Female Rats Do Not Exhibit Free Fatty Acid–Induced Insulin Resistance

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It is well described that excessive lipid metabolism can cause insulin resistance in both animals and humans, and this has been implicated as a causative factor in the development of insulin resistance and type 2 diabetes in humans. Recently, we have shown that intravenous lipid emulsion (liposyn) infusion during a 120-min euglycemic-hyperinsulinemic clamp led to significant reductions in insulin action and fatty acid translocase (FAT/CD36) skeletal muscle protein expression. After reviewing the literature, it became evident that essentially all past studies, including our own, were conducted in male animals. Therefore, to determine whether there were sex determinants of fat-induced insulin resistance, we assessed the impact of free fatty acid (FFA) elevation on insulin action in female rats. Here, we report that a fourfold elevation in plasma FFA concentration induced a 40% reduction in the insulin-stimulated glucose disposal rate, a 30% decline in insulin-stimulated skeletal muscle insulin substrate receptor-1 (IRS-1) phosphorylation, a 48% decrease in IRS-1–associated phosphatidylinositol (PI) 3-kinase activity, and a 50% reduction in muscle FAT/CD36 protein expression in male rats. In striking contrast, we found no effect of FFA elevation to cause insulin resistance, changes in IRS-1/PI 3-kinase, or FAT/CD36 protein levels in female animals. Our findings indicate that female animals are protected from lipid-induced reductions in insulin action. *Diabetes* 51: 1907–1912, 2002

I t is widely held that excessive lipid metabolism, achieved either by high-fat feeding or intravenous infusion of lipid emulsion to raise circulating free fatty acid (FFA) levels, leads to decreased insulin-stimulated glucose uptake (1–10). This lipid-induced decrease in insulin-stimulated glucose disposal is associated with a decline in insulin substrate receptor-1 (IRS-1)-associated phosphatidylinositol (PI) 3-kinase activity in skeletal muscle, and these in vivo and in vitro findings have been reported in several articles (1,6,9,10) and are well supported. Interestingly, after reviewing the litera-

ture, it became apparent that essentially all studies reporting fat-induced insulin resistance in humans or rodents have been conducted in men or male animals. Because the prevailing concept is that fat-induced impairment of glucose disposal is a major risk factor for the development of insulin resistance and type 2 diabetes, and because type 2 diabetes is equally prevalent in men and women, it is important to determine whether the deleterious effects of increased fat metabolism are the same for males and females.

Although women have a lower skeletal muscle mass than men, a review of the literature points to improved glucose tolerance and heightened skeletal muscle insulin sensitivity in normal women compared with men (11–13). Thus, Fernandez-Real et al. (14) have shown that premenopausal women are more insulin sensitive than men when the groups are matched for body fat, and Yki-Jarvinen (13) has shown enhanced insulin sensitivity in women versus men when insulin-stimulated glucose disposal is expressed as a function of muscle mass. In addition to heightened insulin sensitivity, most but not all studies have shown that female rodents are less susceptible to high-fat diet–induced insulin resistance (15–18).

Skeletal muscle is responsible for ~85% of whole-body insulin-stimulated glucose disposal (19,20) and a major component of nonesterified fatty acid (NEFA) uptake (21), demonstrating quantitatively the importance of this tissue in understanding the interaction between glucose and fat metabolism. One of the primary transporters of fatty acids into muscle is the fatty acid translocase (FAT/CD36) (22), and we have previously reported a 50% reduction in skeletal muscle FAT/CD36 protein expression after liposyn infusion into male rats (10), whereas no information is available on FAT/CD36 regulation in females.

In the current studies, we have examined the effects of intravenous lipid infusions (which raise plasma NEFA levels) on insulin action in male and female rats. We conducted euglycemic-hyperinsulinemic clamp studies as well as measurements of FAT/CD36 and insulin-signaling molecules in skeletal muscle samples. Our major findings are that lipid infusions cause insulin resistance in male rats but were without effect on either in vivo or in vitro insulin action in females.

RESEARCH DESIGN AND METHODS

Animals and surgical and experimental clamp procedure. All animals (3 months of age) were chronically cannulated under single-dose anesthesia (42 mg/kg ketamine HCl, 5 mg/kg xylazine, 0.75 mg/kg acepromazine maleate; administered intramuscularly) in the jugular vein for infusion of glucose, tracer, and insulin (dual cannula, internal diameter 0.03 cm; Dow Corning Silastic, Midland, MI) and in the carotid artery (Intramedic polyethylene tubing PE-50; Clay Adams, Becton Dickinson, Sparks, MD) for sampling.

