Impaired insulin-receptor autophosphorylation is an early defect in fat-fed, insulin-resistant rats

JACK F. YOUNGREN,1 JOHN PAIK,2 AND R. JAMES BARNARD2
1Division of Diabetes and Endocrine Research, Department of Medicine, Mount Zion Medical Center, University of California, San Francisco 94143-1616; and 2Department of Physiological Science, University of California, Los Angeles, California 90095-1606

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Youngren, Jack F., John Paik, and R. James Barnard. Impaired insulin-receptor autophosphorylation is an early defect in fat-fed, insulin-resistant rats. J Appl Physiol 91: 2240–2247, 2001.—High-fat feeding results in impaired insulin signaling in skeletal muscle, but the role of the insulin receptor (IR) remains controversial. In the present study, female Fischer 344 rats were fed diets either low in fat [low fat, complex carbohydrate (LFCC)] or high in fat and sucrose (HFS). Insulin-stimulated skeletal muscle glucose transport, measured in purified sarcolemmal vesicles, was lower in rats consuming the HFS diet for 2 and 8 wk compared with LFCC controls (72.9 ± 3.5, 67.6 ± 3.5, and 86.1 ± 3.5 pmol·mg⁻¹·15 s⁻¹, respectively; P < 0.05). Muscle IR content was unchanged in 2-wk HFS animals but was 50% lower in the 8-wk HFS group (P < 0.001). However, compared with LFCC, insulin-stimulated IR autophosphorylation was 26% lower in 2-wk HFS and 40% lower in 8-wk HFS animals (P < 0.005). Total muscle content of the proposed IR inhibitors cytokine tumor necrosis factor-α and membrane glycoprotein PC-1 was not significantly changed in HFS animals at either 2 or 8 wk. These results demonstrate that high-fat feeding induces insulin resistance in muscle concomitant with a diminished IR signaling capacity, although the mechanism remains unknown.

Fat feeding has been demonstrated in both in vivo and in vitro preparations (3, 4, 21, 34), although the mechanisms remain unknown.

The insulin signaling pathway leading to enhanced cellular uptake of glucose begins with binding of insulin to the insulin receptor (IR). After ligand binding to the α-subunit, a conformational change of the β-subunit of the IR induces autophosphorylation of specific tyrosine residues, which then activate the substrate protein tyrosine kinase of the IR (29). The activated IR phosphorylates several intracellular proteins, including IR substrate-1 (IRS-1). Tyrosine phosphorylation of IRS-1 leads to binding of phosphatidylinositol 3-kinase (PI3K) (49) and activation of its enzymatic activity, a necessary step for the translocation of GLUT-4 to the plasma membrane and the elevated rate of cellular glucose uptake in response to insulin (22). Several defects in insulin signaling have been reported in rats fed a high-fat diet. Previous studies in fat-fed rats demonstrated decreased insulin-stimulated PI3K activity and GLUT-4 translocation before the development of obesity (54). This suggests that defects in the proximal insulin signaling pathway develop specifically as a result of increased fat intake. We found that a high-fat, refined-sugar diet impairs IR tyrosine kinase activity (5), but others have reported no effect of fat feeding on IR function (8). Recently, Hansen et al. (21) reported that rats fed a high-fat diet developed a reduced IR tyrosine kinase activity (5), but others have reported no effect of fat feeding on IR function (8). Recently, Hansen et al. (21) reported that rats fed a high-fat diet developed a reduced IR tyrosine kinase activity (5), but others have reported no effect of fat feeding on IR function (8). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

ADDITIONAL INFORMATION

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MATERIALS AND METHODS

Animals. Two-month-old female Fischer 344 rats were obtained from Harlan Sprague Dawley. The animals were kept four per cage, with 12:12-h light-dark cycles. Animals were allowed to acclimate to their environment for 1 wk before the dietary intervention was initiated.

