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
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 expected to be low previously (28), with modifications. Briefly, to prepare cell lysates, we washed the cells with PBS, resuspended them in 300 μl of disruption buffer and subjected to immunoprecipitation. A band was excised. 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 immuno-precipitated as described above. Immunoprecipitates were washed with washing buffer (10 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, 1 mmol/l EDTA, 1% NP-40, 1% phenylmethylsulfonyl fluoride (PMSF), and 10 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.

Metabolic radiolabeling of 3T3-L1 adipocytes. 3T3-L1 adipocytes (day 7) were plated onto six-well plates and were incubated with FCS-free DMEM for 12 h. For metabolic labeling, the cells were washed with PBS and incubated with methionine- and cysteine-free DMEM without serum for 30 min to deplete the intracellular pools. The depletion medium was removed, and the cells were incubated in 1 ml of methionine- and cysteine-free DMEM containing 100 μCi/ml [35S]methionine and [35S]cysteine (Pro-mix [35S] in vitro labeling mix: Amerham) for the specific times indicated (1, 2, and 4 h).

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 μmol/l) or vehicle (ethanol). The medium and cell lysates were collected at indicated times for immunoprecipitation assays.

Immunoprecipitation. For adiponectin immunoprecipitation, we used a polyclonal antibody to adiponectin described previously (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 intraperitonely. Blood samples were obtained at 0 and 1 h after injections, and glucose concentra-
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 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

![Table 1](http://diabetesjournals.org/diabetes/article-pdf/51/9/2734/338929/db0902002734.pdf)
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 meno-

FIG. 3. Effects of castration and testosterone treatment on plasma levels and expression of adiponectin in adipose tissue in male mice. A: Plasma adiponectin concentrations. Plasma samples were collected from the mice described in the legend of Fig. 2. An equal aliquot of plasma sample was subjected to Western blotting as described in RESEARCH DESIGN AND METHODS, and the band intensities were quantified by densitometry. The values of sham-operated and vehicle-treated mice were arbitrarily set as 1.0. Representative blots of individual mice from each group are shown in the upper panel. B: Adiponectin protein levels in subcutaneous white adipose tissues. The protein lysate of subcutaneous white adipose tissues of an individual mouse from each group was isolated and subjected to Western blotting as described in RESEARCH DESIGN AND METHODS. C: Adiponectin mRNA levels in subcutaneous adipose tissues. Total RNA was extracted from the tissue of an individual mouse from each group and subjected to Northern blotting as described in RESEARCH DESIGN AND METHODS. Northern blotting was performed using specific probes for adiponectin or cyclophilin. The band intensities were quantified by densitometry. Note the abundance of adiponectin mRNA relative to that of cyclophilin mRNA. The values of sham-operated and vehicle-treated mice were arbitrarily set as 1.0. Representative blots of the individual mice from each group are shown in the upper panel. Data are mean ± SE.
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).
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 5a-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 hyperinsulinenemic-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 lipaotropic 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 concentrations was 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.

FIG. 6. Plasma levels of adiponectin, glucose, and insulin and HOMA-IR in castrated and ovariectomized mice. Twenty-week-old male ICR mice were castrated (n = 4) or sham-operated (n = 4), and 20-week-old female mice were ovariectomized (n = 3) or sham-operated (n = 4), as described in RESEARCH DESIGN AND METHODS. At 6 weeks after operation, blood samples were collected after a 6-h fast. Plasma adiponectin levels were analyzed by Western blotting. Plasma glucose and insulin concentrations were measured, and HOMA-IR was calculated as described in RESEARCH DESIGN AND METHODS. Data are mean ± SE.
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, estimated by HOMA-IR and insulin sensitivity test. Our results also showed a significant correlation between plasma adiponectin levels and degree of insulin sensitivity when the data of all examined male and female mice were plotted. This was in accordance with our previous observation in humans and monkeys showing 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.

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REFERENCES


20. Shapiro L, Scherer PE: The crystal structure of a complement-1q family

22. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-

23. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K,


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