TABLE 1
Animal characteristics

Variable	Male		Female	
	Control	Lipid	Control	Lipid
n	9	7	8	7
Body weight (kg)	0.305 ± 0.02	0.282 ± 0.02	$0.181 \pm 0.07*$	$0.200 \pm 0.09*$
Plasma glucose concentration (mmol/l)				
Basal	8.0 ± 0.23	8.3 ± 0.43	7.1 ± 0.49	6.9 ± 0.39
Clamp	8.4 ± 0.08	8.49 ± 0.12	8.21 ± 0.09	8.24 ± 0.1
Plasma insulin concentration (nmol/l)				
Basal	0.09 ± 0.018	0.08 ± 0.012	0.06 ± 0.01	0.09 ± 0.02
Clamp	$7.48 \pm 2.5\dagger$	$7.2 \pm 3.2\dagger$	$9.3 \pm 0.53\dagger$	$10.2 \pm 0.43\dagger$
Plasma FFA concentration (mmol/l)				
Basal	0.69 ± 0.05	0.65 ± 0.04	0.53 ± 0.052	0.58 ± 0.082
Clamp	$0.17 \pm 0.013\dagger$	$2.96 \pm 0.25\dagger\ddagger$	$0.31 \pm 0.037\dagger$	$2.88 \pm 0.22\dagger\ddagger$
Intralipid infusion rate (μmol $\cdot$ min^{-1} $\cdot$ kg^{-1})	0	$0.668 \pm 0.04\ddagger$	0	$0.948 \pm 0.02\ddagger*$

Data are means $\pm$ SE. *Between-sex difference (male versus female), within experimental protocol, control or lipid; †difference between basal versus clamp, within sex and infusion protocol; ‡difference between infusion protocols (control versus lipid) within sex.

Cannulas were tunneled subcutaneously, exteriorized at the back of the neck, and encased in silastic tubing (0.2 cm internal diameter) sutured to the skin. Animals were allowed 5 days' recovery from surgery to regain body weight. Animals were fasted 12 h before the euglycemic-hyperinsulinemic clamp. All animals were exposed to the same general glucose clamp protocol. At 90 min before the clamp, animals were weighed and placed into a modified metabolic chamber. Basal samples were drawn at -60 and 0 min. After the basal sample at -60 min, a priming dose of 5 μCi of D-[3-^{3}H]glucose (New England Nuclear, Boston, MA) was administered, followed by tracer constant infusion at 0.16 μCi $\cdot$ min^{-1}. After 60 min of tracer equilibration and basal sampling at time 0, glucose (50% dextrose, variable infusion; Abbott, Chicago, IL) and tracer plus insulin (40 mU $\cdot$ kg^{-1} $\cdot$ min^{-1} Novlin R; Novo Nordisk, Copenhagen, Denmark) infusions were initiated. In addition, a heparinized (30 units/ml) 10% lipid emulsion (Liposyn III; Abbott) was also infused into the jugular vein (0.0167 ml/min). Small blood samples (70 μl) were drawn at 10-min intervals and immediately analyzed for glucose (YSI 2300 Glucose Analyzer; YSI, Yellow Springs, OH) to maintain the integrity of the glucose clamp throughout the duration of the experiment.

Analytical procedures. Plasma glucose was assayed by the glucose oxidase method (YSI). Plasma insulin was measured via radioimmunoassay kit (Linco Research, St. Charles, MO). Plasma glucose specific activity was measured in duplicate after zinc sulfate and barium hydroxide deproteinization. Plasma FFA levels were measured spectrophotometrically using a commercially available kit (NEFA C; Wako, Richmond, VA).

After the glucose clamp, animals were killed with a lethal dose of sodium pentobarbital (100 mg/kg Nembutal; Abbott, Chicago, IL). Muscles were excised, immediately quick-frozen in liquid nitrogen, and stored at -80°C for subsequent analysis. Weeks later, muscle samples (30–100 mg) were homogenized under liquid nitrogen and treated with lysis buffer containing phosphatase and protease inhibitors. After a 10-min incubation, the lysates were clarified by centrifugation (10,000g at 4°C). Supernatants were analyzed for total protein (Biorad, Hercules, CA) before protein separation by SDS-PAGE on 5, 7.5, or 10% polyacrylamide gels.

