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

Hitoshi Nishizawa, Iichiro Shimomura, Ken Kishida, Norikazu Maeda, Hiroshi Kuriyama, Hiroyuki Nagaretani, Morihiro Matsuda, Hidehiko Kondo, Naoki Furuyama, Shinji Kihara, Tadashi Nakamura, Yoshihiro Tochino, Tohru Funahashi, and Yuji Matsuzawa

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 NH_2-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

From the Department of Internal Medicine and Molecular Science, Graduate School of Medicine, Osaka University, Suita, Japan.

Address correspondence and reprint requests to Iichiro Shimomura, Department of Organismal Biosystems, Graduate School of Frontier Bioscience, Department of Medicine and Pathophysiology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: ichi@imed2.med.osaka-u.ac.jp.

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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 23.3 ± 2.6 kg/m^2) and 137 women (age 53.2 ± 12.0; BMI 22.8 ± 2.9). Because plasma adiponectin concentrations were reported to be low in obese subjects (19), the 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.

Blood was drawn after an overnight fast. Plasma adiponectin concentrations were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (19). Plasma glucose was assayed by the glucose oxidase method, and insulin was assayed by immunoradiometric assay using Insulin Riabead (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 dark-light 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: carbohydrate 54.0%, protein 23.8%, and fat 5.1%. Experiments of castration and ovariectomy 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 ovariectomized (OVAR, $n = 3$) 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 testes with epididymal fat pad were exposed and put back, and the incision was closed with suture. For ovariectomy, after abdominal incision, the uterus, oviducts, and ovaries were exposed with the fat tissues around them, the oviduct and blood vessels supplying the ovaries were tied up, and the ovaries were removed through an incision. The uterus and oviducts were put back with the fat tissues around them, and the incision was closed with suture. For the sham operation to ovariectomy, 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 homeostasis 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, and glucose concentrations 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 supplemented with 5 μg/ml insulin, 0.5 mmol/l 1-methyl-3-isobutyl-xanthin, and 1 μmol/l dexamethasone, 2 days after reaching confluence.

Effect of hormones on expression and secretion of adiponectin in 3T3-L1 adipocytes. 3T3-L1 cells on day 7 (7 days after differentiation-induction) were used for the experiments to examine the effects of hormones on the expression and secretion of adiponectin. Cells were washed twice with PBS and incubated with DMEM containing 1.5% BSA and indicated hormones or vehicle for 12 h. The medium was washed and replaced with a freshly prepared medium containing the same concentrations of hormones. After a 12-h incubation, the medium was collected for Western blotting to measure the secretion of adiponectin, and the cells were harvested for RNA and protein analyses.

Northern blotting. Total RNA was isolated from mouse subcutaneous adipose tissues and 3T3-L1 cells using RNA STAT-60 (TEL-TEST) according to the manufacturer's instructions. Isolated total RNA (10 μg) was separated by electrophoresis and transferred to nylon membranes. The membrane was hybridized with adiponectin or cyclophilin DNA probe labeled with [α-^{32}P]dCTP. The hybridized membrane was exposed to X-ray film, X-OMAT AR (Kodak). The band intensities were quantified by densitometry.

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. The subcutaneous adipose tissues of mice were homogenized and sonicated, or 3T3-L1 cells were sonicated in lysis buffer containing 10 mmol/l HEPES (pH 7.5), 142.5 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.2% NP-40, 1 mmol/l Na_3VO_4, 10 mmol/l NaF, 1 mmol/l $Na_2P_2O_7$, 1 mmol/l 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 of L-[^{35}S]methionine and L-[^{35}S]cysteine (Pro-mix L-[^{35}S] in vitro labeling mix; Amersham) 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 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 procedure described previously (32), with modifications. Briefly, to prepare cell lysates, we washed the cells with PBS, resuspended them in 300 μl of disruption buffer (10 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, 5 mmol/l EDTA, 10 mmol/l benzamidine, 1 mmol/l PMSF, 1% Nonidet P-40 [NP-40]), and lysed them with three repetitive freeze-thaw cycles. Cellular debris was removed by centrifugation. The cell lysates were adjusted to equal protein concentration with disruption buffer and subjected to immunoprecipitation. A total of 500 μl of cell lysates (100 μg of total protein) was mixed with an equal volume (500 μl) of disruption buffer lacking EDTA and NP-40 and immunoprecipitated with 5 μl of rabbit polyclonal antibody against murine adiponectin overnight at 4°C, followed by incubation with 40 μl of protein G beads (Amersham) for 2 h at 4°C. For medium, aliquots (450 μl) of the metabolically labeled culture medium were added by 1/10 volume of 10× immunoprecipitation buffer (IPB; 1× IPB = 10 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, 1 mmol/l EDTA, 1% NP-40, 10 mmol/l benzamidine, 1 mmol/l PMSF) and immunoprecipitated as described above. Immunoprecipitates were washed three times with 1× IPB, followed by solubilization with sample buffer, and subjected to SDS-PAGE. After electrophoresis, radiolabeled proteins were autoradiographed. The band intensities were quantified by densitometry.

