

Insulin and Leptin Resistance With Hyperleptinemia in Mice Lacking Androgen Receptor

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Epidemiological evidence suggests that sex differences exist in type 2 diabetes. Men seem to be more susceptible than women to the consequences of obesity and sedentary lifestyle, possibly because of differences in insulin sensitivity and regional body fat deposition. Thus, lacking androgen receptor (AR) in male individuals may promote insulin resistance. To determine whether lacking AR in male individuals contributes to in vivo insulin resistance, an AR knockout model ($AR^{-/y}$) was used to study the correlation between AR and insulin resistance. Progressive reduced insulin sensitivity and impaired glucose tolerance were seen in $AR^{-/y}$ mice with advancing age. Aging $AR^{-/y}$ mice displayed accelerated weight gain, hyperinsulinemia, and hyperglycemia, and loss of AR contributes to increased triglyceride content in skeletal muscle and liver. Leptin is higher in serum of $AR^{-/y}$ mice. Treatment with exogenous leptin fails to stimulate weight loss in $AR^{-/y}$ mice in advanced age, suggesting leptin resistance in the $AR^{-/y}$ mice. Exogenous dihydrotestosterone replacement fails to reverse the metabolic abnormalities and insulin resistance in $AR^{-/y}$ mice. Our in vivo studies demonstrate that androgen-AR plays key roles in the development of insulin and leptin resistance, which may contribute to the development of type 2 diabetes and cardiovascular disease. *Diabetes* 54:1717–1725, 2005

It is estimated that by the year 2030, there will be ~366 million people affected by type 2 diabetes worldwide (1), with many of those affected in the elderly age group (2). Although the primary factors causing this disease are elusive, it is clear that insulin resistance and leptin insensitivity may play a major role in its development (3).

Epidemiological evidence suggests that sex differences exist in type 2 diabetes. The prevalence of type 2 diabetes

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Received for publication 24 June 2004 and accepted in revised form 28 February 2005.

AR, androgen receptor; GTT, glucose tolerance test; ITT, insulin tolerance test; PI3K, phosphoinositide-3-OH kinase; PPAR, peroxisome proliferator-activated receptor; TNF- α , tumor necrosis factor- α ; WAT, white adipose tissue.

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is higher in men than women (1), possibly because of differences in insulin sensitivity and regional body fat deposition (4,5). The detailed mechanisms of how sex hormones influence insulin sensitivity or fat deposition, however, remain unclear. Testosterone and its metabolite, dihydrotestosterone, can activate androgen receptor (AR) to exert their androgenic actions. Proper or maximal androgen action may require interaction with selective coregulators in selective tissues (6,7).

Leptin, the adipocyte-derived adipokine product of the *ob* gene, has been shown to induce a negative energy balance by reducing appetite and increasing energy expenditure (8). Leptin circulates in serum at levels that parallel the mass of body fat. However, obese individuals have been found to be resistant to the negative regulatory function of circulating leptin (9). *Ob/ob* and *db/db* mice that lack leptin or are leptin resistant, respectively, are profoundly hyperphagic and hypometabolic, leading to an obese phenotype, and they manifest numerous abnormalities, such as type 2 diabetes with severe insulin resistance, hypothermia and cold intolerance, infertility, and decrease in lean mass (10–14).

However, to date, the relationship between androgen-AR and insulin sensitivity remains unclear, and little is known about the role of androgen-AR in age-related changes for regulation of leptin production. Therefore, we used a conditional knockout strategy to generate AR knockout mice ($AR^{-/y}$) to study this relationship (15), and here we report the influences of loss of AR on insulin and leptin resistance.

RESEARCH DESIGN AND METHODS

All animal procedures were approved by the animal care and use committee of the University of Rochester School of Medicine, in accordance with National Institutes of Health guidelines. Construction of targeting vectors and generation of the chimera founder mice have been described previously (15). The strains of the mosaic founder mice were C57BL/6 and 129Sv background. β -Actin is a housekeeping gene and is universally expressed in every tissue; therefore, the β -actin promoter-driven Cre (ACTB-Cre; Jackson Laboratories, Bar Harbor, ME) will express and delete floxed AR fragments in all of the tissues. The $AR^{-/y}$ mice were genotyped by PCR, as described previously (15). Animals were housed in pathogen-free facilities, maintained on a 12-h light/dark schedule (light on at 0600), and had free access to standard laboratory chow (no. 5010; PMI Lab Diet, St. Louis, MO) and water.

Histology. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin. Nonadjacent sections, separated by 70–80 μ m, were obtained from perigonadal fat pads and analyzed systematically with respect to adipocyte size and number. Staining of the sections was performed with hematoxylin/eosin. Images were acquired using an E800 microscope (Nikon, Melville, NY) and a SPOT camera (Diagnostic Instruments, Sterling Heights, MI) and were analyzed using SigmaScan Pro software (version 5.0; SPSS, Chicago, IL).

Analytical procedures. Fasting blood samples were taken from mice 14 h after withdrawal of food. Blood samples designated as random-fed state were

TABLE 1

Primers used for real-time PCR assays of mRNA expression of genes related to lipid metabolism in WAT, liver, and skeletal muscle

Gene	Access no.	Forward primer	Reverse primer	Amplicon (bp)
aP2	M13385	AAGGAAAGTGGCAGGCATGG	CACGCCAGTTTGAAGGAAATC	118
C/EBP α	BC028890	CAAGAACAGCAACGAGTACC	AGGCGGTCATTGTCACTG	135
PPAR α	X57638	CGGGAAAGACCAGCAACAAC	TGGCAGCAGTGGGAAGAATCG	142
PPAR γ	NM011146	CGAGGACATCCAAGACAAC	TGTGACGATCTGCCTGAG	124
SREBP1c	NM011480	CTGGCTGAGGCGGGATGA	TACGGGCCACAAGAAGTAGA	290