To improve the quality of the IRS-1 immunoblot, the IRS-1 protein was immunoprecipitated from lysates using anti–IRS-1 antibody (anti–rat carboxy-terminal IRS-1; Upstate Biotechnology, Lake Placid, NY) and protein A-agarose (Upstate Biotechnology). After gel electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-p; Millipore, Bedford, MA) and blotted with mouse monoclonal antiphosphotyrosine antibody (PY-20; Transduction Laboratories, Lexington, KY), anti–insulin receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-CD36 antibody (Serotec, Raleigh, NC) according to the manufacturer's instructions. After incubation with horseradish peroxidase–conjugated secondary antibodies, proteins were visualized by enhanced chemiluminescence. PY-20 blots were stripped and reblotted with anti–IRS-1 antibody (anti–rat carboxy-terminal IRS-1; Upstate Biotechnology) to assess total protein.

Reagents for enhanced chemiluminescence were purchased from Amersham Life Science (Arlington Heights, IL). Band intensities were quantified by densitometry on a Hewlett-Packard ScanJet II using National Institutes of Health (NIH) Image 1.6 software. Immunoblots are expressed as arbitrary densitometry units based on internal comparison to lysates prepared from a control standard male or female rat. No group differences for total muscle protein, as reflected by total α-actin content ($P = 0.32$), were observed.

Measurement of IRS-1–associated PI 3-kinase activity. Muscle lysates were incubated with anti–IRS-1 antibody and recombinant protein A-agarose overnight at 4°C. Bead pellets were washed three times with buffer A (Tris-buffered saline, pH 7.4, 1% nonidet P-40, and 100 μmol/l Na$_3$VO$_4$), three times with buffer B (100 mmol/l Tris, pH 7.4, 500 mmol/l LiCl$_2$, and 100 μmol/l Na$_3$VO$_4$), and twice with buffer C (10 mmol/l Tris, pH 7.4, 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 μmol/l Na$_3$VO$_4$). Pellets were resuspended in buffer C without Na$_3$VO$_4$. As described previously (23,24), PI 3-kinase activity was assessed by the phosphorylation of PI in the presence of 20 μCi of [γ-^{32}P]ATP. The reaction was stopped with 20 μl of 8 N HCl and 160 μl of CHCl$_3$:methanol (1:1). The samples were centrifuged and the organic phase removed and applied to potassium oxalate (1%)-coated silica gel thin-layer chromatography (TLC) plates. After the separation of lipids by TLC using a borate buffer system, PI 3-phosphate was visualized by autoradiography. NIH Image scanning software was used for quantification.

Calculations. Hepatic glucose output (HGO) and glucose disposal rate (GDR) were calculated using Steele's equation (25). Values presented are expressed as the means $\pm$ SE. Statistical analyses were performed using ANOVA with Tukey's post hoc comparison for identification of within- and between-group differences (SPSS graduate pack; SPSS, Chicago, IL). Significance was set a priori at $P < 0.05$.

RESULTS

Table 1 presents some of the physiological characteristics of the age-matched male and female Wistar rats used in these studies. As expected, body weight was significantly lower in the age-matched female animals compared with the males. However, basal insulin and FFA levels were comparable across all groups. Although basal glucose levels were normal in all animals, the values were slightly, but significantly, lower in the female rats compared with the males. Glucose and insulin levels during the euglycemic clamps were identical in all groups. As expected, the FFA levels rose substantially in the lipid-infused animals, and the levels achieved were essentially identical between male (2.96 ± 0.25 mmol/l) and female (2.88 ± 0.22 mmol/l) rats. Interestingly, although the absolute rate of liposyn infusion was the same in male and female animals (0.184 μmol/min), on a per-kilogram basis, the infusion rate was ~30% higher in the female animals compared with males. Because both groups achieved identical steady-state circulating FFA concentrations, the relative clearance of FFAs was greater in the female group.

