Title

Regulation of adiponectin receptor gene expression in diabetic mice

Authors

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Summary

Adiponectin is an adipocyte-derived factor, which plays pivotal roles in lipid and glucose metabolism in muscle and liver. Two adiponectin receptor types were recently identified; AdipoR1 is abundantly expressed in muscle, whereas AdipoR2 is predominantly expressed in the liver. To clarify the regulation of adiponectin receptor gene expression in diabetic states, we examined mRNA levels of AdipoR1 in the muscles of diabetic animals by northern blotting. The level of AdipoR1 mRNA was increased approximately 2.5-fold in muscle of streptozotocin (STZ) diabetic mice, but the normal level was restored by insulin administration, indicating that insulin has an inhibitory effect on AdipoR1 expression. To confirm this inhibitory effect of insulin, we performed in vitro experiments using C2C12 skeletal muscle cells. Insulin treatment for 24 h decreased AdipoR1 expression by approximately 60% in C2C12 cells. In addition, this effect was mediated by the phosphatidylinositol-3 kinase (PI-3 kinase) dependent pathway rather than the mitogen-activated protein kinase (MAPK) pathway. AdipoR1 expression in insulin resistant diabetic mice was also investigated. AdipoR1 expression was decreased by 36% in type 2 diabetic obese db/db mice, as compared to lean mice. In contrast, hepatic AdipoR2 expression was not significantly changed in either STZ mice or genetically obese mice. Our results indicate that regulation of AdipoR1, but not that of AdipoR2, may be involved in glucose and lipid metabolism in diabetic states.
Introduction

In recent years, adipocytes have been recognized to secrete a variety of proteins such as tumor necrosis factor (TNF-α), adipin, plasminogen activator inhibitor-1, leptin, resistin and adiponectin/ACRP30 (9, 10, 13, 22, 24). These proteins are termed adipocytokines, and are likely to be involved in the development of metabolic syndrome (15). Among them, adiponectin is an insulin-sensitizing hormone which is exclusively expressed in adipose tissues. Screening for mutations in the adiponectin gene revealed a patient carrying a missense mutation to have a markedly decreased plasma level of adiponectin, as well as cardiovascular disease (25). Reduced production of this newly identified hormone appears to be related to the pathophysiology of insulin resistance and atherosclerosis (4, 19). Furthermore, a strong negative correlation was observed between plasma adiponectin levels and body mass index (BMI) (2). These data suggest that adiponectin is an important adipocytokine for preventing the development of type 2 diabetes with obesity and vascular disease. Two novel adiponectin receptor types (AdipoR1 and AdipoR2) were very recently identified (30); AdipoR1 is ubiquitously expressed, most abundantly in skeletal muscle, and exhibits high-affinity, whereas AdipoR2 is predominantly expressed in the liver and exhibits intermediate affinity, to ligands. Adiponectin receptors were also demonstrated to mediate increased AMP kinase and PPARα ligand activities, resulting in enhancements of fatty acid oxidation and glucose transport activity, when stimulated with ligands (30).

In the present study, we investigated AdipoR1 expression in skeletal muscle of diabetic mice at the transcriptional level. We found AdipoR1 mRNA to be increased in STZ induced diabetic mice (3), while this effect was reversed by administration of
insulin. To further confirm this inhibitory effect of insulin on AdipoR1 expression, we endeavored to elucidate which insulin signal transduction pathway is involved in AdipoR1 regulation. Furthermore, AdipoR1 expression was significantly suppressed in genetically obese and diabetic (db/db) mice. These findings are important for understanding the mechanisms by which adiponectin signaling and insulin sensitization are modulated in diabetic states.
MATERIALS AND METHODS