drawn 6 h after introducing food into the cages of mice that had been subjected to a preceding 14-h fast. Blood glucose concentrations were measured using a glucometer (One Touch Ultra; Lifescan, Milpitas, CA). Insulin levels and leptin levels were determined in duplicate 5- μ l serum samples, using a mouse insulin and leptin enzyme-linked immunosorbent assay kit (Crystal Chem, Downers Grove, IL) according to the manufacturer's protocol. Serum tumor necrosis factor- α (TNF- α) levels were determined in duplicate in 20- μ l serum samples using a mouse TNF- α enzyme-linked immunosorbent assay kit (eBioscience, San Diego, CA) according to the manufacturer's protocol. For the glucose tolerance test (GTT), after a 14-h fast, mice were given an oral bolus of D-glucose (2 g/kg body wt), and the blood glucose concentration was measured in samples taken at 0, 30, 60, 90, and 120 min after the glucose bolus. Insulin tolerance test (ITT) was performed on 6-h fasting mice by intraperitoneal injection of 1 unit/kg body wt human insulin (Sigma Aldrich, St. Louis, MO). Blood glucose concentration was determined at 0, 30, 60, 90, and 120 min after insulin administration. Triglyceride levels in serum from fasting animals were determined using a GPO-Trinder assay (Sigma Aldrich). Serum free fatty acid levels in fasting animals were measured using a NEFA-Kit-U (Wako Pure Chemical, Richmond, VA). For determination of tissue triglyceride content, 50–100 mg tissue pieces were homogenized on ice in pH 7.3 extraction buffer (20 mmol/l Tris, 1 mmol/l β -mercaptoethanol, 1 mmol/l EDTA). After centrifugation, the glycerol content of the supernatants was determined using a GPO-Trinder assay (Sigma Aldrich) according to the manufacturer's instructions.

Phosphoinositide-3-OH kinase activity. Mice were subjected to 14-h fast, injected with saline or insulin (10 units/kg body wt i.p.) and sacrificed 3 min after injection. Tissues were collected and frozen. Phosphoinositide-3-OH kinase (PI3K) activity was measured in phosphotyrosine immunoprecipitates (*p*-Tyr, Ab-4; EMD Biosciences, San Diego, CA) from white adipose tissue (WAT), skeletal muscle, and liver lysates, as previously described (16).

Intraperitoneal leptin administration. Mice were divided into two groups and were treated once daily with equal volumes of intraperitoneally injected saline or mouse leptin (R&D Systems, Minneapolis, MN) dissolved in saline at doses of 5 μ g/g body wt for 6 days. Food intake and changes in body weight were measured to estimate the effects of exogenous leptin administration.

Real-time quantitative RT-PCR. Mouse WAT, skeletal muscles, and livers from wild-type and $AR^{-/y}$ were dissected and total RNA isolated, using a TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA synthesis and PCR were performed using Superscript RNase H⁻ reverse transcriptase and cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed using iCycler real-time PCR amplifier (Bio-Rad Laboratories, Hercules, CA). Each PCR contained 1 μ l cDNA, 50 μ mol/l primers, and 12.5 μ l iQ SYBR Green Supermix reagent (Bio-Rad Laboratories) and was triplicated, and 18s was used as an internal control. A list of the primer sequences for real-time PCR is available in Table 1.

Dihydrotestosterone replacement study. A 60-day time-release 5 α -dihydrotestosterone (5 mg) or placebo pellet (Innovative Research of America, Sarasota, FL) was inserted into a subcutaneous pocket (posterior neck region) of male wild-type and $AR^{-/y}$ mice, respectively. At 8 weeks after implantation of the pellet, the mice were killed to study serum hormones and metabolic parameters.

Statistical analyses. All values are the means \pm SE. Differences between two groups were assessed using the unpaired two-tailed Student's *t* test or among more than two groups by ANOVA. In all statistical comparisons, *P* < 0.05 was defined as a significant difference. SigmaStat statistics software (version 2.0; SPSS) was used for all calculations.

RESULTS

Loss of AR is associated with fat accumulation and altered metabolic profiles. Using Cre/loxP-mediated recombination by crossing AR-loxP mice with ACTB-Cre mice, we generated mice lacking functional AR in mixed C57BL/6/129Sv/FVB background (15). Male wild-type and

knockout $AR^{-/y}$ mice were used for the present studies. Because the $AR^{-/y}$ mice were phenotypically female in appearance, we also used female wild-type mice as an additional comparison group for phenotypic analysis. The growth curves of male wild-type, female wild-type, and $AR^{-/y}$ mice showed that $AR^{-/y}$ mice exhibited a significant decrease in the body weight gain throughout the 20th week compared with male wild-type mice (Fig. 1A). The body weight of male wild-type and $AR^{-/y}$ mice was significantly greater at all time points after 8 weeks compared with female wild-type littermates. However, body weight gain was significantly greater in $AR^{-/y}$ mice after 22 weeks compared with their male and female wild-type littermates throughout the 40-week study period (male wild-type: 36.0 \pm 1.3 g; female wild-type: 26.35 \pm 0.59 g; $AR^{-/y}$: 40.87 \pm 1.0 g; *P* = 0.009 for male wild-type vs. $AR^{-/y}$; *n* = 9). This was accompanied by significantly enlarged gonadal and perirenal fat pads compared with male and female wild-type counterparts (Fig. 1B and C, Table 2). Aging obesity profiles are consistent with previous findings (17,18). Circulating triglyceride levels of $AR^{-/y}$ mice was increased by twofold compared with male and female wild-type mice (Table 2). Notably, histological analysis of WAT after fixation (Fig. 1B) and quantification of adipocyte size (Fig. 1C) revealed that adipocytes from 35-week-old $AR^{-/y}$ mice were significantly larger than those in wild-type counterparts. The comparison data for muscle and bone mass showed no significant difference in those tissue masses between wild-type and $AR^{-/y}$ mice (data not shown). Although obvious enlargement of adipose tissues can be seen from intra-abdominal appearance in $AR^{-/y}$ mice, this only accounts for \sim 15% increased body weight in $AR^{-/y}$ mice compared with wild-type mice. These results indicate that $AR^{-/y}$ mice had marked increased adiposity at an older age.

Development of insulin resistance in $AR^{-/y}$ mice. Given the excess lipid deposition in WAT, we examined blood glucose and insulin levels in male wild-type, female wild-type, and $AR^{-/y}$ mice. $AR^{-/y}$ mice start to show elevated blood glucose at 20 weeks (Table 3), which persisted to at least 35 weeks under both the fasting and fed conditions (Table 2). The hyperglycemia occurred despite a marked increase in serum insulin levels in the fasting (\sim 60%) and fed (\sim 67%) states, indicating that $AR^{-/y}$ mice were more resistant to insulin than their wild-type counterparts under ambient conditions (Table 2).

