Progressive development of insulin resistance phenotype in male mice with complete aromatase (CYP19) deficiency

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Abstract

Aromatase (CYP19) is a cytochrome P450 enzyme that catalyzes the formation of aromatic C18 estrogens from C19 androgens. It is expressed in various tissues and contributes to sex-specific differences in cellular metabolism. We have generated aromatase-knockout (ArKO) mice in order to study the role of estrogen in the regulation of glucose metabolism. The mean body weights of male ArKO (−/−) mice (n=7) and wild-type littermates (+/+) (n=7) at 10 and 12 weeks of age were 26·7 ± 1·9 g vs 26·1 ± 0·8 g and 28·8 ± 1·4 g vs 26·9 ± 1·0 g respectively. The body weights of the ArKO and wild-type mice diverged between 10 and 12 weeks of age with the ArKO males weighing significantly more than their wild-type littermates (P<0·05). The ArKO males showed significantly higher blood glucose levels during an intraperitoneal glucose tolerance test compared with wild-type littermates beginning at 18 weeks of age. By 24 weeks of age, they had higher fasting blood glucose levels compared with wild-type littermates (133·8 ± 22·8 mg/dl vs 87·8 ± 20·3 mg/dl respectively; P<0·01). An intraperitoneal injection of insulin (0·75 mU insulin/g) caused a continuous decline in blood glucose levels in wild-type mice whereas ArKO males at 18 weeks and older exhibited a rebound increase in glucose levels 30 min after insulin injection. Thus, ArKO male mice appear to develop glucose intolerance and insulin resistance in an age-dependent manner. There was no difference in fasting serum triglyceride and total cholesterol levels between ArKO male mice and wild-type littermates at 13 and 25 weeks of age. However, serum triglyceride and cholesterol levels were significantly elevated following a meal in ArKO mice at 36 weeks of age. Serum testosterone levels in ArKO male mice were continuously higher compared with wild-type littermates. Treatment of ArKO males with 17β-estradiol improved the glucose response as measured by intraperitoneal glucose and insulin tolerance tests. Treatment with fibrates and thiazolidinediones also led to an improvement in insulin resistance and reduced androgen levels. As complete aromatase deficiency in man is associated with insulin resistance, obesity and hyperlipidemia, the ArKO mouse may be a useful animal model for examining the role of estrogens in the control of glucose and lipid homeostasis.

Journal of Endocrinology (2003) **176,** 237–246

Introduction

Aromatase cytochrome P450 (P450 arom) is encoded by the CYP19 gene (*Cyp19*) and is a key enzyme in the biosynthesis of C18 estrogens from C19 androgens (Simpson 2000). It is expressed in the ovary and placenta as well as other tissues including testis, brain, fat, liver and muscle (Simpson 2000). In humans, complete aromatase deficiency is associated with pseudohermaphroditism and pubertal failure with no signs of estrogen action in women (Shozu *et al.* 1991). In males, the absence of estrogen due to aromatase deficiency is associated with tall stature, continued growth, delayed skeletal maturation, osteopenia, large testis and abnormal glucose and lipid metabolism (Faustini-Fustini *et al.* 1999, Grumbach & Auchus 1999, Simpson 2000). In one report, one of two aromatase-deficient men had increased fasting insulin concentrations with normal blood glucose levels (Morishima *et al.* 1995), while in another, the patient had normal insulin and glucose levels (Carani *et al.* 1997). Estrogen receptor mutations can also result in a syndrome of estrogen resistance and have been associated with increased fasting glucose levels and insulin resistance (Smith *et al.* 1994). The relationship between high androgen levels and insulin resistance has been investigated in women (Mauras *et al.* 1998) and estrogen replacement

0022–0795/03/0176–237
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therapy in postmenopausal women has been associated with a reduction in serum lipid levels (Walsh *et al.* 1991, Nabulsi *et al.* 1993). The molecular mechanisms by which estrogens affect carbohydrate and lipid metabolism in men and women are still poorly understood, in part because patients with estrogen deficiency for whatever reason are quite rare.

In mice, estrogen receptor α (ERα) deficiency due to a knockout of the ERα gene (αERKO) leads to increased body weight at 4–8 months of age whereas ERβ deficiency (βERKO) does not (Couse & Korach 1999). Estrogen/estrogen receptor α signaling appears to be critical for regulating white adipose tissue mass (Heine *et al.* 2000). Aromatase knockout (ArKO) mice show a similar phenotype to that of αERKO mice with increased gonadal fat pad weight (Fisher *et al.* 1998). These reports suggest an important role of estrogen action in the function of adipose tissue. Estrogens may also play a role in the regulation of lipid metabolism in other tissues since we observed hepatic steatosis in ArKO males due to the impairment of lipid β-oxidation (Nemoto *et al.* 2000, Toda *et al.* 2001*c*). Here, we further examine the effect of complete aromatase deficiency on carbohydrate and lipid metabolism in estrogen-deficient mice created by knockout of *Cyp19* (ArKO) (Toda *et al.* 2001*c*). ArKO male mice have glucose intolerance and insulin resistance resulting, at least in part, from obesity and high androgen levels. The impaired glucose tolerance could be improved by treatment with estradiol as well as fibrates and thiazolidinediones. Thus, the ArKO mice may be useful for examining the role of estrogens in the regulation of glucose and lipid metabolism.

