Androgens Decrease Plasma Adiponectin, an Insulin-Sensitizing Adipocyte-Derived Protein

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Adiponectin, an adipose-specific secretory protein, exhibits antidiabetic and antiatherogenic properties. In the present study, we examined the effects of sex hormones on the regulation of adiponectin production. Plasma adiponectin concentrations were significantly lower in 442 men (age, 52.6 ± 11.9 years [mean ± SD]) than in 137 women (53.2 ± 12.0 years) but not different between pre- and postmenopausal women. In mice, ovariectomy did not alter plasma adiponectin levels. In contrast, high levels of plasma adiponectin were found in castrated mice. Testosterone treatment reduced plasma adiponectin concentration in both sham-operated and castrated mice. In 3T3-L1 adipocytes, testosterone reduced adiponectin secretion into the culture media, using pulse-chase study. Castration-induced increase in plasma adiponectin was associated with a significant improvement of insulin sensitivity. Our results indicate that androgens decrease plasma adiponectin and that androgen-induced hypoadiponectinemia may be related to the high risks of insulin resistance and atherosclerosis in men. Diabetes 51:2734–2741, 2002

Adipose tissue secretes a variety of biologically active molecules, conceptualized as adipocytokines/adipokines (1–5). We and others have shown that dysregulated secretions of adipocytokines are involved in the development of common metabolic disorders (1,6–11). Increased production and secretion of tumor necrosis factor (TNF)-α and resistin from the accumulated adipose tissues are thought to be partially responsible for the development of insulin resistance in obesity (6–8). Furthermore, overproduction of plasminogen activator inhibitor type 1 from intra-abdominal fat was suggested to contribute to the development of thrombotic vascular diseases highly associated with abdominal fat obesity (1). Leptin, which regulates the amount of body fat by reducing food intake and increasing energy expenditure through its action on the hypothalamus (12), was shown to increases insulin sensitivity in the whole body (13). Therefore, lack of leptin in lipodystrophy and leptin resistance in obesity caused the decrease of insulin sensitivity, resulting in the development of insulin resistance syndrome (14,15).

Adiponectin is an adipocyte-derived plasma protein, which we identified through the human cDNA project targeting on adipose tissue (16). ACRP30 and AdipoQ were isolated from differentiated adipocytes independently by Scherer et al. (17) and Hu et al. (18) and are the mouse counterpart of adiponectin. Adiponectin is expressed specifically in adipose tissue (9,16–18) and exists abundantly in the plasma at the range of 5–30 μg/ml (19). Adiponectin possesses a signal sequence at the NH2-terminal end and is composed of the collagen repeats domain and the COOH-terminal globular domain (18,20). Plasma concentrations of adiponectin correlate inversely with BMI (19) and are reduced in patients with type 2 diabetes compared with those in BMI-matched subjects (10). During the course of development of type 2 diabetes in rhesus monkey, reduction of plasma adiponectin occurs before the incidence of hyperinsulinemia, which is used as a parameter of insulin resistance (9). Hyperinsulinemic-euglycemic clamp studies in human and monkey revealed that plasma concentrations of adiponectin significantly correlate with the degree of insulin sensitivity in the whole body (9,11). Furthermore, several recent studies have shown that the supplement of recombinant adiponectin improved insulin resistance in diabetic mice (21,22). Thus, the above studies demonstrated that adiponectin is an insulin-sensitizing hormone and that hypoadiponectinemia in obesity and type 2 diabetes is involved in the development of insulin resistance. Furthermore, we showed the possible involvement of hypoadiponectinemia on the pathophysiology of atherosclerosis. First, hypoadiponectinemia associated strongly with the incidence of coronary artery diseases (23). Second, the degree of hypoadiponectinemia correlated with the incidence of fatal heart ischemia in the subjects with chronic renal failure (24). Finally, adiponectin had various antiatherogenic moieties to vascular endothelial cells, smooth muscle cells, and macrophages in tissue cultures (23,25,26).

In the present study, we investigated the effect of sex hormones on the production of adiponectin in human subjects, rodents, and cultured cells. We demonstrate for the first time that testosterone reduces the production of...
adiponectin in plasma, which may relate to the high risks of insulin resistance and atherosclerosis in men.

