

Compensatory alterations for insulin signal transduction and glucose transport in insulin-resistant diabetes

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Department of Molecular and Cellular Biochemistry, Loyola University Stritch School of Medicine, Maywood 60153; Research Service, Hines Veterans Affairs Hospital, Hines 60141; and Shock/Trauma Institute, Loyola University Medical Center, Maywood, Illinois 60153; Metabolic Diseases Research, The Upjohn Company, Kalamazoo, Michigan 49001; and Research Division, Joslin Diabetes Center, Boston, Massachusetts 02215

Bonini, James A., Jerry R. Colca, Charlene Dailey, Morris White, and Cecilia Hofmann. Compensatory alterations for insulin signal transduction and glucose transport in insulin-resistant diabetes. *Am. J. Physiol.* 269 (*Endocrinol. Metab.* 32): E759–E765, 1995.—Insulin binding activates the receptor tyrosine kinase toward the insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1 then interacts with the p85 α subunit of phosphatidylinositol 3-kinase (PI3K), Nck, growth factor receptor-bound protein 2 (GRB2), and Syp, thus branching insulin's signal for both mitogenic and metabolic responses. To determine whether the expression of these proteins is altered in insulin resistance, the levels of these proteins were compared in adipose and liver tissues of nondiabetic mice and obese insulin-resistant diabetic KKA ν mice. IR and PI3K p85 α protein levels were significantly lower in KKA ν mice than in control nondiabetic mice, whereas IRS-1 protein levels were not altered. In contrast, the protein levels of GRB2, Nck, Syp, and GLUT-1 were dramatically elevated in KKA ν fat, with less striking changes in liver. Treatment of diabetic animals with pioglitazone, an insulin-sensitizing antihyperglycemic agent, partially corrected the expression of some of these proteins. Taken together, these findings suggest that the insulin-resistant diabetic condition is characterized by changes in expression of insulin signal transduction components that may be associated with altered glucose metabolism.

insulin receptor substrate-1; phosphatidylinositol 3-kinase; growth factor receptor-bound protein 2; Syp; receptor

THE INSULIN RECEPTOR (IR) is a transmembrane protein composed of two α - and two β -subunits. Binding of insulin to the extracellular α -subunit results in tyrosine autophosphorylation of the transmembrane β -subunit for subsequent activation of this β -subunit as a tyrosine kinase toward intracellular substrates (27). One of the major proteins phosphorylated by the IR on insulin stimulation is the IR substrate-1 (IRS-1), a cytosolic protein with an apparent molecular mass of 160–185 kDa (27). Once IRS-1 becomes phosphorylated, it acts as a multisite docking protein via an interaction of its phosphotyrosines with *src*-homology 2 (SH2) domains of several target proteins (27). SH2-containing proteins that interact with IRS-1 on insulin stimulation include the p85 α subunit of phosphatidylinositol 3-kinase (PI3K), the tyrosine phosphatase Syp (also called SH-PTP2 or PTP 1D), growth factor receptor-bound protein 2 (GRB2), and Nck (25, 27).

The insulin-activated binding of these proteins to IRS-1 is thought to represent a point of divergence for insulin's signal to elicit pleiotropic effects (25, 27). PI3K

is composed of two subunits: an 85-kDa regulatory adaptor subunit and a 110-kDa catalytic subunit. Association of the 85-kDa subunit with phosphorylated IRS-1 activates the 110-kDa subunit to phosphorylate phosphatidylinositol moieties at the 3-position of the inositol ring (27). Although the physiological function of these phosphorylated products is unknown, inhibition of PI3K catalytic activity by the fungal toxin wortmannin inhibited insulin-stimulated glucose transport and GLUT-4 translocation to the plasma membrane (10). These findings are consistent with a role for PI3K in insulin-stimulated glucose transport.

