Elevated basal PI 3-kinase activity and reduced insulin signaling in sucrose-induced hepatic insulin resistance

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1Division of Endocrinology, Metabolism and Diabetes, University of Colorado Health Sciences Center, Denver, Colorado 80262; 2Department of Physiology and Biophysics, University of Southern California, Los Angeles, California 90089; and 3Exercise Science Research Institute, Arizona State University, Tempe, Arizona 85287

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Pagliassotti, Michael J., Jione Kang, Jeffrey S. Thresher, Chin K. Sung, and Michael E. Bizeau. Elevated basal PI 3-kinase activity and reduced insulin signaling in sucrose-induced hepatic insulin resistance. Am J Physiol Endocrinol Metab 282: E170–E176, 2002.—Sucrose feeding reduces the ability of insulin to suppress glucose production and hepatic gluconeogenesis. The present study examined the effect of a high-sucrose diet on early insulin-signaling steps in the liver. Rats were provided a high-starch (STD, control diet) or high-sucrose diet (HSD) for 3 wk. On the day of study, overnight-fasted rats were anesthetized and injected with either saline (n = 5/diet group) or insulin (2 mU/kg, n = 5/diet group) via the portal vein. Portal venous blood and liver tissue were harvested 2 min after injections. Portal vein plasma glucose levels were not significantly different among groups, pooled average 147 ± 12 mg/dl. Western blot analysis revealed no significant differences in the amount of insulin receptor (IR), insulin receptor substrates-1 and -2 (IRS-1, IRS-2), and the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase). In contrast, the amount of the p110β subunit of PI 3-kinase was increased ~2-fold in HSD vs. STD (P < 0.05). After saline injection, tyrosine phosphorylation (pY) of IR, IRS-1, and IRS-2 was not significantly different between groups. However, PI 3-kinase activity associated with phosphorylated proteins was increased ~40% in HSD vs. STD (P < 0.05). After insulin injection, pY of the IR was not different between groups, whereas pY of IRS-1 and IRS-2 was reduced (P < 0.05) in HSD vs. STD. In addition, association of IRS-1 and IRS-2 with p85 was significantly reduced in HSD vs. STD. These data demonstrate that an HSD impairs insulin-stimulated early postreceptor signaling (pY of IRS proteins, IRS interaction with p85). Furthermore, the increased amount of p110β and increased basal PI 3-kinase activity suggest a diet-induced compensatory response.

phosphatidylinositol 3-kinase; diet; insulin action; insulin receptor substrates; rats

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METHODS

Animals and diet protocol. Male Sprague-Dawley rats weighing ~180 g were obtained from an institutional breeding colony. Animals were housed individually in a temperature-controlled room with a 12:12-h light-dark cycle and free access to food and water. All procedures for animal use were approved by the Institutional Animal Care and Use Committee at Arizona State University. Upon initiation of the study, all animals were provided a semipurified high-starch diet (STD: 68% of energy as corn starch, 12% as fat, 20% as protein; Table 1) for a 2-wk baseline period. Food intake was measured daily and average food intakes were calculated and reported weekly during the baseline period. Rats either remained on STD or were switched to a high-sucrose diet (HSD: 68% sucrose, 12% fat, 20% protein; Table 1) for 3 wk. We have previously demonstrated that reduced insulin suppression of glucose production, elevated hepatic lipids, and increased saturated fatty acid content of liver sinusoidal membranes were observed after 1 wk of HSD feeding. In addition, 2–8 wk of sucrose feeding produced no significant additional changes in these parameters (21). During these 3 wk, rats were fed 95% of the average food intake measured during the 2nd wk of the baseline period. This feeding regimen was used to ensure similar rates of weight and fat gain throughout the 3-wk dietary period (19, 23).