Experimental design and diet. Sixteen animals were assigned to each of three groups: low-fat, complex-carbohydrate (LFCC) diet; 2-wk high-fat, sucrose (2-wk HFS) diet; or 8-wk high-fat sucrose (8-wk HFS) diet. After 1 wk, during which time all animals consumed the same LFCC diet, 8-wk HFS animals were then started on the HFS diet, whereas the other two groups continued to consume the LFCC diet. After 6 wk, animals assigned to the 2-wk HFS group were placed on the HFS diet. The study continued for an additional 2 wk, so that, at the end of the study, all three groups were the same age and had been on the LFCC diet for 8 wk or the HFS diet for either 2 wk (2-wk HFS) or 8 wk (8-wk HFS). The LFCC and HFS diets were prepared by Purina Test Diets (Richmond, IN). In the HFS diet, fat is supplied in the form of lard, and carbohydrates are from sucrose. Macronutrient and caloric content of the two diets are shown in Table 1. Both diets, as well as water, were provided ad libitum. At the end of the experimental period, animals were studied after an overnight fast. One-half of the animals in each group (n = 8) were injected with insulin, and sarcoplasmal (SL) vesicles were prepared from their pooled hindlimb muscles. In the remaining nonstimulated animals in each group (n = 8), gastrocnemius and quadriceps muscles were excised, quick frozen in liquid nitrogen, and stored at −70°C for subsequent biochemical and immunoblotting analysis.

Insulin stimulation and SL vesicle isolation. Insulin stimulation was performed by intraperitoneal injection of 15 units of regular porcine insulin. After a wait of 30 min for the maximum effect of insulin to occur, the animals were killed, and the hindlimb muscles were removed. SL vesicles were prepared as described initially by Grimditch et al. (17), except that the incubation with DNase was left out, as previously reported (3). After purification through differential and sucrose-gradient centrifugation, the SL vesicle suspension was frozen and stored in liquid N₂ until it was used for determination of protein content, K⁺-stimulated p-nitrophosphatase (KpNPPase) activity, and glucose transport.

KpNPPase activity. The activity of the SL marker enzyme KpNPPase was determined in vesicle preparations as previously described (17).

Glucose transport. The transport of glucose into SL vesicles was determined under equilibrium-exchange conditions, as adapted from Ludvigsen and Jarett (35) and initially reported by Grimditch et al. (17). Transport was measured at 37°C for 15 s at a concentration of 180 μM D- and L-glucose, with samples tested in triplicate.

Total membrane and muscle extract preparation. Frozen hindlimb muscles were processed to produce a soluble extract for determination of tumor necrosis factor (TNF-α) content; a separate solubilized preparation was employed to measure IR content and autophosphorylation, IRS-1 content, and membrane glycoprotein PC-1 enzyme activity; and a total membrane preparation was used to quantify muscle GLUT-4 content.

Samples for the TNF-α ELISA were prepared by homogenizing pooled quadriceps and gastrocnemius muscles in PBS, 0.5% Tween 20, 2 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride, pH 7.6, with 4- and 10-s bursts of a Polytron homogenizer. The homogenate was centrifuged at 35,000 g for 20 min at 4°C. The supernatant was collected and stored at −70°C for subsequent use in the TNF-α ELISA. In addition, a total membrane preparation and a soluble muscle extract were made by homogenizing ~1-g frozen gastrocnemius samples in ~3 ml of homogenization buffer (50 mM HEPES, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μM pepstatin A, pH 7.6). The resultant homogenate was divided in half, with Triton X-100 added to one-half of the preparation to a final concentration of 1% for solubilization. This extract preparation was allowed to solubilize for 1 h at 4°C. Soluble extracts were then centrifuged at 180,000 g for 60 min at 4°C. Supernatants from the extract preparation were collected and stored at −70°C for subsequent biochemical analysis and Western blotting. The total membrane pellet was prepared by diluting the original 1.5-ml homogenate sample 1:1 with additional homogenization buffer and centrifuging it at 227,000 g for 1 h at 4°C. The resultant pellets were resuspended in homogenization buffer and stored at −70°C until they were used to determine GLUT-4 content. Content of protein in each preparation was determined by the method of Bradford (9).