Experimental Animals and Protocols. -Nine-week-old male mice (C57bl/KsJ, n=12) were divided into 4 groups. Control mice (C group, n=3) were maintained on a standard rodent chow. Starved mice (F group, n=3) were fasted for 48h before sacrifice. Diabetes was induced by a single intraperitoneal injection of 0.2 ml of 50 mM sodium citrate solution (pH 4.5) containing streptozotocin (STZ) (250mg/kg) 7 days before sacrifice in 6 mice (S and S+I groups). Five days after STZ-treatment, plasma glucose levels of all 6 mice were measured and diabetes was confirmed (glucose level > 20 mM). Three mice (S+I group) received a mixture of human regular and NPS insulin (1 unit/kg) twice a day intraperitoneally for three days before sacrifice. Ten week-old genetically obese db/db mice (D and D+P groups, n=10) and lean littermates (L and L+P groups, n=10) were purchased from Clea Japan, Inc. (Osaka, Japan), and maintained on a standard rodent chow, then sacrificed at 13 weeks of age. Half of each group of ten (D+P and L+P groups) was fed standard rodent chow containing 0.01% (w/w) pioglitazone (PGZ) for 7 days before sacrifice. All mice were maintained on a 12:12h light-dark cycle, fed the indicated rodent diet ad libitum, and had unlimited access to water. Before sacrifice, the animals were fasted for 8h. Serum adiponectin concentrations were measured using a mouse/rat adiponectin ELISA kit (Otsuka Life Science, Tokyo, Japan).

Cell culture and PI-3 kinase assay. -C2C12 myoblasts were maintained in DMEM containing 10% fetal calf serum (Life Technologies, Inc.) at 37°C in 5% CO₂. After the C2C12 cells had reached subconfluence, differentiation was induced by treatment
with DMEM containing 5% horse serum for 7 days, at which time formation of
myotubes was maximal. Chemicals were added to the medium at the indicated time
before the experiments in DMEM containing 0.2% BSA. After incubation with the
chemicals, cells were washed with ice-cold PBS, and lysed in ice-cold lysis buffer (1%
Nonidet P-40, 10% glycerol, 1 mM vanadate, 10 mM NaF, 10 mM sodium
pyrophosphate, 50 mM Hepes, pH 7.5, 137 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 2
mM EDTA, 2 mg/ml aprotinin and 34 mg/ml PMSF), and were centrifuged at 14,000 x
for 10 min at 4°C. The supernatant was used for PI-3 kinase assay, which was
performed as previously described (31).

Northern blot analysis and real time PCR analysis. -The mice were killed by cervical
dislocation, and soleus muscles were rapidly removed from the hind limbs. Liver and
epididymal fat tissues were also removed. Total RNA was isolated with Isogen
(Nippon Gene, Japan). For Northern blot analysis, aliquots of purified total RNA
were pooled (20 µg), denatured with formaldehyde and formamide, subjected to
electrophoresis in a 1% formaldehyde agarose gel, and transferred to a Hybond N+
membrane (Amersham). cDNA fragments for mouse AdipoR1 and adiponectin were
isolated from mouse skeletal muscle and adipose tissue RNA, respectively, by reverse
transcriptase PCR. PCR oligonucleotide sequences used for AdipoR1 were as
follows: coding strand, 5’-CAGATGTCTTCCCACAAAGGCTCTGCTGCC-3’;
non-coding strand, 5’-TGTCCAGATGTGCAAGCTCTGCTGTGTG-3’. PCR
oligonucleotide sequences used for adiponectin were as follows: coding strand,
5’-AGGATGCTACTGTGGCAGCTCTGCTC-3’; non-coding strand,
5’-CAGTCAGTTGGTATCATGTTAGAG-3’. The cDNAs were subcloned into TA
vectors, pCRII (Invitrogen), sequenced to confirm their identities, and used for cDNA probes. The cDNA fragments were labeled with [α-32P] dCTP with a Megaprime DNA Labeling System (Amersham). The filters were hybridized with 32P-labeled probe for 2h at 65°C using Rapid Hybridization Buffer (Amersham), then washed twice in 2 X SSC/0.1% SDS for 30 min at room temperature, followed by a single wash in 0.5X SSC/0.1% SDS for 30 min at 65°C. Image analysis and quantitation were performed by exposure of the filters to a BAS 2000 Fuji PhosphorImager (Fuji, Tokyo Japan). For quantitative analysis of AdipoR2, we conducted real-time PCR using an ABI PRISM Model 7000 (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. The primer sets and probes for mouse AdipoR2 (Assay ID; Mm01184032 m1) were purchased.

