A High Fructose Diet Affects the Early Steps of Insulin Action in Muscle and Liver of Rats

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ABSTRACT A high fructose diet induces insulin resistance in rats, although the exact molecular mechanism involved is unknown. In this study, we used immunoprecipitation and immunoblotting to examine the levels and phosphorylation status of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1), as well as the association of the IRS-1 with phosphatidylinositol 3-kinase (PI 3-kinase), and phosphotyrosine phosphatase (SHP2) in the liver and muscle of rats fed a control or high fructose diet for 28 d. There were no differences in IR and the IRS-1 protein levels in the liver and muscle of rats fed the control and high fructose diets. However, tyrosine-phosphorylation of the insulin receptor after insulin stimulation was reduced to 71 ± 2% (P < 0.05) of control in the liver of the fructose-fed rats. In samples previously immunoprecipitated with anti-IRS-1 antibody and blotted with antiphosphotyrosine antibody, the insulin-stimulated IRS-1 phosphorylation levels in the liver and muscle of the fructose-fed group were only 70 ± 6% (P < 0.05) and 76 ± 5% (P < 0.05) of those of control rats, respectively. The insulin-stimulated IRS-1 association with PI 3-kinase was reduced to 84% (P < 0.05) in the liver and to 84 ± 4% (P < 0.05) in the muscle of the fructose-fed group compared with control rats. Insulin-stimulated IRS-1 association with SHP2 was reduced to 79 ± 5% (P < 0.05) in liver of the fructose-fed rats. These data suggest that changes in the early steps of insulin signal transduction may have an important role in the insulin resistance observed in these rats. J. Nutr. 130: 1531–1535, 2000.

KEY WORDS: • fructose • insulin receptor • insulin receptor substrate-1 • phosphatidylinositol 3-kinase • phosphotyrosine phosphatase • rats

Insulin plays a central role in the regulation of glucose homeostasis and acts in a coordinated fashion on cellular events that include the regulation of ion and amino acid uptake, protein synthesis and degradation, gene transcription and mRNA turnover, and cellular growth and differentiation (Kahn 1985, Rosen 1987). The insulin receptor (IR)3β-subunit, which contains an intrinsic tyrosine kinase, undergoes tyrosyl autophosphorylation and is activated in response to insulin binding to the extracellular α-subunit (Kasuga et al. 1982). Subsequent steps in insulin signal transduction are mediated via the phosphorylation of specific intracellular proteins, including insulin receptor substrate-1 (IRS-1) (Sun et al. 1991). In peptide motifs with the sequence Tyr-Met-x-Met (YMXXM) or Tyr-x-x-Met (YXXM), tyrosine phosphorylated IRS-1 serves as a docking protein that interacts with signaling proteins containing src homology-2 (SH2) domains, including the 85-kDa subunit of phosphatidylinositol 3-kinase (PI 3-kinase), phosphotyrosine phosphatase (SHP2) and other SH2-containing proteins (White 1997).

An impairment of insulin action (insulin resistance) is involved in many diseases, including noninsulin-dependent diabetes, obesity, hypertension and cardiovascular diseases (O’Doherty et al. 1997). The rats fed a high fructose diet provide an animal model of insulin resistance associated with hyperinsulinemia, hypertriglyceridemia (Sleder et al. 1980, Tobey et al. 1982, Zavaroni et al. 1980 and 1982) and hypertension (Thorburn et al. 1989). Although fructose in the diet alters the activity of several enzymes and regulates hepatic carbohydrate metabolism, leading to hepatic insulin resistance (Blakely et al. 1981, Tuovinen and Bender 1975), the mechanisms by which an excess of fructose produces these effects are unknown. The phosphorylation of the IR and IRS-1, as well as their association with PI 3-kinase and SHP2 “in vivo,” has not been examined in insulin resistance induced by a high fructose diet. In this study, we investigated the levels of insulin-induced IR and IRS-1 tyrosine phosphorylation and the association of the latter with PI 3-kinase and SHP2 in the liver and muscle of fructose-fed rats.
MATERIALS AND METHODS