IR content ELISA. IR content of muscle extracts was determined by specific ELISA as described previously (13), although with different anti-IR antibodies employed to recognize the rat IR. Briefly, microtiter 96-well plates were coated with 2 μg/ml of CT-1, a monoclonal antibody to the IR β-subunit (kindly supplied by K. Siddle, Cambridge, UK), for 18 h at 4°C. After the plate was washed and blocked, solubilized cellular extract containing 10 μg of protein of each sample was added to each well and allowed to bind overnight at 4°C. Samples were loaded in triplicate. Readout of bound IR was accomplished with the sequential addition of biotinylated anti-IR antibody 2G7 (kindly provided by R. Roth, Stanford University), peroxidase-conjugated streptavidin (Pierce, Rockford, IL), ELAST ELISA amplification system (NEN Research Products, Boston, MA) for signal enhancement, and 3,3′,5,5′-tetramethylbenzidine peroxidase substrate system (Kirkegaard & Perry, Gaithersburg, MD) for color development. The absorption at 450 nm of each well was measured in a microtiter plate reader (DuPont-NEN, Boston, MA).

IR autophosphorylation ELISA. The autophosphorylation capacity of gastrocnemius IR was determined in triplicate for soluble extracts using an ELISA specific for IR tyrosine phosphorylation, as described previously (51). In this assay, solubilized cellular extract containing 10 pg of IR was added to each well of a 96-well microtiter plate coated with antibody cardiophrin-1 and allowed to bind overnight. Immunocaptured IR was then incubated in 50 mM HEPES, 150 mM

Table 1. Macronutrient composition (percentage of total calories) of the LFCC and HFS test diets

<table>
<thead>
<tr>
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<th>LFCC</th>
<th>HFS</th>
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<tbody>
<tr>
<td>Energy as carbohydrate, %</td>
<td>59.81</td>
<td>40.12</td>
</tr>
<tr>
<td>Energy as fat, %</td>
<td>12.14</td>
<td>39.09</td>
</tr>
<tr>
<td>Energy as protein, %</td>
<td>28.05</td>
<td>20.72</td>
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<tr>
<td>Fiber, g/100 g</td>
<td>5.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Sucrose, g/100 g</td>
<td>3.68</td>
<td>45.10</td>
</tr>
<tr>
<td>Cholesterol, %</td>
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<td>0.0175</td>
</tr>
<tr>
<td>Saturated fat, %</td>
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<td>7.91</td>
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<tr>
<td>Monounsaturated fat, %</td>
<td>1.58</td>
<td>8.22</td>
</tr>
<tr>
<td>Polyunsaturated fat, %</td>
<td>0.71</td>
<td>3.04</td>
</tr>
<tr>
<td>Physiological energy, kJ/g</td>
<td>13.97</td>
<td>19.63</td>
</tr>
</tbody>
</table>

LFCC, low-fat, complex-carbohydrate diet; HFS, high-fat, sucrose diet.
NaCl, 10 mM MgCl₂, 2 mM MnCl₂, 0.1% Triton X-100, 0.05% BSA, and 10 μM ATP, pH 7.6, with or without 100 nM insulin, for 1 h at 22°C. The tyrosine phosphorylation state was then determined by incubation with a biotinylated anti-phosphotyrosine antibody (UBI, Lake Placid, NY), followed by an identical procedure for color development as employed in the IR content ELISA.

**TNF-α ELISA.** Determination of TNF-α protein levels in soluble extracts of pooled hindlimb muscles was by an ELISA kit specific for rat TNF-α (Genzyme Diagnostics, Cambridge, MA). Samples were assayed in triplicate.