**Western blot analysis.** Muscle samples from mice were homogenized in lysis-buffer (1% Triton/PBS) and centrifuged at 14,000 x g for 10 min at 4°C. Supernatants including tissue protein extracts were resolved on 10% SDS-PAGE gel, followed by electrophoretic transfer to a nitrocellulose membrane. After blotting with a polyclonal antibody against mouse AdipoR1 (Alpha Diagnostic Intl., San Antonio, TX), detection was performed using an ECL chemiluminescent kit (Amersham Pharmacia Biotech, UK) according to the manufacturer’s instructions. Cell lysates from C2C12 cells were also subjected to SDS-PAGE and blotted with anti-phospho-p42/p44 MAPK antibody (Cell Signaling, Beverly, MA). Quantitations were performed using a Molecular Imager (Bio-Rad Laboratories, CA).

**Recombinant adenoviruses.** Recombinant adenovirus containing full length murine
adiponectin cDNA was prepared as reported previously (16). Lac Z adenovirus was used as a control. Mice were treated with recombinant adenovirus containing Lac Z or murine adiponectin cDNA by systemic injection into the tail vein. Three days after virus infection, we confirmed increased serum adiponectin levels by immunoblot analysis using anti-murine adiponectin antibody (CHEMICON International Inc., Temecula, CA), and the quantitatively analyzed AdipoR1 mRNA as described above.

Statistical analysis. -Data are presented as means ± S.D. of 3 or 5 mice and as means ± S.E. of in vitro experiments. Data from the northern blot analysis were normalized for the quantity of 28S RNA, using NIH image software (Version 1.62). Statistical significance in the quantitation of mRNA levels was determined using the Mann-Whitney test ($P$ value < 0.05). Statistical analyses were performed using Stat View software (Version 5.01; SAS Institute, Cary, NC).
RESULTS

Although we obtained AdipoR1/R2 cDNA fragments from mouse mRNA, no specific bands were detected for hepatic mRNA when we performed Northern blot analysis using the AdipoR2 cDNA fragment as a probe. Thus, we performed real-time PCR for the quantitative analysis of AdipoR2 mRNA expression. We present the serum glucose, insulin and adiponectin levels of each group at sacrifice in Table1. In a preliminary study, the effect of starvation on AdipoR1/R2 expression was analyzed. During starvation, fatty acid oxidation in muscle will generally be stimulated in order to transfer stored energy to the liver as material for gluconeogenesis. Adiponectin was previously reported to function to enhance fatty acid oxidation in muscle via activation of AMP kinase (29). Therefore, it is reasonable to postulate that the level of AdipoR1 expression will increase in order to enhance fatty acid oxidation during starvation. As shown in Fig. 1A, Northern blot analysis under high stringency conditions, using a fragment of AdipoR1 cDNA cloned by reverse transcriptase PCR, revealed a 2.0 kb pair AdipoR1 mRNA as reported previously (30). As expected, AdipoR1 mRNA of the F group was significantly increased (2.9-fold) as compared with that of the C group. To investigate the post-translational regulation of AdipoR1, we determined levels of AdipoR1 protein expression in muscles from F and C group mice (Fig. 1B). AdipoR1 protein in the F group was significantly increased as compared with that in the C group (2.2-fold). These results may partially explain the mechanism by which fatty acid oxidation is enhanced in skeletal muscle under fasting conditions. In contrast, hepatic AdipoR2 mRNA, analyzed by real-time PCR, was not significantly changed by starvation (Fig. 1C). Circulating levels of adiponectin are reportedly elevated during
chronic caloric restriction in both humans and mice (4, 11), as was the case in our experiments (Table 1).