To assess whole-body glucose homeostasis, we next performed oral bolus GTTs and intraperitoneal ITTs on these mice. Oral bolus GTTs demonstrated marked glucose intolerance and distinguishable area under the curve in $AR^{-/y}$ mice (Fig. 2A and B). Blood glucose levels of $AR^{-/y}$ mice were greater at all times during the test, and

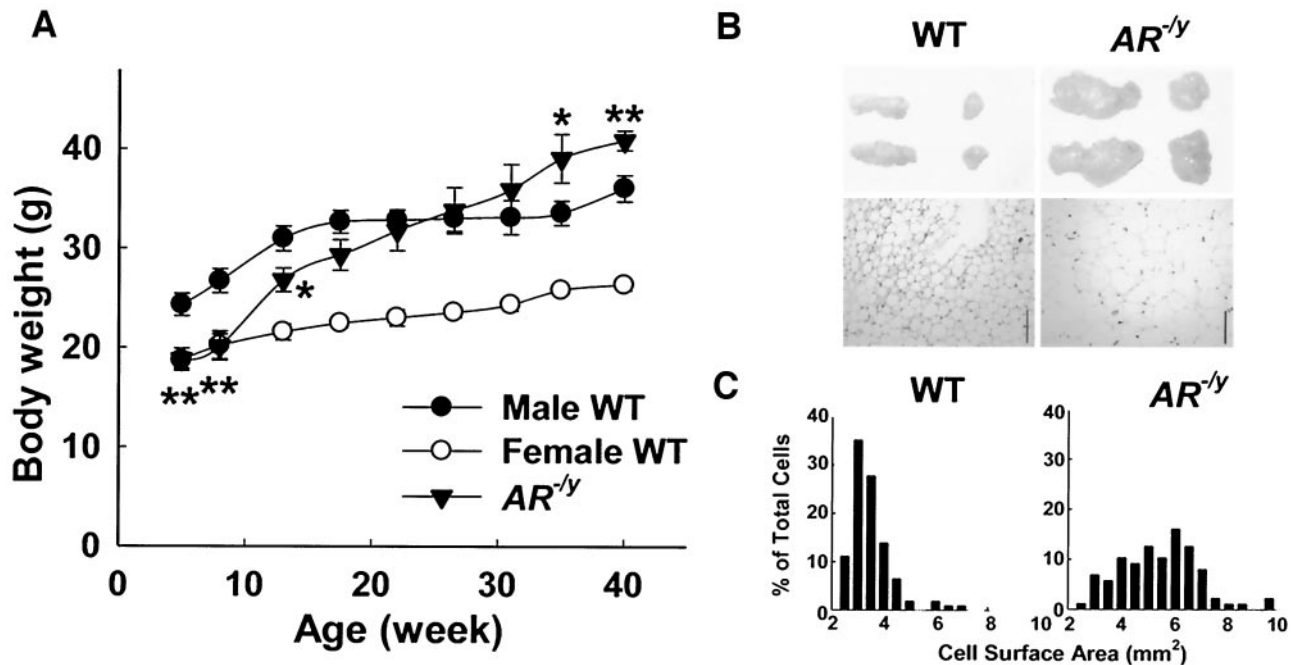


FIG. 1. The $AR^{-/-}$ mice developed excess adiposity. **A**: Growth curves of mice were determined at indicated time points between the ages of 5 and 40 weeks. Values are the means \pm SE from 9–10 mice per group. $*P < 0.05$, $**P < 0.01$, $AR^{-/-}$ vs. male wild-type (WT) mice. **B**: Epididymal and perirenal fat pads isolated from 35-week-old mice (upper panels). Sections of WAT are shown at $40\times$ magnification (lower panels) of 35-week-old mice (scale bar = 200 μ m). **C**: Distribution of cell size of epididymal WAT from 35-week-old mice.

hyperglycemia was still apparent 2 h after the glucose bolus. ITTs showed that $AR^{-/-}$ mice were slightly resistant and highly resistant to the hypoglycemic effect of exogenous insulin at 25 and 35 weeks of age, respectively (Fig. 2C and D). The defect in whole-body insulin sensitivity was not caused by female-like phenotype in $AR^{-/-}$ mice because we detected no differences in response patterns of male and female wild-type mice.

Because insulin resistance can be correlated to the activity of PI3K, a signaling mediator needed for many metabolic effects of insulin, we examined insulin-stimulated PI3K activity using wild-type mice as controls. The PI3K activity on insulin stimulation in $AR^{-/-}$ mice was reduced by 60–63% in insulin target organs, such as skeletal muscle and liver (Fig. 2E), suggesting that $AR^{-/-}$ mice did have skeletal muscle and hepatic insulin resistance and hyperinsulinemia with obesity.

Increased lipid deposition and leptin levels in $AR^{-/-}$ mice. Serum free fatty acid levels in the fasting state are elevated in $AR^{-/-}$ mice (Table 2). Moreover, skeletal muscle and hepatic triglyceride content markedly increased by 2.6- and 1.9-fold, respectively, indicating that insulin resistance was associated with increasing triglyceride deposition in the skeletal muscle and liver (Fig. 2F). As expected from the increased WAT mass, fed serum leptin concentrations were higher in $AR^{-/-}$ mice at both 25 and 35 weeks of age (Fig. 3A). Moreover, serum leptin levels show elevated linear relationships with body weight in $AR^{-/-}$ mice compared with wild-type littermates (Fig. 3B). Surprisingly, fed serum leptin levels were also higher, even though $AR^{-/-}$ mice gained significantly less weight before 20 weeks of age (Fig. 3A), indicating that loss of AR may cause increased leptin earlier than increased body weight. Adiponectin, an insulin-sensitizing adipokine, was