In vivo GDR (mg $\cdot$ kg^{-1} $\cdot$ min^{-1}) was measured in the basal state and at the end of the euglycemic-hyperinsulinemic clamp. Basal GDR was the same in male and female animals (11.2 ± 1.07 and 13.1 ± 1.0 mg $\cdot$ kg^{-1} $\cdot$ min^{-1} in
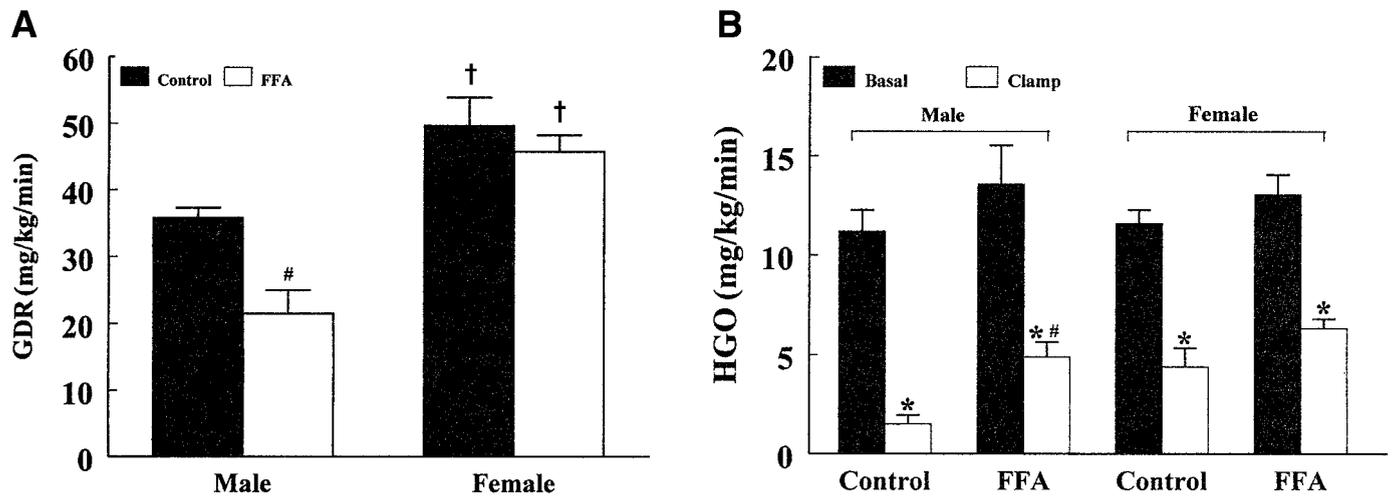
A

B

FIG. 1. Steady-state insulin-stimulated GDR (*A*) and HGO (*B*) (mg · kg^{-1} · min^{-1}) were assessed during the euglycemic-hyperinsulinemic clamps. Values are expressed as the means ± SE for the four experimental groups: male control (*n* = 9), male liposyn-infused (*n* = 7), female control (*n* = 8), and female liposyn-infused (*n* = 7) animals. *Within-sex/protocol difference, basal versus clamp ($P < 0.05$); #within-sex difference between experiments, control versus liposyn infusion ($P < 0.05$); †between-sex difference within experimental protocol ($P < 0.05$).

males and females, respectively) and rose substantially during the control saline infusion clamps. As seen in Fig. 1*A*, the female animals were significantly more sensitive to insulin and achieved an insulin-stimulated GDR of 49 ± 1.43 vs. 36 ± 1.58 mg · kg^{-1} · min^{-1} in males. During the lipid infusion studies, we were able to quantitate the influence of elevated FFA levels on GDR in both sexes. We found that elevated FFAs had a substantial effect to inhibit (40%) insulin-stimulated GDR, leading to a state of insulin resistance in male rats. In striking contrast, elevated FFA levels did not have an inhibitory effect on insulin-stimulated GDR in the female rats. When clamps were performed at a half-maximal insulin concentration (4 mU · kg^{-1} · min^{-1}) or for a more prolonged duration (4 h), comparable results were observed (data not shown).

The suppressive effect of insulin on HGO is well described, and we also quantitated this effect between male and female animals with and without lipid infusion. In the male (saline-infused) rats, insulin led to an 86% inhibition of HGO (from 11.2 ± 1.07 to 1.5 ± 0.45 mg · kg^{-1} · min^{-1}).

During the intralipid infusion, the suppressive effect of insulin on HGO was significantly diminished (4.9 ± 1.0 mg · kg^{-1} · min^{-1}, $P = 0.007$). In the female animals, the suppressive effect of insulin on HGO was not significantly different during the saline versus intralipid infusion studies. Taken together, our results show surprising and striking sex differences in the phenomenon of lipid-induced insulin resistance, with the lipid infusions leading to the predicted state of insulin resistance in male animals, though having no adverse effect on insulin action in females.