RESEARCH DESIGN AND METHODS

Plasma analyses in humans. We studied 579 adult Japanese: 442 men (mean ± SD age 52.6 ± 11.9 years; mean ± SD BMI 23.5 ± 2.6 kg/m²) and 137 women (age 53.2 ± 12.0, BMI 22.8 ± 2.0). Because plasma adiponectin concentration was considered to be low in female subjects (19), only men and women enrolled in the present study were matched for age and BMI. These participants had had annual health checks. A signed informed consent was obtained from all subjects, and study protocols were approved by the Japanese Visceral Fat Syndrome study committee of the Ministry of Health and Welfare of Japan or the Medical Ethics Committee of Osaka University.

Biodata was drawn after an overnight fast. Plasma adiponectin concentrations were measured by sandwich enzyme-linked immunomassay assay (ELISA) as described previously (18). Plasma glucose was assayed by the glucose oxidase method, and insulin was assayed by immunoradiometric assay using Insulin Ribead (Dainabot, Osaka, Japan). Plasma concentrations of TNF-α were measured by ELISA using Quantikine HS (R&D Systems, Minneapolis, MN), and plasma leptin levels were measured by ELISA as described previously (27).

Animals and experimental protocol. ICR mice and C57BL/6J mice were obtained from Clea Japan (Tokyo, Japan) and kept under a 12-h light-dark cycle (lights on 8:00 a.m. to 8:00 p.m.) and constant temperature (22°C) with free access to food and water. The composition of the diet was as follows: 49% carbohydrate 54.0%, protein 23.8%, and fat 51%. Experiments of castration and ovariec- tomy were performed as follows: 20-week-old ICR male mice were castrated (CAST, n = 4) or sham-operated (SHAM, n = 4) and 20-week-old ICR female mice were ovariec- tomized (OVAR, n = 5) or sham-operated (SHAM, n = 4) under pentobarbital sodium anesthesia (50 mg/kg body wt). For castration, after midline abdominal incision, the testes were dissected out with epididymal fat pads, and testicular ducts and blood vessels supplying the testes were isolated and ligated. The testes were then excised through an incision, the epididymal fat pads were put back, and the incision was closed with suture. For the sham castration, after abdominal incision, the uterus, oviducts, and ovaries were exposed with the fat tissues and were put back followed by closure with suture. At 6 weeks after operation, blood samples were collected after a 6-h fast. Glucose and insulin were measured by Glucose CII-Test Wako kit (Wako, Osaka, Japan) and a double-antibody enzyme immunoassay using a Glazyme Insulin EIA Kit (Sanyo Chemical Industries, Kyoto, Japan) with rat insulin as a standard, respectively. The homoeostasis model assessment insulin resistance (HOMA-IR) values were calculated from the fasting concentrations of insulin and glucose using the following formula: fasting plasma insulin (μU/ml) × fasting plasma glucose (mg/dl)/405.

The experiments of castration and testosterone supplement were performed as described previously with minor modifications (28). Briefly, 11-week-old C57BL/6J male mice were castrated (CAST, n = 10) or sham-operated (SHAM, n = 9) under pentobarbital sodium anesthesia as mentioned above. At 6 weeks after operation, randomly selected castrated and sham-operated mice (n = 5 and n = 4, respectively) received one-shot subcutaneous injection of testosterone propionate (Wako) (0.5 mg/100 g body wt) every other day for 2 weeks, whereas other groups of castrated and sham-operated mice received the vehicle (polyethylene glycol) only. After a 6-h fast, mice were killed under anesthesia, and various tissues and blood were collected. This experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Insulin sensitivity test. Insulin sensitivity in mice was estimated as described previously, with minor modifications (29). Briefly, after a 6-h fast, ICR mice received a subcutaneous injection of saline containing epinephrine (1 μg/10 g body wt; Sigma) and propranolol (50 μg/10 g body wt; Sigma) to block the endogenous secretion of insulin. Subsequently, glucose (1 g/10 g body wt) and insulin (0.5 units/kg body wt) were injected intraperitoneally. Blood samples were obtained at 0 and 1 h after injections by cardiac puncture, and glucose concentra- tions were measured. Insulin sensitivity was estimated by percentage changes of plasma glucose concentrations.

Cell culture. 3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS and differentiated with DMEM supple-
Table 1 show the profiles of subjects from the Japanese Visceral Fat Syndrome study. There were no significant differences in plasma glucose and insulin concentrations between men and women. Plasma levels of TNF-α, which was demonstrated to reduce the expression and production of adiponectin (31), showed no sex difference either (Table 1). Figure 1A demonstrates that plasma adiponectin was 35% lower in men than in women. Furthermore, leptin plasma concentrations in men were lower than in women (Fig. 1A), as reported previously (33). Plasma adiponectin concentrations in premenopausal women were not significantly different from those in postmenopausal women (Fig. 1B). In contrast, plasma leptin concentrations in premenopausal women were lower than in postmenopausal women (Fig. 1B), as the increasing effect of estrogen on the leptin production was described previously (34). These results suggested that androgens might reduce plasma adiponectin concentrations in men.