Statistical analysis. Data are expressed as mean ± SE. Statistical analyses, except for data shown in Fig. 7*C*, were performed with unpaired *t* tests. Statistical analysis of the data shown in Fig. 7*C* was performed with Pearson's correlation coefficient test. $P < 0.05$ denoted the presence of a statistically significant difference.

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

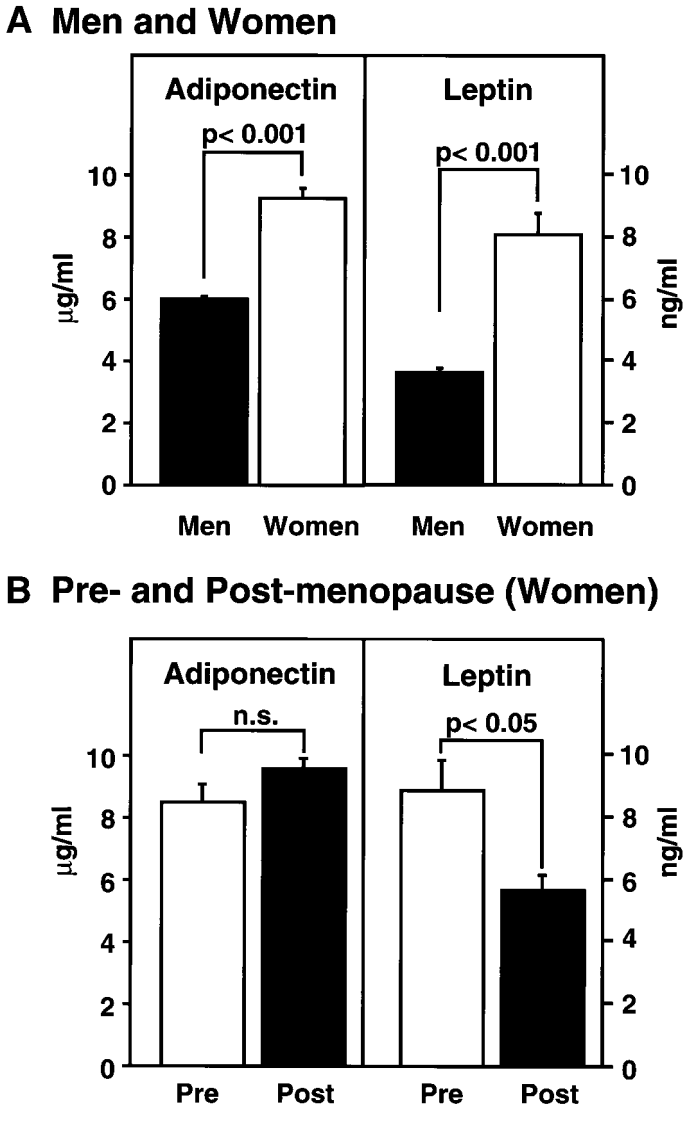

FIG. 1. Plasma adiponectin and leptin concentrations in human subjects. *A*: Plasma adiponectin and leptin concentrations were measured in men and women by ELISA. *B*: Plasma concentrations of adiponectin and leptin in pre- and postmenopausal women. Data are mean ± SE.

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 1*A* demonstrates that plasma adiponectin

TABLE 1
Clinical data in men and women participants

	Men (n = 442)	Women (n = 137)	P
Age	52.6 ± 11.9	53.2 ± 12.0	NS
BMI (kg/m^2)	23.3 ± 2.6	22.8 ± 2.9	NS
Fasting plasma glucose (mmol/l)	5.4 ± 1.0	5.2 ± 1.2	NS
Fasting plasma insulin (pmol/l)	422.8 ± 275.1	418.1 ± 279.3	NS
TNF-α (pg/ml)	4.9 ± 1.8	4.7 ± 1.7	NS

Data are means ± SD.