Skeletal muscle tissue (red quadriceps) was obtained from each animal at the end of the glucose clamp to assess cellular mechanisms underlying the lipid-induced insulin-resistant state. Insulin leads to tyrosine phosphorylation of the insulin receptor (26), and we found no difference in receptor content or tyrosine phosphorylation state among the four experimental groups (data not shown). Measurements of insulin-stimulated IRS-1 tyrosine phosphorylation and IRS-1–associated PI 3-kinase activity in muscle
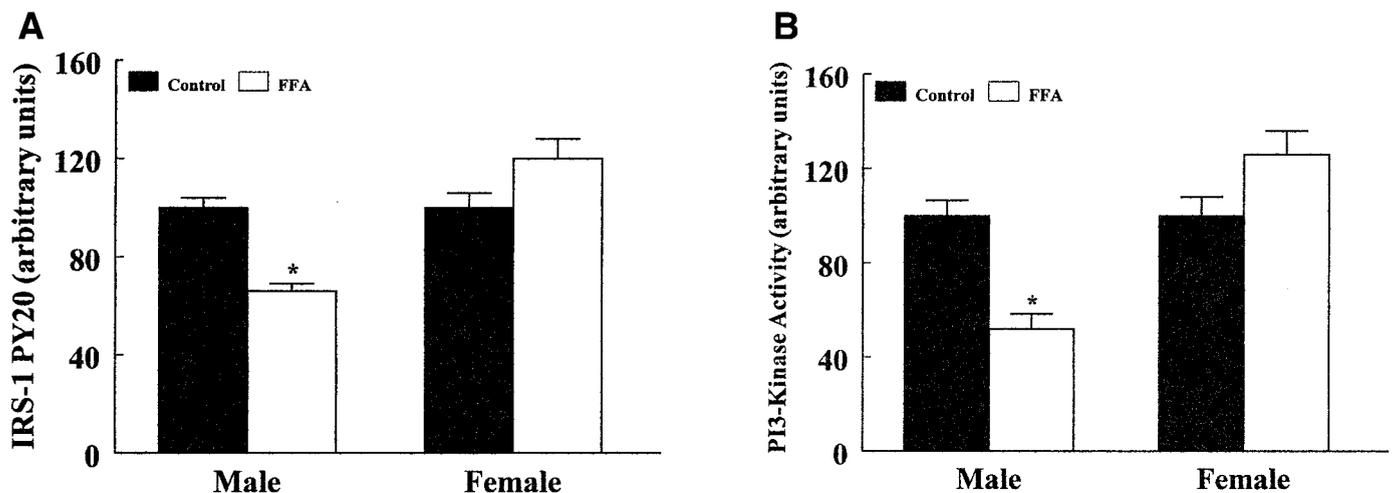
A

