Regulation of adiponectin receptor gene expression in diabetic mice

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Inukai, Kouichi, Youhei Nakashima, Masaki Watanabe, Nobuki Takata, Takahiro Sawa, Susumu Kurihara, Takuya Awata, and Shigehiro Katayama. Regulation of adiponectin receptor gene expression in diabetic mice. Am J Physiol Endocrinol Metab 288: E876–E882, 2005. First published December 21, 2004; doi:10.1152/ajpendo.00118.2004.—Adiponectin is an adipocyte-derived factor that plays pivotal roles in lipid and glucose metabolism in muscle and liver. The following 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 ∼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 ∼60% in C2C12 cells. In addition, this effect was mediated by the phosphatidylinositol 3-kinase-dependent pathway rather than the mitogen-activated protein kinase 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 compared with lean mice. In contrast, hepatic AdipoR2 expression was not significantly changed in type 2 diabetic obese db/db mice compared with lean mice. In addition, 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 four groups. Control mice (C group, n = 3) were maintained on a standard rodent chow. Starved mice (P group, n = 3) were fasted for 48 h before death. Diabetes was induced by a single intraperitoneal injection of 0.2 ml of 50 mM sodium citrate solution (pH 4.5) containing STZ (250 mg/kg) 7 days before death in six mice (S and S + I groups). After STZ treatment (5 days), plasma glucose levels of all six mice were measured, and diabetes was confirmed (glucose level >20 mM). Three mice (S + I group) received a mixture of human regular and neutral protamine Hagedorn insulin (1 U/kg) two times a day intraperitoneally for 3 days before death. 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 (Osaka, Japan), maintained on a standard rodent chow, and then killed at 13 wk of age. One-half of each group of 10 (D + P and L + P groups) was fed standard rodent chow containing 0.01% (wt/wt) pioglitazone (PGZ) for 7 days before death. All mice were maintained on a 12:12-h light-dark cycle, fed the indicated rodent diet ad libitum, and had unlimited access to water. Before death, the animals were fasted for 8 h. Serum adiponectin concentrations were measured using a mouse/ rat adiponectin ELISA kit (Otsuka Life Science, Tokyo, Japan).

Cell culture and phosphatidylinositol 3-kinase assay. C2C12 myoblasts were maintained in DMEM containing 10% FCS (Life Technologies) 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 types (AdipoR1 and AdipoR2) were very recently identified (31); 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 peroxisome proliferator-activated receptor-α ligand activities, resulting in enhancements of fatty acid oxidation and glucose transport activity when stimulated with ligands (31).

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 streptozotocin (STZ)-induced diabetic mice (3), whereas 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.

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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, 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 phenylmethylsulfonyl fluoride), and centrifuged at 14,000 g for 10 min at 4°C. The supernatant was used for phosphorylidyinositol 3-kinase (PI 3-kinase) assay, which was performed as previously described (29).

Northern blot analysis and real-time PCR analysis. The mice were killed by cervical dislocation, and soleus muscles were rapidly removed from the hindlimbs. Liver and epididymal fat tissues were also removed. Total RNA was isolated with Isogen (Nippon Gene). 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 RT-PCR. PCR oligonucleotide sequences used for AdipoR1 were as follows: coding strand, 5'-CAGATGTCCTTCACAAAGGGCTCAGCC-3'; noncoding strand, 5'-TGTCGACAGTTGCGACCTCTTCG-3'. PCR oligonucleotide sequences used for adiponectin were as follows: coding strand, 5'-'AGGATGCTACTGTTGCAAGCTCTC-3'; noncoding strand, 5'-CAGTCAGTTGTATCAGTGGAG-3'. The cDNAs were subcloned into TA vectors, pCRII (Invitrogen), sequenced to confirm their identities, and used for cDNA probes. The cDNA fragments cloned by RT-PCR, revealed a 2.0-kb pair AdipoR1 mRNA of the F group was significantly increased (2.9-fold) relative to D group (‡). ND, not determined.

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 death in Table 1. 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 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 (30). Therefore, it is reasonable to postulate that the level of AdipoR1 expression will increase 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 RT-PCR, revealed a 2.0-kb pair AdipoR1 mRNA, as reported previously (31). As expected, AdipoR1 mRNA of the F group was significantly increased (2.9-fold) 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 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, 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