TABLE 2
Physiological and metabolic parameters of 35-week-old study groups

	Genotype		
	Male wild type	Female wild type	$AR^{-/-}$
Mice (n)	9	10	9
Body weight (g)	33.15 \pm 1.2	25.81 \pm 0.44	39.06 \pm 2.7*
Gonadal fat/body (%)	2.21 \pm 0.14	0.34 \pm 0.08	3.29 \pm 0.23*
Serum total triglycerides (mmol/l)	0.30 \pm 0.04	0.32 \pm 0.08	0.67 \pm 0.1†
Serum nonesterified fatty acids (mEq/l)	0.25 \pm 0.02	0.25 \pm 0.02	0.71 \pm 0.04‡
Serum cholesterol (mmol/l)	2.28 \pm 0.05	2.28 \pm 0.06	2.44 \pm 0.05
Fasting blood glucose (mmol/l)	6.46 \pm 0.31	6.41 \pm 0.27	7.94 \pm 0.38*
Fed blood glucose (mmol/l)	14.03 \pm 0.54	14.93 \pm 0.29	19.75 \pm 0.78‡
Fasting serum insulin (ng/ml)	0.55 \pm 0.13	0.53 \pm 0.33	0.88 \pm 0.33*
Fed serum insulin (ng/ml)	0.92 \pm 0.17	1.12 \pm 0.14	1.54 \pm 0.16*

Data are the means \pm SE. After a 14-h fast, animals were killed. $*P < 0.05$, $\dagger P < 0.01$, and $\ddagger P < 0.001$, AR^{-} vs. male wild type.

TABLE 3
Effects of dihydrotestosterone treatment on serum hormones and metabolic parameters in 20-week-old study groups

	Male wild-type 20-week-old (12–20 weeks) mice		<i>AR</i> ^{-/-} 20-week-old (12–20 weeks) mice		
	Sham	Placebo	Sham	Placebo	Dihydrotestosterone
Mice (<i>n</i>)	4	4	4	4	4
Insulin resistance index	945.6 ± 22.6	1,095.1 ± 73.5	5,540.4 ± 182.7*	4,473.3 ± 255.1*	4,478.4 ± 394.4*
IGTT AUC insulin (ng · ml ⁻¹ · min ⁻¹)	44.50 ± 8.84	48.30 ± 6.55	178.55 ± 12.15*	152.16 ± 9.39*	136.40 ± 12.7*
IGTT AUC glucose (g · dl ⁻¹ · min ⁻¹)	21.25 ± 0.50	22.67 ± 1.52	31.61 ± 0.92†	29.39 ± 1.67*	32.83 ± 2.89†
Serum insulin (ng/ml)	0.25 ± 0.06	0.29 ± 0.02	0.61 ± 0.01‡	0.71 ± 0.10‡	0.68 ± 0.04‡
Blood glucose (mmol/l)	6.16 ± 0.09	5.99 ± 0.36	8.02 ± 0.53†	8.00 ± 0.09†	8.69 ± 0.49‡
Leptin (ng/ml)	0.88 ± 0.17	0.69 ± 0.09	7.32 ± 0.32†	7.82 ± 1.03‡	8.12 ± 0.82‡
Cholesterol (mmol/l)	1.59 ± 0.07	1.73 ± 0.30	1.62 ± 0.11	1.68 ± 0.01	1.72 ± 0.16
Nonesterified fatty acid (mEq/l)	0.18 ± 0.03	0.18 ± 0.02	0.33 ± 0.01*	0.36 ± 0.03*	0.30 ± 0.05
Total triglycerides (mmol/l)	0.15 ± 0.02	0.17 ± 0.04	0.29 ± 0.08	0.28 ± 0.04	0.26 ± 0.02
Adiposity index (%)	1.34 ± 0.03	1.15 ± 0.06	1.72 ± 0.05‡	1.71 ± 0.09‡	1.74 ± 0.02†
Androstenedione (ng/ml)	0.58 ± 0.11	0.62 ± 0.07	0.64 ± 0.05	0.59 ± 0.14	0.63 ± 0.09
Estradiol (pg/ml)	55.97 ± 5.48	54.65 ± 4.37	57.06 ± 1.72	59.82 ± 1.60	54.38 ± 1.41
Dihydrotestosterone (ng/ml)	0.61 ± 0.05	0.58 ± 0.07	0.09 ± 0.03‡	0.12 ± 0.05‡	0.73 ± 0.09

Data are the means ± SE. After a 14-h fast, animals were killed. The adiposity index was the ratio of epididymal adipose tissue to body weight for mice. The insulin resistance index is the product of glucose and insulin areas under the curve. Parentheses indicate the period of pellet implantation. **P* < 0.05, †*P* < 0.01, and ‡*P* < 0.001, vs. sham male wild-type mice. AUC, area under the curve; IGTT, intraperitoneal GTT.

reduced in *AR*^{-/-} mice (Fig. 3C), whereas there was no difference in TNF-α levels (Fig. 3D).

Development of leptin resistance in *AR*^{-/-} mice. We also found that food intake and body weight was significantly reduced after exogenously administered leptin in wild-type mice, but not in *AR*^{-/-} mice (Fig. 4A and B). Furthermore, food intake was not significantly different in *AR*^{-/-} mice compared with wild-type mice before exogenous leptin administration, despite elevated leptin levels, indicating *AR*^{-/-} mice were leptin resistant at 35 weeks of age. However, food intake and body weight were reduced after an exogenous load of leptin in both 20-week-old *AR*^{-/-} mice and wild-type mice, whereas two control groups had similar body weight and adiposity (Fig. 4C and D). These results suggest that *AR*^{-/-} mice develop leptin resistance that may be caused by the development of adiposity and the long-term absence of AR.

Loss of AR altered the lipid metabolic profiles. To determine the mechanism of the increased lipid deposition in WAT, as well as skeletal muscle and hepatic triglyceride accumulation, mRNA from these tissues were further analyzed. The mRNA levels of four lipid metabolism genes, peroxisome proliferator-activated receptor (PPAR)-γ (*PPAR*γ), CCAAT/enhancer-binding protein-α (*C/EBP*α), adipocyte fatty acid-binding protein/adipocyte P2 (*aP2*), and sterol regulatory element-binding protein 1c (*SREBP1c*), were higher in WAT of *AR*^{-/-} compared with wild-type mice (Table 4), suggesting that loss of AR may contribute to the increase of adipogenesis and lipogenesis via stimulation of several genes. Moreover, consistent with triglyceride accumulation, loss of AR reduced mRNA levels of PPAR-α (*PPAR*α) in skeletal muscle and liver (Table 4). These results suggest that AR is directly or indirectly involved in lipid metabolism.