B

FIG. 2. IRS-1 tyrosine phosphorylation (*A*) and IRS-1–associated PI 3-kinase activity (*B*). Immunoblots were quantified via densitometry and expressed as the means ± SE in arbitrary units for the four experimental groups, six animals per group. *Within-sex difference, control versus liposyn infusion ($P < 0.05$).
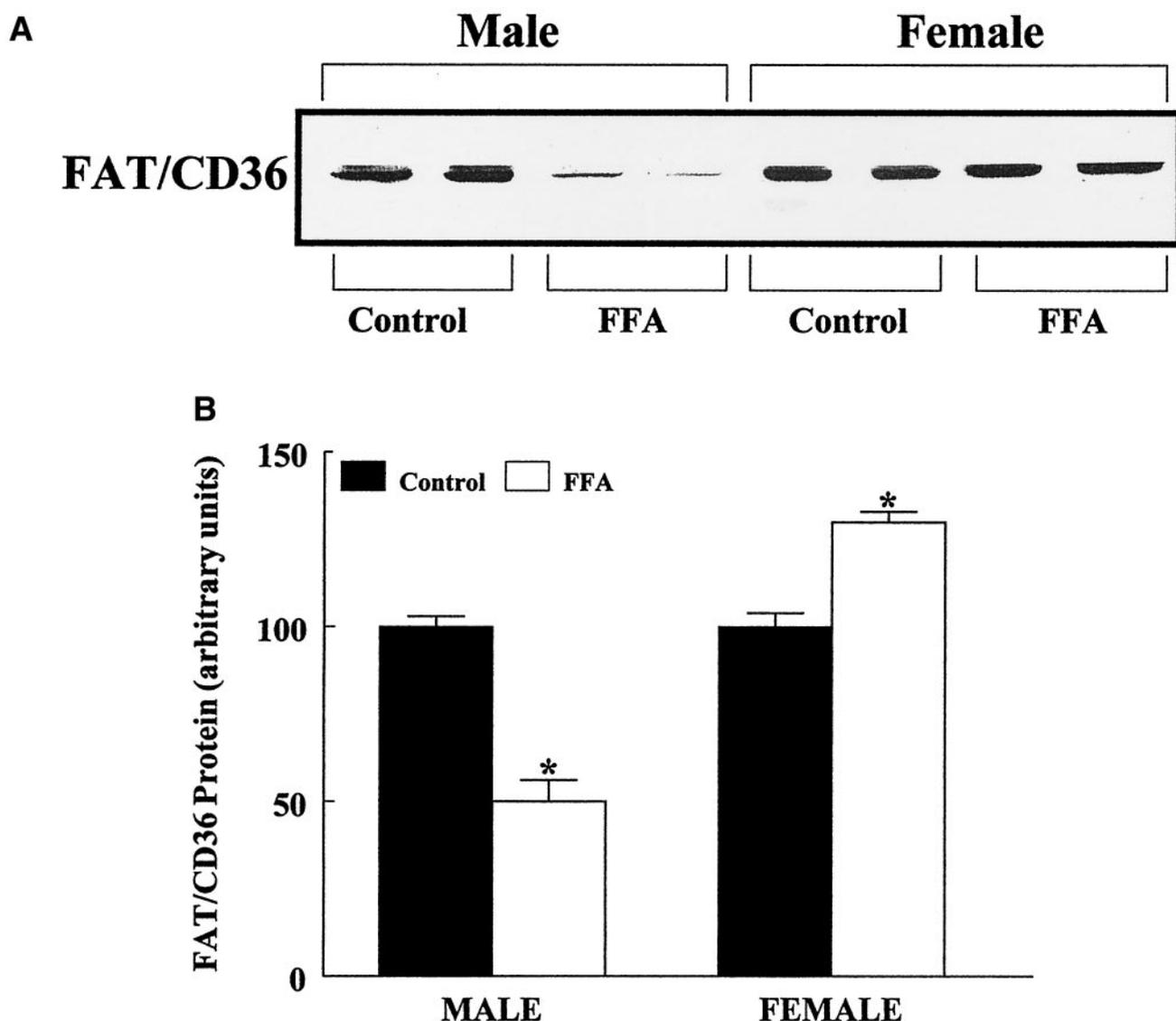
FIG. 3. Skeletal muscle FAT/CD36 protein levels. *A*: Representative immunoblot for FAT/CD36 after a 120-min euglycemic-hyperinsulinemic clamp. *B*: FAT/CD36 total protein expressed as a mean ± SE, in arbitrary units, for the four experimental groups (six animals per group). *Within-sex difference, control versus liposyn infusion ($P < 0.05$).

showed no differences between control saline-infused male versus female animals (Fig. 2*A* and *B*). During lipid infusion, we found a 30% decrease in IRS-1 tyrosine phosphorylation and a 48% decrease in IRS-1–associated PI 3-kinase activity in the male rats. In contrast, lipid infusion had no inhibitory effect on IRS-1 phosphorylation or IRS-1–associated PI 3-kinase activity in the female rats. If anything, these values increased slightly during the lipid infusions, but these differences were not significant. In addition to in vitro analyses of insulin-signaling molecules, we measured the relative expression of skeletal muscle FAT/CD36 after the clamp with and without liposyn infusion in male versus female rats. We found a marked difference in the protein expression levels between control and liposyn-infused males (50% reduction in protein expression after liposyn infusion, $P = 0.001$), but for female animals we found no difference in FAT/CD36 total protein expression between these two experimental groups.

Thus, in our studies we have been able to demonstrate in vitro and in vivo deficits in insulin action associated with lipid administration in male rats. In contrast, female rats appear to be protected from the deleterious effects of lipid administration, at both the cellular and in vivo levels.