Animal and in vitro studies. For validating the above hypothesis, male mice were castrated, treated with or without testosterone, and adiponectin production was analyzed in these animals (Fig. 2). Figure 2A provides a schematic illustration of the experiment. From 6 weeks after the castration or sham operation, mice were treated with subcutaneous injections of testosterone or vehicle solution, every other day, for a total of eight injections. On the next day of the final injection, mice were killed. Figure 2B shows the weights of the body and various organs. The operation procedures and injections did not influence body weight or weight of subcutaneous white fat. Previous studies demonstrated that the weight of kidney was decreased after castration as a result of a fall in testosterone level and that such changes were reversed after testosterone supplement (35,36). In the present study, we observed a significant decrease in kidney and prostate weights by castration (28 and 77% of sham-operated mice, respectively) and found that testosterone treatment restored the weights of these organs to near-normal levels. These results validated the operation procedures and testosterone treatment in this study.

Figure 3A shows plasma adiponectin concentrations measured by Western blotting in these mice. Castration significantly increased the concentrations of plasma adiponectin (170% of sham-operated mice). Furthermore, testosterone injections reduced plasma adiponectin in

### Table 1

<table>
<thead>
<tr>
<th>Clinical data in men and women participants</th>
<th>Men (n = 442)</th>
<th>Women (n = 137)</th>
<th>P</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>52.6 ± 11.9</td>
<td>53.2 ± 12.0</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.3 ± 2.6</td>
<td>22.8 ± 2.9</td>
<td>NS</td>
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<tr>
<td>Fasting plasma glucose (mmol/L)</td>
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<td>5.2 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/L)</td>
<td>422.8 ± 275.1</td>
<td>418.1 ± 279.3</td>
<td>NS</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>4.9 ± 1.8</td>
<td>4.7 ± 1.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are means ± SD.
castrated mice (46% of vehicle-treated castrated mice) and also in sham-operated mice.

We previously showed that adiponectin mRNA was expressed only in adipose tissues in human, monkey, and mouse (9,16–18). Figure 3B and C compare the protein and mRNA levels of adiponectin in subcutaneous adipose tissues of these mice. Castration or testosterone treatment had no effect on adiponectin protein and mRNA levels. Considered together, the above data indicated that testosterone reduced plasma adiponectin concentrations without changing the mRNA and protein levels of adiponectin in the adipose tissue.

We further examined the effect of androgens on adiponectin production in vitro 3T3-L1 adipocytes (Fig. 4). The amount of adiponectin secreted in media was markedly decreased by both testosterone and 5α-dihydrotestosterone (5α-DHT), a metabolite of testosterone that is not converted to estrogen (Fig. 4A). There were no apparent changes in adiponectin protein and mRNA levels in adipocytes treated with testosterone or 5α-DHT (Fig. 4B and C). These results obtained from the 3T3-L1 adipocytes were in agreement with the findings of in vivo studies demonstrating that testosterone reduced the production of adiponectin in plasma without changing the mRNA and protein levels of adiponectin in adipose cells.

To examine further the inhibitory effect of testosterone on adiponectin secretion from 3T3-L1 adipocytes, we performed the pulse-chase study. First, we established the procedure for adiponectin immunoprecipitation. 3T3-L1 adipocytes (day 7) were metabolically radiolabeled for 1, 2, or 4 h, and the cell lysates were collected and were immunoprecipitated with antiadiponectin antibody (Fig. 5A). The labeled adiponectin was increased in a time-dependent manner and not observed with nonimmune rabbit serum instead of adiponectin antibody for immunoprecipitation. Next, after a 2-h radiolabeling, the secretion into culture medium (Fig. 5B) and the amount in the cell lysates (Fig. 5C) of the newly synthesized radiolabeled adiponectin were chased in the presence and absence of testosterone. The secretion of labeled adiponectin was continuously inhibited with testosterone treatment from 1 to 12 h of chasing period (Fig. 5B). The testosterone-mediated inhibition was more significant in the acute phases such as 1 h (0.3-fold) and 2 h (0.5-fold). However, the labeled adiponectin was more retained in cells from 2 to 12 h with testosterone treatment. These results indicated that testosterone inhibited the secretion of adiponectin pool in 3T3-L1 adipocytes.