Materials. The reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Tris, phenylmethylsulfonylfluoride (PSMF), aprotinin, dithiothreitol (DTT), Triton X-100, Tween 20 and glycerol were from Sigma Chemical (St. Louis, MO). Protein A-Sepharose 6 MB was from Pharmacia (Uppsala, Sweden), and protein A was from Amersham (Aylesbury, UK) and nitrocellulose paper (Hybond ECL, 0.45 μm) was from Schleicher & Schuell (Keene, NH). Human recombinant insulin (Humulín R) was from Biobras (Belo Horizonte, Brazil) and sodium thiopental was from Cristália (Itapira, Brazil). Antibodies against phosphotyrosine, IR, IRS-1 and SHP2 were from Santa Cruz Technology (Santa Cruz, CA), and antibodies against the p85 subunit of PI 3-kinase were from Upstate Biotechnology (Lake Placid, NY). The fructose was kindly provided by Vepé Indústria Alimentícia Ltda (Santa Bárbara dos Campos, Brazil)

General protocol. Male Wistar-Hannover rats (~120 g) from the University's Central Animal Breeding Center were randomly distributed into two diet groups (control and high fructose) for the subsequent 28 d. The control diet was a nonpurified diet (Nuvilab--Nuvital, Curitiba, Brazil) containing cornstarch (527 g/kg diet), corn oil (35 g/kg diet), beef and fish flours (220 g/kg diet), sodium (3 g/kg diet) and potassium (10 g/kg diet). The high fructose semipurified diet contained fructose (624 g/kg diet), soybean oil (50 g/kg diet), casein (223 g/kg diet), sodium (1.0 g/kg diet) and potassium (3.6 g/kg diet). The fiber and mineral and vitamin mixtures used in the experimental diets met the criteria specified by the AIN (Reeves et al. 1993). The rats had free access to food and tap water, were maintained under standard conditions (20–22°C and a 12-h light-dark cycle) and were weighed weekly to record their body weight gain. Different groups of rats were used for tissue sampling and the biochemical assays. The experiments were approved by the Faculty’s Committee for Animal Experimentation and the general guidelines established by the Declaration of Helsinki (1964) for laboratory animals were followed throughout the study.

Methods

Tissue extracts. Control and fructose-fed rats in the fed state were anesthetized intraperitoneally (i.p.) with sodium thiopental (80 mg/kg body weight) and used as soon as they were fully anesthetized (loss of pedal and corneal reflexes). The abdominal cavity was opened, the portal vein exposed, and 10 mmol/L insulin was injected. Within 30 s, the liver sample was removed, minced coarsely and homogenized immediately in 10 vol of solubilization buffer (10 mM Tris–HCl [pH 7.4], 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PSMF and 0.1 g of aprotinin/L) at 4°C, using a Polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, NY) operated at maximum speed for 20 s. Approximately 90 s after the insulin injection, hindlimb muscle samples were excised and homogenized as described above. The tissue extracts were centrifuged at 16,000 × g for 4°C for 20 min to remove insoluble material and the supernatants were used in immunoprecipitation experiments.

Protein analysis by immunoblotting. For immunoprecipitations, the supernatants were incubated with antibodies against IR or IRS-1 at 4°C overnight, followed by the addition of protein A-Sepharose 6 MB. The immunoprecipitated proteins were treated with Laemmli buffer (Laemmli 1970) containing 100 mM/L DTT, heated in a boiling water bath for 4 min and subjected to SDS-PAGE (6% acrylamide) in a Bio-Rad mini gel apparatus (Mini-Protein, Bio-Rad). The electrotransfer of proteins from the gels to nitrocellulose membranes was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protein) as described by Towbin et al. (1979), but with 0.02% SDS added to the transfer buffer to enhance the elution of high-molecular-mass proteins. Non-specific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in blocking buffer (50 g/L nonfat dry milk, 10 mM/L Tris, 150 mM/L NaCl and 0.5 mM/L Tween 20). The nitrocellulose blots were incubated for 4 h at 22°C with antibodies against phosphotyrosine, IR, IRS-1, the p85 subunit of PI 3-kinase or SHP2 diluted in blocking buffer (30 g/L nonfat dry milk, 10 mM/L Tris, 150 mM/L NaCl and 0.02% Tween 20) followed by washing for 30 min in blocking buffer without milk. The blots were then incubated with 2 μCi of [125I]protein A (1.11 TBq/g) in 10 mL of blocking buffer (10 g/L nonfat dry milk) for 1 h at 22°C and washed again as described above. [125I]Protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DE) at −80°C for 12–48 h. Band intensities were quantitated by optical densitometry of the developed autoradiographs.