Table 1. Serum glucose, insulin and adiponectin concentrations of each group

<table>
<thead>
<tr>
<th>Glucose, mg/dl</th>
<th>Insulin, ng/ml</th>
<th>Serum Adiponectin, µg/ml</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>134.0 ± 11.3</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>F</td>
<td>81.7 ± 7.8*</td>
<td>0.10 ± 0.05*</td>
</tr>
<tr>
<td>S</td>
<td>491.3 ± 27.3*</td>
<td>ND</td>
</tr>
<tr>
<td>S+1</td>
<td>140.0 ± 17.2</td>
<td>1.41 ± 0.63*</td>
</tr>
<tr>
<td>L</td>
<td>129.7 ± 12.0</td>
<td>0.30 ± 0.08</td>
</tr>
<tr>
<td>L+P</td>
<td>121.6 ± 7.9</td>
<td>0.27 ± 0.10</td>
</tr>
<tr>
<td>D</td>
<td>436.6 ± 46.5*</td>
<td>8.32 ± 1.23*</td>
</tr>
<tr>
<td>D+P</td>
<td>190.0 ± 10.2*</td>
<td>2.01 ± 0.54*</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD of 3–5 mice. C, control group; F, mice fasted for 48 h; S, streptozotocin-treated mice; S+I, mice treated with streptozotocin and insulin; L, lean group; L+P, pioglitazone-treated db/db mice; D, db/db mice; D+P, pioglitazone-treated db/db mice. P < 0.05, significant difference relative to the C group, as determined by Mann-Whitney U-test (*), significant difference relative to L group †, and significant difference relative to D group ‡. ND, not determined.
level of the S group was increased (2.5-fold) compared with that of the C group. To confirm that this was the result of 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 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 similar results were obtained (Fig. 2B), i.e., the AdipoR1 protein level doubled in the S group, whereas 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 compared with 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 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. Because these cells were derived from mice, a similar single band was observed with a height of 2.0 kb. 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 3 h and peaked at 40% after 24 h (Fig. 3A, middle and bottom), whereas no significant changes of AdipoR1 mRNA were observed in the absence of insulin (Fig. 3A, top). Insulin signaling is mediated mainly by two major pathways, i.e., the p42/p44 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 Fig. 3B, middle and bottom, incubation with 50 μM PD-98059, a chemical mitogen/extracellular signal-regulated kinase (MEK) inhibitor, had no influence on the actions of insulin. In contrast, when incubated with 10^{-7} wortmannin, a chemical PI 3-kinase inhibitor, the inhibitory effect of insulin on AdipoR1 expression was abrogated (Fig. 3C, middle and bottom). We confirmed that 50 μM PD-98059 and 10^{-7} wortmannin inhibited insulin-induced activations of MAPK and PI 3-kinase, respectively (Fig. 3, B, top, or C, top). 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 compared with the L group. Next, we administered PGZ, an insulin-sensitizing drug, to these obese mice. Though AdipoR1 expression tended to increase with PGZ treatment, it was not changed significantly in the D+P group compared with the D group (Fig. 4A). An immunoblot analysis (Fig. 4B) confirmed significantly decreased (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 ~32%, in the D group compared with the L group.

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**Fig. 3.** Expression and regulation of AdipoR1 mRNA in C2C12 cells. **A:** time course of insulin (10^{-7} M) effect on AdipoR1 mRNA expression in the presence (middle and bottom) and in the absence (top) of insulin. The experiment was performed four times, and means ± SE are shown. Data on bottom 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 50 μM PD-98059. Cell lysates from C2C12 myocytes treated with the indicated ligands were subjected to SDS-PAGE and immunoblotted with anti-p42/p44 mitogen-activated protein (MAP) kinase antibody (top). Northern blot analyses of AdipoR1 mRNA in C2C12 myocytes are shown (middle and bottom). Representative data from four experiments are presented. Each column shows the mean ± SE 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 phosphatidylinositol 3-kinase (PI 3-kinase) activity (top). Northern blot analyses of AdipoR1 mRNA in C2C12 myocytes are shown (middle and bottom). Representative data from four experiments are presented. Each column shows the mean ± SE 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.
pared with the L group. PGZ treatment significantly increased adiponectin expression, by 1.3-fold, in the D/P groups 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 upregulated in starved mice and downregulated in genetically obese mice, whereas the AdipoR1 expression in response to PGZ or STZ treatment did not correlate with adiponectin expression. These results suggest that AdipoR1 expression in muscles may partially correlate with serum adiponectin levels. To address this issue, we examined the effect of adiponectin on AdipoR1 expression. With the use of adenovirus gene transfer, ectopic overexpression of adiponectin in the liver markedly upregulated serum adiponectin levels. To address this issue, we examined the effect of adiponectin on AdipoR1 expression. With the use of adenovirus gene transfer, ectopic overexpression of adiponectin in the liver markedly upregulated 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 with control mice (Fig. 6A, lane 2), whereas mice injected with $5 \times 10^9$ pfu had serum adiponectin concentrations approximately five 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 $\sim 20\%$ 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 investigated intensively 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 $0.01\%$ 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 downregulation of AdipoR1 expression by insulin in both in vivo and in vitro experiments.

AdipoR2, which has a lower affinity for adiponectin than AdipoR1 and is abundant in the liver, was also identified (31).
For quantitative analysis of AdipoR2 mRNA, we employed a real-time PCR system, since 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 compared with AdipoR1 expression in skeletal muscle. In contrast to AdipoR1 expression, AdipoR2 expression was not significantly changed in our experiments, indicating that AdipoR2 expression 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 sterol response element-binding protein-1c, which serves mainly as a transcription factor for fatty acid synthesis (21). Distinct consensus insulin response sequences (IRSs), through which insulin upregulates gene expression (17), have also been identified. In contrast, insulin also has inhibitory effects on various gene expressions. These genes include phosphoenolpyruvate carboxykinase and glucose 6-phosphatase (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 ∼35%, presumably via the suppressive effect of TNF-α 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 in the promoter region of adiponectin. Because 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...
compared with 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 posttranslational 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