Experimental protocol. Overnight-fasted rats were anesthetized with pentobarbital sodium (~50 mg/kg, ip), placed on a heating pad, and used when pedal and corneal reflexes were absent. The abdominal cavity was opened, the portal vein was exposed, and insulin (2 mU/kg, ip), placed into the abdominal cavity, was injected. Two minutes after injection, a blood sample was drawn from the portal vein, and a sample of liver was freeze-clamped in situ.

Preparation of cell lysates from liver. Livers were homogenized on ice in a buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 10% glycerol, 2 mM EGTA, 1 mM sodium vanadate, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 50 mM β-glycerophosphate, 3 mM benzamidine, 10 μM leupeptin, 5 μM pepstatin, and 10 μg/ml aprotinin. Samples were rotated for 30 min at 4°C and centrifuged at 14,500 rpm for 1 h. Total protein concentration was determined by the Bradford method.

Immunoprecipitation and Western blotting. For immunoprecipitations, equivalent amounts of protein were incubated with antibodies against the insulin receptor (IR), IRS-1, IRS-2, p85, p110β, or protein kinase Bo (Akt1/PI3Kα). Membranes were incubated and analyzed as described.

PI 3-kinase activity. Cell lysates were incubated with anti-pY, IRS-1, or IRS-2 antibodies for 2 h at 4°C following incubation with protein G-agarose for 2 h at 4°C. Pellets were washed with the wash buffer, and the kinase assay was initiated by the addition of a reaction mixture containing PI (0.2 mg/ml) and [γ-32P]ATP (40 μM, 2 μCi), and the incubation was continued for 10 min at 25°C. The reaction was stopped by the addition of 4 N HCl. After chloroform-methanol extraction, 30 μl of extracted lipid products were spotted onto preactivated silica gel 60 plates and air dried. Thin layer chromatography was performed with chloroform-methanol-water-ammonium hydroxide (60:47:11.3:3.2 vol/vol/vol/vol). Lipid products were visualized by autoradiography, and the PI 3-phosphate spot was cut out and quantified in a scintillation counter.

Basal and euglycemic hyperinsulinemic clamps. With rats under general anesthesia (im injection of 5 mg/kg acepromazine, 10 mg/kg xylazine, and 50 mg/kg ketamine), catheters (PE-50, Clay-Adams Intramedic) were implanted in the left carotid artery and right jugular vein (22). Animals were allowed at least 4 days to recover and were required to be at >95% of the presurgical body weights at that time to be studied further. Basal (0–120 min) and hyperinsulinemic (120–240 min, 4 μU·kg−1·min−1) euglycemic clamps were performed on overnight-fasted, conscious rats (n = 4/diet) as described in detail previously (19, 21, 22).

Other assays. Plasma glucose was determined using a Beckman glucose analyzer (Fullerton, CA). Plasma insulin was determined by radioimmunoassay (Linco Research, St. Charles, MO).

Calculations. Rates of appearance and disappearance were calculated as described in detail previously (19, 21, 22).

Statistics. Data are expressed as means ± SE. Group means were compared using an unpaired t-test with an α-level of P = 0.05.

RESULTS

Basal and hyperinsulinemic euglycemic clamps. Consistent with our previous studies (19, 21, 22), 3 wk of sucrose feeding reduced the ability of insulin to suppress glucose appearance and stimulate glucose disappearance (Table 2).

General animal characteristics. Body weights on the day of study were 411 ± 5 g in STD and 421 ± 9 g in HSD. Body weight gain over the 3-wk dietary feeding period was not significantly different between groups.

<table>
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<tr>
<th>Table 1. Diet composition</th>
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<tr>
<td>Cornstarch</td>
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<tr>
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<tr>
<td>Sucrose</td>
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<td>Choline bitartrate</td>
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Diet composition is expressed in g/kg. Maltodextrin 10 is enzyme-converted cornstarch with a dextrose equivalence of 10%. Salt and vitamin mix are based on American Institute of Nutrition guidelines (25). All diets were formulated by Research Diets, New Brunswick, NJ.
Portal vein plasma glucose levels in saline-injected rats were 151 ± 6 and 148 ± 6 mg/dl in STD and HSD, respectively. Portal vein glucose levels in insulin-injected rats were 143 ± 6 and 139 ± 6 mg/dl in STD and HSD, respectively.

