

Adiponectin gene therapy of streptozotocin-induced diabetic mice using hydrodynamic injection

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Abstract

Background Adiponectin (Adipo), an adipocyte hormone involved in the regulation of glucose and lipid metabolism, has already been identified as a potential therapeutic target for the treatment of diabetes. However, successful delivery of Adipo to the receptors is difficult due to their peptide characteristics. Receptors for Adipo are abundantly expressed in the liver and skeletal muscle.

Methods Uptake of 2-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) in hepatoblastoma HepG2 cells expressing Adipo was examined. Adipo-expressing plasmid DNA (10–50 µg) in saline solution (0.1 ml/g body weight) was rapidly injected into the tail vein of 4-week-old diabetic mice after 4–6 weeks of treatment with streptozotocin (STZ). Uptake of glucose in diabetic mice also was measured using a planar positron imaging system featuring 18-fluorodeoxyglucose.

Results HepG2 cells expressing Adipo exhibited significantly increased 2-NBDG uptake compared with cells transfected with control plasmid even in the absence of insulin. STZ-induced diabetic mice showed decreased serum Adipo levels compared with non-diabetic mice. A single hydrodynamic injection of 10–50 µg Adipo-expressing plasmid DNA into diabetic mice led to approximately 10–15-fold elevation in serum Adipo levels, and resulted in decreased serum levels of glucose and triglyceride. As well as exhibiting higher levels of Adipo expression, diabetic mice also had higher hepatic glucose uptake than similar mice injected with control plasmid.

Conclusions We report that STZ-induced diabetic mice exhibited decreased Adipo levels and hyperglycemia which may be alleviated by hydrodynamic injection of the Adipo gene. This type of gene delivery system to the liver offers a different approach in developing novel treatments for type 1 and 2 diabetes. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords adiponectin; gene expression; glucose-lowering effect; STZ-induced diabetic mice; glucose uptake; receptor targeting

Introduction

Adiponectin (Adipo) is an adipocyte hormone involved in glucose and lipid metabolism [1–4]. Impaired glucose and lipid metabolism, hallmarks of obesity and type 2 diabetes [5], promote excessive lipid storage in insulin target tissues, such as muscle and the liver, thereby leading to insulin



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resistance [6]. Cell-surface receptors of Adipo (AdipoR1 and AdipoR2) have been cloned [7] and their expression patterns revealed: AdipoR1 was abundantly expressed in skeletal muscle, whereas AdipoR2 was principally expressed in the liver [7]. The insulin-sensitizing effects of Adipo in insulin-resistant mice correlated with an increase in the activity of AMPK (5'-AMP-activated protein kinase), which could be directly related to the binding of Adipo to AdipoR1 and AdipoR2.

Adipo has already been identified as a potential therapeutic target for the treatment of diabetes and other obesity-related illnesses [8]. However, successful delivery of Adipo to these receptors is difficult due to their peptide characteristics. Use of adeno-associated virus vectors to mediate Adipo expression led to an improvement in insulin sensitivity in diet-induced obese rats [9] compared to non-obese rats [10], and this was due to an increase in AMPK activity as well as modulation of hepatic gluconeogenesis [10]. Recent advances in gene therapy have already targeted receptors. The hydrodynamic injection method can provide a route for efficient hepatic expression of transgenes in mice just by systemic administration of naked DNA [11–14]. However, to our knowledge, hydrodynamic injections of Adipo genes targeted to liver have not yet been reported. Therefore, we used this method to evaluate the usefulness of Adipo gene therapy to the liver for diabetes.

The effects of Adipo on mice with type 2 diabetes have been extensively studied [8,15], but the effects of Adipo on mice with type 1 diabetes are less clear. Therefore, at first we investigated Adipo levels in streptozotocin (STZ)-induced diabetic mice. We aimed to induce transfection of full-length Adipo plasmid DNA (pCMV-Ad) in liver, via hydrodynamic injection since full-length Adipo protein has a higher binding affinity to hepatic membrane fractions than globular Adipo [16].

We found that STZ-induced diabetic mice showed decreased Adipo levels, and that the gene delivery of pCMV-Ad in STZ-induced diabetic mice via hydrodynamic injection significantly increased serum and hepatic concentrations of Adipo, and decreased serum glucose and triglyceride levels in these mice. This glucose-lowering effect was likely to be directly related to an increase in glucose uptake in the liver.

Materials and methods

Cell culture

Human hepatoblastoma (HepG2) cells were obtained from the Riken Cell Bank (Ibaraki, Japan) and were grown in RPMI-1640 medium supplemented with 10% heat-inactivated serum.

Plasmid constructions

Complementary DNA was synthesized from the total RNA in mouse adipose tissue (Biochain Institute, CA,

USA). In the construction of pCMV-Ad, cDNA coding for bp 1–744 of mouse Adipo was amplified by polymerase chain reaction (PCR) using the following Adipo-specific primers: Adipo forward primer (5'-GAAAAGCTTaccATGCTACTGTTGCAAGCTCTC); Adipo reverse primer (5'-GAATCTAGATCAGTTGGT-ATCATGGTAGA). The forward primer contained a 3-bp optimal Kozak sequence (in lower-case letters) together with a *Hind* III restriction site (underlined). The reverse primer coding for bp 723–744 of Adipo had an *Xba* I restriction site (underlined). Cytomegalovirus (CMV) promoter DNA was amplified and digested with *Kpn* I and *Hind* III as previously reported [17]. After amplification, Adipo cDNA was digested with *Hind* III and *Xba* I, and ligated into a *Hind* III/*Xba* I-digested pGL3-enhancer (Promega, Madison, WI, USA). The plasmid was then digested with *Kpn* I and *Hind* III, and ligated with CMV promoter DNA [17]. In the construction of the plasmid pCMV-luc encoding luciferase gene, pGL3 enhancer was digested with *Kpn* I and *Hind* III, and was ligated with the CMV promoter. pGL3-basic encoding the luciferase gene without a promoter was obtained from Promega, and used as a control plasmid. A protein-free preparation of the plasmid was purified after alkaline lysis in Maxiprep columns (Qiagen, Hilden, Germany) without contamination with LPS (less than 0.1 EU/1 µg plasmid DNA).