Next, we investigated the effects of sex hormones on plasma adiponectin concentration and insulin sensitivity in mice. Male ICR mice were castrated and female mice were ovariectomized, and plasma adiponectin concentrations were measured by Western blotting (Fig. 6). In sham-operated mice, plasma adiponectin concentrations were lower in male mice than in female mice, as was observed in human subjects (Fig. 1A). In male mice, castration resulted in increased plasma adiponectin levels, as observed in Fig. 3A. However, in female mice, there was no significant difference in adiponectin plasma concentrations between ovariectomized and sham-operated mice similarly to the female subjects before and after meno-
pause, which indicated no effect of estrogen on the adiponectin production in plasma.

Fasting plasma concentrations of glucose and insulin were measured in these mice, and HOMA-IR was calculated. In male mice, castration reduced plasma glucose, insulin, and HOMA-IR to levels close to those of sham-operated female mice. These results indicated that androgens decreased insulin sensitivity, as described previously (37–39). The mirror image of adiponectin levels and HOMA-IR in Fig. 6 support the previously described concept in human, monkey, and rodents that adiponectin is an insulin-sensitizing hormone (9–11,21,22).

FIG. 4. Effects of testosterone and 5α-DHT on secretion and levels of adiponectin protein and mRNA in 3T3-L1 adipocytes. 3T3-L1 cells on day 7 (7 days after differentiation induction) were treated with the indicated concentrations of testosterone, 5α-DHT, or vehicle (ethanol) as described in RESEARCH DESIGN AND METHODS. A: Adiponectin levels secreted in culture media. Adiponectin level secreted into the medium over 12 h were analyzed by Western blotting, as described in RESEARCH DESIGN AND METHODS. The value of vehicle (ethanol)-treated 3T3-L1 cells was arbitrarily set as 1.0. B: Adiponectin protein levels in cells. Two micrograms of total protein from 3T3-L1 cell lysate was subjected to Western blotting, as described in RESEARCH DESIGN AND METHODS. C: Adiponectin mRNA levels in cells. Ten micrograms of total RNA extracted from the 3T3-L1 cells was subjected to Northern blotting and quantified and normalized relative to the 18s rRNA signal. The value of vehicle (ethanol)-treated 3T3-L1 cells was arbitrarily set as 1.0.

FIG. 5. Pulse-chase experiments of 35S-labeled adiponectin. A: Specificity of adiponectin immunoprecipitation and time-dependent metabolic labeling of adiponectin. 3T3-L1 adipocytes (day 7) were metabolically radiolabeled for 1, 2, or 4 h as described in RESEARCH DESIGN AND METHODS. Adiponectin was immunoprecipitated from the cell lysates, separated by SDS-PAGE, and visualized by autoradiography. non, nonimmune rabbit serum. The result obtained from duplicate was represented. B and C: 3T3-L1 adipocytes were labeled for 2 h, and then labeling medium was replaced with culture medium containing testosterone (300 nmol/l) or vehicle (ethanol). The medium (B) and cell lysates (C) were collected at indicated times from an individual well, subjected to immunoprecipitation, and visualized as described in A. The band intensities were quantified by densitometry and plotted. A representative blot is shown. The similar results were obtained in two other independent experiments.
Using the mice described in Fig. 6, we also performed an insulin sensitivity test based on the steady-state plasma glucose concentration method, as described previously (Fig. 7). In this test, endogenous production of insulin is blocked by administration of epinephrine and propranolol, and glucose and insulin are injected to compare the insulin-mediated decrease in plasma glucose (Fig. 7A). In Fig. 7B, the ability of insulin to suppress plasma glucose concentration was much less in sham-operated male mice than in sham-operated female mice. In male mice, castration augmented insulin sensitivity to reduce plasma glucose concentration to levels close to those of sham-operated female mice. Ovariectomy did not affect insulin sensitivity in female mice. Figure 7C shows a plot of plasma adiponectin concentrations and the percentage change of the decrease in plasma glucose in the insulin sensitivity test, using data of all mice examined in Fig. 7B. There was a close correlation between plasma adiponectin concentrations and insulin sensitivity. Plasma leptin concentrations were similar in sham-operated and castrated male mice and, hence, showed no correlation to the increase of insulin sensitivity (data not shown). Taken together, these results suggest that testosterone-mediated suppression of adiponectin closely associates with less insulin sensitivity in male mice.

