Effects of Antidiabetic Treatment with Metformin and Insulin on Serum and Adipose Tissue Adiponectin Levels in \textit{db/db} Mice

HIROKI FUJITA, HIROMI FUJISHIMA, JUN KOSHIMURA, MIHOKO HOSOBA, NAOYI YOSHIOKA, TAKASHI SHIMOTOMAI, TSUKASA MORII, TAKUMA NARITA, MASAFUMI KAKEI AND SEIKI ITO

Division of Endocrinology, Metabolism and Geriatric Medicine, Department of Internal Medicine, Akita University School of Medicine, Akita 010-8543, Japan

Abstract. Decreased circulating levels of adiponectin, a novel adipose-derived adipocytokine, in obesity possibly contribute to the development of insulin resistance which is a major factor in the pathogenesis of type 2 diabetes. The present study was conducted to examine whether circulating and adipose tissue adiponectin levels are modulated by chronic treatment with metformin and intensive treatment with insulin in murine models of obesity and type 2 diabetes, \textit{db/db} mice with a C57BL/KsJ genetic background. Nine-week-old male \textit{db/db} mice were treated with metformin, insulin, and vehicle for 4 weeks. Expectedly, metformin treatment led to inhibition of weight gain and improvement of hyperinsulinemia. Insulin treatment lowered fasting blood glucose levels to normal values, although it sustained hyperinsulinemic state. However, after 4 weeks of treatment, serum adiponectin levels were not significantly elevated in either metformin-treated or insulin-treated \textit{db/db} mouse group (14.2 ± 0.7 and 16.7 ± 1.0 \mu g/ml, respectively) compared to vehicle-treated group (14.9 ± 0.6 \mu g/ml). Similarly, adipose tissue adiponectin levels determined by Western blot analysis were not increased in either metformin-treated or insulin-treated group relative to vehicle-treated group. Recent studies have shown that adiponectin possibly has the same physiological effects on lipid and glucose metabolism that metformin has. Therefore, an elevation in blood concentration of metformin following the treatment might lead to suppression in adiponectin synthesis in adipose tissue, independent of inhibition in weight gain and improvement in hyperinsulinemia by metformin treatment. The present results indicate that adiponectin is not involved in the mechanism by which metformin treatment enhances insulin sensitivity. Moreover, our results suggest that adiponectin synthesis in adipose tissue may be suppressed under hyperinsulinemic state sustained by insulin treatment, even though hyperglycemia is markedly reduced. We conclude that antidiabetic treatment with metformin and insulin does not affect circulating and adipose tissue adiponectin levels.

Key words: Adiponectin, Metformin treatment, Insulin treatment, Obesity, Insulin resistance

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**OBESITY** is a major risk factor for the development of type 2 diabetes. An accumulation of excess body fat in obesity frequently contributes to the development of insulin resistance which is a major factor in the pathogenesis of type 2 diabetes. Adipose tissue is currently known to work not simply as an organ for energy storage, but also as an endocrine and secretory organ. Various proteins with endocrine functions such as leptin [1, 2], tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) [3], and plasminogen-activator inhibitor-1 (PAI-1) [4], which are shown to regulate insulin sensitivity, are secreted from adipose tissue into circulating blood. These proteins are collectively called adipocytokines. Although the mechanism by which obesity causes insulin resistance has not been fully elucidated, changes in the expression, secretion, and action of the adipocytokines in obesity are possibly implicated in the development of insulin resistance.

Adiponectin (also known as Acrp30) is a novel adipocytokine that has been recently identified [5–8]. It is exclusively expressed in adipose tissue [5] and abundantly released into circulating blood [9]. More recently, the findings that obesity decreases plasma
adiponectin levels have been reported in humans [9–11] and experimental animals [12, 13]. Moreover, it has been shown that hypoadiponectinemia is closely related to insulin resistance [14, 15]. Interestingly, administration of recombinant adiponectin to obese mice improves insulin resistance and lowers blood glucose levels [16, 17]. These lines of evidence suggest that a reduction in adipose and circulating adiponectin levels in obesity may greatly contribute to the development of insulin resistance, and that adiponectin may be working as a key regulator of insulin sensitivity.

