Insulin/Foxo1 Pathway Regulates Expression Levels of Adiponectin Receptors and Adiponectin Sensitivity

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Adiponectin/Acrp30 is a hormone secreted by adipocytes, which acts as an anti-diabetic and anti-atherogenic adipokine. We reported previously that AdipoR1 and -R2 serve as receptors for adiponectin and mediate increased fatty acid oxidation and glucose uptake by adiponectin. In the present study, we examined the expression levels and roles of AdipoR1/R2 in several physiological and pathophysiological states such as fasting/refeeding, obesity, and insulin resistance. Here we show that the expression of AdipoR1/R2 in insulin target organs, such as skeletal muscle and liver, is significantly increased in fasted mice and decreased in refed mice. Insulin deficiency induced by streptozotocin increased and insulin replenishment reduced the expression of AdipoR1/R2 in vivo. Thus, the expression of AdipoR1/R2 appears to be inversely correlated with plasma insulin levels in vivo. Interestingly, the incubation of hepatocytes or myocytes with insulin reduced the expression of AdipoR1/R2 via the phosphoinositide 3-kinase/Foxo1-dependent pathway in vitro. Moreover, the expressions of AdipoR1/R2 in ob/ob mice were significantly decreased in skeletal muscle and adipose tissue, which was correlated with decreased adiponectin binding to membrane fractions of skeletal muscle and decreased AMP kinase activation by adiponectin. This adiponectin resistance in turn may play a role in worsening insulin resistance in ob/ob mice. In conclusion, the expression of AdipoR1/R2 appears to be inversely regulated by insulin in physiological and pathophysiological states such as fasting/refeeding, insulin deficiency, and hyperinsulinemia models via the insulin/phosphoinositide 3-kinase/Foxo1 pathway and is correlated with adiponectin sensitivity.

Adiponectin/Acrp30 (1–4) is a hormone secreted by adipocytes, which acts as an anti-diabetic (5–12) and anti-atherogenic (8, 12, 13) adipokine. This insulin-sensitizing effect of adiponectin appears to be mediated by an increase in fatty acid oxidation via activation of the 5'-AMP-activated protein kinase (AMPK)1 (10, 11) and peroxisome proliferator-activated receptor-α (5, 6, 12). Very recently, we have reported the cloning of complementary DNAs encoding adiponectin receptors AdipoR1 and -R2 by expression cloning (14). AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver. AdipoR1 and -R2 are predicted to contain seven transmembrane domains (14) but to be structurally and functionally distinct from G-protein-coupled receptors (15–17). AdipoR1 and -R2 serve as receptors for globular and full-length adiponectin and mediate increased AMPK (10, 11), peroxisome proliferator-activated receptor-α ligand activities (12), and fatty acid oxidation and glucose uptake by adiponectin (14). It has not yet been determined whether the expressions of AdipoR1 and -R2 are altered in physiological and pathophysiological states. To address these questions, we first studied the expressions of AdipoR1 and -R2 during fasting and refeeding. We also analyzed the expressions of AdipoR1/R2 in an insulin deficiency model by streptozotocin (STZ) treatment (18). Moreover, we studied these expressions in a hyperinsulinemia model because of obesity-linked insulin resistance. The results of these studies reveal that insulin negatively regulates the expression levels of adiponectin receptors and adiponectin sensitivity and that obesity-linked insulin resistance/hyperinsulinemia can cause down-regulation of adiponectin receptors and adiponectin resistance. We have also demonstrated that down-regulation of adiponectin receptors by insulin may be mediated via the phosphoinositide 3-kinase (PI3-kinase)/Foxo1 dependent pathway.
EXPERIMENTAL PROCEDURES

Chemicals—All materials were obtained from the sources given in Refs. 6, 10, 14, and 19. One-week-old ob/ob mice and their wild-type C57BL/6 mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Male mice were housed in colony cages and maintained on a 12-h light/12-h dark cycle. Their high fat diet contains oil (1152 g of safflower oil/100 g), sucrose (633.6 g of No. 13 from Oriental Yeast), vitamin mix (50.4 g of No. 20 from Oriental Yeast [AIN76]), cellulose powder (20.16 g of No. 19 from Oriental Yeast), DL-methionine (18 g from Wako Pure Chemicals, Osaka, Japan), and water 360 g/ml, total, 3600 g (19). Plasma insulin was measured by an insulin immunoassay (Shibayagi, Gunma, Japan). The animal care and procedures were approved by the Animal Care Committee of the University of Tokyo.

