences in the plasma glucose levels between the two groups (70.8 ± 7.0 vs. 72.8 ± 4.4 mg/dl, $n = 13$, $P = 0.81$). The plasma insulin concentration tended to be higher in the siAdipoR1-treated mice, though the difference did not reach a statistical significance (3221 ± 814.4 vs. 5378 ± 1041 pg/ml, $n = 13$, $P = 0.102$). On the other hand, the suppression of AdipoR2 did not alter SREBP1c expression in the liver, while the expressions of ACO and glucokinase (GCK) were significantly reduced (*right panel*, Fig. 3B). These results indicate that the suppression of SREBP1c by adiponectin is mediated by its functional receptor, and that AdipoR1, not AdipoR2, could account for the action. These are consistent with our recent report, showing that AdipoR1 knockout mice and AdipoR1/R2 double knockout mice showed elevated hepatic SREBP1c expression, while AdipoR2 knockout mice did not [24].

Adiponectin suppressed SREBP1c expression via activating AMPK in hepatocytes

Next, we investigated the mechanisms whereby adiponectin suppressed SREBP1c. It has been reported that adiponectin activates AMPK via AdipoR1 [12,24]. 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a pharmacological activator of AMPK, suppresses SREBP1c in hepatocytes [25], while the disruption of LKB1, one of the major upstream kinases of AMPK, caused a nearly complete loss of AMPK phosphorylation in the liver and led to significant elevation of the lipogenic genes [17]. These data prompted us to investigate whether adiponectin suppressed SREBP1c in hepatocytes via LKB1/AMPK pathway. LKB1 disruption in the liver was conducted by injecting an adenovirus expressing Cre recombinase to *LKB1*$^{lox/lox}$ mice, which led to robust inhibition of the basal AMPK phosphorylation (*upper panel*, Fig. 4A). LKB1 deletion led to elevated basal SREBP1c expression, and completely abolished the adiponectin-induced suppression of SREBP1c (*lower panel*, Fig. 4A). We also overexpressed the dominant negative mutant of AMPKα1 subunit (DN-AMPK) in Fao cells, and confirmed that the adiponectin-induced suppression of SREBP1c was abolished (Fig. 4B). These data suggest that adiponectin suppresses SREBP1c expression through the pathway composed of AdipoR1, LKB1 and AMPK, and also show that the AMPK activation by adiponecctin necessitates LKB1 as its upstream kinase.

What are the implications of SREBP1c suppression by adipnectin? Under physiological conditions, plasma glucose and insulin stimulates SREBP1c expression and fatty acid synthesis in the fed state, whereby excess energy is stored in the form of triglycerides in the liver. In our observation, plasma adiponectin concentration and the expressions of AdipoRs are elevated in the fasted state (unpublished data), suggesting that adiponectin action is physiologically more potent in the fasted state. Accordingly it is hypothesized that the suppression of SREBP1c by adiponectin could minimize excess energy storage in the liver in the fasted state, thereby allowing peripheral tissues to efficiently utilize the lipids as an energy source.

In contrast, under pathological conditions, the low expression of adiponectin and its receptors is thought to contribute to the pathogenesis of insulin resistance and the metabolic syndrome [8–11,26]. As shown in the current study, the attenuated action of adiponectin leads to upregulated SREBP1c expression, which is supposed to be one of the causal factors of fatty liver and insulin resistance. Interestingly, fatty acid synthesis is rather increased due to the compensatory hyperinsulinaemia, despite the downregulation of many insulin actions [5,27]. Our data suggest that the decreased adiponectin action could also, at least in part, contribute to the development of this paradoxical increase in SREBP1c expression and hepatic lipid accumulation in obesity-induced insulin resistance, although the precise elucidation awaits further experiments.

In summary, we here report for the first time that adiponectin suppresses SREBP1c expression in hepatocytes via AMPK activation through AdipoR1. The possible regulation of fatty acid synthesis by adiponectin, together with enhanced fatty acid oxidation, could be one of the mechanisms whereby adiponectin maintains insulin sensitivity.

Acknowledgments

We thank Dr. H. Shimano for the reporter plasmid used for the measurement of SREBP1c promoter activity, and F. Takahashi and H. Chiyonobu for their excellent technical assistance. This work was supported by a Grant-in-Aid for Creative Scientific Research from the Japan Society for the Promotion of Science (10NP0201) (to T.K.), a grant from Special Coordination Funds for Promoting Science and Technology, a grant from the Juvenile Diabetes Foundation International (1-2003-746) (to T.K.), a Grant-in-Aid for the Development of Innovative Technology from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T.K. and K.U.), Health Science Research grants (Research on Human Genome and Gene Therapy) from the Ministry of Health and Welfare, a grant from Takeda Science Foundation (to K.U.).

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