Other assays. Serum triacylglycerol, cholesterol and insulin concentrations were determined in 9-wk-old fructose-fed and control rats after 4 wk of diet treatment. The rats were deprived of food for 6 h, and then anesthetized with sodium thiopental (80 mg/kg body weight, i.p.) after which blood samples were collected by cardiac puncture. Serum triacylglycerol and cholesterol concentrations were determined by the glycerol phosphate oxidase and cholesterol oxidase methods, respectively, using commercial kits (Labtest Diagnóstica, Lagoa Santa, Brazil) and serum insulin was determined by double-antibody radioimmunoassay using a rat-specific kit (Diagnostic Products, Los Angeles, CA).

The effect of fructose on the ability of insulin to stimulate glucose disposal was estimated by the intravenous insulin tolerance test (ITT). The ITT was determined in control and fructose-fed rats after 14 h of food deprivation. The rats were anesthetized with thiopental (80 mg/kg body weight, i.p.); tail blood samples were drawn before insulin (10−5 mol/L) injection (0 or basal) and at 4, 8, 12 and 16 min after hormone administration. The blood samples collected before insulin infusion were used to determine the basal glucose concentrations. Serum glucose concentrations were determined by the glucose oxidase method using a commercial kit (Labtest Diagnóstica). The rate constant for plasma glucose disappearance (Kitt) was calculated using the formula 0.693/t1/2. The plasma glucose t1/2 was calculated by the slope of the least-squares analysis of the plasma glucose concentrations during the linear decay phase (Bonnora et al. 1989).

Statistical analysis. The results are presented as means ± SEM for the number of rats (n) per experimental condition. The experiments were always performed by analyzing samples from fructose-fed rats in parallel with a control group. Student’s t test for unpaired analyses was used for comparisons. The level of significance was set at P < 0.05.

RESULTS

Animal characteristics. After 28 d of treatment, the body weights did not differ between groups. The food intake was 20 ± 2 g/d for rats fed the high fructose diet and 23 ± 1.5 g/d for those fed the control diet. Serum triacylglycerol concentrations were significantly higher in the fructose group than in the controls (Table 1). The basal serum glucose, cholesterol and insulin concentrations did not differ between groups after 4 wk of diet treatment. The glucose disappearance rate (Kitt) during the ITT was significantly lower in fructose-fed rats than in control rats, demonstrating a moderate state of insulin resistance.

Tyrosine phosphorylation of IR and IRS-1, and IRS-1 association with PI 3-kinase or SHP2 in the liver of fructose-fed rats. There were no differences in the insulin receptor and IRS-1 protein levels (Fig. 1A, E) in the liver of control and fructose-fed rats, as determined by immunoblotting with anti-IR and anti-IRS-1 antibodies. However, after stimulation with insulin, samples previously immunoprecipitated with anti-IR antibody and immunoblotted with antiphosphotyrosine (anti-PY) antibody showed IR autophosphorylation that was reduced to 71 ± 2% of the control values (P < 0.05) in fructose-fed rats (Fig. 1C). In liver samples previously immunoprecipitated with anti-
IRS-1 antibody and immunoblotted with anti-PY antibody (Fig. 1G), the insulin-induced IRS-1 phosphorylation in the fructose-fed group was reduced to 70 ± 6% (P < 0.05) of the control group value.

After stimulation with insulin, the intensity of the band with the expected molecular weight of the regulatory subunit of PI 3-kinase (85 kDa) was greater in anti-IRS-1 antibody immunoprecipitates of the liver samples from both groups of rats compared with the basal bands. This observation was consistent with a stable association between IRS-1 and PI 3-kinase. However, comparison of the bands stimulated by insulin revealed that the association between IRS-1 and PI 3-kinase was slightly reduced (84 ± 3% of control; P < 0.05) in the liver of fructose-fed rats compared with the control group (Fig. 1I).