STZ Treatments—Diabetes was induced by a double intraperitoneal injection of 0.2–0.3 ml of 50 mM sodium citrate solution (pH 4.5) containing STZ (200 mg/kg body weight). Control (non-STZ) mice were administered 50 mM sodium citrate solution (pH 4.5) and 45% linoleic acid (18:2n-6) from total fatty acids (from Benihana, Tokyo, Japan) and 0.2 ml of saline was administered to the STZ + insulin group. The mice in the STZ + vehicle and non-STZ groups received 0.2 ml of saline injected intraperitoneally. After the injection of insulin or saline, the animals were fasted for 6 h, and then the hind limb skeletal muscles were removed.

Fasting and Refeeding—In the refeeding experiment, food was reintroduced after 48 h of fasting. The animals were killed 6 h after refeeding to isolate the tissues.

Mice Primary Hepatocytes—Hepatocytes were isolated from fasting 8-week-old male C57BL/6 mice by the collagenase perfusion method (20). Aliquots of 0.8 × 10^6 cells were plated onto collagen I-coated 12-well dishes (Iwaki, Chiba, Japan) in Williams medium E supplemented with 1% bovine serum albumin for 6 h. The cells were then switched to Williams medium E supplemented with 1% bovine serum albumin and 100 μg/ml streptomycin. After incubation at 37 °C in 5% CO₂ for 5 h, the cells were washed twice with phosphate-buffered saline and incubated with Williams medium E supplemented with 1% bovine serum albumin for 6 h. The cells were then switched to Williams medium E supplemented with 1% bovine serum albumin and 100 μg/ml insulin and harvested 6 h after the addition of insulin to isolate total RNA.

Studies with C2C12 Cells—Induction of myogenic differentiation was carried out according to a method described previously (10). In brief, the mouse C2C12 myoblasts were grown in 90% Dulbecco’s modified high glucose Eagle’s medium, 10% (v/v) horse serum, 10 μM 2-mercaptoethanol, 1 nM insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. Five days later, the cells were treated with the indicated concentrations of insulin. The cells were harvested 6 h after the addition of insulin to isolate total RNA.

Adenovirus-mediated Gene Transfer of Foxo1—Murine cDNAs encoding Foxo1-T24A/S253D/S316A were used (21, 22). C2C12 cells were transfected with adenovirus containing the cDNAs mentioned above as described previously (10) with some modifications. Induction of differentiation was carried out according to the methods described above. Five days later, the cells were treated with the indicated concentrations of insulin and then analyzed.

Quantitative Analysis of Adiponectin—Real-time PCR—Total RNA was prepared from cells or tissues with TRIzol (Invitrogen) according to the manufacturer’s instructions. A real-time PCR method was used to quantify the Adiponectin mRNA levels (14). The primer sets and conditions for the real-time PCR were as follows. The forward primer for murine AdipoR1 was 5’-aagttggagatctcgcct-3’, the reverse primer, 5’-ctctctgctggatgcgataa-3’, and the probe, 5’-ctctctgctggatgcgataa-3’. The forward primer for murine AdipoR2 was 5’-tctcagaggtggaatctcgc-3’, the reverse primer, 5’-tctcagaggtggaatctcgc-3’, and the probe, 5’-atgtccccgctcctacaggccc-3’. The relative amount of each Adiponectin transcript was normalized to the amount of β-actin transcript in the same cDNA (14).

Binding Assay—Synthetic adiponectin was 125I-labeled by IODO-beads (Pierce) in the presence of NaCl (2,000 Ci/mmol, American Biosciences) according to the manufacturer’s protocol. Membrane fractions of muscles were purified by sucrose gradient ultracentrifugation and incubated at 4 °C for 1 h with binding buffer (ice-cold phosphate-buffered saline/10% skim milk) containing the designated concentrations of [125I]adiponectin (2,000 cpm/ng of protein) plus unlabeled competitors. The binding equilibrium was judged to have been established when the binding assay was conducted at 4 °C for 2 h. The membrane fractions were then washed three times with ice-cold phosphate-buffered saline, and the membrane-bound radioactivity was determined using a γ-counter (10, 14). Nonspecific binding was determined using a 200-fold excess of unlabeled adiponectin. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The values presented under “Results” represent an average of triplicate determinations of three experiments.

