

Adiponectin suppresses endoplasmic reticulum stress in nonalcoholic steatohepatitis

TAKATO UENO¹, ANNA NAKAMURA¹, HITOMI NAKAYAMA², SHUICHI OTABE², XIAOHONG YUAN², TOMOKA FUKUTANI², HIDEKI IWAMOTO³, TORU NAKAMURA³, HIRONORI KOGA³, TAKUJI TORIMURA³, MICHIO SATA³ and KENTARO YAMADA²

¹Research Center for Innovative Cancer Therapy, Kurume University; ²Division of Endocrinology and Metabolism, and ³Division of Gastroenterology, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Received July 24, 2011; Accepted August 22, 2011

DOI: 10.3892/etm.2011.348

Abstract. In this study, we examined whether adiponectin suppresses endoplasmic reticulum (ER) stress in nonalcoholic steatohepatitis (NASH) using male transgenic mice expressing nSREBP-1c in adipose tissue, nSREBP-1c/adiponectin double-transgenic mice expressing human adiponectin in the liver, and wild-type male mice as the control. Histological findings similar to those observed in liver specimens from patients with NASH were observed in the livers from the nSREBP-1c transgenic mice at 30 weeks of age. By contrast, the NASH-like liver histology was markedly attenuated in age-matched nSREBP-1c/adiponectin double-transgenic mice. The nSREBP-1c/adiponectin double-transgenic mice showed human adiponectin production in the liver and a restored circulating human adiponectin level. Human adiponectin messenger ribonucleic acid (mRNA) expression in the liver was identified in the nSREBP-1c/adiponectin double-transgenic mice, but adiponectin receptor 1 and 2 mRNA expression in the liver was normal. TNF α mRNA was decreased in the liver of the nSREBP-1c/adiponectin double-transgenic mice compared with the nSREBP-1c transgenic mice. The protein expressions of X-box-binding protein-1, activating transcription factor 4, acetyl-CoA carboxylase, TNF α and NF κ B were down-regulated in liver tissues from the nSREBP-1c/adiponectin double-transgenic mice. Mouse adiponectin and activating transcription factor 6 expressions were almost the same in the three groups. Post-load plasma glucose levels were significantly lower in the nSREBP-1c/adiponectin double-transgenic mice compared with the nSREBP-1c transgenic mice. These results indicate that adiponectin expressed in the liver suppresses ER stress and attenuates hepatic steatosis, inflammation and insulin resistance in NASH. Adiponectin may open the way to novel therapies for human NASH.

Introduction

The liver plays a central role in whole-body lipid metabolism by governing the synthesis, oxidation, transport and excretion of lipids. Unfolded protein response (UPR) was identified as a signal transduction system that is activated by endoplasmic reticulum (ER) stress (1). ER stress and activation of UPR have been linked to numerous human disorders, including obesity, type 2 diabetes and cancer (2). In addition, Rutkowski *et al* showed that unresolved ER stress contributes to metabolic dysfunction and hepatic steatosis (3).

UPR is a signaling system emanating from ER that is activated when ER protein folding is disturbed (4). Previous studies have revealed novel diverse functions of mammalian UPR, including its role in hepatic lipid metabolism (3). UPR activation has been observed in fatty liver diseases, suggesting the induction of ER stress in these conditions (5). Previous studies have demonstrated that ER stress activates the sterol regulatory element-binding proteins (SREBPs), transcription factors involved in *de novo* lipid biosynthesis (6). SREBPs play a significant role in cholesterol metabolism and LDL receptor expression (SREBP-2), as well as fatty acid and triglyceride biosynthesis (SREBP-1) (7). We have previously reported that transgenic mice expressing nuclear SREBP-1c (nSREBP-1c) in adipose tissue under the control of the aP2 promoter, an inherited lipodystrophic model with insulin resistance and fatty acids, spontaneously develop steatohepatitis (8).

Adiponectin is a hormone mainly produced by adipose tissue (9). Experimental studies have suggested that adiponectin plays a major role in the pathophysiology of insulin resistance and metabolic lipid storage and lipolysis in insulin-sensitive tissues, which may induce an increased flux of free fatty acids from adipose tissue to the liver and cause steatosis (10). Adiponectin plays a major role in the pathophysiology of insulin resistance and metabolic syndrome.