Dihydrotestosterone replacement failed to reverse the metabolic abnormalities and insulin resistance in *AR*^{-/-} mice. Because serum testosterone levels were markedly decreased as a result of atrophic testes in *AR*^{-/-} mice, we could not exclude the possibility that the insulin resistance and metabolic abnormalities in *AR*^{-/-} mice

simply reflected the low levels of androgens. To address this issue, nonaromatizable androgen, dihydrotestosterone, was given to both 26- and 12-week-old *AR*^{-/-} and wild-type littermates. Several serum hormones and metabolic parameters were assessed after 8 weeks of pellet implantation. Dihydrotestosterone replacement restored serum dihydrotestosterone levels to within the physiological range (0.6–0.9 ng/ml) in *AR*^{-/-} mice. It is known that estradiol is converted not only from estrone but also testosterone; therefore, we could not exclude the possibility that the abnormalities in *AR*^{-/-} mice simply reflected less estrogen converted from testosterone, although we found that serum estradiol levels, as well as levels of the prohormone androstenedione, remained unchanged in *AR*^{-/-} mice compared with wild-type mice (Tables 3 and 5). Dihydrotestosterone replacement couldn't reverse the metabolic abnormalities and insulin resistance in *AR*^{-/-} mice in either age study group (Tables 3 and 5), which suggests that androgen actions directly mediated via AR are significant in insulin sensitivity.

DISCUSSION

Cross-sectional epidemiological studies have indicated a direct correlation between serum testosterone concentrations and insulin sensitivity (19). Low testosterone levels are associated with an increased risk of type 2 diabetes in men (20,21). Because most androgens need to bind to AR to exert their androgenic biological functions, AR have been thought to function as a modulator of insulin sensitivity. Our current results demonstrate that mice lacking AR develop insulin and leptin resistance at an advanced age. Late onset of obesity, as we observed in *AR*^{-/-} mice, was reported to be associated with insulin resistance (22). The marked hyperinsulinemia and hyperglycemia in *AR*^{-/-} mice clearly demonstrate that loss of AR may reduce insulin sensitivity. A relatively small increase in body weight (~15%) is associated with a remarkable reduction in insulin sensitivity (~65%) in *AR*^{-/-} mice and with insulin resistance that occurs as early as 20 weeks of age