Next, in order to investigate the effect of insulin on AdipoR1/R2 expression, we prepared STZ-mice and conducted a similar analysis. The plasma glucose level of the S group showed marked hyperglycemia, which was attributed to insulin deficiency (Table 1). As shown in Fig. 2A, the AdipoR1 mRNA level of the S group was increased (2.5-fold) as compared with that of the C group. To confirm that this was due to insulin deficiency, we investigated whether or not the S group increase was reversed by insulin administration. After confirming that plasma glucose decreased to euglycemic (fasting glucose < 9 mM) levels with insulin treatment, AdipoR1 mRNA of the S+I group was analyzed. As expected, AdipoR1 expression was restored to a level comparable to that of the C group (Fig. 2A), indicating that AdipoR1 expression is suppressed by insulin. These results were also confirmed by AdipoR1 protein levels, for which the similar results were obtained (Fig. 2B), i.e. the AdipoR1 protein level doubled in the S group, while insulin treatment decreased AdipoR1 protein to the C group level. On the other hand, hepatic AdipoR2 mRNA levels of the S and S+I groups were not significantly changed as compared to that of the C group (Fig. 1C). We measured plasma adiponectin levels in the S and S+I group mice and neither showed significant changes in plasma adiponectin levels as compared with the C group (Table 1).

To further confirm the inhibitory effect of insulin on AdipoR1 expression, we used cultured C2C12 skeletal muscle cells for Northern blot analysis. Since these cells were derived from mice, a similar single band was observed with a height of 2.0kb. The alterations in AdipoR1 mRNA in response to treatment with $10^{-7}$ M insulin at the
indicated times are shown in Fig. 3A. Suppression of AdipoR1 expression was detected after 3h and peaked at 40% after 24h (Fig. 3A, middle and lower panels), while no significant changes of AdipoR1 mRNA were observed in the absence of insulin (Fig. 3A, upper panel). Insulin signaling is mediated mainly by two major pathways, i.e. the p42/p44 mitogen-activated protein kinase (MAPK) and the PI-3 kinase pathway. On the basis of our observation that insulin inhibited AdipoR1 expression, we next investigated the contributions of these pathways to insulin-induced suppression of AdipoR1 expression. As shown in the middle and lower panels of Fig. 3B, incubation with 50μM PD98059, a chemical MEK1 inhibitor, had no influence on the actions of insulin. In contrast, when incubated with 10⁻⁷ wortmannin, a chemical PI-kinase inhibitor, the inhibitory effect of insulin on AdipoR1 expression was abrogated (Fig. 3C, middle and lower panels). We confirmed that 50μM PD98059 and 10⁻⁷ wortmannin inhibited insulin-induced activations of MAPK and PI-3 kinase, respectively (Fig. 3B or C, upper panels). The results of these experiments suggest that insulin-induced suppression of AdipoR1 expression depends on the PI-3 kinase pathway rather than the MAPK pathway.