We have previously reported that the nSREBP-1c/adiponectin double-transgenic mice show hepatic adiponectin transgenically expressed in the liver, and nonalcoholic steatohepatitis (NASH)-like hepatic lesions were markedly attenuated in age-matched double-transgenic mice (11). In addition, Awazawa *et al* showed that adiponectin suppresses hepatic SREBP1c expression in an adiponectin receptor

Correspondence to: Dr Takato Ueno, Research Center for Innovative Cancer Therapy, Kurume University, 67 Asahi-machi, Kurume, Fukuoka 830-0011, Japan
E-mail: takato@med.kurume-u.ac.jp

Key words: nonalcoholic steatohepatitis, adiponectin, endoplasmic reticulum stress

(AdipoR)1/LKB1/AMPK-dependent pathway (12). In this study, we examined whether adiponectin suppresses ER stress in NASH.

Materials and methods

Preparation of nSREBP-1c transgenic mice and nSREBP-1c/adiponectin double-transgenic mice. Transgenic mice (C57BL/6 background) expressing nSREBP-1c in adipose tissue (13) were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and bred in our laboratory by crossing with wild-type C57BL/6 mice (Nippon Clea, Shizuoka, Japan). Generation of transgenic mice expressing full-length human adiponectin in the liver of C57BL/6 background mice was previously described (14). Female mice that expressed nSREBP-1c in adipose tissue were crossed with adiponectin-expressing male mice to produce a nSREBP-1c/adiponectin double-transgenic line. The double-transgenic mice were identified by polymerase chain reaction (PCR) of tail DNA using nSREBP-1c-specific primers (5'-CTA CATTCGCTTTCTGCAAC-3' and 5'-ATAGAAGGACACC TAGTCAG-3') and human adiponectin transgenic specific primers (5'-TGAATTCGGGCTCAGGATGCTGTTGCT-3' and 5'-AGGATCCTGATCAGTTGGTGTTCATGGTA-3'). Male mice heterozygous for nSREBP-1c and human adiponectin were used in the following experiments. All mice were fed standard mouse chow (347 kcal/100g, protein 24.9 g/100 g, fat 4.6 g/100 g; Nippon Clea) and water *ad libitum*. Body weight was measured prior to sacrifice. All procedures were approved by the Ethics Review Committee for Animal Experimentation of Kurume University School of Medicine.

Biochemical assays. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride and total cholesterol levels were determined by spectrophotometric enzyme assays using peroxidase, lipoprotein lipase and cholesterol oxidase, respectively (Wako Ltd., Osaka, Japan). Glucose tolerance was assessed using an intraperitoneal glucose tolerance test (IPGTT). The IPGTT was performed by injecting glucose (1 g/kg in 10% solution) intraperitoneally in overnight-fasted mice. Glucose levels in blood obtained from the tail veins were measured by glucose dehydrogenase methods using Free Style (Nipro, Osaka, Japan) at 0, 30, 60 and 120 min following glucose injection. Serum levels of mouse leptin and adiponectin were measured with enzyme-linked immunosorbent assay kits from R&D (Oxon, United Kingdom) and AdipoGen (Seoul, Korea), respectively.

Light microscopy. Paraffin-embedded sections of the liver were stained with hematoxylin and eosin for standard microscopy or the Azan-Mallory stain to observe the location of the extracellular matrix in the liver tissues. The specimens were reviewed by a hepatopathologist. Each specimen was assigned to one of the following histological subgroups for the purposes of comparative analysis: type 1, fatty liver alone, which was predominantly macrovesicular in more than 33% of the lobules; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning hepatocytes; type 4, fat accumulation, ballooning hepatocytes, and either Mallory's hyaline or fibrosis. We dealt with types 3 and 4 as NASH, as previously described by Matteoni *et al* (5).

Electron microscopy. Passaged and cryopreserved HSCs were fixed in 1% glutaraldehyde for 1 h at 4°C, and post-fixed in 1% osmic acid for 1 h at 4°C, dehydrated with ethanol and embedded. They were then sectioned and stained with lead citrate. The samples were then observed under an electron microscope (H-7650, Hitachi, Tokyo, Japan).