Adipo expression and AMPK activity

HepG2 cells were cultured on 35-mm dishes. These cells were transfected with 2 µg of pCMV-Ad, or pGL3-basic as a control, using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA), and then incubated for 24 h. To reveal AMPK activation, the cells were treated with 1 mM 5-aminoimidazole-4-carboxamide-1-D-riboside (AICAR) (Wako, Osaka, Japan) for 0, 10, 30, 60 or 180 min. Cell protein extracts were prepared with 1 ml of sampling buffer containing 1% (v/v) Triton X-100, protease inhibitor cocktail set III and protein tyrosine phosphatase inhibitor II (Calbiochem, Darmstadt, Germany) in phosphate-buffered saline (PBS). They were separated by 7.5 or 12.5% (w/v) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Nippon Genetics, Tokyo, Japan). Membranes were blocked at room temperature for 1 h with PBS containing 0.1% (v/v) Tween-20 and 5% (w/v) skimmed milk, and subsequently incubated at room temperature for 1 h with rabbit anti-mouse adiponectin polyclonal antibody (Assay pro, MO, USA) or rabbit anti-phospho-AMPK-α (Thr-172) monoclonal antibody (Cell Signaling Technology, MA, USA). Membranes were then incubated with a horseradish peroxidase-conjugated, goat, anti-rabbit, IgG antibody (Santa Cruz Biotechnology, CA, USA) as a secondary antibody. To enhance the antibody signal, all antibodies were diluted with Can Get Signal (Toyobo, Osaka, Japan). The signal was

detected using a SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA). Mouse Adipo levels in cells and culture media were determined by Mouse/Rat adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan).

2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (2-NBDG) uptake

HepG2 cells were cultured on 96-well black cell culture dishes. In AICAR experiments, cells were incubated with 1 mM AICAR for 60 min. In transfection experiments, cells were incubated for 24 h after transfection of pGL3-basic or pCMV-Ad using Lipofectamine 2000. After AICAR treatment or DNA transfection, all cells were incubated with 50 μ M 2-NBDG (Molecular Probes, OR, USA) in PBS for 15 min, and then washed with additional PBS to remove excess 2-NBDG. Fluorescence in the cells was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Wallac ARVO SX 1420 multi-label counter (Perkin Elmer Life Sciences, Japan, Co. Ltd., Kanagawa, Japan).

Animal studies

Male ICR mice (aged 4 weeks, Tokyo Laboratory Animal Science, Co., Ltd., Tokyo, Japan) were used in this study. The mice were maintained in accordance with institutional guidelines of the Hoshi University Animal Care and Use committee. Each mouse received an intravenous injection of 200 mg/kg STZ to induce diabetes [18]. Mice were fasted for 3 h before commencing the experiment and prior to taking blood samples. Glucose concentration of each sample was measured using Medisafe Mini (Terumo, Tokyo, Japan). Mice with blood glucose levels over 500 mg/dl and 20–30g body weight were selected for testing 4 to 6 weeks after STZ treatment. To transfect pCMV-Ad into mice, we used a hydrodynamic injection method: Injection into the tail vein in 5 s with 2.5 ml of saline solution containing 1, 5, 10 or 50 μ g of pCMV-luc, pGL3-basic or pCMV-Ad [11,12]. Serum concentrations of insulin, glucagon and triglyceride (TG) were measured using an Ibis insulin ELISA kit (Sibayagi, Gunma, Japan), a glucagon EIA kit (Yauchihara, Shizuoka, Japan) and a triglyceride E test (Wako, Osaka, Japan), respectively. Serum Adipo levels were measured by ELISA, as described above. Twenty-four hours after plasmid injection, mice were anesthetized and perfused with saline through the left ventricle, and those that had received pGL3-basic or pCMV-Ad were sacrificed. Liver and skeletal muscle were harvested, homogenized with sampling buffer and centrifuged at 15 000 g. Supernatant fractions were subjected to Western blot analysis to detect expression of Adipo and phosphor-AMPK- α , and the concentration of Adipo was measured using the above method.

Distribution of 18-fluorodeoxyglucose (FDG) using a planar positron imaging system

An anesthetized mouse, which had been fasting for at least 12 h, was fixed onto the stage plate [19,20]. FDG, a short-lived glucose analogue labeled with fluorine-18 ($t_{1/2}$ = 110 min) (4 MBq/20 g), was injected intravenously into the STZ-induced diabetic mouse through the tail vein 18 to 24 h after hydrodynamic injection of 10 μ g of pGL3-basic or pCMV-Ad. After FDG injection, image data were acquired at 1-min frame intervals for 60 min with a PPIS-4800 planar positron imaging system (Hamamatsu Photonics, Hamamatsu, Japan). The distribution and kinetics of FDG *in vivo* were analyzed from a composite image accumulated during the last 20 min of data acquisition. The region of interest (ROI) was preset on the composite image, and the time-activity curves of radioactivity in the heart, liver and muscle tissues were monitored for 60 min after FDG injection.