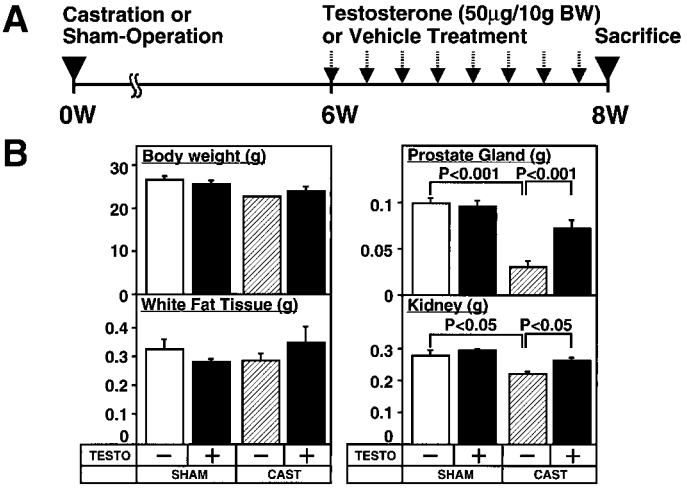

FIG. 2. Castration and testosterone treatment of male mice and weight changes in body and organs. *A*: Experimental design of castration and testosterone treatment. Surgery and treatment with testosterone or vehicle were performed as described in RESEARCH DESIGN AND METHODS. *B*: Weights of body and indicated organs were measured in sham-operated (n = 5), sham-operated and testosterone-treated (n = 4), castrated (n = 5), and castrated and testosterone-treated (n = 5) mice. Data are mean ± SE.

was 35% lower in men than in women. Furthermore, leptin plasma concentrations in men were lower than in women (Fig. 1*A*), as reported previously (33). Plasma adiponectin concentrations in premenopausal women were not significantly different from those in postmenopausal women (Fig. 1*B*). In contrast, plasma leptin concentrations in postmenopausal women were lower than in premenopausal women (Fig. 1*B*), 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 2*A* 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 2*B* 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 3*A* 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