**Western blotting.** Gastrocnemius GLUT-4 content was determined by SDS-PAGE, using 25 μg of total membrane protein loaded onto an 8–16% polyacrylamide minigel. The samples were loaded as single determinations and run through the gel at 30 mA for 1 h. The proteins were electrophoretically transferred to a nitrocellulose membrane at 30 V for 1.6 h. After transfer, the membranes were incubated for 30 min in SuperBlock blocking buffer (Pierce). The membranes were incubated at 4°C with a polyclonal antibody to GLUT-4 (East Acres Biologicals, Southbridge, MA) diluted 1:1,000 in PBS with 0.05% Tween 20 (PBST). After an overnight incubation, the membranes were washed with distilled water. Next, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG and diluted 1:3,000 in PBS. The membranes were then incubated at 4°C with a biotinylated anti-phosphotyrosine antibody (UBI, Lake Placid, NY), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG and diluted 1:3,000 in PBST for 1.5 h at 22°C. Membranes were washed again with water and then with PBST. GLUT-4 protein was visualized by enhanced chemiluminescence (Pierce). Determination of gastrocnemius IRS-1 content was determined in soluble extracts by SDS-PAGE, using 25 μg of protein on an 8–16% gel. The immunoblotting protocol was identical to that for GLUT-4, except that nitrocellulose membranes were probed with an anti-rat IRS-1 (pleckstrin homology domain) antibody (UBI) at 0.4 μg/ml in PBST.

**PC-1 enzymatic activity.** Alkaline phosphodiesterase I activity of glycoprotein PC-1 was determined by hydrolysis of the specific substrate thymidine 5′-monophosphate p-nitrophenyl ester (PNTP) (53). In a 96-well microtiter plate, muscle homogenate (50 μg) was incubated at 37°C in a total volume of 125 μl reaction buffer [0.1 M 2-amino-2-methyl-1-propanol and 7.5 mM Mg(OAc)₂, pH 9.4] containing 4.2 mM PNTP. The concentration of p-nitrophenol produced by the enzyme activity was measured on a plate reader at 401 nm at several time points (20, 40, and 60 min) to ensure linearity of the reaction. PNTP hydrolyzing activity was calculated after subtracting out the time 0 blanks for each sample. Samples were assessed in triplicate, and activity was expressed as nanomoles of p-nitrophenol produced per milligram of protein per minute.

**Statistical analyses.** Data are expressed as means ± SE. For each variable measured, data represent values from eight animals in each diet group. The effects of diet were determined by performing an analysis of variance, with post hoc analysis of significant diet effects by repeated-measures t-test. Statistical significance was accepted at P < 0.05.

**RESULTS**

**Body weight.** As our laboratory reported previously for female Fischer rats (3, 5), there was no significant effect of diet on body weight at the end of the 8 wk [LFCC: 167.4 ± 2.7 g; 2-wk HFS: 169.6 ± 6.4 g; 8-wk HFS: 174.4 ± 4.5 g; P = not significant (NS)].

**Glucose transport.** Purity and yield of the SL vesicle preparations were determined by analysis of KpNPPase activity, an enzyme marker specific for the sarcolemma (17). KpNPPase activity was not different among preparations from LFCC, 2-wk HFS, and 8-wk HFS groups (4.03 ± 0.27, 3.93 ± 0.38, and 4.32 ± 0.46 μmol·mg⁻¹·h⁻¹, respectively). In addition, the yield of SL protein per gram tissue was not different among groups (data not shown). Thus differences in SL glucose transport could not be attributed to differences in the yield or purity of the SL preparations.

The HFS diet negatively impacted the ability of insulin to activate the skeletal muscle glucose transport system. Specific transport of glucose into SL vesicles prepared from insulin-stimulated rats was 86.1 ± 3.5 pmol·mg⁻¹·15 s⁻¹ in the LFCC controls. Insulin-stimulated glucose transport was significantly lower in animals fed the HFS diet for 2 and 8 wk (72.9 ± 3.5 and 67.6 ± 3.5 pmol·mg⁻¹·15 s⁻¹, respectively; P < 0.05) (Fig. 1). This insulin resistance was reflected by elevations in fasting plasma insulin levels in the fat-fed animals. After 8 wk on the HFS diet, plasma insulin levels were significantly greater (42 ± 6 μU/ml) than that of 2-wk HFS (18 ± 4 μU/ml) or LFCC controls (8 ± 2 μU/ml) (P < 0.01). The difference between LFCC control and 2-wk HFS animals did not reach statistical significance (P < 0.09).