Thiazolidinediones (TZDs) are used as insulin-sensitizing drugs to treat type 2 diabetic patients with insulin resistance. The insulin-sensitizing effects of TZDs are considered to be mediated by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) which is abundant in adipose tissue. Recent studies have demonstrated that treatment with TZDs increases plasma adiponectin levels in type 2 diabetic patients [18–21] and murine models of obesity and type 2 diabetes, db/db mice [22], and even in nondiabetic subjects [21, 22]. Moreover, treatment with a TZD, rosiglitazone, has been shown to increase adiponectin mRNA and protein expression in adipose tissue of db/db mice [22]. Therefore, TZDs are likely to enhance adiponectin synthesis and secretion in adipose tissue and subsequently increase plasma adiponectin levels. This may be postulated as a mechanism by which TZDs ameliorate insulin-resistant state. Thus, TZDs have an intrinsic effect on the regulation of circulating and adipose tissue adiponectin levels.

In addition to TZDs, various antidiabetic drugs such as insulin, sulfonylurea, metformin, and α-glucosidase inhibitor are currently used to treat type 2 diabetic patients. However, it still remains unclear whether these drugs except TZDs affect circulating and adipose tissue adiponectin levels. Similar to TZDs, metformin is also regarded as an insulin-sensitizing drug and widely used to treat type 2 diabetic patients with insulin resistance. While metformin has been shown to improve hepatic insulin resistance and reduce hyperglycemia by reducing hepatic gluconeogenesis [23, 24] and glycogenolysis [25, 26], it is not well understood whether metformin treatment enhances insulin action by increasing circulating and adipose tissue adiponectin levels. On the other hand, insulin treatment, as monotherapy or in combination with TZDs or metformin, is often started in type 2 diabetic patients with insulin resistance failing to respond to treatment with TZDs or metformin, and actually improves glycemic control in those patients. However, there are no data regarding the effects of insulin treatment on circulating and adipose tissue adiponectin levels.

In the present study, we examined the effects of chronic metformin treatment and intensive insulin treatment on circulating and adipose tissue adiponectin levels in murine models of obesity and type 2 diabetes, db/db mice.

**Materials and Methods**

**Experimental animals**

Male obese diabetic db/db mice, with a C57BL/KsJ genetic background, and their lean non-diabetic heterozygous littermates, db/m mice, were purchased from Clea Japan (Tokyo, Japan) at 8 weeks of age. The mice were housed (n = 3–4 per cage) in a room with relative humidity of 50% and a 12/12-h light/dark cycle at 20 to 22°C, and allowed unrestricted access to standard rodent chow and water.

**Antidiabetic treatment with metformin and insulin**

At 9 weeks of age, the db/db mice were divided into the following 3 groups: metformin group (n = 7), insulin group (n = 7), and control group (n = 7). The db/db mice of metformin group were intraperitoneally injected once daily with metformin (250 mg/kg; Nippon Shinyaku, Tokyo, Japan) in saline for 4 weeks. The db/db mice of insulin group received daily morning and evening subcutaneous injections of neutral protamine Hagedorn (NPH) insulin (Humulin N, Eli Lilly, Indianapolis, IN, USA) in doses individually adjusted to maintain fasting blood glucose levels between 60 and 100 mg/dl for 4 weeks. The db/db mice of insulin group received daily morning and evening subcutaneous injections of neutral protamine Hagedorn (NPH) insulin (Humulin N, Eli Lilly, Indianapolis, IN, USA) in doses individually adjusted to maintain fasting blood glucose levels between 60 and 100 mg/dl for 4 weeks. The db/db mice of control group were intraperitoneally injected once daily with vehicle (saline). Fasting blood glucose levels were monitored using blood samples collected through tail bleeds at least once a week. Age-matched db/m mice (n = 4) served as lean non-diabetic controls. After 12 h fast at the end of the treatment period, the db/db and db/m mice were sacrificed, and their epididymal adipose tissues were removed. Blood samples collected at the time the mice were sacrificed were used for determination of blood glucose, serum insulin, and serum adiponectin levels. Blood glucose levels were mea-
sured using a glucose monitoring device, Tide (Miles-Sankyo, Tokyo, Japan). Serum insulin levels were determined using a commercially available radioimmunoassay kit (Eiken Chemical, Tokyo, Japan). Serum adiponectin levels were measured using a commercially available ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan). All animals were treated in accordance with the Animal Welfare Guidelines of Akita University, and all procedures were approved by the Committee on Animal Experimentation of Akita University.