To investigate the regulation of AdipoR1/R2 in insulin-resistant diabetic states, we analyzed AdipoR1/R2 expression in skeletal muscles and liver tissues from db/db obese mice. As shown in Fig. 4A, AdipoR1 mRNA was significantly decreased, by 36%, in the D group as compared with the L group. Next, we administered pioglitazone (PGZ), an insulin-sensitizing drug, to these obese mice. Though AdipoR1 expression tended to increase with PGZ treatment, it was not significantly changed in the D+P group as compared with the D groups (Fig. 4A). An immunoblot analysis (Fig. 4B) confirmed significantly deceased (by 32%) expression of AdipoR1 in genetically obese mice. To
investigate whether AdipoR2 expression compensates for the decreased expression of AdipoR1, we analyzed AdipoR2 expression in several tissues from these obese mice. However, no significant change was observed in the liver (Fig. 4C), epididymal fat or skeletal muscles (data not shown), suggesting that AdipoR2 does not compensate for the decreased AdipoR1 expression in insulin-resistant diabetic states. To investigate whether AdipoR1 expression levels correlate with those of adiponectin, we analyzed adiponectin mRNA in epididymal fat (Fig. 5) from obese mice. Adiponectin mRNA was significantly decreased, by approximately 32%, in the D group as compared with the L group. PGZ treatment significantly increased adiponectin expression, by 1.3-fold, in the D+P groups as compared with the D groups. The plasma adiponectin results were similar to those for adiponectin mRNA except for the marked increase induced by PGZ treatment in obese mice (Table 1).

AdipoR1 and adiponectin genes are both up-regulated in starved mice and down-regulated in genetically obese mice, while the AdipoR1 expression in response to PGZ or STZ treatment did not correlate with adiponectin expression. These results suggest the AdipoR1 expression in muscles may partially correlate with serum adiponectin levels. To address this issue, we examined the effect of adiponectin on AdipoR1 expression. Using adenovirus gene transfer, ectopic overexpression of adiponectin in the liver markedly up-regulated serum adiponectin (Fig. 6A). The mice injected with 5 \times 10^8 plaque-forming units (pfu) of recombinant adenovirus containing adiponectin cDNA had approximately double the serum adiponectin concentration compared of control mice (Fig. 6A, lane 2), while mice injected with 5 \times 10^9 pfu had serum adiponectin concentrations approximately 5-times those of controls (Fig. 6A, lane 3). Such a high adiponectin concentration (lane 3) is likely to be unphysiological.
When we analyzed the AdipoR1 mRNA expressed in the muscles of these mice, those with higher serum adiponectin concentrations showed increases in AdipoR1 mRNA of approximately 20% as compared with control mice (Fig. 6B). This increase did not, however, reach statistical significance (p=0.117, Mann-Whitney u test). We thus conclude that adiponectin has no significant effect on AdipoR1 expression,
DISCUSSION

Although the pharmacological properties of adiponectin have been intensively investigated in recent years, the mechanisms by which physiological effects are exerted on adiponectin target tissues remain unknown. Adiponectin was observed to accumulate in the walls of injured vessels, but not in intact vascular walls, and was also shown to bind to several types of collagen, which are present in the vascular intima (11). Moreover, adiponectin is a relatively abundant plasma protein, accounting for approximately 0.01% of total plasma protein (18), making it much more abundant than other cytokines and markedly exceeding the concentrations of circulating hormones. Based on these observations, it was postulated that adiponectin has no specific receptors via which its pharmacological actions are exerted. In this sense, the discovery of adiponectin receptors was somewhat of a surprise. Further studies are needed to investigate whether any other pathways convey the adiponectin signal in addition to these newly identified receptors. In the present study, we investigated AdipoR1 expression in skeletal muscles of diabetic mice. We found AdipoR1 expression to be regulated differentially in various mouse models and by various treatments, raising the possibility that AdipoR1 expression is influenced by several hormones, cytokines and signaling molecules. Among these, we demonstrated the down-regulation of AdipoR1 expression by insulin in both in vivo and in vitro experiments.

Another type of adiponectin receptor (AdipoR2), which has a lower-affinity for adiponectin than AdipoR1 and is abundant in the liver, was also identified (30). For quantitative analysis of AdipoR2 mRNA, we employed a real-time PCR system as no
specific bands were detected when we performed Northern blot analysis using this fragment as a probe. Although the reason for this is unknown, it is possible that AdipoR2 expression is extremely limited in the liver as compared with AdipoR1 expression in skeletal muscle. In contrast to the AdipoR1 expression, AdipoR2 expression was not significantly changed in our experiments, indicating that AdipoR1, but not AdipoR2, is involved in glucose or lipid metabolism. A number of recent studies, which have focused on the relation between AdipoR1/R2 and insulin sensitivity, are consistent with this notion (6, 23, 28).