There was no evidence that the development of insulin resistance was the result of added weight in the animals. Neither insulin-stimulated glucose transport nor fasting insulin was related to body weight of the animals (r = −0.14 and −0.02, respectively, P = NS).

**IR autophosphorylation.** The tyrosine phosphorylation state of skeletal muscle IR was determined by tyrosine phosphorylation ELISA with immunocaptured IR incubated in vitro in the presence or absence of 100 nM insulin. Basal IR tyrosine phosphorylation was significantly elevated in the 8-wk HFS animals compared with the LFCC and 2-wk HFS groups [0.067 ± 0.010 vs. 0.022 ± 0.005 and 0.025 ± 0.003 optical density (OD) units, respectively; P < 0.01], (Fig. 2). Maximal autophosphorylation capacity of IR was significantly impaired with the HFS diet. Receptor

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**Image:** Fig. 1. Effects of a high-fat, sucrose (HFS) diet on insulin-stimulated sarcolemmal glucose transport. Sarcolemmal vesicles were prepared from hindlimb muscles following maximal in vivo insulin stimulation. Values are means ± SE. Insulin-stimulated glucose transport was significantly lower in 2- and 8-HFS groups compared with low-fat, complex-carbohydrate (LFCC) control diet, *P < 0.05.
autophosphorylation after stimulation with 100 nM insulin was significantly lower than that of LFCC control (0.720 ± 0.047 OD units) in both 2- and 8-wk HFS groups (0.547 ± 0.022 and 0.433 ± 0.058 OD units, respectively) (P < 0.005). Maximal autophosphorylation was significantly lower in both 2- and 8-wk HFS groups than in LFCC controls, *P < 0.005. Maximal autophosphorylation did not differ between 2- and 8-wk HFS groups.

**IR content.** The IR content of LFCC animals was 7.3 ± 0.5 ng/mg protein. This value was not changed by 2 wk on the HFS diet but was 50% lower in 8-wk HFS animals (7.9 ± 3.6 vs. 3.6 ± 0.4 ng/mg, respectively; P < 0.001) (Fig. 3A).

**IRS-1.** Compared with that in LFCC controls, the IRS-1 content of gastrocnemius muscle was unchanged in 2-wk HFS animals but was significantly lower in the 8-wk HFS group (OD values: 90 and 59% of LFCC; P = NS, P < 0.005, respectively) (Fig. 3B).

**GLUT-4.** There were no differences in gastrocnemius GLUT-4 protein levels among LFCC, 2-wk HFS, and 8-wk HFS animals (OD values: 91 and 84% of LFCC controls, respectively) (Fig. 4).

**TNF-α content.** The TNF-α content was determined in pooled hindlimb muscles. There were no significant differences in TNF-α content among any groups (2.30 ± 0.18, 2.68 ± 0.23, and 2.84 ± 0.18 ng/g muscle tissue for LFCC, 2-wk HFS, and 8-wk HFS, respectively) (Fig. 5A).