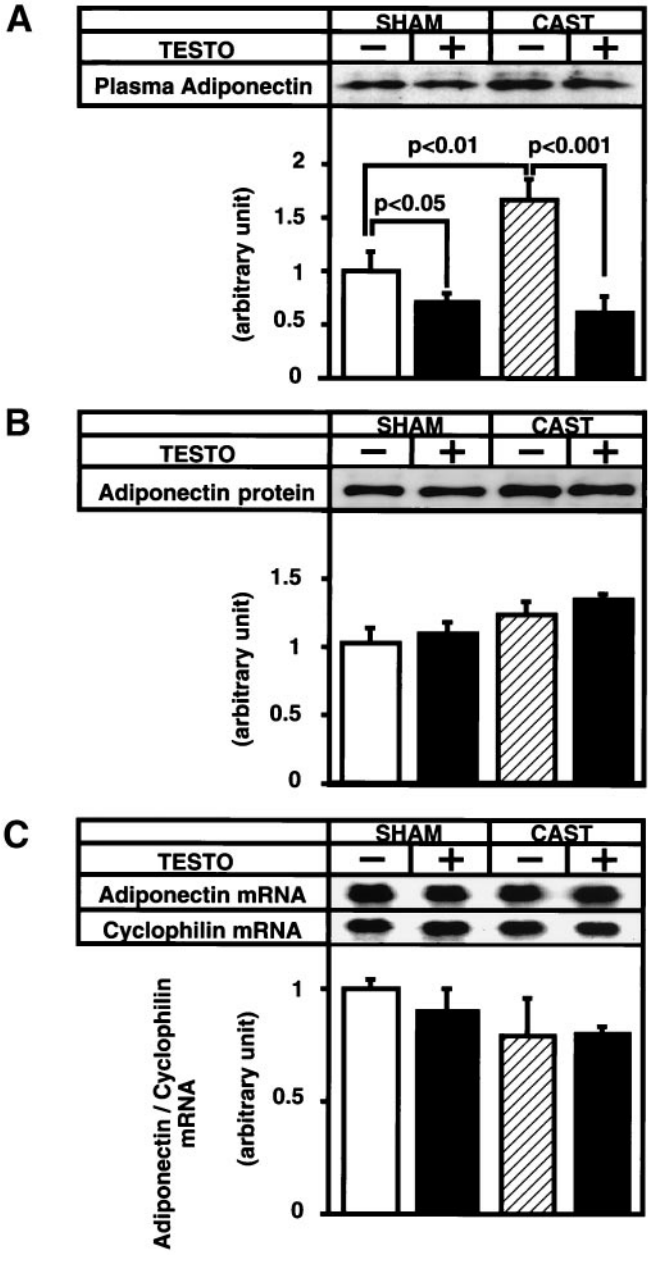

FIG. 3. Effects of castration and testosterone treatment on plasma levels and expression of adiponectin in adipose tissue in male mice. *A*: Plasma adiponectin concentrations. Plasma samples were collected from the mice described in the legend of Fig. 2. An equal aliquot of plasma sample was subjected 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 tissue 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.

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 3*B* 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. 4*A*). There were no apparent changes in adiponectin protein and mRNA levels in adipocytes treated with testosterone or 5α-DHT (Fig. 4*B* 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. 5*A*). 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. 5*B*) and the amount in the cell lysates (Fig. 5*C*) 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. 5*B*). 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. 1*A*). In male mice, castration resulted in increased plasma adiponectin levels, as observed in Fig. 3*A*. 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-

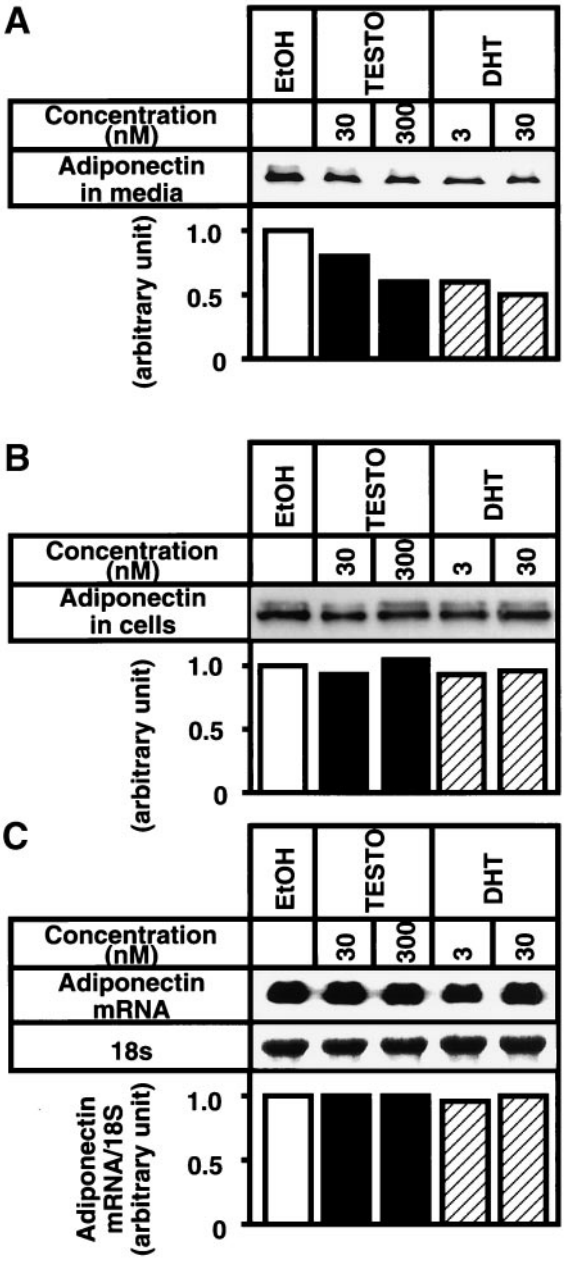

FIG. 4. Effects of testosterone and 5α-DHT on secretion and levels of adiponectin protein and mRNA in 3T3-L1 adipocytes. 3T3-L1 cells on day 7 (7 days after differentiation induction) were treated with the indicated concentrations of testosterone, 5α-DHT, or vehicle (ethanol) as described in RESEARCH 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.