Insulin actually mediates its actions involved in glucose and lipid metabolism by regulating a number of gene expressions. For example, insulin enhances the transcription of glucokinase, which is a key enzyme in glycolysis (14). Insulin also stimulates expression of SREBP1c, which serves mainly as a transcription factor for fatty acid synthesis (21). Distinct consensus insulin response sequences (IRSs), through which insulin up-regulates gene expression (17), have also been identified. In contrast, insulin also has inhibitory effects on various gene expressions. These genes include phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase) (20, 27), both of which play important roles in hepatic gluconeogenesis. Insulin has recently been shown to inhibit the expressions of these genes via phosphorylation and nuclear export of the forkhead transcription factor (FKHR) in a PI-3 kinase dependent manner (5, 26). In our study, AdipoR1 expression was also proved to be suppressed by insulin action. Furthermore, when the PI-3 kinase pathway was inhibited by the chemical PI-3 kinase inhibitor wortmannin, the inhibitory effect of insulin on AdipoR1 expression was blocked, whereas it was not blocked by a MEK1 inhibitor. Thus, the activation of PI-3 kinase, but not that of MAPK, is
required for the regulation of AdipoR1 expression via insulin signaling. These results support our hypothesis that IRSs exist in the promoter regions of AdipoR1 and certain transcription factors, such as FKHR, that may serve as negative regulators of AdipoR1 transcription downstream from PI-3 kinase.

To investigate AdipoR1 expression in an insulin-resistant diabetic state, we employed genetically obese mice (db/db), which exhibit overt hyperglycemia and hyperinsulinemia. Compared with lean control mice, adiponectin mRNA in fat tissues of these diabetic mice was significantly decreased, by approximately 35%, presumably via the suppressive effect of TNFα on adiponectin production (8). In addition, when these obese mice were treated with PGZ, the adiponectin mRNA level rose significantly. These observations were consistent with those of a previous report (7) and also confirm recent work (12), clearly demonstrating peroxisome proliferative responsive elements (PPRE) in the promoter region of adiponectin. As PI-3 kinase activity in skeletal muscle was markedly decreased in db/db mice (1), AdipoR1 expression in these mice should be increased given that insulin signaling suppresses AdipoR1 expression. Contrary to expectation, AdipoR1 expression was decreased as compared to that in lean control mice. Another mechanism, which is dominant over insulin signaling, presumably regulates AdipoR1 gene expression. Not only the decrease in adiponectin expression in adipose tissues, but also that in AdipoR1 expression in skeletal muscle, may partially explain the deterioration of insulin sensitivity in obese diabetic mice.

In summary, we analyzed AdipoR1 regulation in skeletal muscles of diabetic mice at both the transcriptional and the post-translational level and demonstrated insulin signaling to have an inhibitory effect on AdipoR1 expression both in vivo and in vitro. These results contribute to our understanding of the regulation of adiponectin signaling
and insulin sensitization.
REFERENCES


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FIGURE LEGENDS

Table 1. Serum glucose, insulin and adiponectin concentrations of each group
Data are presented as means ± S.D. of 3-5 mice. *Significant difference (P <0.05) relative to the C group, as determined by Mann-Whitney U test. **Significant difference (P <0.05) relative to L group. †Significant difference (P <0.05) relative to D group.