**PC-1 enzymatic activity.** In soluble gastrocnemius extracts, the hydrolysis of PNTP, a specific substrate for PC-1, was not different among the groups (12.5 ± 0.5, 12.6 ± 0.5, and 12.8 ± 0.4 nmol·mg⁻¹·min⁻¹ for LFCC, 2-wk HFS, and 8-wk HFS animals, respectively) (Fig. 5B).
In our studies of the effects of diet on insulin action, we have employed female Fischer 344 rats, a strain that is relatively resistant to obesity due to dietary intervention or aging (4, 37). Thus we are able to study the effects of the diet per se, distinct from the negative impact of adiposity on insulin action. Our laboratory has previously characterized carefully the changes in caloric intake and development of obesity in female Fischer 344 fed an ad libitum diet high in fat and refined sugar (2, 3). After 2 wk on the HFS diet, there was no increase in omental fat cell volume in female Fischer rats (2, 3, 6). At 8 wk, the second time point examined in the present study, fat cell hypertrophy was present, although there was still no significant change in percent body fat in the animals (3). In the present study, body weight was not significantly different among any of the diet groups, and there was no correlation between body weight and fasting insulin levels or insulin-stimulated glucose transport. Thus the insulin resistance produced in this model is, at least initially, independent of the overt obesity that develops as a result of the HFS diet.

In the present study, we found that an impaired capacity for autophosphorylation by skeletal muscle IR is an early event in the development of insulin resistance with fat feeding. Two weeks of HFS feeding resulted in maximal in vitro IR autophosphorylation levels that were 26% lower than those of controls. Maximal autophosphorylation after 8 wk on the HFS diet was 40% lower than that of LFCC controls. In our laboratory’s previous study, employing the IR autophosphorylation ELISA to measure IR function in human muscle biopsies, we demonstrated that IR autophosphorylation capacity was significantly correlated with the protein tyrosine kinase activity of immunocaptured IR (51). Thus this technique is an effective surrogate measure of the tyrosine kinase activity of IR on exogenous substrates, although with greater accuracy and sensitivity than traditional substrate radiolabeling techniques. The application of this assay to skeletal muscle of rats fed on a high-fat diet has demonstrated that impaired IR signaling capacity is an early result of the HFS diet, appearing concurrently with decreased insulin stimulation of glucose transport.

Whereas evidence for proximal defects in insulin signaling in dietary insulin resistance has been widely reported, studies of IR function have produced conflicting results. Decreased IR tyrosine kinase activity has also been reported for rats fed a very-low-carbohydrate diet (7.5% of calories) for 14 days (25). This dietary regimen is associated with increased adiposity but little or no increased weight gain. In a study of Sprague-Dawley rats consuming the HFS diet, our laboratory has previously demonstrated an impaired IR tyrosine kinase activity after longer consumption (8 and 12 wk) of the HFS diet (5). Furthermore, decreased IR tyrosine kinase activity associated with high-fat diets has been reported in fat and liver tissues (8, 48). However, Hansen et al. (21) found no decrease in IR autophosphorylation and tyrosine kinase activity after 8-wk high-fat diets, and Boyd et al. (8) found no effect of 4 wk of high-fat feeding on skeletal muscle IR tyrosine kinase activity. The diets employed in these studies were very similar to the HFS diet of the present study. As these studies involved rodent strains more susceptible to obesity and relatively long dietary interventions, the fat-fed animals in both studies were significantly heavier than the chow-fed controls. Diminished IR tyrosine kinase activity is associated with obesity in humans and rodents (24, 34, 52); therefore, it is unclear why the rats in these studies were less susceptible to downregulation of IR function than the normal-weight rats in the present study. In the study by Hansen et al. (21), IR autophosphorylation and tyrosine kinase activity were lower in HFS animals after 30 wk on the diet, without any change at 8 wk, despite the presence of insulin resistance and obesity. Careful examination of their data reveals that these conclusions could have resulted from abnormally low values for insulin-stimulated phosphorylation of the IR in the 8-wk control group.