Materials and Methods

Mice

The derivation of the ArKO mice used in this study has been described previously (Toda *et al.* 2001*c*). The genotypes of the mice were determined by PCR using DNA from tail tips (Toda *et al.* 2001*a*). Wild-type (+/+) male littermates were used as controls. The mice were fed a diet which had 12·5% of calories as fat, 54·9% of calories as carbohydrate and 32·6% of calories as protein (Oriental Yeast Co., Tokyo, Japan). After birth, ArKO male mice were divided into five groups. The first group was a control group without any treatment. The second group was treated with 17β-estradiol (E2) beginning after birth. The third group was treated with E2 from 24–36 weeks of age. The fourth group was treated with a fibrate (bezafibrate) from 20–24 weeks of age. The fifth group was treated with a thiazolidinedione (pioglitazone) for ten days before the mice became 36 weeks of age. This study was approved by the Animal Use and Care Committee of Kochi Medical School and was carried out in accordance with institutional animal care regulations.

Physiological measurements

The body weights were determined every 2 weeks beginning at 6 weeks of age. Blood samples were collected by tail cut. The blood glucose levels were measured using Glutest Ace and Glutest Sensor (Sanwa Kagaku Kenkyusho Co., Nagoya, Japan). Serum triglyceride and total cholesterol concentrations were measured by an enzymatic method using glycerol-3-phosphate oxidase and cholesterol oxidase respectively (Hitachi 7350, Tokyo, Japan). Serum insulin concentrations were measured using an enzyme immunoassay kit (Pharmacia Amersham Biotech., Tokyo, Japan). Serum androgen levels were measured using a DPC total testosterone kit (Diagnostic Products Co., Los Angeles, CA, USA). The food intake per day was measured in ArKO males and wild-type littermates from 8–19 weeks of age.

Insulin and glucose tolerance tests

Insulin sensitivity was assessed using an insulin tolerance test (ITT). Mice were given an intraperitoneal injection of human regular insulin (Humulin, 0·75 mU/g) and blood glucose concentrations were measured at 0, 15, 30, 45, 60 and 90 min. Glucose tolerance was determined using an intraperitoneal glucose test (1·5 mg glucose/g) (IPGTT) after a 16-h fast with blood glucose measurements at 0, 30, 60, 90 and 120 min. ITT and IPGTT studies were carried out at 12, 18, 24 and 36 weeks of age.

Effects of estradiol, bezafibrate and pioglitazone on glucose tolerance and insulin action

ArKO males (second group above) were injected with 7·5 μg E2 every three days for the first 3 weeks after birth and once a week thereafter with 0·75 μg E2 (Toda *et al.* 2001*a*). In the third group of ArKO mice, E2 (0·75 μg/mouse) was given every week beginning at 24 weeks of age until 36 weeks of age when ITT and IPGTT studies were carried out. In the fourth group, bezafibrate, which acts to decrease triglyceride and cholesterol levels (Balfour *et al.* 1990), was added to the diet (0·5%, w/w) of ArKO males in a dose which corresponds to 500 mg/kg body weight/day as an average daily dose of bezafibrate; treatment began at 20 weeks and continued for a month, following which ITT and IPGTT studies were carried out. Pioglitazone (10 mg/kg body weight/day), a member of the thiazolidinedione class of drugs (Saltiel & Olefsky 1996), was given to ArKO males (the fifth group) directly per os for 10 days before the mice became 36 weeks of age after which time ITT and IPGTT studies were carried out.

Statistical analysis

All values are reported as means ± S.E.M. Statistical significance was determined using paired *t*-test or split plot

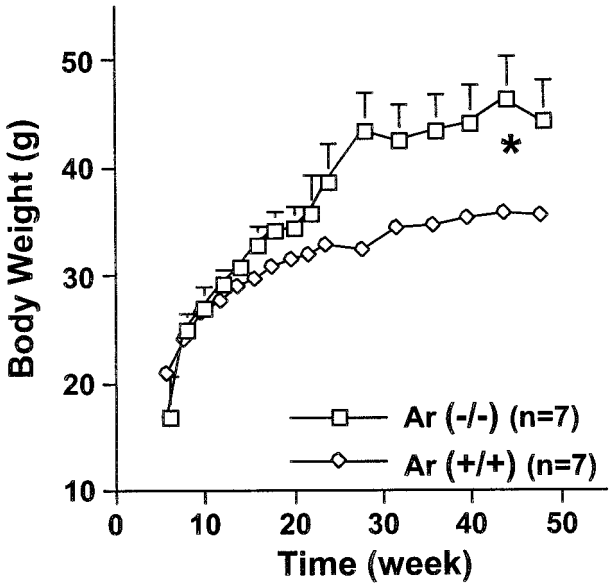

Figure 1 Growth curves. Body weights were measured every two weeks beginning at 6 weeks of age. There is a significant difference in weight gain between ArKO (−/−) males (n=7) and wild-type littermates ArKO (+/+) (n=7) over time. Statistical comparison was made by split plot ANOVA. *P<0·01.

ANOVA, with differences considered as significant at P<0·05.