Adipo receptor mRNA expression in the liver

Total liver RNA was isolated using the Quickprep Total RNA extraction kit (Amersham Biosciences, NJ, USA) 24 h after hydrodynamic injection of pGL3-basic or pCMV-Ad. UV spectrometry measurements at 260 nm and 280 nm were used to assess RNA yield, and RNA purity was analyzed by electrophoresis. Complementary DNA was synthesized from the total amount of RNA extracted from the liver, real-time PCR was performed with the iCycler MyiQ detection system (Bio-Rad Laboratories, Hercules, CA, USA), and a SYBR Green I assay (iQTM SYBER Green Supermix, Bio-Rad Laboratories) was used for quantification. PCR amplification required denaturation at 94 °C for 0.5 min, primer annealing at 58 °C for 0.5 min, and elongation at 72 °C for 1 min for 40 cycles. PCR amplification of the housekeeping gene β -actin, and AdipoR1 and AdipoR2 mouse Adipo receptors, were performed within the same cycle for all samples. For the amplification of mouse AdipoR1, the primers AdipoR1-FW, 5'-TGATGTGCTTCCTGACTGGCTGAAAGAC-3', and AdipoR1-RW, 5'-TCAAGCCAAGTCCCAGGAACATCCTGC-3', were used. For the amplification of mouse AdipoR2, the primers AdipoR2-FW, 5'-CGAATGGAAGAGTTTGTTT-GTAAGGTGT-3', and AdipoR2-RW, 5'-GATTCCACTCAG-GCCTAAGCCCACGAAC-3', were used. For the amplification of mouse β -actin, the primers β -actin-FW, 5'-ACCCACACTGTGCCATCTA-3', and β -actin-RW, 5'-CTGCTTGCTGATCCACATCT-3', were used. Samples were analyzed in triplicate and the expression level of Adipo receptor mRNA was normalized for the amount of β -actin in the same sample. A difference of one cycle was calculated to be the equivalent of a 2-fold change in gene expression.

Statistical analysis

Significant differences in mean values were evaluated using Student's unpaired *t*-test. A *P*-value of less than 0.05 was considered to be significant.

Results

Adipo gene transfection in HepG2 cell cultures

ELISA analysis was used to quantify Adipo levels in HepG2 cells transfected with pCMV-Ad, and its surrounding culture medium. High levels of Adipo protein were detected in cells that had been transfected with pCMV-Ad, whereas Adipo protein was not detected at all in both non-transfected and pGL3-basic-transfected cells (Figure 1). Samples taken 24 h post-transfection showed Adipo levels to be 183 ± 30 ng/ml in HepG2 cells and 107 ± 25 ng/ml in the culture medium when measured by ELISA (Figure 1).

Adipo stimulates the phosphorylation of AMPK and glucose uptake in HepG2 cells

Yamauchi *et al.* reported that Adipo activity requires liver AMPK activation in mice [16]. To investigate Adipo activity in pCMV-Ad-transfected HepG2 cells, the level of AMPK activation was studied. Cell phosphorylation of AMPK (p-AMPK) was measured using Western blot analysis. As a control, HepG2 cells were treated with AICAR, a cell-permeable activator of AMPK, which sustained the phosphorylation of Thr 172 in the α -subunit of AMPK (α -AMPK) in proportion to the incubation time (Figure 2a). Cells transfected with pCMV-Ad also exhibited an increase in p-AMPK (Figure 2b).

Next, to investigate glucose uptake by AMPK activation, uptake of 2-NBDG, a metabolizable fluorescent derivative of glucose, in HepG2 cells treated with AICAR or transfected with pGL3-basic or pCMV-Ad was measured. There was no increase in cellular uptake of 2-NBDG 1 h after treatment with 1 mM AICAR, but the level of uptake in cells transfected with pCMV-Ad had significantly increased compared with cells transfected with pGL3-basic (Figure 2c).

Reduction of Adipo levels in STZ-induced diabetic mice

Prior to transfection, time-dependent changes in serum Adipo levels in non-diabetic and STZ-induced diabetic mice were investigated for 4–6 weeks. As a murine model of type 1 diabetes, serum Adipo levels in diabetic mice declined within 2 weeks of STZ treatment and continued for a further 5 weeks, exhibiting overall lower serum

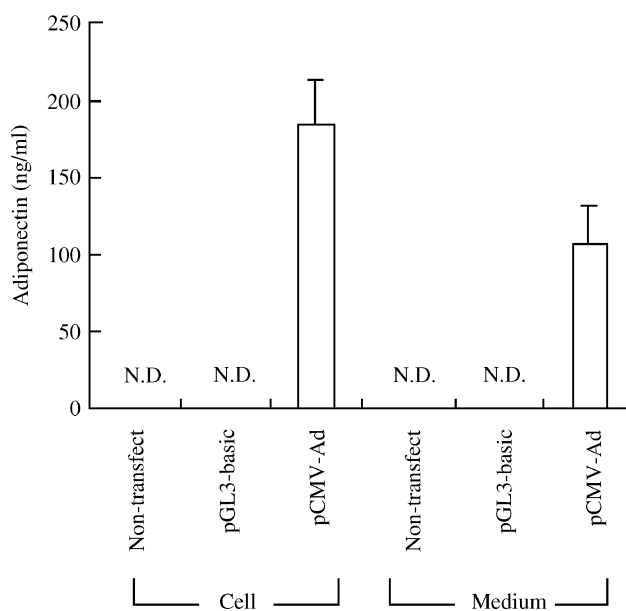


Figure 1. Adipo expression in HepG2 cells transfected with pCMV-Ad. Adipo levels in HepG2 cells and culture medium 24 h after transfection with pCMV-Ad were determined by ELISA; *n* = 3 for each sample. Each bar represents the mean \pm standard deviation (S.D.) N.D.: not detected

Adipo levels than non-diabetic mice (Figure 3a). This effect appeared 4–6 weeks after the treatment with STZ in mice aged 4 weeks, despite persistently high serum glucose levels (Figure 3b). Serum Adipo levels in non-diabetic mice were 15.0 ± 2.08 μ g/ml, while those in STZ-induced diabetic mice 4–6 weeks after STZ treatment were 7.10 ± 1.88 μ g/ml (Figure 3c). The concentration of serum glucose in non-diabetic mice was in the range of 150 to 200 mg/dl, while those of STZ-induced diabetic mice were over 500 mg/dl 1 week after STZ treatment (Figure 3b). Average serum concentration of insulin in non-diabetic mice was 2.60 ± 0.37 ng/ml, but the presence of insulin was not detected at all in serum samples from STZ-induced diabetic mice 4–6 weeks after treatment with STZ (Figure 3d).