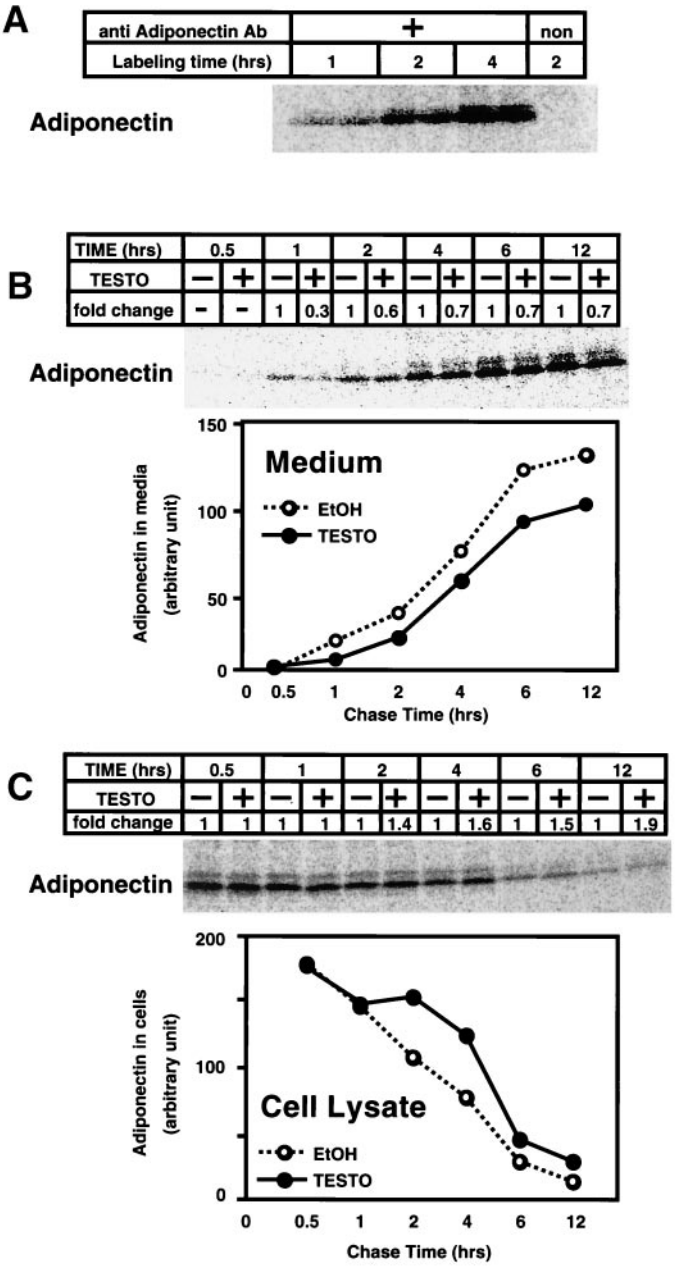

FIG. 5. Pulse-chase experiments of ^{35}S-labeled adiponectin. *A*: Specificity of adiponectin immunoprecipitation and time-dependent metabolic labeling of adiponectin. 3T3-L1 adipocytes (day 7) were metabolically radiolabeled for 1, 2, or 4 h as described in RESEARCH DESIGN AND METHODS. Adiponectin 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.

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).