Results

Body weight

We measured the body weights of ArKO (−/−) male mice (n=7) and wild-type (+/+) littermates (n=7) from 6 to 48 weeks of age (Fig. 1). The mean body weight of male ArKO (−/−) (n=7) and wild-type littermates (+/+) (n=7) were similar at 10 weeks of age (26·7 ± 1·9 g vs 26·1 ± 0·8 g respectively) but by 12 weeks of age the ArKO mice weighed consistently more than their wild-type littermates (28·8 ± 1·4 g vs 26·9 ± 1·0 g respectively at 12 weeks, P<0·05). Overall, there was a significant difference in the growth curves and rate of weight gain between ArKO males and wild-type littermates from 6 to 48 weeks of age (P<0·01; Fig. 1). This increase in body weight was associated with an increase in weight of gonadal and perirenal fat pads. At 22 weeks of age, the gonadal and perirenal fat pad weights of ArKO males were increased compared with wild-type littermates (419·3 ± 44·5 mg vs 312·8 ± 26·7 mg and 400·7 ± 186·7 mg vs 87 ± 6·2 mg respectively). There was also accumulation of fat in the liver (hepatic steatosis) of the ArKO males beginning at 10 weeks of age (Nemoto *et al.* 2000) although there was no difference in liver weight at 12 weeks of age. Thus, ArKO males gradually accumulate abdominal fat and develop hepatic steatosis. There was no difference in food intake per day between ArKO males (n=6) and wild-type littermates (n=8) from 8 to 19 weeks of age.

Serum androgen levels

Mean serum testosterone levels in ArKO males and wild-type littermates at age 24 and 36 weeks were 1585·0 ± 478·9 ng/dl vs 481·5 ± 215·8 ng/dl and 1173·3 ± 362·5 ng/dl vs 565·9 ± 117·6 ng/dl respectively. When ArKO males were treated with bezafibrate or pioglitazone, serum testosterone levels decreased to 199·4 ± 80·7 ng/dl and 253·4 ± 107·0 ng/dl respectively, as compared with those before treatment (Table 1).

Fasting blood glucose levels

There was no difference in fasting blood glucose levels between ArKO males and wild-type littermates at 12 and 18 weeks of age (Fig. 2). Fasting blood glucose levels were significantly higher in ArKO males than in age-matched wild-type littermates at 24 weeks of age (133·8 ± 9·3 mg/dl and 87·8 ± 9·1 mg/dl, P<0·01) and persisted at 36 weeks of age (157 ± 12·1 mg/dl and 73·2 ± 5·3 mg/dl, P<0·001, Fig. 2).

Glucose tolerance

There was no significant difference in glucose tolerance between ArKO and wild-type mice at 12 weeks of age.

Table 1 Serum testosterone levels (ng/dl) in ArKO males before and after bezafibrate and pioglitazone treatment

	ArKO (−/−)		**Wild-type** (ArKO (+/+))	
	Before	After	Before	After
Treatment				
Bezafibrate	1585·0 ± 478·9 (n=4)	199·4 ± 80·7* (n=4)	481·5 ± 215·8 (n=5)	103·0 ± 44·1 (n=5)
Pioglitazone	1173·3 ± 362·5 (n=6)	253·4 ± 107·0* (n=6)	565·9 ± 117·6 (n=7)	217·7 ± 108·1 (n=7)

*P<0·05 after treatment vs before treatment.