Real-time PCR analysis. For real-time quantitative PCR, the messenger ribonucleic acid (mRNA) levels of mouse adiponectin and AdipoR1 and 2, and human adiponectin were assayed using the 7000 sequence detection system ABI Prism sequence detector (Applied Biosystems, Tokyo, Japan), and the double-strand specific dye SYBR-Green (Applied Biosystems). The PCR conditions and cycles were as follows: initial DNA denaturations for 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 sec, followed by an annealing step and then extension at 60°C for 1 min. Each point was performed in triplicate. To ensure that the primers produced a single and specific PCR amplification product, a dissociation curve was generated during the PCR cycle and only primers with a unique dissociation peak were selected, followed by migration on a 2% agarose gel to ensure that the PCR product was unique. The amplification efficiency for each primer pair was calculated. The expression level of each gene was adjusted by the level of GAPDH and expressed as the ratio to GAPDH.

Western blot analysis. To determine whether there was any synergy between human and mouse adiponectins, TNF receptor 1 (TNFR1), pNFκB, acetyl-CoA carboxylase (pACC) and ER stress-related agents, such as X-box-binding protein-1 (XBP-1) and activating transcription factor-4 (ATF-4) and ATF-6, we performed Western blot analysis of whole cell protein extracts. Cells were lysed in RIPA buffer. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel, and then blotted on a PVDF membrane. The membrane was immunoblotted. Immunoreactive bands were visualized using ECL detection reagents. The cells were incubated in a RIPA lysis buffer for 20 min. Following determination of protein concentrations using a BCA protein assay kit (Pierce, Rockford, IL, USA), the samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes. First they were reacted with primary antibodies and then with peroxidase-conjugated secondary antibody (dilution 1:1000) (GE Healthcare, Buckinghamshire, UK). Antigens were visualized by enhanced chemiluminescence using the ECL Western blotting detection system (Amersham, San Francisco, CA, USA).

Statistical analysis. Numerical data were expressed as the means ± SD. Unpaired Student's t-test was performed to assess statistical significance between groups. A value of P<0.05 was considered to be statistically significant.

Results

Biochemical assays. At 20 weeks of age, serum AST and ALT levels were elevated in the nSREBP-1c transgenic mice compared with the wild-type and nSREBP-1c/adiponectin transgenic mice. AST levels were highest in the nSREBP-1c

transgenic mice, and were significantly reduced in the nSREBP-1c/adiponectin transgenic mice. ALT elevation was attenuated to normal levels in the nSREBP-1c/adiponectin transgenic mice, although the difference was not statistically significant (Fig. 1A). No significant difference was observed in total cholesterol or triglyceride levels between the nSREBP-1c transgenic mice and the nSREBP-1c/adiponectin transgenic mice (Fig. 1B).

The intraperitoneal glucose (1 g/kg in 10% solution) tolerance test performed at 20 weeks of age showed significantly lower plasma glucose levels in the nSREBP-1c/adiponectin transgenic mice than in the nSREBP-1c transgenic mice at 30 and 60 min after glucose load (Fig. 2).

Circulating endogenous mouse adiponectin levels were slightly but significantly higher in the nSREBP-1c/adiponectin transgenic mice than in the nSREBP-1c transgenic mice (Fig. 3A). Human adiponectin levels in the nSREBP-1c/adiponectin transgenic mice at 20 weeks of age were significantly higher than in the other two groups (Fig. 3B).

Light microscopy. For light microscopy, sections were stained with hematoxylin and eosin staining or Mallory's Azan. Fat droplets, cell infiltration and pericellular fibrosis were decreased in the nSREBP-1c/adiponectin transgenic mice (Fig. 4A and B) compared with the nSREBP-1c transgenic mice (Fig. 4C and D).

Electron microscopy. The livers of SREBP-1c transgenic mice contain hepatocytes with degenerated nuclei, and small and large fat droplets. Enlarged ERs are visible in the magnified image (Fig. 5A). However, hepatocytes in the livers of the nSREBP-1c and adiponectin double-transgenic mice contain a few small fat droplets in the cytoplasm. However, the nucleus and organelles, such as mitochondria and ER, are similar to those of normal hepatocytes. These findings show that the ER stress was decreased in the double-transgenic mice (Fig. 5B).

Real-time PCR analysis. Real-time PCR analysis showed that human adiponectin expression was significantly increased in the liver of nSREBP-1c/adiponectin transgenic mice compared with the wild-type and nSREBP-1c transgenic mice (Fig. 6A). However, there was no significant difference in the mouse adiponectin mRNA expression between the wild-type, nSREBP-1c transgenic mice and nSREBP-1c/adiponectin transgenic mice (Fig. 6B).

