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

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Received for publication 19 January 2002 and accepted in revised form 22 May 2002.

5α-DHT, 5α-dihydrotestosterone; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; HOMA-IR, homeostasis model assessment insulin resistance; IPB, immunoprecipitation buffer; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; TNF, tumor necrosis factor.
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 found to be lower previously (29), with modifications. Briefly, to prepare cell lysates, we washed the cells with PBS, resuspended them in 300 μl of cell lysates (100 μg/ml aprotinin, followed by centrifugation. Equal amounts (2 μg) of the protein were subjected to SDS-PAGE. Western blotting was performed as described previously (31). The band intensities were quantified by densitometry.

**Northern blotting.** Total RNA was isolated from mouse subcutaneous adipose tissues and 3T3-L1 cells using TRIzol (GIBCO, Life Technologies, Grand Island, NY), and plasma leptin levels were measured by ELISA as described previously (19). Western blotting. Plasma adiponectin concentration of mice, adiponectin secreted from 3T3-L1 cells into the media, and protein amounts in subcutaneous adipose tissues and in 3T3-L1 cells were analyzed by quantitative Western blotting. An equal aliquot of plasma (1 μl × 1/250) or medium sample (7 μl) was lysed in sample buffer (30) and subjected to SDS-PAGE. For the immunoprecipitation of radiolabeled adiponectin in medium and cell lysates, the metabolic labeling was performed for 2 h. Then, the labeling medium was replaced with 1 ml of complete DMEM (FCS-free) with testosterone (300 nmol/l) or vehicle (ethanol). The medium and cell lysates were collected at indicated times for immunoprecipitation assays.

**Immunoprecipitation.** For adiponectin immunoprecipitation, we used a 3T3-L1 adipocyte culture described previously (7). We labeled culture medium with 100 μCi/ml of [3H]leucine, and the cells were harvested for RNA and protein analyses.

**RESULTS**

**Human studies.** First, to investigate the relationship to sex in adiponectin, we compared plasma concentrations of adiponectin between men and women. Figure 1 and
Table 1 shows 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 postmenopausal women were lower than in premenopausal 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 (Figs. 2 and 3). 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

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**TABLE 1**

Clinical data in men and women participants

<table>
<thead>
<tr>
<th></th>
<th>Men (n = 442)</th>
<th>Women (n = 137)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age (yrs)</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>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>5.4 ± 1.0</td>
<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 amounts 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 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 menop-
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).

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**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.

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**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 lipoatrophic 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
adiponectin may account, at least in part, for the higher incidence of atherosclerotic diseases in men.

Previous studies indicated that androgens reduced insulin sensitivity in humans and rodents (37–39). Female patients with polycystic ovary syndrome, which is characterized by hyperandrogenemia, also exhibit insulin resistance, although the exact mechanism remains undetermined. In the present study, castration-mediated increase in plasma adiponectin was accompanied by improvement of insulin sensitivity in male mice, estimated by HOMA-IR and insulin sensitivity test. Our results also showed a significant correlation between 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 promoters (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.

ACKNOWLEDGMENTS

This work was supported in part by “Research for the Future” Program from the Japan Society for the Promotion of Science (JSPS-RFTF97L00801), Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (12307022, 12557090, and 12671084), and a grant from Fuji Foundation for Protein Research.

We thank Dr. Shoji Tajima (Osaka University) for invaluable suggestions to the pulse-chase study. We also thank Sachiyo Tanaka and Yuko Matsukawa of our laboratory for helpful technical assistance.

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