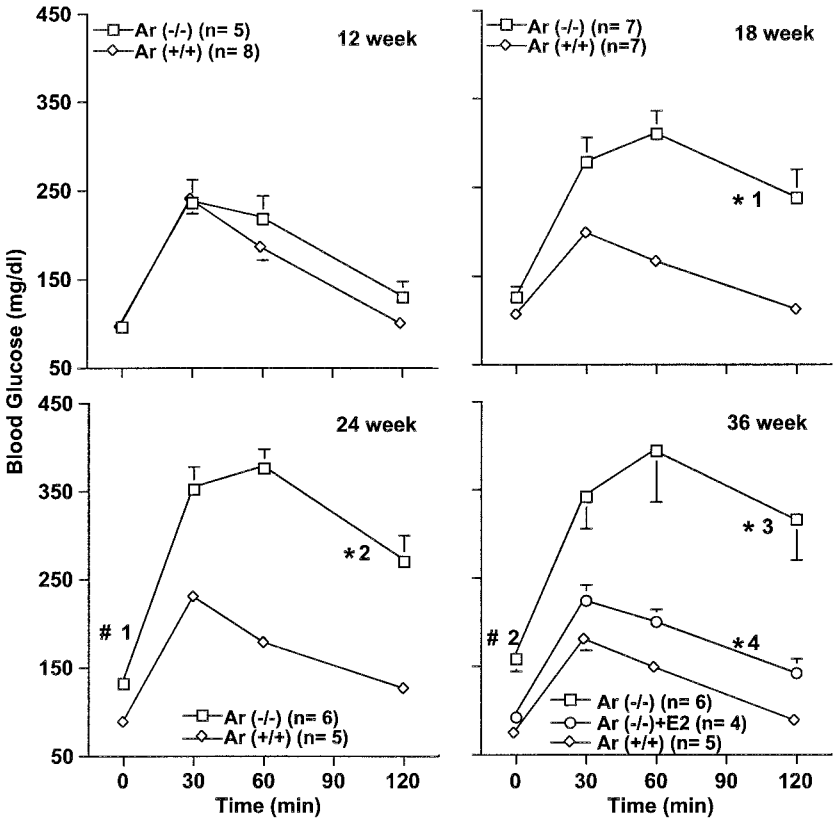

Figure 2 Intraperitoneal glucose tolerance testing in male mice. After an overnight fast (16 h), glucose (1·5 mg/g i.p.) was administered to 12-, 18-, 24- and 36-week-old male mice and blood glucose levels were measured at 0, 30, 60, 90 and 120 min after injection. (Top left) Blood glucose levels in ArKO (−/−) males (Ar(−/−)) (n=5) and ArKO (+/+) males (Ar(+/+)) (n=8) at 12 weeks of age. (Top right) Blood glucose levels in ArKO (−/−) males (n=7) and ArKO (+/+) males (n=7) at 18 weeks of age. (Bottom left) Blood glucose levels in ArKO (−/−) males (n=6) and ArKO (+/+) males (n=5) at 24 weeks of age. (Bottom right) Blood glucose levels in ArKO (−/−) males (n=6), ArKO (−/−) males treated with E2 (n=4) and ArKO (+/+) males (n=5) at 36 weeks of age. Fasting glucose levels in ArKO (−/−) males were significantly higher than those of wild-type littermates at 24 and 36 weeks of age (P<0·01 and P<0·001 respectively). Areas under the glucose × time curve of ArKO (−/−) males and ArKO (+/+) males at 12, 18, 24 and 36 weeks of age and of ArKO (−/−) males treated with E2 were 22 371 ± 2132 vs 19 896 ± 1088, 31 472 ± 2943 vs 18 446 ± 685, 37 625 ± 2767 vs 19 980 ± 874, 39 856 ± 5030 vs 15 765 ± 1113 and 21 345 ± 1055 respectively. After glucose administration, ArKO (−/−) males at 18, 24 and 36 weeks of age showed significantly larger areas under the glucose × time curve compared with wild-type littermates. E2 treatment showed significantly smaller areas under the curve compared with ArKO (−/−) mice without treatment. #1 P<0·01, #2 P<0·001, ArKO (−/−) vs ArKO (+/+); *1 P<0·01; *2 P<0·001, ArKO (−/−) vs ArKO (+/+); *3 P<0·05, ArKO (−/−)+E2 vs ArKO (−/−); and *4 P<0·01, ArKO (−/−)+E2 vs ArKO (+/+).

However, the ArKO males showed a significant impairment in glucose tolerance during the course of an IPGTT at 18 weeks and thereafter as shown by the statistical analysis calculating areas under the glucose × time curve (Fig. 2).

Insulin action

The ArKO males at 12 weeks of age showed a small but significant decrease in blood glucose levels after intraperitoneal insulin injection compared with wild-type littermates (P<0·05; Fig. 3), suggesting that ArKO males at this age have better insulin tolerance than their wild-type littermates. However, after 18 weeks of age, ArKO males showed a lower rate of fall in blood glucose levels and a nadir in the blood glucose × time concentration curve at 30 min after insulin injection during ITT (Fig. 3) in marked contrast to wild-type littermates which showed a continuous decrease in blood glucose levels over the next