RESULTS

Fasting Increased, but Refeeding Decreased AdipoR1/R2 Gene Expression—To clarify whether the expressions of AdipoR1 and AdipoR2 would be regulated under physiological and/or pathophysiological states, we first examined the expression levels of AdipoR1 and AdipoR2 in insulin and as adiponectin target organs such as skeletal muscle and liver in mice under ad libitum fed, fasted, and refeed conditions. At the start of the night cycle (9:00 p.m.), the animals were either provided with food or deprived of food for 48 h. A third group of mice was refed for 6 h after the 48-h fast. As can be seen in Fig. 1, A and B, the levels of AdipoR1 and AdipoR2 mRNA expression in the liver increased dramatically after the 48-h fast, and refeeding rapidly restored these to a level equal to the original fed state. We observed similar regulation of AdipoR1 and AdipoR2 mRNA by fasting and refeeding in skeletal muscle (Fig. 1, C and D).

Plasma Insulin Levels Appear to Be Inversely Correlated with Expression Levels of the AdipoR1/R2 Gene—The particular hormones or nutrients responsible for this response to fasting and feeding are of considerable interest. Because insulin is the classic hormone of the fed state, we next measured plasma insulin levels under the ad libitum fed, fasted, and refeed states. As expected, fasting decreased the plasma insulin levels (Fig. 1F), whereas they were increased by refeeding in a parallel fashion with the plasma glucose levels (Fig. 1E). These findings suggested that plasma insulin levels may appear to be inversely correlated with the expression levels of the AdipoR1/R2 gene and raised the possibility that insulin may control AdipoR1/R2 expression.

STZ Treatment Increased, but Insulin Decreased AdipoR1/R2 Expression—To address this issue, we next studied the effects of insulin on AdipoR1/R2 expression in mice treated with STZ. To eliminate the effects of insulin on food intake, we withdrew food from all of the mice and killed the animals 6 h later. STZ treatment abolished plasma insulin (Fig. 2A) and, at the same time, caused a significantly marked increase in plasma glucose (Fig. 2B, lanes 1 and 2). The animals treated with STZ plus insulin exhibited reduced plasma glucose levels compared with the animals treated with STZ alone (Fig. 2B, lanes 2 and 3).

We next measured the amounts of mRNA for AdipoR1 and AdipoR2 in skeletal muscle and liver extracts. The results revealed...
To further address the possibility that insulin may reduce AdipoR1/R2 mRNA levels, we next studied whether insulin would reduce AdipoR1/R2 mRNA levels in C2C12 myocytes in vitro. Insulin treatment at 10 nM did indeed decrease AdipoR1/R2 expression after 6 h (Fig. 3, C and D).

Insulin-mediated Reduction in AdipoR1/R2 mRNA Is Suppressed by Inhibitors of PI3-kinase—The level of AdipoR1/R2 mRNA in C2C12 myocytes was investigated 6 h after the addition of insulin to myocytes incubated with various inhibitors of insulin signaling pathways (Fig. 3, C and D). The PI3-kinase inhibitor LY294002 abolished the decreased effect of insulin. In contrast, the response to insulin was essentially unaffected by PD98059, an inhibitor of mitogen-activated protein kinase activation. The inhibitory effects shown in Fig. 3, C and D, were maximal for all of the drugs tested, a point that was verified by dose-response experiments (results not shown).

Fox1 Increased AdipoR1/R2 mRNA Levels and Blocked Insulin-induced Reduction of the Levels—Insulin-dependent repression of the genes, which harbor a cis-acting element capable of binding FoxO1 in their promoter, might depend, at least in part, on PI3-kinase/Akt-mediated phosphorylation and inactivation of this forkhead transactivator (21, 22). Expression of the constitutively active form of FoxO1 (FoxO1-T24A/S253D/S316A) caused a marked increase in AdipoR1/R2 mRNA levels. Insulin could not reverse the effect of FoxO1 on AdipoR1/R2 mRNA levels (Fig. 3, E and F). These findings suggest that FoxO1 increased the expression levels of AdipoR1/R2 and that insulin repressed AdipoR1/R2 mRNA expressions via inactivation of FoxO1.