DISCUSSION

In our studies of the effects of diet on insulin action, we have employed female Fischer 344 rats, a strain that is relatively resistant to obesity due to dietary intervention or aging (4, 37). Thus we are able to study the effects of the diet per se, distinct from the negative impact of adiposity on insulin action. Our laboratory has previously characterized carefully the changes in caloric intake and development of obesity in female Fischer 344 fed an ad libitum diet high in fat and refined sugar (2, 3). After 2 wk on the HFS diet, there was no increase in omental fat cell volume in female Fischer rats (2, 3, 6). At 8 wk, the second time point examined in the present study, fat cell hypertrophy was present, although there was still no significant change in percent body fat in the animals (3). In the present study, body weight was not significantly different among any of the diet groups, and there was no correlation between body weight and fasting insulin levels or insulin-stimulated glucose transport. Thus the insulin resistance produced in this model is, at least initially, independent of the overt obesity that develops as a result of the HFS diet.
Insulin-stimulated IR autophosphorylation was far less in the 8-wk control group than in the 30-wk control group; just the opposite of what is expected. Compared with 30-wk controls, insulin stimulation in the high-fat group was low at both 8 and 30 wk, which agrees with the glucose transport data. In a previous study of Wistar rats fed a high-fat, low-carbohydrate diet for 2 wk, our laboratory was unable to demonstrate a significant decrease in autophosphorylation of IR stimulated in vivo during a 2-h hyperinsulinemic, euglycemic clamp (39). The discrepant results among these studies could be due to the very different protocols for stimulating the IR or to significant differences in the diets and animal strains employed. Certainly, the Western blot and substrate labeling techniques are less accurate than the ELISA method employed in the present study.

The mechanisms responsible for inducing impaired IR autophosphorylation are not known. Membrane glycoprotein PC-1 and cytokine TNF-α have both been shown to decrease IR tyrosine kinase activity. Overexpression of PC-1 results in diminished IR autophosphorylation capacity and tyrosine kinase activity (36, 52), and, in humans, muscle PC-1 content is inversely related to insulin action (13, 52, 53) and IR tyrosine kinase activity. Cytokine TNF-α has also been hypothesized to play a causal role in impaired IR function through activation of an intracellular serine kinase (24). TNF-α was originally thought to operate through a paracrine effect, because adipocyte hypertrophy resulted in an increased production of TNF-α and an increased concentration of TNF-α in the plasma of obese animals (24). However, as primary muscle cells cultured from insulin-resistant subjects overproduce TNF-α and are themselves resistant to insulin (42), it is possible that TNF-α may impair muscle IR function through an autocrine effect.

In the present study, we found no evidence that either PC-1 or TNF-α plays a role in the impaired IR autophosphorylation capacity. The negative results for PC-1 are in agreement with our laboratory's previous study of a short-term, high-fat, very-low-carbohydrate diet, although in that study muscle IR function was also unchanged (39). Similarly, the findings that muscle TNF-α does not contribute to diet-induced insulin resistance are in agreement with recent reports showing that transgenic mice lacking either the p55, or p75, or both TNF-α receptors were not protected from high-fat-induced insulin resistance (43).

Several potential mechanisms could be responsible for the decreased IR autophosphorylation capacity induced by the high-fat diet. Incubation of cultured myotubes with palmitate results in reductions in insulin-stimulated IR autophosphorylation and reduced glucose transport (47). Dietary-induced insulin resistance in sand rats is accompanied by reduced IR signaling that is associated with overexpression of protein kinase C (PKC)-ε (44). PKC-θ has also been implicated in free fatty-induced inhibition of the insulin signaling pathway (15). PKC activity can downregulate IR autophosphorylation and tyrosine kinase activity via serine phosphorylation of the IR (11, 23).

The HFS diet did lead to downregulation of specific proteins of the insulin signaling pathway, although only after insulin resistance and impaired IR autophosphorylation had been observed. In the present study, we found that muscle levels of IR and IRS-1 protein were lower in HFS than control animals only after 8 wk on the diet, well after the development of insulin resistance. Decreased IRS-1 levels in response to a diet higher in fat and lower in carbohydrates than the present study have been reported (36). IRS-1 downregulation in muscle has also been demonstrated in several models of human and rodent obesity (1, 12, 14, 30). Beyond the total amount of fat in the diet, the type of fat can impact muscle IRS-1 expression. Kim et al. (32) reported IRS-1 levels significantly decreased in rats fed a diet high in beef tallow vs. one with safflower oil as the fat source. It is not clear, therefore, why Hansen et al. (21) found no decrease in muscle IRS-1 levels in rats, even at 30 wk on a HFS diet high in lard and similar to that employed in the present study.