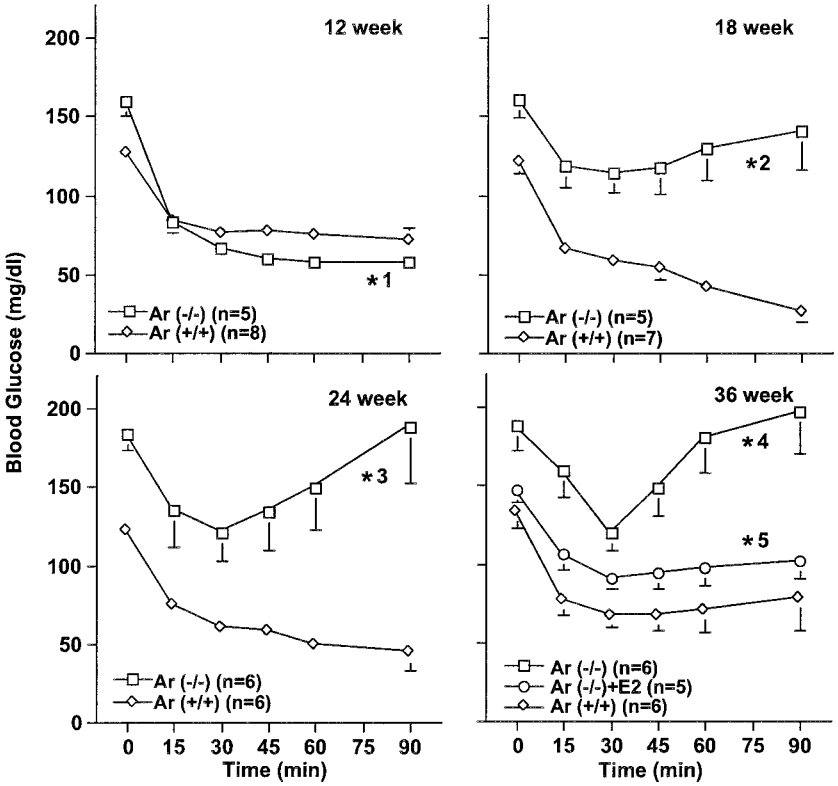

Figure 3 Insulin tolerance testing in male mice. Insulin (0·75 mU/g i.p.) was administered to 12-, 18-, 24- and 36-week-old mice and blood glucose levels were measured at 0, 15, 30, 45, 60 and 90 min after injection. (Top left) Blood glucose levels in ArKO (−/−) males (Ar(−/−)) (n=5) and ArKO (+/+) males (Ar(+/+)) (n=8) at 12 weeks of age. (Top right) Blood glucose levels in ArKO (−/−) males (n=5) and ArKO (+/+) males (n=7) at 18 weeks of age. (Bottom left) Blood glucose levels in ArKO (−/−) males (n=6) and ArKO (+/+) males (n=6) at 24 weeks of age. (Bottom right) Blood glucose levels in ArKO (−/−) males (n=6), ArKO (−/−) males with E2 (n=5) and ArKO (+/+) males (n=6) at 36 weeks of age. After insulin administration, ArKO (−/−) males showed significantly lower blood glucose levels compared with wild-type littermates at 12 weeks of age (P<0·05). The decrease in blood glucose was significantly less in ArKO (−/−) males compared with wild-type littermates at 18, 24 and 36 weeks of age (P<0·01, P<0·001 and P<0·05 respectively). ArKO (−/−) males with E2 treatment showed a significantly larger decrease in blood glucose levels than ArKO (−/−) males without treatment at 36 weeks of age (P<0·05). Statistical comparison was made by split plot ANOVA. *1 P<0·05, *2 P<0·01, *3 P<0·001, *4 P<0·05, ArKO (−/−) vs ArKO (+/+); *5 P<0·05, ArKO (−/−)+E2 vs ArKO (−/−).

60 min. The abnormal glucose response during the ITT persisted in the ArKO males and was also evident at 24 and 36 weeks of age (18, 24 and 36 weeks, P<0·01, P<0·001 and P<0·05 respectively).

Fasting triglyceride, cholesterol and insulin levels

There were no differences in fasting serum triglyceride and total cholesterol concentrations between ArKO males and age-matched wild-type littermates at 13 or 25 weeks of age (Fig. 4). Fasting serum insulin levels of ArKO and wild-type males at 18, 24 and 36 weeks of age were 14·0 ± 3·2 ng/ml vs 8·3 ± 4·2 ng/ml, 24·6 ± 10·2 ng/ml vs 24·4 ± 6·6 ng/ml, and 45·4 ± 17·0 ng/ml vs 21·7 ± 2·8 ng/ml respectively. While the insulin levels in the ArKO mice were higher than their wild-type littermates, the differences were not significant due to marked inter-animal variability. There was a positive correlation between fasting insulin concentration and body weight and fasting blood glucose level in the ArKO mice (r=0·428, P<0·05 and r=0·480, P<0·01 respectively).

Glucose tolerance and insulin action in ArKO mice after E2 treatment

We carried out IPGTT and ITT on ArKO male mice at 36 weeks of age following treatment with E2 from birth

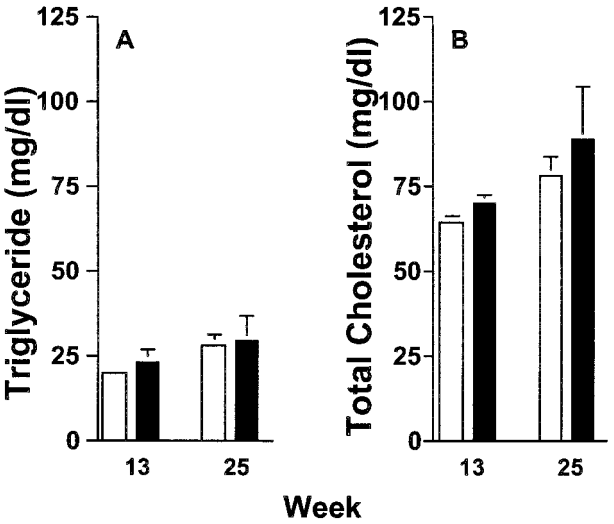

Figure 4 Fasting serum lipid concentrations in ArKO and wild-type mice. (A) Serum triglyceride concentrations in ArKO (− / −) males (n=6) (solid bars) and wild-type littermates (n=5) (open bars) were measured at 13 and 25 weeks of age. (B) Serum total cholesterol concentrations of ArKO (− / −) males (n=6) (solid bars) and wild-type littermates (n=5) (open bars) were measured at 13 and 25 weeks of age.