Protein levels and pY of IR, IRS-1, and IRS-2.

IR, IRS-1, and IRS-2 protein levels were not significantly different between STD and HSD (Fig. 1). pY of IR, IRS-1, and IRS-2 in saline-injected rats was not significantly different between STD and HSD. Insulin-stimulated (difference between saline- and insulin-injected) pY of the IR was not significantly different between STD and HSD (Fig. 2). In contrast, insulin-stimulated pY of IRS-1 and IRS-2 was significantly reduced in HSD vs. STD (Fig. 2).

Association of p85α with IRS proteins and PI 3-kinase activity.

Insulin-stimulated p85α association with IRS-1 and IRS-2 was significantly reduced in HSD vs. STD (Fig. 3). PI 3-kinase activity in anti-pY immunoprecipitates was increased by ~50% (P < 0.05) in saline-injected HSD vs. saline-injected STD (Fig. 4). In contrast, PI 3-kinase activity in anti-IRS-1 and anti-IRS-2 immunoprecipitates was not significantly differ-

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### Table 2. Clamp data

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<tr>
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<td>Insulin, µU/ml</td>
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<td>Ra, mg·kg⁻¹·min⁻¹</td>
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Values are means ± SE; n = 4/diet. STD, rats fed the starch diet for 3 wk; HSD, rats fed the sucrose diet for 3 wk; Ra, rate of appearance; Rd, rate of disappearance. Ra and Rd were determined from the infusion of [3-3H]glucose. Values for glucose, Ra, and Rd represent the average of 3 samples taken during the final 30 min, steady-state period of the basal (90–120 min), and clamp (210–240 min) periods. Insulin data represent the results from a single sample taken at the end of the basal and clamp periods. *Significantly different from STD (P < 0.05).

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Fig. 1. Results of immunoblot analysis of the insulin receptor (IR), insulin receptor substrate-1 (IRS-1), and IRS-2 in livers from starch (STD) and sucrose (HSD)-fed rats. Bar graphs represent means ± SE; n = 10/diet group. Representative (n = 3/group) immunoblot exposures are provided at top.

Fig. 2. Tyrosine phosphorylation of the IR, IRS-1, and IRS-2 in livers from saline- (−) and insulin-injected (+) STD- and HSD-fed rats. Liver supernatants were immunoprecipitated with antibodies to the IR, IRS-1, or IRS-2 and then immunoblotted with an anti-phosphotyrosine (pY) antibody as described in METHODS. Bar graphs represent means ± SE; n = 5/diet group. Representative (n = 2/group) immunoblot exposures are provided at top. *Significant difference from STD (P < 0.05).

Fig. 3. Association of IRS proteins (IRS-1, IRS-2) with the p85 subunit of phosphatidylinositol (PI) 3-kinase in livers from saline- (−) and insulin-injected (+) STD- and HSD-fed rats. Liver supernatants were immunoprecipitated with antibodies to IRS-1 or IRS-2 and then immunoblotted with an antibody to the p85 subunit of PI 3-kinase as described in METHODS. Bar graphs represent means ± SE; n = 5/diet group. Representative (n = 2/group) immunoblot exposures are provided at top. *Significant difference from STD (P < 0.05).
between HSD and STD (data not shown). Insulin-stimulated PI 3-kinase activity in anti-pY (Fig. 4), anti-IRS-1 and anti-IRS-2 immunoprecipitates was significantly reduced in HSD vs. STD.