Western blot analysis

Adipose tissue adiponectin levels were determined by Western blot analysis. The removed epididymal adipose tissues were homogenized in PBS containing 0.5% sodium deoxycholate. Homogenates were incubated for 24 h at 37°C. After the incubation, the homogenates were centrifuged at 15,000 g for 10 min. The fat cake was removed by suction, and adipose tissue extracts (supernatants) were used for Western blot analysis. Aliquots of the tissue extracts (10 μg of protein) prepared in SDS sample buffer were incubated for 5 min at 100°C. Denatured proteins were separated by SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated with 1 : 10,000 dilution of mouse anti-mouse adiponectin monoclonal antibody (Chemicon International, Temecula, CA, USA) for 12 h at room temperature and then incubated with 1 : 5,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (DAKO, Glostrup, Denmark) for 1 h at room temperature. After the incubation, the membranes were soaked in chemiluminescence solution using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membranes were exposed to X-ray film, and the adiponectin protein was thus visualized. The signals from X-ray film were quantified using Scion Image software (Chicago, IL, USA). Data are presented as means ± SE. Statistical significance was evaluated with ANOVA. Post hoc comparisons of group pairs were performed by Scheffé’s multiple comparison test after ANOVA had revealed significant differences among groups. A P-value of less than 0.05 was considered statistically significant.

Results

Clinical parameters before and after antidiabetic treatment with metformin and insulin

At baseline, control, metformin, and insulin groups were matched with respect to body weight (40.6 ± 0.5, 42.0 ± 0.5, and 41.6 ± 0.6 g, respectively; Fig. 1A) and fasting blood glucose (244 ± 21, 245 ± 12, and 224 ± 31 mg/dl, respectively; Fig. 1B). After 4 weeks of antidiabetic treatment, body weight and epididymal fat weight in metformin group (42.1 ± 0.8 g and 2.46 ± 0.05 g, respectively) were significantly decreased as compared with those in control group (50.4 ± 0.4 g and 3.07 ± 0.07 g, respectively), whereas body weight and epididymal fat weight in insulin group (47.1 ± 0.8 g and 2.90 ± 0.10 g, respectively) were not significantly different from those in control group (Figs. 1A, 2A). After the antidiabetic treatment, fasting blood glucose was significantly lower in metformin and insulin groups (214 ± 7 and 73 ± 6 mg/dl, respectively) than in control group (302 ± 13 mg/dl; Fig. 1B). In particular, the intensively insulin-treated db/db mice achieved normal levels of fasting blood glucose. As expected, fasting serum insulin levels were significantly decreased in metformin and insulin groups (214 ± 7 and 73 ± 6 mg/dl, respectively) than in control group (302 ± 13 mg/dl; Fig. 1B). In particular, the intensively insulin-treated db/db mice achieved normal levels of fasting blood glucose. As expected, fasting serum insulin levels were significantly decreased in metformin group (360 ± 30 pmol/l) relative to control group (589 ± 79 pmol/l; Fig. 2B). This finding was considered to reflect an improvement in insulin sensitivity by metformin treatment. Insulin group showed remarkably high levels of fasting serum insulin (679 ± 70 pmol/l), similar to control group (Fig. 2B). The db/m mice with a heterozygous diabetogenic gene (db) exhibited lean and non-diabetic characteristics compared to control db/db mice throughout the period of study.

Serum and adipose tissue adiponectin levels after antidiabetic treatment with metformin and insulin

Serum and adipose tissue adiponectin levels in
Control, metformin, and insulin groups and inagematched db/m mice are shown in Figs. 3 and 4. Adiponectin protein in adipose tissue was visualized as a band of 30 kDa by Western blot analysis. Serum adiponectin levels were not significantly elevated in either metformin or insulin group (14.2 ± 0.7 and 16.7 ± 1.0 µg/ml, respectively) compared to control group (14.9 ± 0.6 µg/ml). Similarly, adiponectin levels in adipose tissue were not increased in either metformin or insulin group relative to control group. Thus, despite inhibition in weight gain and improvement in hyperinsulinemia, metformin treatment did not affect serum and adipose tissue adiponectin levels. Moreover, although intensive insulin treatment lowered fasting blood glucose levels in db/db mice to normal values, it did not affect the adiponectin levels. Expectedly, serum adiponectin levels in db/m mice (23.9 ± 1.9 µg/ml), which served as lean non-diabetic controls, were significantly higher than those in control db/db mice, and adipose tissue adiponectin levels in db/m mice were also increased by approximately 150% relative to those in control db/db mice.
Discussion