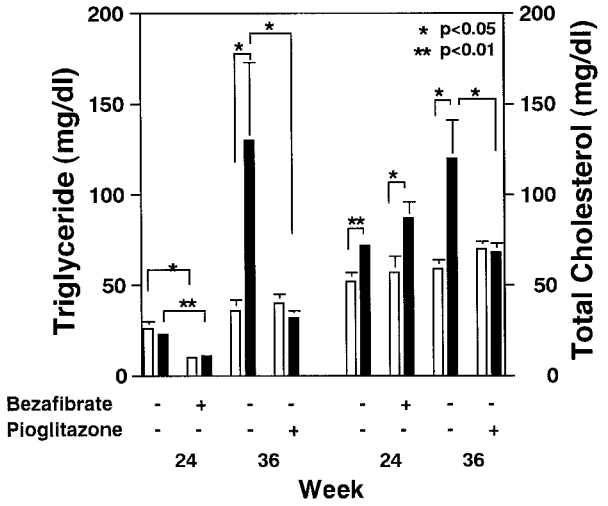

Figure 5 Serum lipid concentrations after a meal in ArKO and wild-type mice treated with bezafibrate and pioglitazone. (Left hand graphs) Serum triglyceride concentrations of ArKO (− / −) males (n=6) (solid bars) and wild-type littermates (n=5) (open bars) after a meal were measured at 24 and 36 weeks of age. Bezafibrate treatment showed significantly decreased serum triglyceride levels in both ArKO (− / −) males and wild-type littermates compared with those without treatment at 24 weeks of age (P<0·01 and P<0·05 respectively). Serum triglyceride levels in ArKO (− / −) males without treatment, which were significantly higher than wild-type littermates (P<0·05), significantly decreased with pioglitazone treatment at 36 weeks of age (P<0·05). (Right hand graphs) Serum total cholesterol concentrations in ArKO (− / −) males (n=7) (solid bars) and wild-type littermates (n=5) (open bars) were measured at 24 and 36 weeks of age. Serum total cholesterol levels in ArKO (− / −) males both with and without bezafibrate were significantly higher than in wild-type littermates at 24 weeks of age (P<0·05 and P<0·01 respectively). ArKO (− / −) males showed significantly higher serum total cholesterol levels than wild-type littermates after a meal at 36 weeks of age (P<0·05). Serum total cholesterol levels in ArKO (− / −) males were significantly decreased with pioglitazone treatment compared with those without treatment at 36 weeks of age (P<0·05). *P<0·05, **P<0·01.

(Figs 2 and 3). There was a marked improvement in both glucose tolerance and insulin sensitivity in the E2-treated mice. ArKO males treated with E2 for a 12-week period beginning at 24 weeks of age showed a similar degree of improvement in glucose tolerance and insulin sensitivity compared with animals treated from birth (data not shown).

Serum triglyceride and cholesterol levels after bezafibrate and pioglitazone treatment

Bezafibrate treatment was associated with decreased serum triglyceride levels after a meal in ArKO males and age-matched wild-type littermates at 24 weeks of age (P<0·01 and P<0·05 respectively; Fig. 5, left part of figure). Serum total cholesterol levels were higher in ArKO males than age-matched wild-type littermates (P<0·01) and were not reduced by treatment with bezafibrate at 24 weeks of age (Fig. 5, right part of figure).

Pioglitazone treatment led to a significant reduction of serum triglyceride and total cholesterol levels in ArKO males (P<0·05, Fig. 5) at 36 weeks of age when serum triglyceride and cholesterol levels after a meal were significantly higher in ArKO males compared with wild-type littermates without treatment (P<0·05, Fig. 5).

Glucose metabolism in ArKO mice after treatment with bezafibrate and pioglitazone

ArKO males treated with bezafibrate at 24 weeks of age had significantly lower blood glucose levels during an IPGTT compared with untreated ArKO males (P<0·01, Fig. 6A). Similarly, ArKO males treated with pioglitazone at 36 weeks of age had lower blood glucose levels during an IPGTT compared with untreated ArKO males (P<0·05, Fig. 6B). However, the pioglitazone-treated males still had an abnormal response relative to wild-type littermates (P<0·01, Fig. 6B). Statistical analysis was carried out for calculating areas under the glucose × time curve (Fig. 6A,B).

There was a significant difference in the decrease in glucose levels during an ITT in bezafibrate-treated ArKO males compared with untreated ArKO controls (P<0·01, Fig. 6C). However, the bezafibrate-treated males still had an abnormal response relative to wild-type littermates (P<0·001, Fig. 6C). Pioglitazone treatment at 36 weeks of age also led to an improvement in the glucose response of ArKO males during an ITT compared with ArKO males without treatment (P<0·05, Fig. 6D).

Discussion

Complete aromatase deficiency in male mice appears to lead to obesity, glucose intolerance and decreased insulin sensitivity. These phenotypes develop progressively beginning at 10 to 12 weeks of age. Obesity due to estrogen insufficiency has been reported in another line of ArKO mice (Jones *et al.* 2000) and also in αERKO mice (Heine *et al.* 2000). In the present study, we show that there is no difference in body weight or accumulation in visceral fat deposits between ArKO and wild-type male mice younger than 10 weeks of age. The increase in body weight and visceral fat deposits as well as hepatic steatosis develop gradually with aging. At 12 weeks of age, the mean body weight in ArKO males began to increase relative to wild-type littermates without any difference in fasting glucose levels or glucose response during an IPGTT or ITT. Beginning at 18 weeks of age, ArKO males showed decreased insulin sensitivity during an ITT suggesting decreased peripheral glucose utilization. The increase in blood glucose levels observed in ArKO males at 30 min during the ITT may be due to increased hepatic glucose production suggesting that hepatic dysfunction in ArKO males may contribute to the development of insulin resistance in this model (Nemoto *et al.* 2000). The higher blood glucose levels in ArKO males during an IPGTT at 18, 24 and 36 weeks of age are consistent with a lack of suppression of hepatic glucose production which leads to increased fasting blood glucose levels after 24 weeks of age. The results suggest that the increased adiposity in the ArKO males including hepatic steatosis play an important role in the development of insulin resistance in peripheral tissues and liver in this model system.