Expression of Adipo in STZ-induced diabetic mice

To confirm gene expression had occurred in the liver post-hydrodynamic injection, plasmid DNA encoding luciferase (pCMV-luc) was administered by the same method into STZ-induced diabetic mice and non-diabetic control mice. Twenty-four hours post-injection, mice from both cohorts were sacrificed, and luciferase activity in the liver, kidneys, lungs, and spleen was measured. Luciferase activity in the liver was 100–1000-fold higher than the kidneys, lungs or spleen, but was not detected in the muscle in both non-diabetic and STZ-induced diabetic mice (data not shown). This result correlated with published data and indicated that hydrodynamic injection was an effective method for gene delivery to the liver [11,12].

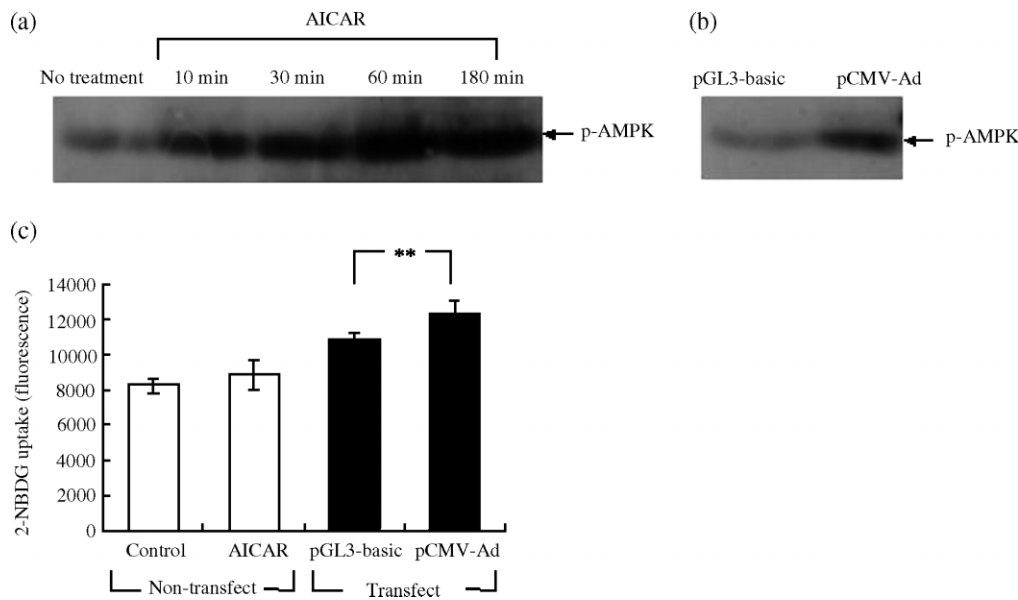


Figure 2. Western blot analysis showing increased AMPK activity in HepG2 cells treated with AICAR (a) or transfected with pCMV-Ad (a, b), and cells transfected with pCMV-Ad showing 2-NBDG uptake (c). (a) Phosphorylation of AMPK in HepG2 cells treated with 1 mM AICAR for 0, 10, 30, 60 and 180 min. (b) Phosphorylation of AMPK in HepG2 cells 24 h after transfection with 10 μ g of pGL3-basic or pCMV-Ad. (c) Cellular uptake of 2-NBDG for 15 min after 1 h treatment with 1 mM AICAR, or after 24 h transfection with either pGL3-basic or pCMV-Ad. Each bar represents the mean \pm S.D.; $n = 4$. ** $P < 0.01$; compared with pGL3-basic

The relationship between dose dependency and Adipo expression of plasmid DNA in STZ-induced diabetic mice was examined by administering hydrodynamic injections of pGL3-basic as control plasmid DNA, with 1, 5, 10 or 50 μ g of pCMV-Ad. Results showed that the transfection of pGL3-basic and 1 μ g of pCMV-Ad did not affect serum Adipo levels in STZ-induced diabetic mice, but transfection of 5 μ g or 10 μ g of pCMV-Ad significantly raised serum Adipo levels approximately 4-fold and 15-fold, respectively, 24 h after injection, when compared with pre-injection measurements (Figure 4a). Elevated Adipo levels were sustained up to the 48-h mark, before declining gradually during the 48–72-h post-injection interval (Figure 4a). Administration of 50 μ g of pCMV-Ad induced slightly higher serum Adipo levels than that of 10 μ g of pCMV-Ad at the 48-h and 72-h stage (Figure 4a). Comparing the effects of 10 μ g and 50 μ g of pCMV-Ad, no significant difference in Adipo levels was seen (142.8 ± 26.1 vs. 143.9 ± 29.1 μ g/ml) 24 h post-injection. Since Adipo levels seemed to plateau (Figure 4a), 10 μ g of pCMV-Ad was used in subsequent experiments except for the experiment shown in Figure 5a. All mice had been fasted for 3 h prior to pCMV-Ad injection or blood sampling.