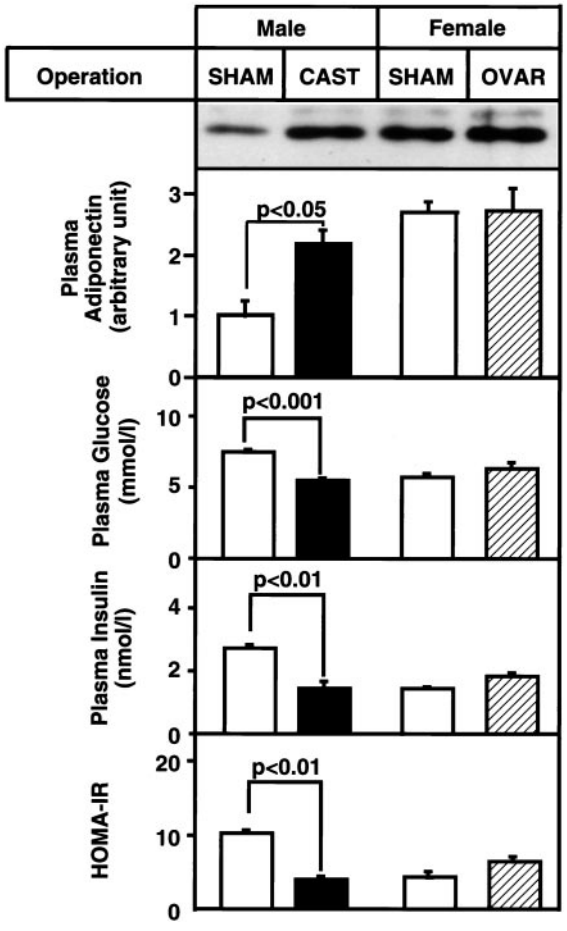

FIG. 6. Plasma levels of adiponectin, glucose, and insulin and HOMA-IR in castrated and ovariectomized mice. Twenty-week-old male ICR mice were castrated (n = 4) or sham-operated (n = 4), and 20-week-old female mice were ovariectomized (n = 3) or sham-operated (n = 4), as described in RESEARCH DESIGN 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.

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. 7*A*). In Fig. 7*B*, 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 7*C* 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. 7*B*. 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

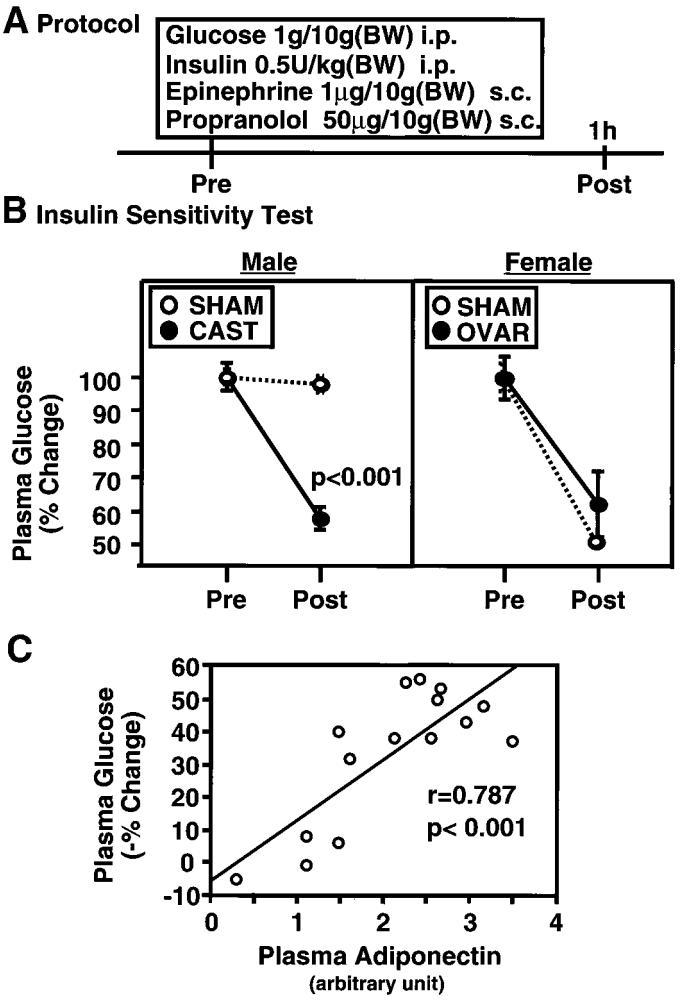

FIG. 7. Insulin sensitivity in castrated and ovariectomized mice. *A*: Experimental design for the insulin sensitivity test. *B*: Insulin sensitivity test was performed using the mice described in the legend of Fig. 5, as described in RESEARCH DESIGN AND METHODS. The percentage changes in insulin-mediated decrease in plasma glucose were compared in sham-operated and castrated male mice and in sham-operated and ovariectomized female mice, as described in RESEARCH DESIGN AND METHODS. The initial plasma glucose concentrations in this study were 8.2 ± 0.2, 6.0 ± 0.2, 6.2 ± 0.2, and 6.9 ± 0.8 mmol/l (mean ± SE) in sham-operated male mice, castrated male mice, sham-operated female mice, and ovariectomized-female mice, respectively. Data are mean ± SE. *C*: Correlation between plasma adiponectin levels and the degree of insulin sensitivity. Plasma adiponectin levels and percentage change of the decrease in plasma glucose in the insulin sensitivity test in all mice examined in Figs. 5 and 6*B* are plotted. r, correlation coefficient.