AdipoR1/R2 Expression Levels Were Down-regulated in ob/ob Mice—We studied the expression of AdipoR1 and -R2 in insulin-sensitive tissues (such as liver), skeletal muscle (such as the soleus muscle and extensor digitorum longus (EDL)), and adipose tissues (such as white adipose tissue (WAT) and brown adipose tissue (BAT)) in control C57BL/6 mice and insulin-resistant ob/ob mice. As shown in Fig. 4, A and B, in control C57BL/6 mice, AdipoR1 was most abundantly expressed in skeletal muscle, whereas AdipoR2 was most abundantly ex-

**A. Tsuchida, T. Yamauchi, and T. Kadowaki, manuscript in preparation.
pressed in the liver. Interestingly, both AdipoR1 and -R2 were more abundantly expressed in the EDL than in the soleus muscle. Moreover, both WAT and BAT expressed AdipoR1 and -R2.

The expressions of both AdipoR1 and -R2 were significantly decreased in most of the insulin-sensitive tissues examined, except the liver, in ob/ob mice as compared with control C57BL/6 mice (Fig. 4, A and B). The expressions of AdipoR1 in the liver, soleus, EDL, WAT, and BAT from ob/ob mice were 79.4, 54.5, 50.5, 31.9, and 28.1% of those from the wild-type mice, respectively (Fig. 4A). The expressions of AdipoR2 in the liver, soleus, EDL, WAT, and BAT from ob/ob mice were 63.3, 51.4, 59.5, 7.9, and 29.4% of those from the wild-type mice, respectively (Fig. 4B).

**Decreased Expression Levels of AdipoR1 and R2 in ob/ob Mice Appear to Be Correlated with Decreased Adiponectin Sensitivity**—To clarify whether the expression levels of AdipoR1/R2 are associated with the effects of adiponectin in each tissue, we measured adiponectin binding to the membrane fractions of skeletal muscle and AMPK activation by adiponectin. Interestingly, adiponectin had a higher binding to the membrane fractions of wild-type mice than ob/ob mice (Fig. 5, A and B). Scatchard plot analysis revealed that both of the high affinity and low affinity binding sites for globular adiponectin and adiponectin binding in skeletal muscles of ob/ob mice were reduced compared with those of wild-type mice, suggesting that the numbers of both AdipoR1 and AdipoR2 were reduced (Fig. 5, A and B) (14). Moreover, adiponectin was able to activate AMPK in skeletal muscle of wild-type mice, whereas adiponectin was unable to activate AMPK in skeletal muscles of ob/ob mice (Fig. 5, C and D). These observations suggest that the expression levels of AdipoR1/R2 might regulate adiponectin binding and AMPK activation by adiponectin referred to as adiponectin sensitivity.

**DISCUSSION**

We have recently cloned the adiponectin receptors AdipoR1 and AdipoR2, which appear to mediate the insulin-sensitizing actions of adiponectin (14). In the present study, we examined the expression levels of AdipoR1 and -R2 to explore the potential role of altered expression of adiponectin receptors in the
regulation of insulin and/or adiponectin sensitivity. Interestingly, we showed for the first time in this study that fasting increased, whereas refeeding decreased, the expression levels of AdipoR1 and -R2.

During fasting, increased plasma catecholamines and glucocorticoids can raise cAMP levels, resulting in the activation of the transcription factor cAMP response element-binding protein through protein kinase A (24). This factor then functions in a variety of fasting-sensitive genes through a canonical cAMP response element-binding protein binding site. Fasting also leads to an elevation of glucocorticoids, which control gene expression mainly if not exclusively through the glucocorticoid receptor and its well-defined cognate sequence (25). However, neither cAMP nor glucocorticoids appear to have marked effects on the expression levels of AdipoR1/R2.3

Plasma insulin levels are decreased by fasting and increased by refeeding. Thus, we hypothesized that insulin can negatively regulate the expression levels of AdipoR1/R2. To test this hypothesis, we employed another animal model, STZ-induced insulin deficiency. Interestingly, STZ treatment diminished plasma insulin and at the same time caused a marked increase in AdipoR1/R2 mRNA levels, and replenishment of insulin into STZ-treated mice reduced the levels to those observed in mice without STZ treatment, indicating that insulin reversed the effect of STZ on AdipoR1/R2 mRNA levels. Insulin greatly decreased AdipoR1/R2 expressions at the mRNA level and did so at doses of insulin consistent with those functioning through the insulin receptor. Of course, it is entirely possible that other hormones or nutrients in addition to insulin participate in the regulation of AdipoR1/R2 in this in vivo context.