Previous studies have reported no effect of diet on muscle IR content or insulin binding sites (8, 21, 25, 39). In the present study, the first dietary study to measure IR content by ELISA, we observed a 50% lower muscle IR content in rats after 8 wk on the HFS diet. In adipose and other cells, it has long been recognized that the number of IR far exceeds the quantity required for the full biological effects of insulin (27). The presence of these “spare receptors” suggests that a significant loss of IR would not significantly affect the maximal response to insulin but would only decrease the sensitivity to insulin (27). Whether skeletal muscle contains spare IR is controversial (10, 18). Whereas the physiological significance of a lower IR content is unclear, when combined with a decreased autophosphorylation capacity per receptor, this reduction in muscle IR number results in a dramatic loss of insulin signal transduction per cell after 8 wk on the HFS diet. Insulin-stimulated glucose transport values in this group are not significantly reduced compared with that in the 2-wk HFS-fed rats, although fasting insulin levels are significantly elevated. Transgenic mice overexpressing dominant-negative, kinase-deficient IR in muscle, as well as muscle-specific IR knockout transgenic mice, display significant muscle insulin resistance (31, 38). The functional significance of more physiological reductions in muscle IR content and function remains unclear. In cultured muscle cells, downregulating IR signaling capacity by incubating with palmitate leads to reduced insulin stimulation of glucose uptake, whereas TNF-α does not impair glucose uptake, despite inhibiting IR tyrosine kinase activity, IRS-1 phosphorylation, and PI3K activation (47).

In contrast to the downregulation of IR and IRS-1 proteins, we found no effect of a high-fat diet on skeletal muscle GLUT-4 content. The discrepant results of most studies of GLUT-4 regulation in high-fat feeding are apparently due to different dietary protocols. A diet-induced reduction in muscle GLUT-4 is appar-
ently related to a low level of carbohydrate intake. Muscle GLUT-4 levels are unchanged, irrespective of the development of obesity, in animals fed a high-fat, refined-sugar diet, as in the present study (20), or a relatively low-carbohydrate diet (20–24% of calories) consumed ad libitum (41, 54). However, in very-low-carbohydrate diets (5% of calories), muscle GLUT-4 levels are reduced (26). GLUT-4 levels are also decreased when total carbohydrate intake is reduced on a high-fat, moderate-carbohydrate (25% of calories) diet provided isocalorically to a control chow diet, as in the study of Kim et al. (33). In this study design, muscle GLUT-4 levels are reduced in the absence of weight gain (33). It is unlikely, however, that the reductions in cellular GLUT-4 observed with reduced carbohydrate intake play a causative role in insulin resistance. Decreased total content of GLUT-4 in muscle is not the likely cause of a diminished translocation of the intracellular pool of GLUT-4. Muscle GLUT-4 levels are unchanged in human obesity (45), as well as in genetically obese rodents (50).

In summary, by studying early time points in an obesity-resistant strain of rat, the present study provides the first evidence that impairments in IR autophosphorylation capacity are an early response to a diet high in fat and refined sugar, appearing before other established derangements of the insulin signaling pathway and before any measurable increase in adiposity. Subsequently, after 8 wk on the HFS diet, muscle content of IR and IRS-1, but not GLUT-4, was significantly decreased without further decrement in insulin-stimulated glucose transport. Finally, we found no effect of the HFS diet on muscle content of PC-1 or TNF-α, two reported inhibitors of IR function.

Together, these results suggest that a decreased ability of the IR to autophosphorylate may be the initial defect responsible for insulin resistance resulting from high-fat feeding. The mechanism of diminished IR autophosphorylation remains unknown.

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REFERENCES