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 the 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. 4*B*, total protein levels of adiponectin were not affected with testosterone treatment, although the newly synthesized adiponectin was more retained (Fig. 5*C*), 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

1. Shimomura I, Funahashi T, Takahashi M, Maeda K, Kotani K, Nakamura T, Yamashita S, Miura M, Fukuda Y, Takemura K, Tokunaga K, Matsuzawa Y: Enhanced expression of PAI-1 in visceral fat: possible contributor to vascular disease in obesity. *Nat Med* 2:800–802, 1996
2. Spiegelman BM, Flier JS: Adipogenesis and obesity: rounding out the big picture. *Cell* 87:377–389, 1996
3. Hotamisligil GS, Spiegelman BM: Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 43:1271–1278, 1994
4. Friedman JM, Halaas JL: Leptin and the regulation of body weight in mammals. *Nature* 395:763–770, 1998
5. Saltiel AR, Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414:799–806, 2001

6. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM: Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest* 95:2409–2415, 1995
7. Hotamisligil GS, Shargill NS, Spiegelman BM: Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. *Science* 259:87–91, 1993
8. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA: The hormone resistin links obesity to diabetes. *Nature* 409:307–312, 2001
9. Hotta K, Funahashi T, Bodkin NL, Ortmeyer HK, Arita Y, Hansen BC, Matsuzawa Y: Circulating concentrations of the adipocyte protein adiponectin are decreased in parallel with reduced insulin sensitivity during the progression to type 2 diabetes in rhesus monkeys. *Diabetes* 50:1126–1133, 2001
10. Hotta K, Funahashi T, Arita Y, Takahashi M, Matsuda M, Okamoto Y, Iwahashi H, Kuriyama H, Ouchi N, Maeda K, Nishida M, Kihara S, Sakai N, Nakajima T, Hasegawa K, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Hanafusa T, Matsuzawa Y: Plasma concentrations of a novel, adipose-specific protein, adiponectin, in type 2 diabetic patients. *Arterioscler Thromb Vasc Biol* 20:1595–1599, 2000
11. Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, Tataranni PA: Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. *J Clin Endocrinol Metab* 86:1930–1935, 2001
12. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM: Positional cloning of the mouse obese gene and its human homologue. *Nature* 372:425–432, 1994
13. Kamohara S, Burcelin R, Halaas JL, Friedman JM, Charron MJ: Acute stimulation of glucose metabolism in mice by leptin treatment. *Nature* 389:374–377, 1997
14. Shimomura I, Hammer RE, Ikemoto S, Brown MS, Goldstein JL: Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. *Nature* 401:73–76, 1999
15. Frederich RC, Lollmann B, Hamann A, Napolitano-Rosen A, Kahn BB, Lowell BB, Flier JS: Expression of ob mRNA and its encoded protein in rodents: impact of nutrition and obesity. *J Clin Invest* 96:1658–1663, 1995
16. Maeda K, Okubo K, Shimomura I, Funahashi T, Matsuzawa Y, Matsubara K: cDNA cloning and expression of a novel adipose specific collagen-like factor, apM1 (adipose most abundant gene transcript 1). *Biochem Biophys Res Commun* 221:286–289, 1996
17. Scherer EP, Williams S, Fogliano M, Baldini G, Lodish HF: A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 270:26746–26749, 1995
18. Hu E, Liang P, Spiegelman BM: AdipoQ is a novel adipose-specific gene dysregulated in obesity. *J Biol Chem* 271:10697–10703, 1996
19. Arita Y, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y: Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 257:79–83, 1999
20. Shapiro L, Scherer PE: The crystal structure of a complement-1q family protein suggests an evolutionary link to tumor necrosis factor. *Curr Biol* 8:335–338, 1998
21. Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N, Ezaki O, Akanuma Y, Gavrilova O, Vinson C, Reitman ML, Kagechika H, Shudo K, Yoda M, Nakano Y, Tobe K, Nagai R, Kimura S, Tomita M, Froguel P, Kadowaki T: The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nat Med* 7:941–6, 2001