We observed no significant difference between the nSREBP-1c transgenic mice and nSREBP-1c/adiponectin transgenic mice in mouse AdipoR1 and 2 expression levels (Fig. 6C and D).

Western blot analysis. In Western blot analysis, TNFR1 and phosphorylated NF κ B were decreased in the liver of the nSREBP-1c/adiponectin transgenic mice compared with the nSREBP-1c transgenic mice. It can be assumed that adiponectin suppresses the inflammation through the TNFR1 and NF κ B pathway in the liver of the nSREBP-1c/adiponectin transgenic mice (Fig. 7A).

In addition, the protein expressions of XBP-1, ATF-4 and phosphorylated ACC were decreased in the liver of the nSREBP-1c/adiponectin transgenic mice compared with

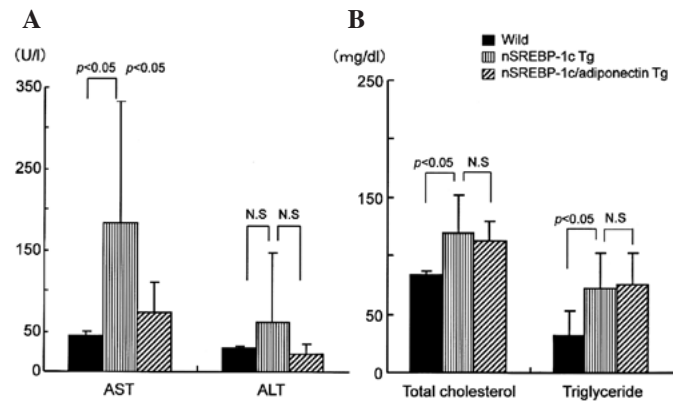


Figure 1. Serum transaminase and lipid levels at 20 weeks of age. (A) Serum AST and ALT levels were elevated in the nSREBP-1c transgenic mice compared with the wild-type and nSREBP-1c/adiponectin transgenic mice. AST levels were highest in the nSREBP-1c transgenic mice, and were significantly reduced in the nSREBP-1c/adiponectin transgenic mice. ALT level was normal in nSREBP-1c/adiponectin transgenic mice, although the difference compared with nSREBP-1c transgenic mice was not statistically significant. (B) No significant difference was observed in the total cholesterol or triglyceride levels between the nSREBP-1c transgenic mice and the nSREBP-1c/adiponectin transgenic mice.

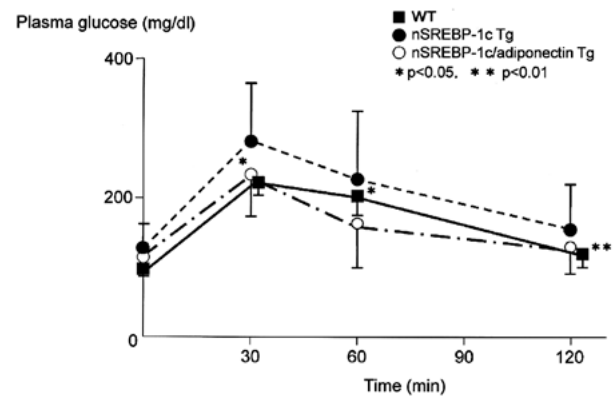


Figure 2. Intraperitoneal glucose tolerance test at 20 weeks of age. Intraperitoneal glucose (1 g/kg in 10% solution) tolerance test performed at 20 weeks of age showed significantly lower plasma glucose levels in the nSREBP-1c/adiponectin transgenic mice than in the nSREBP-1c transgenic mice at 30 and 60 min after glucose load.