When the same blots containing liver samples previously immunoprecipitated with anti-IRS-1 antibody were incubated subsequently with antibodies directed against SHP2 (Fig. 1L), an immunoreactive band was seen in the basal state of control and fructose-fed rats. The intensity of this band increased after insulin stimulation, indicating a stable association of IRS-1 with SHP2 in both groups of rats. However, comparison of the insulin-stimulated bands revealed that the amount of SHP2 associated with IRS-1 in the liver of fructose-fed rats was 79 ± 5% that of the controls (P < 0.05).

Tyrosine phosphorylation of IR and IRS-1, and IRS-1 association with PI 3-kinase or SHP2 in skeletal muscle of fructose-fed rats. As in liver, the fructose diet did not affect the IR and IRS-1 protein levels in rat skeletal muscle (Fig. 1B, F). However, in contrast to liver, stimulation with insulin did not alter the extent of phosphorylation of the IR in the fructose-fed rats compared with the control group (Fig. 1D). Insulin-induced IRS-1 phosphorylation in fructose-fed rats was reduced to 76 ± 5% (P < 0.05) of the control group (Fig. 1H). As expected, in both groups, the 85-kDa subunit of PI 3-kinase was present in the IRS-1 immunoprecipitates after exposure to insulin. A comparison of the bands revealed a significant reduction in the fructose group to 84 ± 4% (P < 0.05) of the control rats (Fig. 1J).

When the blots from these experiments were incubated with antibody directed against SHP2, immunoreactivity was detected in the basal state of both groups. After insulin stimulation, the intensity of this 66-kDa band increased in both groups of rats. However, comparison of the bands stimulated by insulin revealed that the amount of SHP2 associated with IRS-1 did not differ in the muscle of both groups (Fig. 1M).

**DISCUSSION**

The insulin resistance in animals fed a high fructose diet is associated with glucose intolerance, increased serum triacyl-
glycerol and insulin concentrations, and decreased sensitivity to insulin (Blakely et al. 1981, Imura et al. 1995, Thorburn et al. 1989, Tobey et al. 1982, Zavaroni et al. 1980 and 1982). Our results showed a state of moderate insulin resistance in the fructose-fed rats, as demonstrated by the reduced glucose disappearance rate after insulin infusion and the greater serum triacylglycerol concentration. The discrepancies between the metabolic serum glucose and insulin concentrations in our study, compared with previous reports (Sleder et al. 1980, Tobey et al. 1982), may be related to the duration of the fructose feeding and the lipid content of the diets (Sleder et al. 1980, Zavaroni et al. 1982).

Despite the normal number of insulin receptors in the liver and muscle of fructose-fed rats, there was a 29% reduction in insulin receptor autophosphorylation in the liver after stimulation with insulin “in vivo.” The decrease observed here may be of biological importance because a reduction in receptor phosphorylation has been correlated with insulin resistance in different animal models (Carvalho et al. 1996, Saad et al. 1993 and 1997). Our results differed from those of Deutsch et al. (1993), who reported no change in insulin receptor kinase activity in fructose-fed rats. In that study, the insulin receptors were purified from liver samples and the kinase assay was performed “in vitro” using an exogenous phosphoacceptor (Shepherd et al. 1998). This difference likely reflected the amount and mode of feeding, as well as variations in assay conditions.