Although PI3K appears to be involved principally in metabolic signaling, the downstream effects of Nck, Syp, and GRB2 appear to be mainly mitogenic. Nck is a 47-kDa protein composed of one SH2 and three SH3 domains and is likely to be involved in cell proliferation (17). Syp, a 65- to 72-kDa protein tyrosine phosphatase with two SH2 domains in its NH₂-terminals, has recently been linked to insulin stimulation of DNA synthesis (28). GRB2 is a 24-kDa protein that is composed of one SH2 domain flanked by two SH3 domains and has no known catalytic function of its own (27). However, association of GRB2 with tyrosine-phosphorylated IRS-1 results in activation of several downstream molecules, including ras, MAP kinase, and dynamin (1, 27). As a result, signaling through GRB2 leads to stimulation of DNA synthesis (27), increased expression and activity of the GLUT-1 glucose transporter (6, 14), and possibly IR internalization (1).

Cellular defects leading to insulin-resistant diabetes are currently unknown. Non-insulin-dependent diabetes mellitus (NIDDM) is usually associated both with insulin resistance of peripheral tissues (primarily adipose and muscle) and with a defect in insulin production and secretion by the pancreatic β -cells (9). Some cases of insulin resistance have been associated with IR mutations; however, the majority of insulin-resistant individuals likely have other defects, presumably in postreceptor signaling in target tissues (liver, muscle, and adipose) (9). Such defects could attenuate insulin's signaling for normal intracellular effects.

We have previously shown that target cells from KKA ν mice are insulin resistant and express less GLUT-4 than cells from nondiabetic mice and that these differences were removed after treatment of the diabetic mice with an experimental insulin sensitizer (8). We now have asked whether differences in insulin sensitivity in these

models are associated with differences in the expression of molecules thought to be involved in the early aspects of insulin signaling (IR, IRS-1, PI3K p85 α , GRB2, Nck, or Syp) and whether there was any alteration in the expression of these signaling molecules after treatment with an experimental drug that has been shown to improve insulin responsiveness under these conditions (8, 18). In addition, because of the potential link between GRB2 and GLUT-1, the ubiquitous glucose transporter responsible for basal glucose uptake, we measured GLUT-1 expression in adipose tissue from these animals. In the present study, insulin resistance was associated with reduced expression of the IR and PI3K p85 α , whereas GRB2, Nck, Syp, and GLUT-1 were expressed at a higher level in insulin-resistant animal tissues than those from nondiabetic controls. Furthermore, partial correction of the expression of these insulin-signaling components was observed in insulin-resistant mice treated with pioglitazone, an antihyperglycemic agent known to sensitize cells to insulin (8).

EXPERIMENTAL PROCEDURES

Reagents. Anti-GRB2, anti-Nck, and anti-Syp monoclonal antibodies were purchased from Transduction Laboratories (Lexington, KY). Anti-p85 α monoclonal antibodies were purchased from Upstate Biotechnologies (Lake Placid, NY), and anti-GLUT-1 antibodies were purchased from East Acres Biologicals (Southbridge, MA). Anti-IRS-1 monoclonal and polyclonal antibodies, IR β -subunit polyclonal antibodies, and IRS-1 cDNA were produced at the Joslin Diabetes Center (Boston, MA). Anti-mouse and anti-rabbit secondary antibodies conjugated to horseradish peroxidase, enhanced chemiluminescence (ECL) reagents, and cytidine 5'-[³²P]triphosphate were purchased from Amersham (Arlington Heights, IL). Protein A-Sepharose was purchased from Pharmacia (Milwaukee, WI). Nytran nylon membranes (Schleicher and Schuell, Keene, NH) and Immobilon polyvinylidene fluoride membranes (Millipore, Bedford, MA) were used for Northern and Western blotting, respectively. Riboprobe preparation kits were obtained from Promega (Madison, WI). IR cDNA was obtained from Graeme Bell (Chicago, IL), and p85 α cDNA was acquired from Lewis Cantley (Boston, MA). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Animal models and treatment. Three animal groups were studied: nondiabetic mice, insulin-resistant KKA^y mice, and pioglitazone-treated KKA^y mice. KKA^y mice result from a cross between glucose-intolerant black KK female mice and yellow obese male A^y mice. The yellow offspring KKA^y mice are an excellent model of NIDDM because they are obese and insulin resistant and display marked hyperinsulinemia and hyperglycemia (4). Male KKA^y mice 10–12 wk of age were obtained from the Upjohn colony. To study the effects of improvement of the diabetic state, animals were fed ad libitum for 4 days before the experiment with or without pioglitazone delivered as a food admixture at ~ 20 mg \cdot kg⁻¹ \cdot day⁻¹. It was previously determined that this dosage and delivery method produced maximal changes in blood glucose and insulin sensitivity in mice within 4 days (data not shown). On the 5th day from the start of drug treatment, the mice were bled from the orbital sinus for measurement of blood glucose concentrations and were decapitated, and liver and epididymal fat pads were extracted for protein and RNA isolation. As in previous studies, C57 Bl-J6 male mice of the same age were obtained from Charles River (Portage, MI) and used as nondiabetic controls (4, 8, 24, 26).