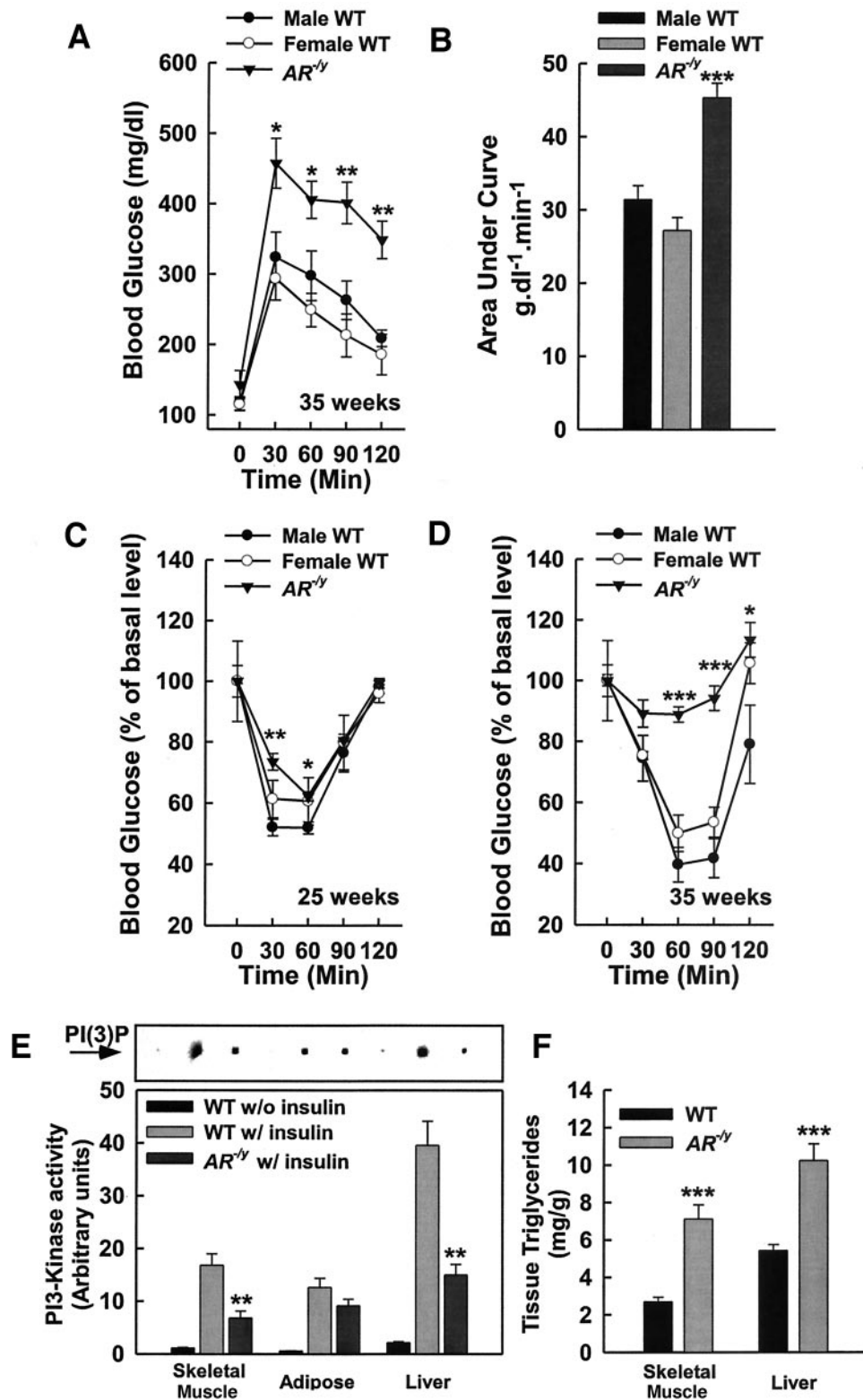


FIG. 2. $AR^{-/-}$ mice are insulin resistant and glucose intolerant. **A:** GTT (oral bolus 2 mg/g body wt) of 35-week-old mice after 14-h fast. **B:** area under the curve analysis of GTT. **C:** ITT (1 unit/kg body wt i.p.) of 25-week-old mice. **D:** ITT (1 unit/kg body wt i.p.) of 35-week-old mice. Values are the means \pm SE from 5–6 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $AR^{-/-}$ vs. male wild-type mice. **E:** In vivo PI3K activity was measured in phosphotyrosine immunoprecipitates prepared from skeletal muscles, adipose tissues, and livers from 14 h-fasted 35-week-old mice. Tissues were collected 3 min after intraperitoneal injection of insulin or isotonic saline. The results were quantified by phosphoimaging. Values are representative of three mice from each group. ** $P < 0.01$, $AR^{-/-}$ with insulin vs. wild type with insulin. **F:** Skeletal muscles and livers were removed from 35-week-old ad libitum-fed mice. Values of each group were converted into milligrams of triglyceride per gram of tissue (wet weight) after comparison with a glycerol standard. Values are the means \pm SE from five mice per group. *** $P < 0.001$, $AR^{-/-}$ vs. wild-type mice. PI(3)P, phosphatidylinositol-3-phosphate; w/, with; w/o, without; WT, wild type.

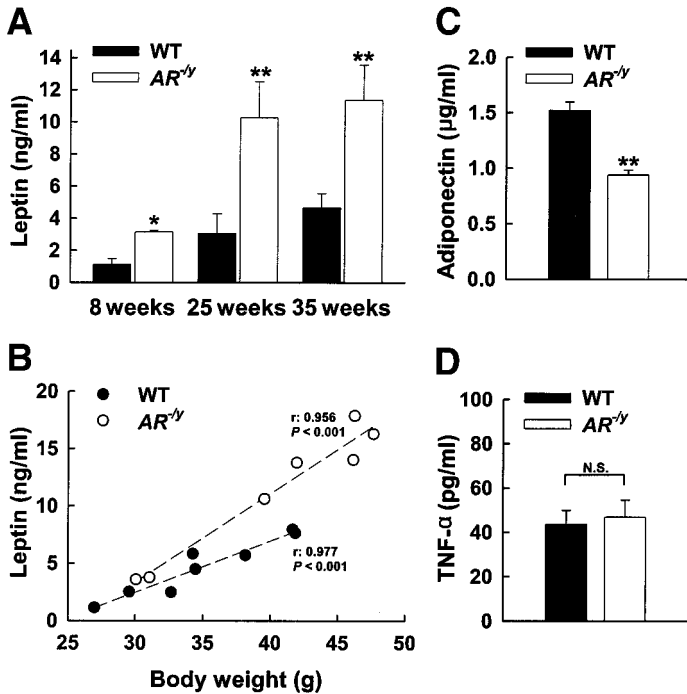


FIG. 3. Effects of AR deficiency on adipocyte-derived adipokine. **A:** Fed leptin concentrations in 8-, 25-, or 35-week-old mice. **B:** Relationship between fed leptin concentrations and body weight of 35-week-old mice. **C:** Adiponectin concentrations in 35-week-old mice. **D:** TNF- α concentrations in 35-week-old mice. Values are the means \pm SE from five mice per group. In **D**, $n = 8$ for wild-type mice and $n = 7$ for $AR^{-/-}$ mice. Regression lines for both groups are shown. * $P < 0.05$, ** $P < 0.01$, $AR^{-/-}$ vs. wild type. N.S., no significance; WT, wild type.

in nonobese $AR^{-/-}$ mice, suggesting that loss of AR may directly reduce insulin sensitivity in target tissues without first increasing body weight significantly.

The phenotypically female appearance of $AR^{-/-}$ mice is similar to that of Tfm (testicular feminized) mice, in which AR is functionally deficient by introduction of the Tfm mutation in the AR gene (23). Therefore, we added female wild-type mice for comparison, and these mice remain smaller and have less adiposity than both male wild-type and $AR^{-/-}$ mice. In addition, we found no significantly distinguishable metabolic pattern between female and male wild-type mice, whereas $AR^{-/-}$ mice exhibited severe insulin resistance and obesity. A previous study has shown that db/db Tfm/Y males develop severe diabetes. In contrast, female db/db littermates only exhibit mild hyperglycemia (24).

The excess fat mass in $AR^{-/-}$ mice may be caused by an impaired ability of skeletal muscle to use lipids as a fuel substrate and of liver to catabolize lipids, leading to a shunting of lipids to adipose tissue. In fact, the unchanged food intake in $AR^{-/-}$ mice suggests that the excess weight gain and adiposity may be caused by normal energy input coupled with reduced lipid oxidation and increased lipid storage. Indeed, impairment in hepatic lipid oxidation resulted in elevated circulating free fatty acid and hepatic steatosis in PPAR- α null mice (25). Activation of PPAR- α by fibrates reduces adiposity in fa/fa ZDF (Zucker diabetic fatty) rats via activation of several peroxisomal and mitochondrial fatty acid oxidation genes (26). Given that skeletal muscle and liver are the major sites of lipid oxidation and deposition, respectively, it is possible that