Figure 1. Expression and regulation of AdipoR1/R2 in 48h starved mice
A. Northern blot analysis of AdipoR1 mRNA in the skeletal muscles of control (C group, lanes 1-3) and 48h fasted (F group, lanes 4-6) mice. Ethidium bromide (EtBr) staining is shown as a control for loading and integrity of total RNA. B. Immunoblot analyses of AdipoR1 protein in control (C group, lanes 1-3) and 48h fasted (F group, lanes 4-6) mice were performed using an anti-mouse AdipoR1 antibody. C. Real-time PCR analysis of hepatic AdipoR2 mRNA in control (C group, lanes 1-3) and 48h fasted (F group, lanes 4-6) mice. Each column shows the mean ± S.D. obtained from three animals in each group. *Significant difference (P <0.05) relative to the C group, as determined by Mann-Whitney U test.

Figure 2. Expression and regulation of AdipoR1/R2 in STZ-treated mice
A. Northern blot analysis of AdipoR1 mRNA in the skeletal muscles of control (C group, lanes 1-3), STZ-treated (S group, lanes 4-6) and STZ+insulin treated (S+I group, lanes 7-9) mice. Ethidium bromide (EtBr) staining is shown as a control for loading and integrity of total RNA. B. Immunoblot analyses of AdipoR1 protein in the skeletal muscles of control (C group, lanes 1-3), STZ-treated (S group, lanes 4-6) and STZ+insulin treated (S+I group, lanes 7-9) mice. C. Real-time PCR analysis of hepatic AdipoR2 mRNA in control (C group, lanes 1-3),
STZ-treated (S group, lanes 4-6) and STZ+insulin treated (S+I group, lanes 7-9) mice. Each column shows the mean ± S.D. obtained from three animals in each group. *Significant difference ($P < 0.05$) relative to C group, **Significant difference ($P < 0.05$) relative to S group, as determined by Mann-Whitney $U$ test.

**Figure 3. Expression and regulation of AdipoR1 mRNA in C2C12 cells**

(A) Time curve of insulin ($10^{-7}$ M) effect on AdipoR1 mRNA expression in the presence (middle and lower panels) and in the absence (upper panel) of insulin. The experiment was performed four times and the mean ± S.E. is shown. The data in the lower panel were normalized to the value at the same time point without the insulin group. *Significant difference ($P < 0.05$) relative to AdipoR1 mRNA in the absence of insulin, as determined by Mann-Whitney $U$ test. (B) C2C12 cells were incubated in the presence or absence of $10^{-7}$ insulin, with or without 50µM PD98059. Cell lysates from C2C12 myocytes treated with the indicated ligands were subjected to SDS-PAGE and immunoblotted with anti-pp42/44 MAP kinase antibody (upper panel). Northern blot analyses of AdipoR1 mRNA in C2C12 myocytes are shown (middle and lower panels). Representative data from four experiments are presented. Each column shows the mean ± S.E. obtained from these experiments. *Significant difference ($P < 0.05$) relative to AdipoR1 mRNA in the absence of insulin, as determined by Mann-Whitney $U$ test. (C) C2C12 cells were incubated in the presence or absence of $10^{-7}$ insulin, with or without $10^{-7}$ wortmannin. Cell lysates from C2C12 myocytes treated with the indicated ligands were assayed for PI-3 kinase activity (upper panel). Northern blot analyses of AdipoR1 mRNA in C2C12 myocytes are shown (middle and lower panels). Representative data from four experiments are presented. Each column shows the mean ± S.E. obtained from these experiments. *Significant difference ($P < 0.05$) relative to
AdipoR1 mRNA in the absence of insulin, as determined by Mann-Whitney $U$ test.