DISCUSSION
In the present study, we demonstrated that plasma concentrations of adiponectin, an adipocyte-derived plasma protein, were lower in men than in women but were not different between pre- and postmenopausal women. These data suggested that androgens act to reduce plasma adiponectin concentrations. In animal experiments, castration increased and testosterone supplement reduced plasma adiponectin concentrations in male mice. In cultured 3T3-L1 adipocytes, testosterone and 5α-DHT suppressed the secretion of adiponectin into the culture media. These results indicated that androgen decreased plasma adiponectin concentrations through its effect on adipocytes.

We reported previously the presence of low concentrations of plasma adiponectin in human subjects of obesity and type 2 diabetes (10,11,19). In a longitudinal study of rhesus monkeys that had free access to food and became obese and subsequently developed type 2 diabetes, the reduction of plasma adiponectin preceded the development of hyperinsulinemia and hyperglycemia (9). We also demonstrated, in monkey and human, that plasma adiponectin levels correlated significantly with insulin sensitivity in the whole body using the hyperinsulinemic-euglycemic glucose clamp technique (9,11). Recent studies demonstrated that adiponectin increased fatty acid oxidation (40), augmented insulin action in the muscle and liver, and further improved insulin resistance in lipotropic and genetically obese mice, both of which had hypoadiponectinemia (21,22). These observations indicate that adiponectin is an insulin-sensitizing hormone and that hypoadiponectinemia induces insulin resistance.

In addition, we showed that plasma adiponectin levels were associated with atherosclerosis: plasma adiponectin concentrations were low in patients with coronary artery disease (23). Adiponectin levels in plasma correlated inversely with the incidence of ischemic heart diseases in subjects with chronic renal failure (24). These results suggested that hypoadiponectinemia is also involved in the pathophysiology of atherosclerosis. In tissue-cultured cell experiments, adiponectin suppressed TNF-α-induced mRNA expression of several adhesion molecules, including vascular cell adhesion molecule-1, intracellular adhesion molecule-1, and E-selectin in vascular endothelial cells, and monocyte attachment to endothelial cells (23,25). Adiponectin also reduced lipid accumulation in human monocyte-derived macrophages presumably through its suppressive effect on the expression of macrophage scavenger receptor (26). It also inhibited the proliferation of vascular smooth muscle cells (Y. Arita, unpublished data). Moreover, we observed that in mice with hyperadiponectinemia induced by adiponectin-adenovirus, mechanical injury of vascular walls resulted in suppression of proliferation of vascular smooth muscle cells (M.M., unpublished data). These results demonstrate the antiatherogenic property of adiponectin and the involvement of hypoadiponectinemia in the pathophysiology of atherosclerosis. On the basis of these results, it is possible that testosterone-mediated reduction of plasma...
plasma adiponectin levels and insulin sensitivity in the whole body (9,11). In the present study, plasma levels of leptin, which was shown to modify insulin sensitivity, were similar in male mice with or without castration. Taken together, testosterone-mediated suppression of plasma adiponectin may be responsible, at least in part, for the lower insulin sensitivity in males than in females.

In the present study, testosterone reduced adiponectin levels in plasma of mice and in media of 3T3-L1 adipocytes. These results were accounted for by the inhibitory effect of testosterone on the secretion of adiponectin from adipocytes, shown in the pulse-chase studies (Fig. 5). In Fig. 4B, total protein levels of adiponectin were not affected with testosterone treatment, although the newly synthesized adiponectin was more retained (Fig. 5C), suggesting that amount of adiponectin secreted into media was much smaller when compared with the intracellular stores. Although testosterone, classically, binds to its cognate nuclear receptor and enhances the transcription of the target genes by binding to the specific cis-element of the promotors (41), the adiponectin mRNA was not affected by the testosterone treatment in vivo and in vitro. Existence of another factor involved in the secretion machinery of adiponectin, whose activity is modulated by testosterone, may be suggested.

From preventive and therapeutic aspects, understanding the mechanisms involved in the regulation of adiponectin secretion may allow the design of a new class of agents to combat insulin resistance and atherosclerosis. In this regard, identification of the responsible factor for adiponectin secretion, whose transcription or activity is modulated by testosterone, should provide useful information for the therapeutic strategy.

In conclusion, the current study revealed that testosterone reduces plasma concentration of adiponectin, an adipose tissue-derived antidiabetic and antiatherogenic protein. Our finding may relate to the high risks of diabetes and atherosclerosis in men.

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REFERENCES