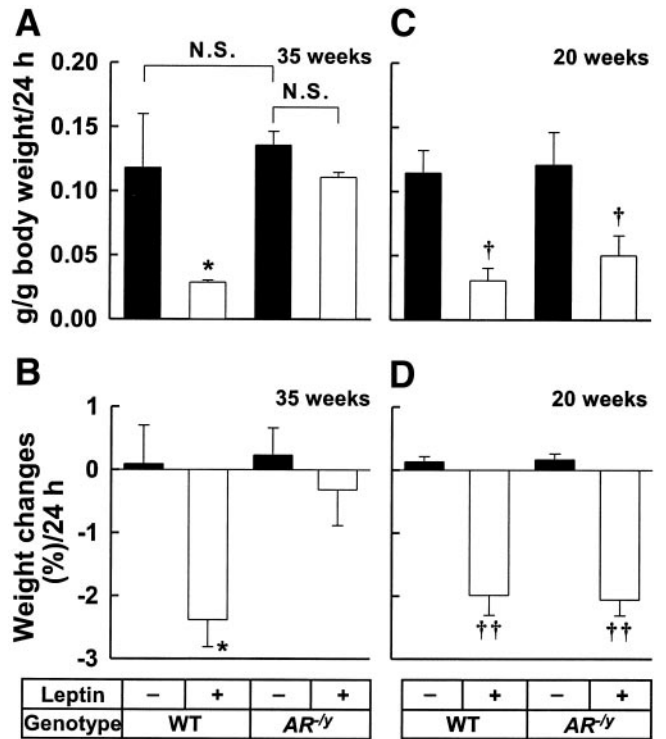


FIG. 4. Aging $AR^{-/-}$ mice developed leptin resistance but not younger mice. Two groups of 35- or 20-week-old mice received an intraperitoneal injection of either leptin (5 μ g/g body wt) or isotonic saline. **A:** Food intake per 24 h of 35-week-old mice. **B:** Weight changes per 24 h of 35-week-old mice. **C:** Food intake per 24 h of 20-week-old mice. **D:** Weight changes per 24 h of 20-week-old mice. Values are the means \pm SE from 3–4 mice per group. * $P < 0.05$, wild type with leptin vs. control; † $P < 0.05$, leptin treatment vs. control; †† $P < 0.001$, leptin treatment vs. control. N.S., no significance; WT, wild type.

loss of PPAR- α could produce such an effect. Consistent with this possibility, decreased PPAR- α expression in skeletal muscle, decreased hepatic lipid metabolism, and subsequent alterations in expression of genes that stimulate adipocyte differentiation (PPAR γ [22], C/EBP α [27], and SREBP1c [28]) and lipid accumulation (aP2 [29]) in WAT were found in $AR^{-/-}$ mice. Furthermore, liver-specific AR knockout mice, which are more susceptible to high-fat diet-induced insulin resistance, showed lower hepatic PPAR- α expression (H.-Y.L., C.C., unpublished observations). Recent studies using hyperinsulinemic-euglycemic clamps have shown that PPAR- α null mice are

TABLE 4
Expression of lipid metabolism genes in WAT, liver, and skeletal muscle in $AR^{-/-}$ mice as quantified by real-time RT-PCR

Gene	$\Delta\Delta C_T$ (average ΔC_T - average $\Delta C_{T,WT}$)	Normalized amount relative to wild type $2^{-\Delta\Delta C_T}$
WAT		
PPAR γ	-1.40 \pm 0.81	2.6 (1.5–4.6)
C/EBP α	-3.98 \pm 0.22	15.8 (13.5–18.4)
SREBP1c	-2.28 \pm 0.58	4.9 (3.2–7.3)
aP2	-0.43 \pm 0.17	1.35 (1.20–1.52)
Liver		
PPAR α	3.13 \pm 0.45	0.11 (0.08–0.16)
Skeletal muscle		
PPAR α	2.36 \pm 0.13	0.19 (0.18–0.21)

C_T , threshold cycle; $C_{T,WT}$, threshold cycle for wild type.

TABLE 5
Effects of dihydrotestosterone treatment on serum hormones and metabolic parameters in 34-week-old study groups

	Male wild-type 34-week-old (26–34 weeks) mice		<i>AR</i> ^{-/-} 34-week-old (26–34 weeks) mice		
	Sham	Placebo	Sham	Placebo	Dihydrotestosterone
Mice (<i>n</i>)	4	4	4	4	6
Insulin resistance index	1,893.1 ± 27.4	1,742.1 ± 228.4	4,918.4 ± 390.6*	5,302.9 ± 237.1*	5,381.8 ± 329.2*
IGTT AUC insulin (ng · ml ⁻¹ · min ⁻¹)	73.15 ± 10.77	76.45 ± 5.59	144.83 ± 8.40*	151.60 ± 18.29*	148.7 ± 1.30*
IGTT AUC glucose (g · dl ⁻¹ · min ⁻¹)	25.88 ± 0.37	22.78 ± 2.98	33.96 ± 2.69†	34.98 ± 1.56†	36.19 ± 2.21†
Serum insulin (ng/ml)	0.48 ± 0.08	0.46 ± 0.02	0.78 ± 0.03‡	0.80 ± 0.01‡	0.80 ± 0.10‡
Blood glucose (mmol/l)	6.21 ± 0.32	6.25 ± 0.17	8.41 ± 0.45†	8.42 ± 0.53†	8.75 ± 0.82†
Leptin (ng/ml)	1.19 ± 0.19	1.44 ± 0.58	9.83 ± 1.98*	9.70 ± 0.80*	10.38 ± 0.26*
Cholesterol (mmol/l)	1.83 ± 0.30	2.03 ± 0.64	2.01 ± 0.49	2.08 ± 0.05	2.08 ± 0.26
Nonesterified fatty acids (mEq/l)	0.23 ± 0.02	0.20 ± 0.03	0.54 ± 0.12†	0.53 ± 0.01†	0.52 ± 0.07‡
Total triglycerides (mmol/l)	0.19 ± 0.01	0.18 ± 0.06	0.55 ± 0.15†	0.60 ± 0.08†	0.62 ± 0.08†
Adiposity index (%)	1.56 ± 0.09	1.51 ± 0.19	2.76 ± 0.18*	2.79 ± 0.12*	2.43 ± 0.14*
Androstenedione (ng/ml)	0.65 ± 0.06	0.71 ± 0.12	0.67 ± 0.08	0.66 ± 0.11	0.70 ± 0.06
Estradiol (pg/ml)	58.94 ± 3.89	54.35 ± 3.07	57.02 ± 1.91	52.22 ± 2.27	55.44 ± 4.03
Dihydrotestosterone (ng/ml)	0.81 ± 0.01	0.77 ± 0.07	0.10 ± 0.03*	0.12 ± 0.05*	0.77 ± 0.14

Data are means ± SE. After a 14-h fast, animals were killed. The adiposity index was the ratio of epididymal adipose tissue to body weight for mice. The insulin resistance index is the product of glucose and insulin areas under the curve. Parentheses indicate the period of pellet implantation. **P* < 0.05, †*P* < 0.01, and ‡*P* < 0.001, vs. sham male wild-type mice. AUC, area under the curve; IGTT, intraperitoneal GTT.

not protected against high-fat diet-induced insulin resistance (30). Thus, loss of PPAR- α expression in *AR*^{-/-} mice may be a potential mechanism contributing to the decreased insulin sensitivity observed.