Western blot analysis and ELISA were used to quantify the induced Adipo that had been expressed in the serum, liver and muscle of STZ-induced diabetic mice. After transfection of 10 μ g of pGL3-basic, Adipo was not detected in liver tissue, and the Adipo level in muscle was 5.24 ± 1.34 μ g/g (Figure 4b). After transfection of 10 μ g of pCMV-Ad, Adipo levels were 112.7 ± 41.3 μ g/g in liver and 16.04 ± 2.56 μ g/g in muscle (Figure 4b). Twenty-four hours after injection of 10 μ g of pCMV-Ad, non-diabetic mice exhibited Adipo levels of $156.0 \pm$

1.68 μ g/ml in blood serum, 273.1 ± 13.5 μ g/g in liver tissues, and 6.64 ± 0.86 μ g/g in muscle tissues (data not shown). The level of Adipo in liver was significantly higher than that in muscle, in STZ-induced diabetic mice as well as non-diabetic mice, after injection of 10 μ g of pCMV-Ad, which suggested that pCMV-Ad was mainly expressed in the liver and the induced Adipo protein was then secreted from that organ into the bloodstream.

Hepatic Adipo expression reduces serum glucose and triglyceride levels

The effect of hepatic Adipo expression on glycemia was observed by measuring serum glucose levels in STZ-induced diabetic mice after hydrodynamic injections of 10 μ g of pCMV-luc, 10 μ g of pGL3-basic, or 1, 5, 10 and 50 μ g of pCMV-Ad (Figure 5a). Mice transfected with either pGL3-basic or pCMV-Ad showed high glucose levels of over 600 mg/dl prior to injection. By 24 h, glucose concentration in mice transfected with 1 or 5 μ g of pCMV-Ad 24 h had decreased to 77 and 75% of the pre-injection levels, and those injected with 10 or 50 μ g of pCMV-Ad had decreased to 58 and 49% (531 ± 21 and 410 ± 73 mg/dl, respectively, $P < 0.05$) of the pre-injection concentration, while those injected with pGL3-basic or pCMV-luc did not exhibit such a marked reduction (802 ± 47 mg/dl). Although the glucose-lowering effect caused by 10 μ g of pCMV-Ad did not extend beyond 48 h, the effect of 50 μ g of pCMV-Ad was sustained beyond 48 h and up to 72 h, after which serum glucose concentration returned to pre-injection levels. Furthermore, the glucose-lowering effect of a dose of 10 μ g of pCMV-Ad was evident in non-diabetic mice because, at 24 h post-injection,

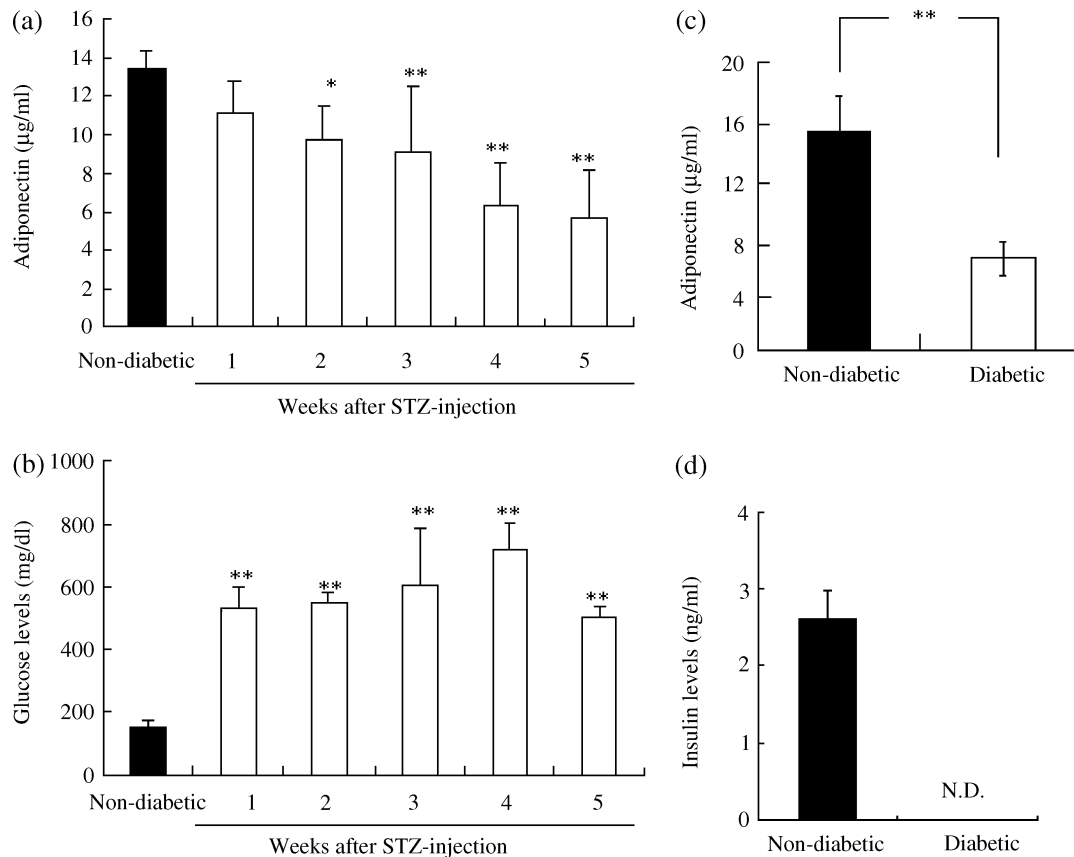


Figure 3. Serum Adipo, glucose and insulin levels in non-diabetic and type 1 diabetic model mice. Time-dependent change of serum Adipo, glucose levels in non-diabetic and type 1 diabetic model mice after treatment with STZ. Serum Adipo levels (a) and glucose levels (b) in non-diabetic and STZ-induced diabetic mice; $n = 7$ for non-diabetic mice (■); $n = 5$ for diabetic mice (□) (* $P < 0.05$; ** $P < 0.01$, compared with non-diabetic mice). Serum Adipo level in STZ-induced diabetic mice was measured once a week for 5 weeks after STZ treatment. Each bar represents the mean \pm S.D. (c) Serum Adipo levels in non-diabetic (■) and STZ-induced diabetic mice (□); $n = 4$ for non-diabetic mice; $n = 7$ for diabetic mice 4 to 6 weeks after treatment of STZ (** $P < 0.01$). Each bar represents the mean \pm S.E. (d) Serum insulin levels in non-diabetic (■) and STZ-induced diabetic mice (□) 4 to 6 weeks after treatment of STZ; $n = 3$ for each group. Each bar represents the mean \pm S.D. For (a–d), each animal was fasted for 3 h before a blood sample was taken. N.D.: not detected. In subsequent experiments, mice held for 4 to 6 weeks after treatment with STZ were used as STZ-induced diabetic mice

serum glucose had dropped from 170.4 ± 9.96 mg/dl to 110.8 ± 11.7 mg/dl (data not shown).