Protein levels of PI 3-kinase subunits. There were no significant differences in p85 protein levels between groups (Fig. 5). In contrast, p110β was increased about twofold ($P < 0.05$) in HSD vs. STD (Fig. 5). When samples were immunoprecipitated with anti-p85 antibody and subsequently immunoblotted with anti-p110β antibody, no significant differences were observed between groups (Fig. 6).

Phospho-Akt/PKBα. Phospho-Akt/PKBα was significantly increased in saline-injected HSD vs. saline-injected STD (Fig. 7). Insulin stimulation of phospho-Akt/PKBα was significantly reduced in HSD vs. STD (Fig. 7).

**DISCUSSION**

In multiple previous studies, we have observed reduced insulin suppression of glucose production (19, 21, 22) and gluconeogenesis (20) after 1–8 wk of high-sucrose diet feeding. The present study also demonstrates that 3 wk of high-sucrose diet feeding reduced the ability of insulin to suppress glucose appearance (hepatic insulin resistance) and stimulate glucose disappearance (peripheral insulin resistance). In addi-
tion, the present study establishes impaired postreceptor insulin signaling as a mechanism leading to sucrose-induced hepatic insulin resistance. Impairments in postreceptor insulin signaling include tyrosine phosphorylation of IRS-1 and IRS-2. In addition, insulin association with the p85 subunit of PI 3-kinase, and insulin-mediated activation of PI 3-kinase activity and Akt/PKB. An unexpected result from the present study was the observation that basal PI 3-kinase activity and the amount of the p110β of PI 3-kinase were increased by the sucrose-rich diet.

Sucrose feeding did not lead to reductions in the amount of insulin receptor protein or tyrosine phosphorylation of the insulin receptor. The preservation of “normal” insulin receptor autophosphorylation in the present study is different from that in a previous study (2), where a 64% fructose diet reduced insulin receptor autophosphorylation by ∼30%. It is important to note that >3 wk of HSD feeding are required to elicit significant fasting hyperinsulinemia (21), and thus down-regulation of insulin receptor number or impairments in function due to hyperinsulinemia would not be expected in the present study.

Insulin stimulation of tyrosine phosphorylation of IRS proteins, association of IRS proteins with the p85 subunit of PI 3-kinase, and PI 3-kinase activity were reduced in HSD vs. STD. Although the present study does not elucidate the mechanisms leading to these impairments, likely candidates include coupling of IRS proteins to the insulin receptor, elevated tyrosine phosphatase activity, and/or elevated serine/threonine kinase activity. Efficient coupling of IRS proteins to the insulin receptor and maintenance of tyrosine phosphorylation of IRS proteins may be influenced by the lipid environment of the cell (10, 16). The sucrose-rich diet used in the present study increases hepatic triglycerides and membrane lipids after only 1 wk of feeding (23). Diet-induced upregulation of phosphotyrosine phosphatases or serine/threonine kinases could also contribute to the sucrose-induced reduction in tyrosine phosphorylation of IRS proteins (6, 24). However, the association of IRS-1 with SHP-2 [a phosphotyrosine phosphatase that was shown to dephosphorylate IRS-1 in some (12), but not all (17), studies] was decreased after 4 wk of high-fructose diet feeding (2). Serine/threonine phosphorylation of IRS proteins attenuates their tyrosine phosphorylation (5, 24). In skeletal muscle, increased activity of protein kinase C isozymes has been observed after exposure to high-fat (27) and high-fructose (8) diets. Because hepatic triglycerides are increased after 1 wk on the sucrose-rich diet (21), it would be anticipated that lipid precursors of triacylglycerol (i.e., diacylglycerol, fatty acyl-CoA) that activate conventional and novel forms of protein kinase C would be increased in this dietary model. The extent to which simple sugars influence protein kinase C and other serine/threonine kinases in the liver is currently being investigated.