DISCUSSION

Clearly, the female animals were protected from fat-induced insulin resistance, and the potential mechanisms of this protection are of considerable interest. Because the lipid infusion rates were greater in the female animals on a per-kilogram basis, despite similar steady-state FFA levels, FFA clearance is apparently enhanced in female versus male rats. CD36/FAT is a major FAT protein in skeletal muscle (22). Recently, transgenic overexpression of this protein in muscle was shown to enhance fatty acid uptake and oxidation (27). In addition, CD36/FAT-null mice exhibit diminished FFA oxidation and elevated FFA concentrations (28). However, a direct link between the expression of this protein and the development of insulin resistance has not been established. As seen in Fig. 3*A* and

B, we measured CD36/FAT protein by immunoblot analysis and found that whereas basal levels of this protein were the same in male and female rats, after lipid infusion, a striking 50% decrease in CD36/FAT protein content was observed in the male animals, with no decline in females. It seems reasonable to suggest that this difference in CD36/FAT protein levels in skeletal muscle may account for the enhanced FFA clearance in the female rats and also participate in the protective effect against fat-induced insulin resistance. Perhaps fatty acids taken up through CD36/FAT are shuttled to a metabolic pathway that does not lead to cellular defects in insulin action.

A number of previous studies have been conducted examining sex effects on various aspects of insulin and glucose metabolism in rodents. For example, high-sucrose feeding is known to cause insulin resistance in rodents, and Kim et al. (29) have reported that the effect of high-sucrose feeding to impair insulin action is comparable in male and female rats. On the other hand, Horton et al. (30), conducting similar studies, found that high-sucrose feeding effectively produced insulin resistance in male animals, but it had no influence on insulin action in females. High-fat feeding is another dietary means to produce insulin resistance, and, again, sex effects have been shown, at least in some studies (15–17). Many reports have demonstrated the effect of high-fat feeding to cause insulin resistance in male rodents, but relatively few studies have also studied females. For example, Zierath et al. (17) measured insulin-stimulated 2-deoxyglucose uptake in isolated muscle strips from male and female rats. They found that female rats were more responsive to insulin than male rats on normal chow diets, but when fed high-fat diets, insulin responsiveness decreased in both groups; however, the females remained more responsive than the males. Furthermore, it is known that the propensity to develop insulin resistance and diabetes is lower in female compared with male Zucker diabetic fatty rats, and it has been shown that high-fat diets have a greater effect to cause insulin resistance and glucose intolerance in male than in female ZDF animals (15). On the other hand, other studies have found clear effects of high-fat diets to cause insulin resistance in female rats. Obviously, the age of the animals, dietary composition, and methods for determining insulin sensitivity differ among the studies, and there is no easy explanation to reconcile these disparate reports. In the current study, we have found that experimental elevation of circulating FFA levels by lipid/heparin infusions leads to insulin resistance in male but not female rats. Whether lipid/heparin infusions and high-fat diets produce insulin resistance through similar mechanisms remains to be determined.

Although our studies do not elucidate the cellular mechanisms of insulin resistance caused by FFA elevation, certain speculations related to the sex differences we have demonstrated are possible. Shulman and colleagues (31) have proposed that fatty acid stimulation of protein kinase C-θ (PKC-θ) activity is at least one mechanism of fat-induced insulin resistance, and they have shown that PKC-θ knockout animals do not display decreased insulin sensitivity on high-fat diets. PKC-θ is an upstream activator of IKKB, which in turn stimulates nuclear factor-κB (NF-κB)-mediated gene expression changes (32). Recently, it has been demonstrated that the NF-κB pathway is a mediator of insulin resistance and that IKKB knockout animals are protected from the effects of high-fat diets to cause decreased insulin action (33). Coupled with our findings, it is possible that sex differences in the molecular components of these pathways may underlie the protection from fat-induced insulin resistance in females.

The concept that increased fat consumption, through either oral ingestion or intravenous administration, can cause insulin resistance has substantial experimental support (1–6). However, upon review of the literature, essentially all studies showing this effect were conducted in men or male animals. Here, we show that there are strong sex differences in the expression of this phenomenon. In addition, we have recently provided information showing that fatty acid infusions do not lead to insulin resistance in women, although they do in men (34). Further studies of this sex difference could lead to new insights into the mechanisms of fat-induced insulin resistance in males and the reasons for protection against this phenomenon in estrogen-replete females.

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