The effect of increasing serum Adipo on serum glucose levels was at its peak 24 h post-injection (Figure 5a) and serum concentrations of insulin, glucagon and triglyceride (TG), as well as AdipoR1 and AdipoR2 mRNA in the liver, were measured at this stage of the experiment. No insulin was detected in STZ-induced diabetic mice 24 h after transfection with either pGL3-basic or pCMV-Ad (data not shown). Serum glucagon levels of STZ-induced diabetic mice injected with 10 µg of pCMV-Ad showed a 40% increase in serum glucagon levels before injection, and this effect could be associated with a recovery in glucose levels (Figure 5b). Moreover, serum TG levels decreased 60% ($P < 0.05$, compared with before injection; Figure 5c), and AdipoR1 and AdipoR2 mRNA levels in the liver did not change (data not shown). These results demonstrated that high levels of Adipo in the liver had reduced the serum concentrations of glucose and TG, and that these effects had occurred even when insulin was not detected.

Adipo enhances glucose uptake in the liver

The glucose-lowering effect of Adipo was investigated by measuring glucose uptake in heart, liver and muscle tissues using a planar positron imaging system to visualize 18-fluorodeoxyglucose (FDG) after *in vivo* injections of either pGL3-basic or pCMV-Ad. FDG injected into the tail vein quickly reached the heart, then distributed from the liver toward the kidneys and, finally, toward the bowels. Mice with STZ-induced diabetes that had been injected with pCMV-Ad showed higher glucose accumulation in the liver, but not in muscle, for the last 20 min of the 60 min scan, when compared to similar mice injected with control plasmid (Figure 6a). In contrast, glucose accumulation in the liver was not seen after injecting non-diabetic mice with pCMV-Ad or control plasmid (data not shown). Liver FDG levels in STZ-induced diabetic mice transfected with pCMV-Ad were higher than those injected with pGL3-basic. Twenty-four hours post-injection, hepatic tissues of diabetic mice injected

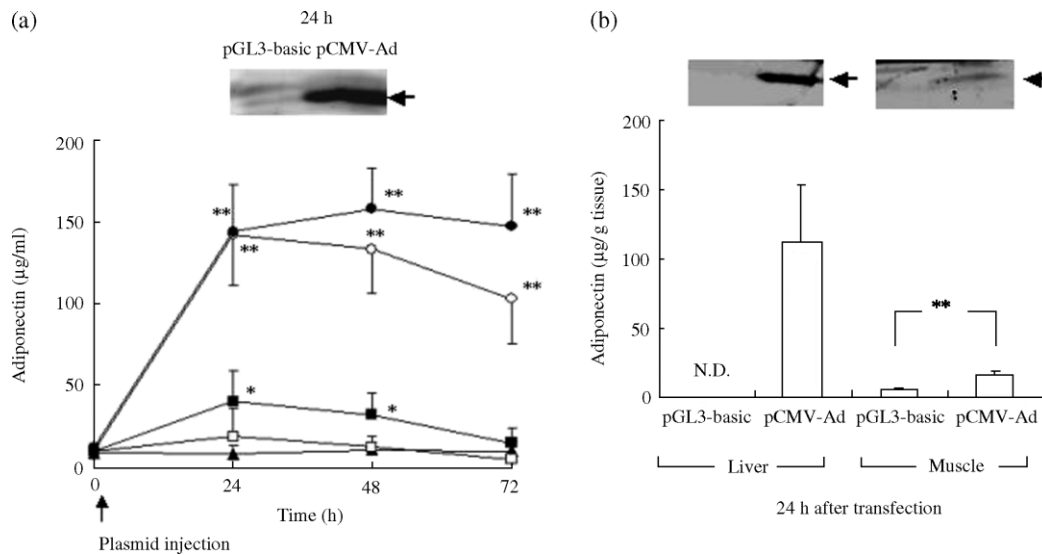


Figure 4. Adipo expression after hydrodynamic injection of pCMV-Ad in STZ-induced diabetic mice. (a) Serum Adipo levels after injection of 10 µg of pGL3-basic (▲) or 1 µg (□), 5 µg (■), 10 µg (○) or 50 µg of pCMV-Ad (●); $n = 3-6$ for each group. Each bar represents the mean \pm S.D. (* $P < 0.05$; ** $P < 0.01$, compared with before injection). Western blot analyses of Adipo in serum 24 h after injection of 10 µg of pGL3-basic or pCMV-Ad. (b) Adipo levels and Western blot analyses of Adipo in liver and muscle 24 h after injection of 10 µg of pGL3-basic or pCMV-Ad; $n = 3$ for each group. Each bar represents the mean \pm S.D. (** $P < 0.01$, compared with pGL3-basic). N.D.: not detected

with pCMV-Ad showed slightly stronger AMPK activation than similar mice injected with pGL3-basic (data not shown). Data suggests that high levels of Adipo expression in the liver were associated with increased hepatic glucose uptake (Figure 6b).