An alternative explanation is that skeletal muscle and hepatic insulin resistance in *AR*^{-/-} mice might be secondary to altered release of adipokines. Leptin increased insulin sensitivity and concomitantly reduced triglyceride content by promoting lipid oxidation both in animal models of insulin resistance and in humans with lipodystrophic diabetes (31). The *AR*^{-/-} mice with elevated leptin, however, have significant leptin resistance when exogenous leptin is administered. Related results have been observed in ZDF rats, *ob/ob* mice that lack leptin, *db/db* mice that have an inactive leptin receptor, and the livers of such mice are all steatotic (32,33). In ZDF rats, the skeletal muscle, pancreas, and heart are also steatotic (34). A strong correlation between intracellular triglyceride content and insulin resistance has been established in both human and animal studies of obesity-related insulin resistance and type 2 diabetes (35). Consistent with this possibility, a substantial ectopic deposition of triglycerides in nonadipocytes, such as skeletal muscle and liver, in *AR*^{-/-} mice is likely a central element. Triglyceride overload of nonadipocytes causes insulin resistance, lipotoxic heart disease, and adipogenic type 2 diabetes (36). We have also demonstrated, through PI3K activity assay after injection of insulin, that skeletal muscle and liver in *AR*^{-/-} mice are insulin resistant. Interestingly, PPAR- α is necessary for the lipopenic action of hyperleptinemia on white adipose and liver tissues. In PPAR- α null mice infused with adenovirus-leptin, upregulation of carnitine palmitoyl transferase-1 mRNA in fat, downregulation of acetyl CoA carboxylase in liver, and upregulation of PPAR- γ coactivator-1 α mRNA in both tissues are abolished, as is the reduction in their triglyceride content, suggesting that leptin action may be mediated through PPAR- α (37). Thus, loss of PPAR- α in *AR*^{-/-} mice may reduce leptin action,

despite elevated leptin levels. In contrast, we found decreased adiponectin and no elevation of serum TNF- α (another adipokine that contributes to insulin resistance) (38) in *AR*^{-/-} mice at 35 weeks of age, whereas mice lacking adiponectin exhibited severe insulin resistance (39). However, it is known that other adipokines also mediate the metabolism of lipids and contribute to the pathogenesis of insulin resistance. Further studies are needed to determine the mechanism of lipid profile elevation in *AR*^{-/-} mice.

Although there is no evidence that androgen-AR can directly affect PPAR- α , indirect interactions of AR and PPAR- α through nuclear receptor coregulators are possible. ARA70 (AR-associated coregulator 70), a coactivator of AR, was able to physically interact with PPAR- α , as determined by coimmunoprecipitation. In the adrenal cell line Y1, ARA70 behaved as a repressor of PPAR- α (40). The mechanism of how leptin is regulated by androgen-AR remains elusive. An early study indicated that in primary rat adipocytes expressing AR, testosterone and dihydrotestosterone were able to suppress leptin mRNA and leptin secretion (41), suggesting a direct effect of testosterone on the regulation of leptin secretion in adipocytes. *AR*^{-/-} mice not only have an AR deficiency, but they also have decreased serum levels of androgens. Thus, in *AR*^{-/-} mice, treatment with nonaromatizable dihydrotestosterone restored the physiological serum dihydrotestosterone levels, whereas estrogen levels remained unchanged compared with wild-type mice. Therefore, the possibility that a lack of estrogen receptor- α activation results in increased WAT, as seen in male mice deficient in either estrogen receptor- α or aromatase, can be excluded. Importantly, we found no reversed effect after dihydrotestosterone treatment, indicating that insulin resistance in *AR*^{-/-} mice, as seen in the present study, is mediated directly via AR.

Taken together, these data suggest that loss of AR may contribute to an increase of leptin levels and leptin resis-

tance, which may play important roles for the development of obesity and insulin resistance. A previous study using transgenic mice overexpressing leptin demonstrated that loss of leptin effectiveness in older transgenic mice might contribute to the accumulation of adipose mass (42). Consistent with this possibility, the $AR^{-/y}$ mice were progressively desensitized to leptin because of long-term elevated circulating leptin throughout the 40-week study period. In rats, testosterone was reported to be associated negatively with serum leptin, independent of BMI (43). Leptin levels are higher in aging men with lower testosterone (44). Furthermore, low testosterone may lead to the accumulation of visceral fat (45). As total body fat mass increases, resistance develops to both leptin and insulin. Elevated leptin fails to stimulate weight loss, and the hypogonadal-obesity cycle ensures further visceral obesity and insulin resistance. In our present study, $AR^{-/y}$ mice progressively develop leptin resistance and insulin resistance, resulting in obesity. This differs from the ob/ob and db/db mice that display early onset genetic obesity and have leptin inactivity from a very early stage (46). $AR^{-/y}$ mice have milder obesity because of the late onset of leptin and insulin resistance. However, leptin resistance in $AR^{-/y}$ mice is similar to that of db/db mice, and having dysfunctional leptin receptors is likely to be a major contributing factor. Progressive insulin resistance can then lead to type 2 diabetes and other diseases, such as high triglyceride-low HDL pattern dyslipidemia and metabolic syndrome X, as well as an increased risk of cardiovascular diseases (45). It has been reported that hypotestosteronemia may be a risk factor for coronary artery disease in men (47).

Interestingly, along with loss of AR, for which we demonstrated a resulting metabolic syndrome, both loss of estrogen receptor- α (48) and aromatase deficiency, which causes loss of the ability to synthesize estrogen (49), result in metabolic syndromes. These observations indicate the possibility that loss of both androgen and estrogen responsiveness disrupts energy homeostasis.

In summary, $AR^{-/y}$ mice provide an in vivo model showing that loss of AR increases serum leptin concentration and skeletal muscle/hepatic triglyceride content, which may result in the development of obesity, leptin resistance, and insulin resistance. Because obesity and progressive insulin resistance may lead to type 2 diabetes and an increased risk of cardiovascular disease (50), a better understanding of the molecular mechanisms involved and dissection of the roles of androgen-AR in insulin and leptin resistance may help in the development of better therapeutic approaches to type 2 diabetes, obesity, and cardiovascular diseases.

ACKNOWLEDGMENTS

This work was supported by grants from the George Whipple Endowment, the National Institutes of Health (DK60948), and the Dr. Shu-Shi Chang Neuroendocrinology Fund.

We thank K. Wolf, L. Collins, and T. Chang for manuscript help.

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