High androgen levels have been associated with peripheral insulin resistance in women (Peiris *et al.* 1989, Polderman *et al.* 1994). However, administration of testosterone to men caused an inhibition of triglyceride uptake and the prevention of lipid retention in adipose tissue particularly in the abdominal region (Marin *et al.* 1995). Administration of pharmacological doses of testosterone for 6 weeks, which caused a threefold increase in serum testosterone, did not impair glucose tolerance or alter insulin secretion in normal men (Friedl *et al.* 1989). These clinical studies suggest that hyperandrogenicity seems to affect insulin sensitivity differently in men and women. This gender difference may be due to the androgen/estrogen ratio and its effects on cellular lipid utilization. The insulin resistance in untreated ArKO males appears to be related to the high androgen levels. Thus, we presume that elevated serum testosterone levels with undetectable estrogen levels may lead to the development of the insulin resistance phenotype in ArKO males.

Continuous E2 treatment beginning at birth led to an improvement in the glucose tolerance and insulin sensitivity in ArKO males, which did not develop fatty liver and obesity as described previously (Nemoto *et al.* 2000). Therefore, the improvement of insulin sensitivity after estrogen replacement was correlated with an improvement in fatty liver. E2 treatment beginning at 24 weeks of age for 12 weeks could also ameliorate the abnormality in the carbohydrate metabolism to a similar degree as with continuous E2 treatment. A short period of treatment with E2 may be sufficient to lead to an improvement in glucose metabolism in ArKO mice.

There were no differences in fasting serum triglyceride and total cholesterol levels at 13 and 25 weeks of age between ArKO males and wild-type littermates. However, serum triglyceride and total cholesterol levels in ArKO males after a meal were significantly higher than those in wild-type littermates. We examined the effect of drugs that modify lipid metabolism on insulin resistance in ArKO male mice. Bezafibrate, a synthetic ligand for peroxisome proliferator-activated receptor α (PPARα), stimulates β oxidation activity (Staels *et al.* 1995, Schoonjans *et al.* 1996) by activation of PPARα which is expressed predominantly in the liver (Isemann & Green 1990, Braissant *et al.* 1995). Fibrates were reported to improve insulin sensitivity without having adverse effects on body weight and adipose tissue mass in animal models of insulin resistance (Guerre-Millo *et al.* 2000). Bezafibrates substantially reduced the hepatic steatosis in ArKO males (Toda *et al.* 2001*b*, Yoshikawa *et al.* 2002), and improved glucose tolerance during IPGTT and insulin sensitivity during ITT. Pioglitazone, a thiazolidinedione, interacts with PPARγ to enhance the actions of insulin with resulting improvement in insulin-dependent glucose disposal and reduction in hepatic glucose output (Lehmann *et al.* 1995, Kawamori *et al.* 1998). Treatment with pioglitazone also improved the abnormality in carbohydrate metabolism in ArKO males and restored the insulin sensitivity in ITT. Additionally, the reduced synthesis of testosterone by thiazolidinediones through activated PPARγ (Pamela *et al.* 2002) and by fibrates through the PPARα pathway in Leydig cells (Braissant *et al.* 1995, Boujrad *et al.* 2000, Gazouli *et al.* 2002) might contribute to the reduction in serum testosterone levels in ArKO males, and these might be partially related with a reduction in peripheral insulin resistance in ArKO males. Both bezafibrate and pioglitazone could overcome the insulin resistance associated with lack of aromatase activity.

In insulin resistance, the ability of insulin to act on muscle and fat to stimulate glucose uptake and metabolism or inhibit hepatic glucose output is decreased (Kahn 1994, DeFronzo 1997). Insulin resistance is a prominent feature of type 2 diabetes and is present maximally in skeletal muscle in the earliest phase of this disorder (DeFronzo 1997). Genetically engineered mice with targeted disruption of the insulin receptor gene in skeletal muscle have normal blood glucose and serum insulin levels and glucose tolerance (Bruning *et al.* 1998). In contrast, mice with liver-specific disruption of the insulin receptor gene have