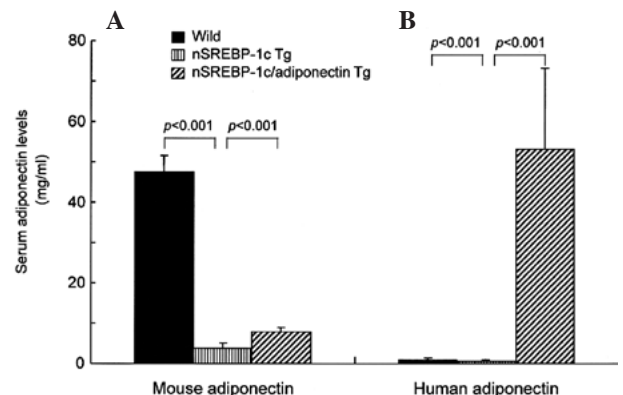


Figure 3. Circulating endogenous mouse adiponectin levels at 20 weeks of age. (A) Circulating endogenous mouse adiponectin levels were slightly but significantly higher in the nSREBP-1c/adiponectin transgenic mice than in the nSREBP-1c transgenic mice. (B) Human adiponectin levels of the nSREBP-1c/adiponectin transgenic mice at 20 weeks of age were significantly higher than in the other two groups.

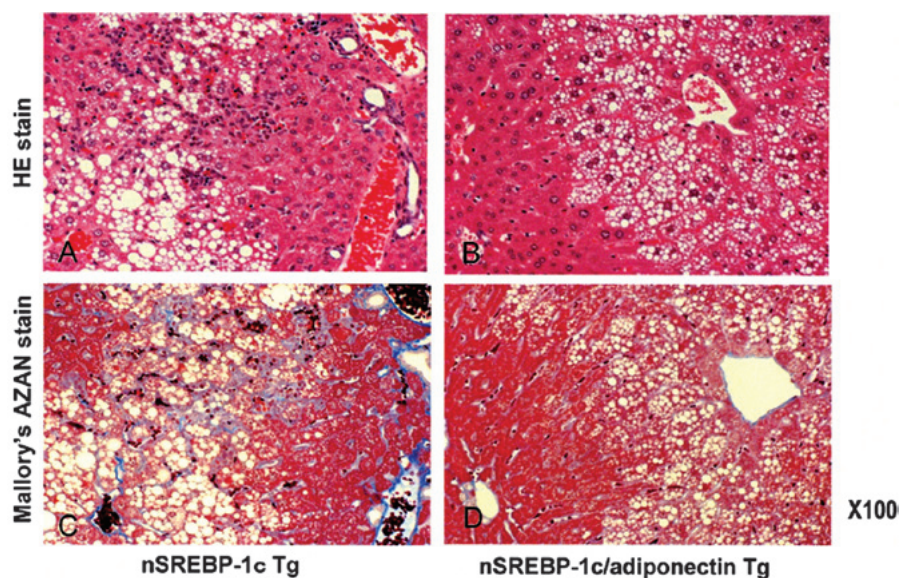


Figure 4. Light microscopy findings. (A and B) Fat droplets, cell infiltration and pericellular fibrosis were decreased in the nSREBP-1c/adiponectin transgenic mice compared with the (C and D) nSREBP-1c transgenic mice.