There was a significant reduction in the level of hepatic and muscle IRS-1 tyrosine phosphorylation, followed by a reduction in IRS-1/PI 3-kinase association in liver and muscle. In previous studies of fructose-fed rats, a reduced ability of insulin to suppress hepatic glucose production has been implicated as one of the elements of insulin resistance (Thorburn et al. 1989, Tobey et al. 1982). The IRS-1/PI 3-kinase association induced by insulin is necessary, and in some cases sufficient to elicit many of the insulin effects on glucose and lipid metabolism. The lipid products of PI 3-kinase act as both membrane anchors and allosteric regulators, serving to locate and activate downstream enzymes and their protein substrates (Shepherd et al. 1998). Glucose 6-phosphatase is a key enzyme of glucose homeostasis because it catalyzes the ultimate reaction of both glycogenolysis and gluconeogenesis. Insulin may regulate this enzyme through competitive inhibition of the microsomal glucose 6-phosphatase by phosphatidylinositol 3,4,5-trisphosphate (PIP₃), the main lipid product of PI 3-kinase (Mithieux et al. 1998). As shown previously, rats fed a high fructose diet for 4 or 12 wk had increased liver glucose-6-phosphatase levels (Tuovinen and Bender 1975). Phosphoenolpyruvate carboxykinase (PEPCK) is another regulatory enzyme in gluconeogenesis. Inhibitors of PI 3-kinase block the effects of insulin on PEPCK gene transcription in liver cells (Sutherland et al. 1995), suggesting that the control of PEPCK by insulin is mediated by PI 3-kinase. PEPCK activity is greater in animals fed high fructose diets (Blakely et al. 1981). Together with our results, these findings suggest that a reduction in the IRS-1/PI 3-kinase association in the liver of rats fed a high fructose diet can reduce the effects of insulin on glucose-6-phosphatase activities and PEPCK expression. In addition, a correlation between PI 3-kinase activity and glycogen metabolism has been described (Van Weeren et al. 1998). Indeed, a reduction in the hepatic glycogen concentration has been reported in this model (Rawana et al. 1993, Thorburn et al. 1989). Glycogen synthase kinase 3 (GSK3) is an enzyme that regulates glycogen synthesis through the inactivation of glycogen synthase by phosphorylation. One of the downstream effectors of PIP₃ lipid signaling is the serine/threonine protein kinase B (PKB or Akt) (Shepherd et al. 1998). PKB activation is necessary and sufficient for insulin-induced GSK3 inactivation (Van Weeren et al. 1998). Thus, the reduction in IRS-1/PI 3-kinase association in the liver of fructose-fed rats may have a role in their insulin resistance.

Regulation of the blood glucose concentration is one of the most important physiologic functions of insulin. An important aspect of this process is the ability of insulin to stimulate the translocation of GLUT4 from an intracellular pool to the plasma membrane, thus increasing the rate of glucose uptake in insulin-responsive tissues, including muscle and adipose tissue (Gould and Holman 1993). Although the molecular mechanism that regulates the intracellular trafficking of GLUT4-containing vesicles is not yet completely known, several effector molecules have recently been identified, including the well-documented targeting and/or activation of PI 3-kinase involved in GLUT4 translocation (Shepherd et al. 1998). In addition to the reduced glucose disappearance rate (Kₘ), the association of IRS-1/PI 3-kinase in muscle samples was reduced in rats fed a high fructose diet. These observations suggest that there are probably alterations in the PI 3-kinase-dependent pathways for glucose transport translocation.

IRS-1 contains >30 potential serine/threonine phosphorylation sites in motifs recognized by various kinases such as serine/threonine protein kinase C (PKC), mitogen activating protein kinases, and cyclic AMP- and cyclic GMP-dependent protein kinases (Fea and Roth 1997, Mothe and Obberrgen 1996, Sun et al. 1991). The phosphorylation of IRS-1 on serine/threonine residues has an inhibitory effect on insulin signaling. The enhanced synthesis of triacylglycerol observed in rats fed a high fructose diet is associated with an increase in 1,2-diacylglycerol and an increased expression of one iso-enzyme of PKC in these rats (Donnelly et al. 1994). Thus, an increase in PKC activity may play an important role in altering the phosphorylation level of the IRS-1.

As with the activation of PI 3-kinase, the association of SHP2 with IRS-1 may activate this phosphatase (Ugi et al. 1996). SHP2 may modulate postreceptor signaling by dephosphorylating the phosphotyrosyl forms of cellular proteins that are substrates for the insulin receptor (White and Kahn 1994). Recent data suggest that SHP2 plays a role in insulin-stimulated mitogenesis and also indicate that this phosphatase is necessary for the insulin-stimulated expression of GLUT4 (Hausdorff et al. 1995). SHP2 can also dephosphorylate IRS-1 (Kuhné et al. 1994, Yamauchi et al. 1995), although this has not been observed uniformly (Milarski and Saltiel 1994, Naguchi et al. 1996). Our results showed that the association of IRS-1/SHP2 after insulin stimulation was reduced in the liver of fructose-fed rats, suggesting that an alteration in this association does not account for the reduction in IRS-1 phosphorylation in these rats.

In summary, a reduction in the phosphorylation of IRS-1 in the liver and muscle of fructose-fed rats led to a reduction in IRS-1/PI 3-kinase association. These changes may help to explain some aspects of insulin resistance in this animal model.

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LITERATURE CITED