In comparison between obese \( db/db \) mice and age-matched lean \( db/m \) mice, expectedly, both serum and adipose tissue adiponectin levels were significantly decreased in the obese \( db/db \) mice (Figs. 3, 4). These results suggest that obesity inhibits adiponectin synthesis and secretion in adipose tissue and subsequently lowers circulating adiponectin levels. Therefore, improvement of obesity, in other words, weight reduction may normalize circulating and adipose tissue adiponectin levels. Regarding this suggestion, two recent studies demonstrated the effect of weight reduction on circulating adiponectin levels. One study showed that weight reduction by calorie-restricted therapy increased plasma adiponectin levels [27]. The other study showed that weight reduction in obese patients who received gastric partition surgery led to an elevation in plasma adiponectin levels [28].

Metformin treatment is well known to cause weight loss by reducing adipose tissue in obese patients with type 2 diabetes [23, 29]. Also in the current study, chronic treatment with metformin in \( db/db \) mice inhibited the gains in body weight and epididymal fat weight (Figs. 1A, 2A). In addition, metformin treatment reduced hyperinsulinemia in \( db/db \) mice, which indicates an improvement in insulin resistance (Fig. 2B). Nevertheless, serum and adipose tissue adiponectin levels were not significantly increased in metformin-treated \( db/db \) mice compared to vehicle-treated \( db/db \) mice (Figs. 3, 4). As far as we know, only one study has previously investigated the effects of chronic metformin treatment in combination with sulfonylurea, not as monotherapy, on the adiponectin levels. Consistent with our results, the previous study showed that chronic treatment with a combination of metformin and sulfonylurea in obese type 2 diabetic patients did not significantly change serum and adipose tissue adiponectin levels [20]. However, it has not been examined whether chronic metformin treatment as monotherapy affects the adiponectin levels. In our study, monotherapy with metformin was carried out in murine models of obesity and type 2 diabetes, and therefore our results can be assessed without consider-
ing the confounding effects of other antidiabetic drugs which would be caused under metformin treatment in combination with other antidiabetic drugs. While weight reduction has been shown to enhance circulating adiponectin levels [27, 28], it still remains unclear why the inhibition of weight gain by metformin treatment fails to increase circulating and adipose adiponectin levels. However, the findings of three recent studies may help to explain this reason. Adiponectin has been shown to cause weight reduction through its ability to stimulate lipid oxidation [30]. Moreover, adiponectin has been reported to inhibit both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production [31]. Regarding the mechanism of metformin action, it has been reported that metformin induces lipid (fatty acid) oxidation and reduces hepatic glucose production by activating AMP-activated protein kinase (AMPK) [32]. Based on these lines of evidence, adiponectin possibly has the same physiological effects on lipid and glucose metabolism that metformin has, and this suggests that adiponectin may work in the body like metformin. Therefore, an elevation in blood concentration of metformin following the treatment might lead to suppression in adiponectin synthesis in adipose tissue, independent of inhibition in weight gain and improvement in hyperinsulinemia by metformin treatment. Further studies are required to clarify the relationship between adiponectin and metformin.

On the other hand, although intensive treatment with insulin lowered fasting blood glucose levels of db/db mice to normal values (Fig. 1B), this treatment did not increase circulating and adipose tissue adiponectin levels (Figs. 3, 4). Therefore, adiponectin synthesis in adipose tissue may be suppressed under hyperinsulinemic state sustained by insulin treatment, even though hyperglycemia is markedly reduced.

We conclude that adiponectin is not involved in the mechanism by which metformin treatment enhances insulin sensitivity. Finally, we suggest that antidiabetic treatment with metformin and insulin does not affect circulating and adipose tissue adiponectin levels.

References