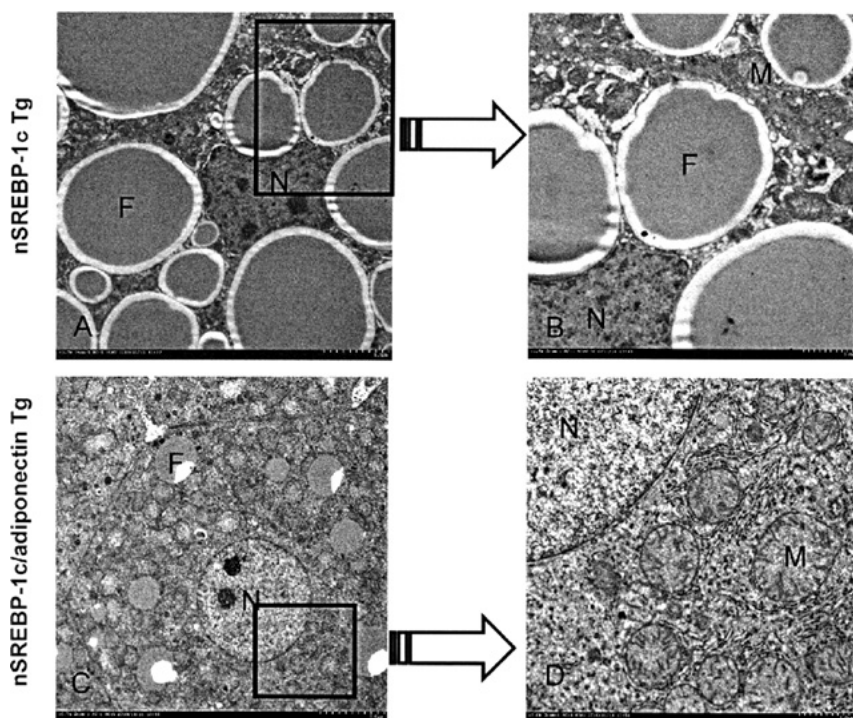


Figure 5. Electron microscopy findings. (A and B) In the liver of the SREBP-1c transgenic mice, hepatocytes with degenerated nuclei, small and large fat droplets, and, in the magnified image, enlarged endoplasmic reticula are visible. (C and D) However, in the liver of the nSREBP-1c and adiponectin double-transgenic mouse, only a few small fat droplets are located in the cytoplasm of the hepatocytes. However, the nuclei and organelles, such as mitochondria and ER, are similar to those of normal hepatocytes.

nSREBP-1c transgenic mice. However, ATF-6 protein expression in the three groups was almost the same (Fig. 7B).

Discussion

Our study shows that ER stress is enhanced in the liver of nSREBP-1c transgenic NASH model mice. Our findings also indicate that adiponectin suppresses ER stress through the XBP-1, ATF-4 and ACC pathway in the liver of the nSREBP-

1c/adiponectin transgenic mice. Adiponectin is known to suppress ER stress in obesity and diabetes (16,17). Recent studies have linked ER stress to obesity, insulin action and type 2 diabetes (18,19), and the activation of the UPR is governed by three ER transmembrane proteins: inositol-requiring kinase-1 (IRE1), protein kinase-like ER kinase (PERK) and activating transcription factor-6 (ATF6). Upon ER stress, IRE1 endonuclease facilitates the splicing of XBP-1 mRNA, leading to the translation of an activated version of the transcription

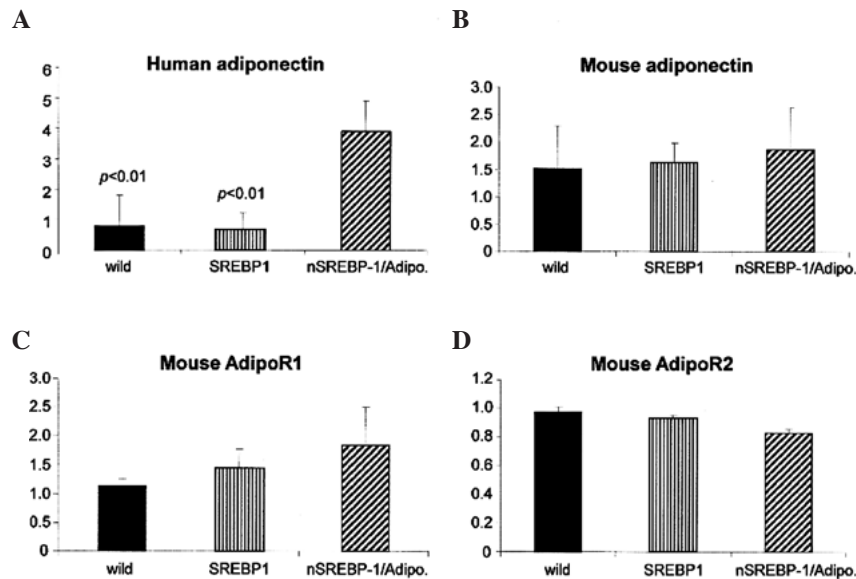


Figure 6. Real-time PCR analysis. (A) Human adiponectin expression was significantly increased in the liver of nSREBP-1c/adiponectin transgenic mice compared with the wild-type and nSREBP-1c transgenic mice. (B) However, there was no significant difference in the mouse adiponectin mRNA expression between the wild type, nSREBP-1c transgenic mice, and nSREBP-1c/adiponectin transgenic mice. (C and D) There was no significant difference between the nSREBP-1c transgenic mice and nSREBP-1c/adiponectin transgenic mice in mouse AdipoR1 and 2 expression levels.