**Figure 4. Expression and regulation of AdipoR1/R2 mRNA in db/db mice**

A. Northern blot analysis of AdipoR1 mRNA in skeletal muscles of lean counterparts (L group, lanes 1-5), db/db mice (D group, lanes 6-10) and pioglitazone treated db/db mice (D+P group, lanes 11-15). Ethidium bromide (EtBr) staining is shown as a control for loading and integrity of total RNA. B. Immunoblot analysis of AdipoR1 protein in skeletal muscles of lean counterparts (L group), pioglitazone treated db/db mice (L+P group), db/db mice (D group) and pioglitazone treated db/db mice (D+P group). Quantitations were performed using a Molecular Imager (Bio-Rad Lab, CA). C. Real-time PCR analysis of hepatic AdipoR2 mRNA in lean counterparts (L group), pioglitazone treated lean counterparts (L+P group), db/db mice (D group) and pioglitazone treated db/db mice (D+P group). Each column shows the mean ± S.D. obtained from five animals in each group. *Significant difference ($P <0.05$) relative to C group, as determined by Mann-Whitney $U$ test.

**Figure 5. Expression and regulation of adiponectin mRNA in epidydymal fat tissues**

Northern blot analysis of adiponectin mRNA in epidydymal fat tissues of lean counterparts (L group, lanes 1-5), db/db mice (D group, lanes 6-10) and pioglitazone treated db/db mice (D+P group, lanes 11-15). Ethidium bromide (EtBr) staining is shown as a control for loading and integrity of total RNA. Each column shows the mean ± S.D. obtained from five animals in each group. *Significant difference ($P <0.05$) relative to L group, **Significant difference ($P <0.05$) relative to D group, as determined by Mann-Whitney $U$ test.

**Figure 6. Effects of adiponectin overexpression on AdipoR1 expression in skeletal muscles.**
A. Immunoblot analysis of serum adiponectin using anti-murine adiponectin antibodies in mice treated with recombinant adenoviruses, 3 days after virus infection, confirmed the increased serum adiponectin levels. Quantitative data were obtained using a Molecular Imager (Bio-Rad Laboratories, CA). B. Quantitative analysis of AdipoR1 mRNA in each group. Each column shows the mean ± S.D. obtained from five animals in each group. The significance of differences was determined by Mann-Whitney U test (p<0.05). Mice were systemically injected, with 5 x 10^9 pfu of recombinant adenovirus containing Lac Z (lane 1) or 5 x 10^8 pfu of recombinant adenovirus containing adiponectin cDNA (lane 2) or 5 x 10^9 pfu of recombinant adenovirus containing adiponectin cDNA (lane 3), via the tail vein.
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<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
</table>

18S

2.0fold increase

<table>
<thead>
<tr>
<th>C group</th>
<th>S group</th>
<th>S+I group</th>
</tr>
</thead>
</table>

**B**

Immunoblot; anti-adipoR1 Ab

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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45kDa

45kDa fold increase

<table>
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<tr>
<th>C group</th>
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</table>

**C**

<table>
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</table>

fold increase

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</table>

N.S.
**A**

2.0kb

Insulin (-)  Insulin (+)

0min 10min 30min 2hr 12hr 24hr

B

Immunoblot; anti-pp42/44 MAP kinase Ab

p42  p44

2.0kb

**C**

PI(3)P

 ori

2.0kb

Insulin (-)  (+)  (+)  (-)  

Wortmannin (-)  (-)  (+)  (+)  

PD98059 (-)  (-)  (+)  (+)
**AdipoR1**

- **2.0kb**
- **28S**
- **18S**

**Fold Increase**

<table>
<thead>
<tr>
<th>Group</th>
<th>L</th>
<th>L+P</th>
<th>D</th>
<th>D+P</th>
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<tbody>
<tr>
<td><strong>0.5</strong></td>
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<tr>
<td><strong>1</strong></td>
<td>*</td>
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<tr>
<td><strong>N.S.</strong></td>
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**45kDa**

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**Fold Increase**

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**Legend**

- L: Low group
- D: Dose group
- D+P: Dose + Prodrug group

*P-value significance marker*
2.0kb

L group        D group  D+P group

0.5fold increase

1.0

fold increase

L group  D group  D+P group

*  **
2.0 kb

AdipoR1

A

30kDa

Adiponectin

fold increase

10

5

1  2  3

N.S.

B

1  2  3

N.S.

N.S.