22. Berg AH, Combs TP, Du X, Brownlee M, Scherer PE: The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nat Med* 7:947–953, 2001
23. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y: Novel modulator for endothelial adhesion molecules: adipocyte-derived plasma protein, adiponectin. *Circulation* 100:2473–2476, 1999
24. Zoccali C, Mallamaci F, Tripepi G, Benedetto FA, Cutrupi S, Parlongo S, Malatino LS, Bonanno G, Seminara G, Rapisarda F, Fatuzzo P, Buemi M, Nicocia G, Tanaka S, Ouchi N, Kihara S, Funahashi T, Matsuzawa Y: Adiponectin, metabolic risk factors, and cardiovascular events among patients with end-stage renal disease. *J Am Soc Nephrol* 13:134–141, 2002
25. Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y: Adiponectin, adipocyte-derived plasma protein, inhibits endothelial NF-κB signaling through cAMP-dependent pathway. *Circulation* 102:1296–301, 2000
26. Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigami M, Kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T, Matsuzawa Y: Adipocyte-derived plasma protein, adiponectin, suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation* 103:1057–1063, 2001
27. Hattori A, Uemura K, Miura H, Ueda M, Tamaya N, Iwata F, Muraguchi M, Ohmoto Y, Iguchi A: Gender-related difference in relationship between insulin resistance and serum leptin level in Japanese type 2 diabetic and non-diabetic subjects. *Endocr J* 47:615–621, 2000
28. Serazin-Leroy V, Morot M, de Mazancourt P, Giudicelli Y: Androgen regulation and site specificity of angiotensinogen gene expression and secretion in rat adipocytes. *Am J Physiol Endocrinol Metab* 279:E1398–E1405, 2000
29. Taketomi S, Ikeda H, Ishikawa E, Iwatsuka H: Determination of overall insulin sensitivity in diabetic mice, KK. *Horm Metab Res* 14:14–18, 1982
30. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685, 1970
31. Maeda N, Takahashi M, Funahashi T, Kihara S, Nishizawa H, Kishida K, Nagaretani H, Matsuda M, Komuro R, Ouchi N, Kuriyama H, Hotta K, Nakamura T, Shimomura I, Matsuzawa Y: PPARγ ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 50:2094–2099, 2001
32. Azuma H, Mima N, Shirakawa M, Miyamoto K, Yamaguchi H, Mitsui T, Shigekiyo T, Saito S: Molecular pathogenesis of type I congenital plasminogen deficiency: expression of recombinant human mutant plasminogens in mammalian cells. *Blood* 89:183–190, 1997
33. Saad MF, Damani S, Gingerich RL, Riad-Gabriel MG, Khan A, Boyadjian R, Jinagouda SD, el-Tawil K, Rude RK, Kamdar V: Sexual dimorphism in plasma leptin concentration. *J Clin Endocrinol Metab* 82:579–584, 1997
34. Rosenbaum M, Nicolson M, Hirsch J, Heymsfield SB, Gallagher D, Chu F, Leibel RL: Effects of gender, body composition, and menopause on plasma concentrations of leptin. *J Clin Endocrinol Metab* 81:3424–3427, 1996
35. Broulik PD, Schreiber V: Effect of alloxan diabetes on kidney growth in intact and castrated mice. *Acta Endocrinol (Copenh)* 99:109–111, 1982
36. Avdalovic N, Bates M: The influence of testosterone on the synthesis and degradation rate of various RNA species in the mouse kidney. *Biochim Biophys Acta* 407:299–307, 1975
37. Shoupe D, Lobo RA: The influence of androgens on insulin resistance. *Fertil Steril* 41:385–388, 1984
38. Toprak S, Yonem A, Cakir B, Guler S, Azal O, Ozata M, Corakci A: Insulin resistance in nonobese patients with polycystic ovary syndrome. *Horm Res* 55:65–70, 2001
39. Rincon J, Holmang A, Wahlstrom EO, Lonnroth P, Bjorntorp P, Zierath JR, Wallberg-Henriksson H: Mechanisms behind insulin resistance in rat skeletal muscle after oophorectomy and additional testosterone treatment. *Diabetes* 45:615–621, 1996
40. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF: Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. *Proc Natl Acad Sci U S A* 98:2005–2010, 2001
41. Roy AK, Lavrovsky Y, Song CS, Chen S, Jung MH, Velu NK, Bi BY, Chatterjee B: Regulation of androgen action. *Vitam Horm* 55:309–352, 1999