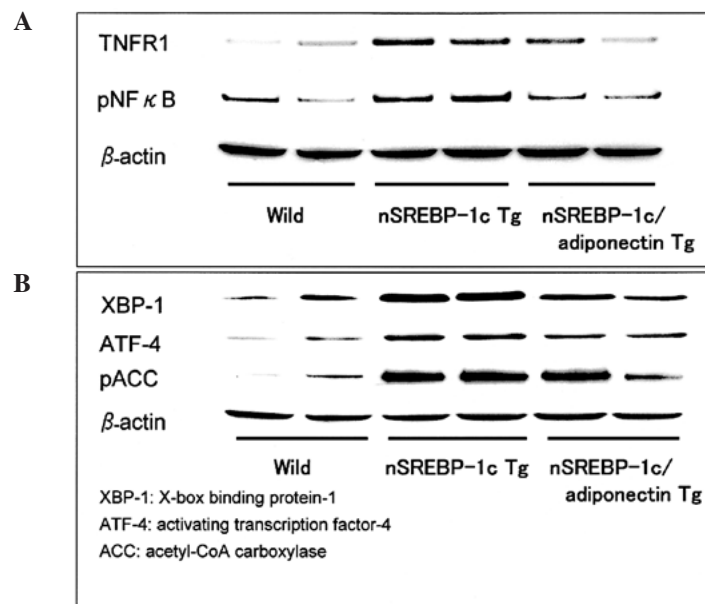


Figure 7. Western blot analysis. TNFR1 and phosphorylated NFκB were decreased in the liver of the nSREBP-1c/adiponectin transgenic mice compared with the nSREBP-1c transgenic mice. (A) It can be assumed that adiponectin suppresses the inflammation through the TNFR1 and NFκB pathway in the liver of the nSREBP-1c/adiponectin transgenic mice. In addition, the protein expression of XBP-1, ATF-4 and phosphorylated ACC were decreased in the liver of the nSREBP-1c/adiponectin transgenic mice compared with nSREBP-1c transgenic mice. (B) However, ATF-6 protein expression in the three groups was almost the same.

factor XBP-1. XBP-1's binding of one of the PI3K regulatory subunits, p85α or p85β, enhances nuclear entry of this transcription factor and induction of UPR target genes (20,21). The p85 subunit does not directly modulate other arms of the UPR, including the association of IRE1 with TRAF2 and the subsequent activation of JNK and PERK phosphorylation of eIF2 (p-eIF2), which represses protein synthesis coincident with preferential translation of the UPR transcriptional activator ATF4. However, the ATF6 arm of the UPR, which is involved

in proteolytic cleavage and release of the transcription factor into the nucleus, may be activated through association with p85 (22).

Although hypoadiponectinemia has been implicated in the development of human nonalcoholic fatty liver disease (NAFLD) and NASH (23), there was no direct evidence that adiponectin has preventive effects on spontaneously occurring NASH. In this study, the double-transgenic mice producing adiponectin in the liver showed normal or slightly elevated

serum levels of adiponectin. The double-transgenic mice normally expressed the two subtypes of AdipoRs, that is, AdipoR1 and 2, in the liver. Adiponectin expressed in the liver stimulates adenosine monophosphate-activated protein kinase activation and PPAR α signaling pathways through AdipoR1 and 2, respectively.

Adiponectin may also exert a protective effect through its anti-inflammatory action (9). Adiponectin is an adipocytokine secreted by mature adipocytes, and its receptors are widely distributed in a number of tissues, including the liver. Adiponectin has direct actions in the liver with prominent roles in improving hepatic insulin sensitivity, increasing fatty acid oxidation and decreasing inflammation. Adiponectin-null mice exhibit impaired liver regeneration and increased hepatic steatosis (24). Yoshiuchi *et al* directly evaluated the effects of the diabetic agent pioglitazone on *in vivo* ER stress under diabetic conditions. Pioglitazone treatment reduced the accumulation of fat droplets in the liver and attenuated the development of insulin resistance, that is, pioglitazone suppresses ER stress in the liver (25). Consequently, adiponectin may directly improve the pathogenesis of NAFLD, including NASH.

In conclusion, the transgenic mouse expressing nSREBP-1c in adipose tissue may serve as a unique model of NASH. The transgene of adiponectin in the liver of the nSREBP-1c transgenic mice induced the improvement of insulin resistance, ER stress, and intralobular inflammation and fibrosis. These observations may lead to the development of novel therapies for NASH in humans.

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