Most, if not all, of the known actions of insulin can be attributed to the receptor-mediated tyrosine phosphorylation of insulin receptor substrates 1 and 2, which in turn triggers kinase cascades involving the PI3-kinase/Akt pathway and the mitogen-activated protein kinase pathway (26–28). With respect to AdipoR1/R2 gene expression, insulin signaling via several possible redundant pathways remains an intriguing possibility. The insulin effect on AdipoR1/R2 gene expression was virtually unaffected by the mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, PD98059, and thus does not appear to require activation of the mitogen-activated protein kinase cascade. By contrast, results with LY294002 clearly implicated the PI3-kinase signaling pathway. These results of experiments using an inhibitor of PI3-kinase lead us to propose that the induction of AdipoR1/R2 gene expression is most likely mediated via the PI3-kinase branch of the insulin signaling pathways.

The PI3-kinase signaling pathway is thought to be involved in the regulation of several genes by insulin, notably the gene for the insulin-like growth factor-binding protein 1 (29). Recently, Akt, which is the downstream effector of PI3-kinase, was shown to phosphorylate several transcriptional activators of the forkhead family, including Foxo1, in vitro and in intact cells. The phosphorylation of specific residues in these factors inhibited their ability to activate the transcription of target genes as a result of the sequestration of the phosphorylated factors in the cytoplasm (30–32). Thus, the insulin-dependent repression of the insulin-like growth factor-binding protein 1 gene, which harbors a cis-acting element capable of binding Foxo1 in its promoter, is thought to depend (at least in part) on Akt-mediated phosphorylation and inactivation of this forkhead transactivator (29).

The DNA-binding characteristics are noteworthy because several (but not all) identified insulin response elements appear to be recognized by Foxo1. Visual and computer-assisted examination reveals many potential responsive sites (a sequence such as an insulin response element) in the AdipoR1/R2 promoter. Thus, we next examined whether Foxo1 would regulate the AdipoR1/R2 expressions. Interestingly, Foxo1 increased the AdipoR1/R2 expressions, and insulin was unable to reduce them, suggesting that Foxo1 increased and insulin reduced the AdipoR1/R2 expressions via down-regulation of Foxo1 activity. To date, there are no data indicating that ectopic expression of other factors can regulate AdipoR1/R2 expression. Further studies will be required to clarify this point.

Finally, we found that the expressions of AdipoR1 and AdipoR2 were down-regulated in ob/ob mice, a model of insulin resistance linked to obesity. We have shown previously that plasma adiponectin levels were decreased in ob/ob mice (12) and that these alterations in plasma adiponectin levels may play causal roles in the regulation of insulin sensitivity. These in combination raise the interesting possibility that altered expressions of AdipoR1/R2 in addition to plasma adiponectin levels may play a causal role in the regulation of insulin sensitivity. This is consistent with our previous observation that decreased expressions of AdipoR1 or AdipoR2 by their respective small interfering RNA were associated with the reduced insulin-sensitizing effects of adiponectin (14). We provided the first evidence that the decreased expressions of AdipoR1 or AdipoR2 in these mouse models are associated with reductions in the insulin-sensitizing effects of adiponectin in vivo.

Chronic overfeeding and its attending elevation of insulin would be expected to result in the decreased expression of AdipoR1/R2. This is likely to cause both adiponectin resistance and hence insulin resistance. The decrease in AdipoR1/R2 mRNA leads to a decrease in adiponectin binding, and this in turn leads to a decrease in the adiponectin effects, termed adiponectin resistance, the so-called vicious cycle. These findings may provide a novel molecular mechanism for the well-known ability of insulin to induce insulin resistance, down-regulation of adiponectin receptors, in addition to the previously recognized down-regulation of insulin receptors.

In conclusion, the expressions of AdipoR1/R2 in insulin target tissues appear to be inversely correlated with plasma insulin levels. The present study is the first to demonstrate that insulin negatively regulates the expression levels of adiponectin receptors via the PI3-kinase/Foxo1 pathway. Our data also suggest that not only agonism of AdipoR1/R2 but also strategies to increase AdipoR1/R2 may be logical approaches to provide a novel treatment modality for insulin resistance and type 2 diabetes.

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REFERENCES