Discussion

Serum Adipo levels in STZ-induced diabetic mice were lower than non-diabetic mice, and could be increased by hydrodynamic injection of pCMV-Ad, which reduced serum glucose and TG levels in the absence of insulin. Furthermore, diabetic mice with higher levels of Adipo expression by injection exhibited higher hepatic glucose uptake than those featuring low levels of Adipo expression.

Adipo levels in STZ-induced diabetic mice were less than those in non-diabetic mice 4–6 weeks after treatment with STZ in mice aged 4 weeks. This effect neither appeared up to 1 week after the treatment with STZ in mice aged 4 weeks, despite persistently high serum glucose levels. Although circulating levels of Adipo may be diminished in insulin-resistant illnesses, such as obesity [21] and type 2 diabetes [22,23], Adipo levels in type 1 diabetes increased [24] and did not [25]. Adipo levels in STZ-induced diabetic mice after treatment with STZ in mice did not change significantly [26,27]. However, recently, it has been reported that Adipo levels in STZ-induced diabetic rats were suppressed when compared with non-diabetic rats [28]. Age and weight of mice treated with STZ, as well as time after treatment, may greatly affect Adipo levels in STZ-induced diabetic mice. Adipo has multiple biological functions and the exact

mechanism behind these differences remains unclear. The reduction of serum Adipo levels in STZ-induced diabetic mice may be related to atrophy of adipocytes and/or other diseases that might be induced by diabetes mellitus.

Hydrodynamic injection has proven to be a very efficient method for hepatic delivery of genes into mice [11,12]. In this study, serum and hepatic Adipo levels in STZ-induced diabetic mice were elevated after hydrodynamic injection of pCMV-Ad had caused high levels of Adipo expression (Figures 4a and 4b). High levels of Adipo expression were sustained for 3 days post-injection and peak expression (142.8 ± 26.1 µg/ml at 10 µg dose/mouse) was seen at the 24-h mark. Corresponding with changes in Adipo expression seen at this mark, serum glucose levels in STZ-induced diabetic mice transfected with 10 and 50 µg of pCMV-Ad had been reduced by more than 50%. Adenovirus-mediated Adipo transfection into Wistar rats increased serum Adipo levels to approximately 70 µg/ml 7 days after intravenous injection, but did not decrease glucose levels [10]. Intraperitoneal injection of recombinant Adipo protein caused a 2- to 3-fold increase in circulating Adipo levels to over 125 µg/ml after 4 h, and triggered a transient decrease in glucose levels in non-diabetic mice, STZ-induced diabetic mice, and non-obese diabetic mice (NOD) (more than 50% decrease before injection for all of them) [26,29]. Consequently, the delivery of Adipo genes to the liver has been shown to be effective in decreasing glucose levels, compared to an injection of Adipo protein. This study provides the first *in vivo* evidence that Adipo expression via pCMV-Ad genes can reduce serum glucose levels in the absence of insulin.

Expression of pCMV-Ad brought about a decrease in serum glucose without changing the concentration of

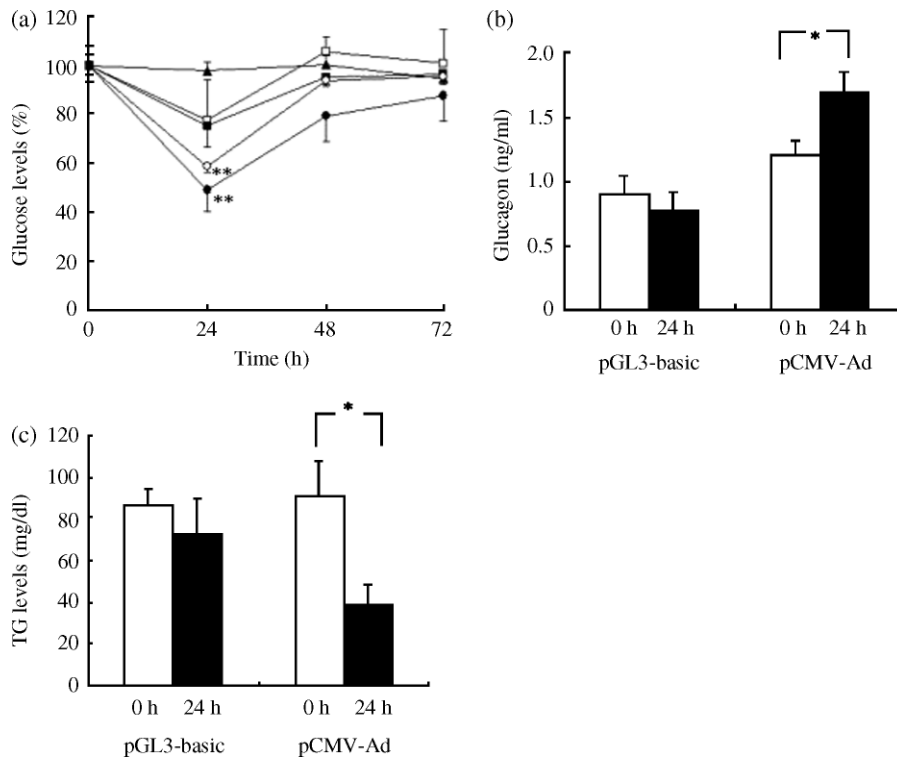


Figure 5. Hydrodynamic injection of pCMV-Ad decreased glucose and TG levels in serum in STZ-induced diabetic mice. (a) Serum glucose levels after injection of 10 µg of pGL3-basic (▲) or 1 µg (□), 5 µg (■), 10 µg (○) or 50 µg of pCMV-Ad (●). Each bar represents the mean ± S.E.; $n = 3-8$. (b) Serum glucagon levels at 0 h (□) or 24 h (■) after injection of 10 µg of pGL3-basic or pCMV-Ad. Each bar represents the mean ± S.E.; $n = 4$. (c) Serum TG levels 0 h (□) or 24 h (■) after injection of 10 µg of pGL3-basic or pCMV-Ad. Each bar represents the mean ± S.E.; $n = 4-5$. (* $P < 0.05$; ** $P < 0.01$, compared with before injection). Each animal shown was fasted for 3 h before the experiment and before a blood sample was taken