The activity of PI 3-kinase in anti-tyrosine phosphorylation immunoprecipitates was increased in saline-injected HSD rats. This activity appeared to influence downstream signaling, since phospho-Akt/PKBα was also increased in saline-injected HSD rats. The lipid kinase activity of PI 3-kinase, via the lipid product PI 3,4,5-P3, inhibits glucose-6-phosphatase (G-6-Pase) activity (18). PI 3,4,5-P3 inhibition of G-6-Pase activity is three times more efficient than its precursor, PI 4,5-P2, with an apparent inhibition constant of ∼1.7 µM (18). High-sucrose diets increase the maximal activity of G-6-Pase (4) but do not increase basal glucose production in vivo (19, 21). Thus elevated basal PI 3-kinase activity and increased provision of PI 3,4,5-P3 may be required in sucrose-fed animals to maintain normal rates of basal glucose production in vivo.

Immunoblot analysis of the p85α and p110β subunits of PI 3-kinase revealed that only the latter was significantly increased by the HSD. In previous studies, overexpression of the catalytic subunit of PI 3-kinase in 3T3-L1 adipocytes increased glucose transport activity and GLUT-4 translocation in the absence and presence of insulin (11, 14). However, in these studies, the exogenously expressed p110α subunit appeared to bind to the regulatory p85 subunit of PI 3-kinase. In the present study, when liver samples were immunoprecipitated with antibodies to the p85 subunit and immunoblotted with antibodies to the p110β subunit, no differences were observed between starch- and sucrose-fed rats. If the immunoblot analysis of p110β represents a real increase in content in sucrose-fed rats, these data suggest that it may not be bound to the regulatory subunit or receive signals transmitted through IRS proteins (31). Because the p85 subunit of PI 3-kinase can inhibit p110 activity (31), it is possible that the increase in p110 contributes to increased basal PI 3-kinase activity. Alternatively, changes in the amount of p110 may contribute to sucrose-induced insulin resistance. Recent studies have demonstrated that the expression of a constitutively active mutant p110 targeted to cellular membranes can desensitize specific insulin-signaling pathways in 3T3-L1 cells (9). In addition, it is possible that the increased amount of p110 increases the protein kinase activity of PI 3-kinase. It should be noted that the increase in p110β observed in sucrose-fed rats may also represent a preferential recognition by the antibody due to changes in the protein. However, this possibility seems unlikely, because other antibodies to p110β provided similar results (data not shown). Whether dietary nutrients influence the cellular distribution of these insulin-signaling proteins and/or upregulate serine/threonine kinases is under investigation.

Several features of this high-sucrose diet model make it useful for the study of hepatic insulin resistance. First, adaptations to the diet have been well characterized. These adaptations include reduced insulin suppression of glucose production and hepatic gluconeogenesis (20, 22), increased capacity for gluconeogenesis (4, 20), increased phosphoenolpyruvate...
carboxykinase, and G-6-Pase activity (4, 22), elevated hepatic triglycerides (15, 21), and increased saturated fatty acid content of liver triglycerides and sinusoidal membrane lipids (23). Importantly, these adaptations occur after only 1 wk of high-sucrose diet feeding (21). Moreover, lower-sucrose diets (18% of energy as sucrose) also produce many of the aforementioned adaptations but simply require a longer period of time to do so, >16 wk (19). Second, high-sucrose diet feeding can produce these adaptations independently of obesity and positive energy balance (19, 21, 22). Thus this dietary model provides a tool to understand the roles of dietary nutrients and cellular lipids in insulin signaling in the liver.

The present study demonstrates that impairments in postreceptor insulin signaling events, involving tyrosine phosphorylation of IRS proteins and activation of PI 3-kinase, contribute to sucrose-induced hepatic insulin resistance. High-sucrose diet feeding also increases basal PI 3-kinase activity and, perhaps, the amount of the p110β subunit of PI 3-kinase in the liver. It is hypothesized that the increased basal PI 3-kinase activity contributes to the maintenance of normal rates of basal glucose production in this model of insulin resistance.

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