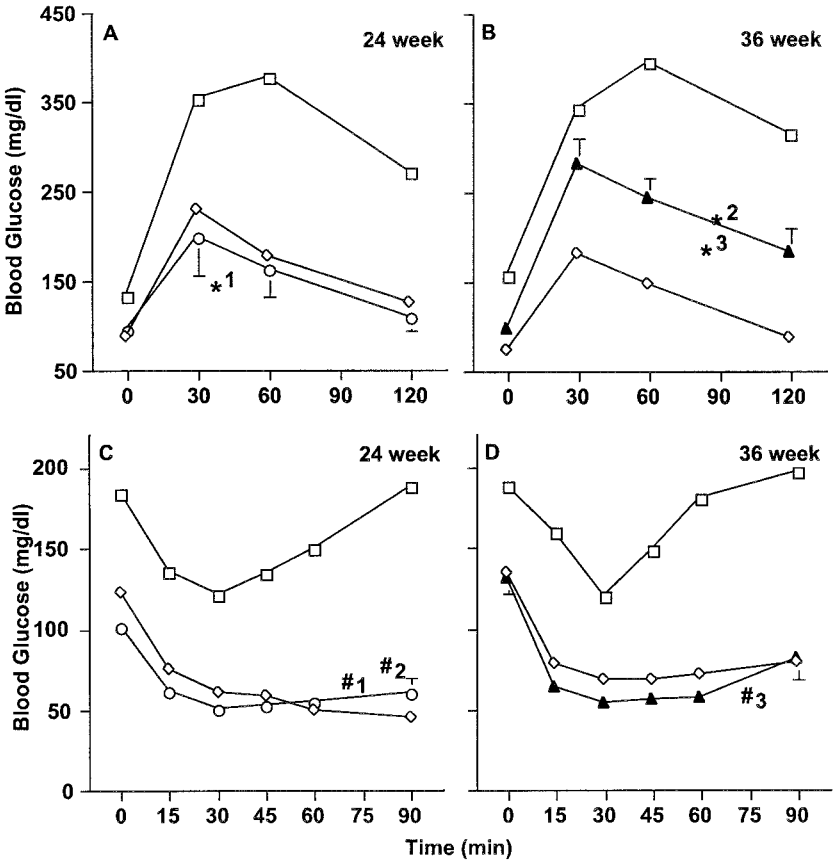

Figure 6 Effects of bezafibrate and pioglitazone on glucose metabolism in ArKO males. (A) Effects of bezafibrate on male mice in an intraperitoneal glucose tolerance test at 24 weeks of age. Areas under the glucose × time curve of ArKO (−/−) males (□), ArKO (−/−) males with bezafibrate (○) and ArKO (+/+) males (◇) were 37 625 ± 2767, 17 985 ± 3067 and 19 980 ± 874 respectively. ArKO (−/−) males with bezafibrate showed significantly smaller areas under the glucose × time curve than ArKO (−/−) males without treatment (P<0·001). (B) Effects of pioglitazone on male mice in intraperitoneal glucose tolerance test at 36 weeks of age. Areas under the glucose × time curve of ArKO (−/−) males (□), ArKO (−/−) males with pioglitazone (▲) and ArKO (+/+) males (◇) were 39 858 ± 5030, 26 328 ± 2613 and 15 765 ± 1113 respectively. Pioglitazone treatment showed significantly smaller areas under the glucose × time curve compared with ArKO (−/−) males without treatment (P<0·05) and significantly larger areas compared with ArKO (+/+) males (P<0·01). Only in mice with bezafibrate or pioglitazone treatment were the S.E. for blood glucose levels shown. (A and B) *1 P<0·001, ArKO (−/−)+bezafibrate vs ArKO (−/−); *2 P<0·05, ArKO (−/−)+pioglitazone vs ArKO (−/−); *3 P<0·01, ArKO (−/−)+pioglitazone vs ArKO (+/+). (C) Effects of bezafibrate on male mice in an insulin tolerance test at 24 weeks of age. ArKO (−/−) males treated with bezafibrate (○) showed a significantly smaller decrease in blood glucose than wild-type littermates (◇) (P<0·001) and a larger decrease in blood glucose than ArKO (−/−) males without treatment (□) (P<0·01). (D) Effects of pioglitazone on male mice in the insulin tolerance test. Pioglitazone treatment (▲) induced a significantly larger decrease in blood glucose compared with ArKO (−/−) males without treatment (□) (P<0·05). Only in mice with bezafibrate or pioglitazone treatment were the S.E. for blood glucose levels shown. Statistical comparison was made by split plot ANOVA. (C and D) #1 P<0·01, ArKO (−/−)+bezafibrate vs ArKO (−/−); #2 P<0·001, ArKO (−/−)+bezafibrate vs ArKO (+/+); #3 P<0·05, ArKO (−/−)+pioglitazone vs ArKO (−/−).

extremely high blood glucose levels (Michael *et al.* 2000). The results of these two studies highlight the importance of the action of insulin in the liver to maintain normal blood glucose levels and are consistent with clinical studies in type 2 diabetic patients (Lewis *et al.* 1999, Basu *et al.* 2000). The present study suggests that hepatic dysfunction

due to estrogen deficiency contributed to the hyperglycemia in ArKO males. As discussed above, hepatic steatosis and abnormal glucose and insulin tolerance appear to develop concurrently in ArKO males. Activation of hepatic lipid metabolism by bezafibrate treatment led to improvement in glucose and insulin tolerance in ArKO males. Thus, reducing hepatic steatosis might be an important action of these drugs which, in turn, leads to improved glucose tolerance in ArKO males.

In summary, our results suggest that insulin resistance appears to be a consequence of obesity and androgenized conditions in ArKO (−/−) male mice. 17β-Estradiol supplementation either continuously after birth or for a short period led to an improvement in insulin sensitivity. Treatment with fibrates or thiazolidinediones also ameliorated glucose and insulin tolerance in this animal model. The improvement in the response to insulin caused by these drugs may be a consequence of their activation of lipid metabolism and might be partially related with the inhibition of androgen biosynthesis. Estrogen appears to play an important role in maintaining normal lipid and glucose homeostasis, and the ArKO mouse may be a useful model for studying the pathogenesis of the estrogen-deficient state.

Acknowledgements

The authors wish to thank Mrs K Shiraishi, Y Okada and E Ohara for technical assistance and Dr K Ichihara for his assistance with statistical analysis. This study was partially supported by a grant from the Nakatomi Foundation to Dr K Toda.

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Received 16 October 2002
Accepted 24 October 2002