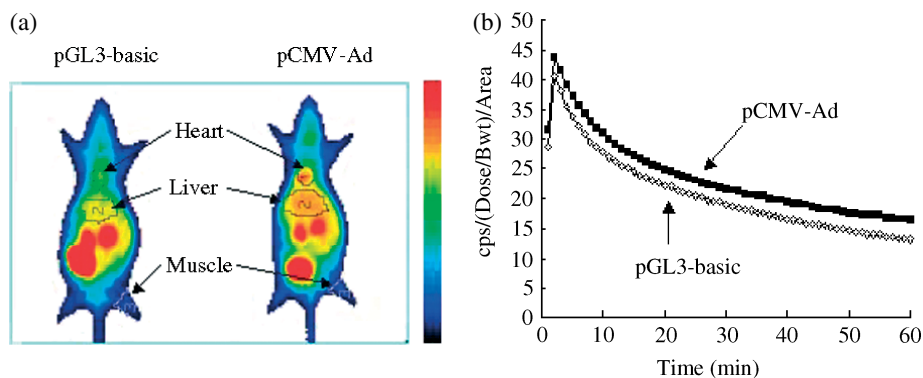


Figure 6. Dynamic change in FDG uptake, measured using a planar positron imaging system (PPIS), 18–24 h after hydrodynamic injection of 10 µg of pGL3-basic or pCMV-Ad into STZ-induced diabetic mice. (a) Distribution of FDG in heart, liver and muscle tissues of STZ-induced diabetic mice. Each image is a composite of accumulated data for the last 20 min of the session. (b) Time-activity curves (TAC) of radioactivity in the liver from 0 to 60 min; $n = 3$ for each group. Variations between experiments are shown at each point. Each animal was fasted for more than 12 h before the experiment. CPS: counts per second of the liver frame in (a) were normalized by dividing body weight (g) and unit area (mm^2)

AdipoR1 or AdipoR2 mRNA in the liver. Inukai *et al.* and Tsuchida *et al.* showed that treatment with STZ led to a reduction in plasma insulin and, at the same time, caused a marked increase in AdipoR1 and AdipoR2 mRNA levels in muscle tissues [27,30], without significantly altering AdipoR2 mRNA levels in the liver [27,31]. The decrease in serum glucose concentration triggered a subsequent rise in glucagon levels. Since serum insulin was not detected in STZ-diabetic mice, it seems that the stimulating effect

of glucagon on glucose level may be significant, and at 24 h after transfection of Adipo, the glucagon level was significantly elevated. This increase of glucagon level in the absence of insulin may act continuously, thereby resulting in restoration of the glucose level at 48 h. This finding correlated with a previous investigation by Berg *et al.* [26], which showed that glucagon and other potential counter-regulatory mechanisms were capable of instigating a recovery in basal glucose levels, and

that over-expression of Adipo caused a subsequent down-regulation in serum TG levels. In the end, the presence of Adipo resulted in a reduction of liver TG in obese and type 2 diabetic mice [32] by increasing fatty-acid oxidation.

The glucose-lowering effect of recombinant Adipo protein in NOD mice may be due to an increase in sensitivity to insulin [26]. In this study, STZ-induced diabetic mice exhibited undetectable insulin levels (<0.25 ng/ml), as well as high levels of Adipo expression, and experienced a decrease in glucose levels after transfection of pCMV-Ad.

Serum Adipo may play a role in the regulation of glucose homeostasis. Adipo expression decreased glucose levels and suppressed hepatic glucose production [29]. Under these conditions, serum glucose levels were predominantly governed by hepatic glucose output and input. Diabetic mice with high levels of Adipo expression by injection were able to increase their hepatic glucose uptake above those with low Adipo expression. In conjunction with AMPK activation, Adipo stimulated fatty-acid oxidation and glucose uptake in skeletal muscle [33–35], reduced the availability of gluconeogenesis components, such as glucose-6-phosphatase in hepatocytes [36], and reduced serum glucose levels in mice [16,26,31,37]. Increased rates of glucose uptake in diabetic mice have been seen in muscle tissues, but not in the liver. Here, it seems that Adipo expression actually stimulated glucose uptake in the liver.

When investigating the insulin-independent glucose-lowering effect, treatment with AICAR or transfection with pCMV-Ad confirmed the presence of AMPK activation in HepG2 cells. Cells treated with AICAR did not increase glucose uptake, but cells transfected with pCMV-Ad significantly increased glucose uptake when compared with those transfected with pGL3-basic (Figure 2c). These findings suggest that Adipo-associated increases in hepatic glucose uptake may not be mediated by AMPK activation in diabetic mice expressing Adipo. In addition, the Adipo gene delivery to the liver in diabetic mice was able to alter hepatic glucose uptake, and the glucose-lowering effect exerted by Adipo on hepatocytes took place even in the absence of insulin. Although this glucose-lowering effect did not seem to be dependent on insulin, we cannot rule out the possibility that very low insulin levels in STZ-induced diabetic mice (<0.25 ng/ml) might be present and could still affect the ability of Adipo to influence serum glucose levels [26].

Thus, we have shown for the first time that hyperglycemia in type 1 diabetes can be alleviated by delivery of Adipo plasmid DNA to liver. Recently, alternative techniques to hydrodynamic injection for clinical applications were reported [38,39]. This type of gene delivery to the liver provides a different approach to gene therapy of type 1 and type 2 diabetes.

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