Western blot analysis of protein from epididymal fat and liver tissue. After treatment, animals were killed and tissues were removed for protein preparation or RNA isolation. Total cell lysates of the tissues destined for protein preparation were generated with an adaptation of a method previously described by Rice et al. (21), in that the tissue samples were homogenized in lysis buffer [tris(hydroxymethyl)aminomethane-buffered saline pH 7.6, 1% Triton X-100, 0.03 M sodium pyrophosphate, 1 mM vanadate, 10 mM E-64, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM EDTA] using a Tekmar Tissue-mizer (Cincinnati, OH) before centrifugation. For measurement of membrane GLUT-1 proteins, particulate fractions of cells were prepared essentially as previously described (3). Equal amounts of sample protein were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (15) and transferred to Immobilon membranes (Millipore) by use of the Bio-Rad Mini Protean II Dual Slab Cell and Mini Transblot apparatus (Richmond, VA). Blots were then immunostained for the desired protein using the appropriate antibodies and the ECL (Amersham) according to the recommendations of the supplier. For detection of IRS-1, 1 mg total protein from each sample was immunoprecipitated with a polyclonal anti-IRS-1 antibody directed against a baculovirus-overexpressed IRS-1, followed by Western blotting and immunostaining with a monoclonal anti-IRS-1 antibody. Signals were quantitated using the Ambis image acquisition analysis system (San Diego, CA), and loading correction was based on quantitation of duplicate Coomassie blue-stained blots, as previously described (8).

Northern blot analysis of RNA from fat and liver tissues. Total RNA was prepared, electrophoresed, and Northern-blotted as previously described (3, 8), followed by hybridization with ³²P-labeled antisense RNA probes using the Riboprobe system according to the method provided by the supplier (Promega). A mouse construct for the insulin receptor cDNA in pUC was obtained from Graeme Bell (Chicago, IL), and a 775 bp insert was subcloned into the *Eco*R I site of pGEM-3Z. A 5.5-kb construct for IRS-1 (in pBluescript) from Dr. Morris White (Boston, MA) was used, and a 1.55-kb cDNA for PI3K (in pBluescript) was obtained from Dr. Louis Cantley. The rat GLUT-1 cDNA (7) was obtained from Graeme Bell in pSPGT-1 and subcloned into the *Bam*H I site of pGEM-4Z. Loading corrections were done as previously described (2). The hybridization and washes were carried out under highly stringent conditions (8), and membranes were rinsed with ribonuclease (RNase) A (3 μ g/ml) and RNase T1 (0.005 U/ml) to remove nonspecific background when appropriate. The RNase rinse was particularly important for the GLUT-1 Northern blots to eliminate any possible cross hybridization with the comigrating GLUT-4 transcript. The blots were autoradiographed on X-ray film, and signals were quantitated as described above.

RESULTS

Protein levels of insulin-signaling mediators. To determine whether the protein levels of IR, IRS-1, p85 α , GRB2, Nck, and Syp were altered in insulin-resistant animals compared with control nondiabetic animals and to determine whether pioglitazone treatment corrected these alterations, protein extracts of epididymal fat and liver tissue from nondiabetic C57 mice, obese KKA^y insulin-resistant mice, and KKA^y mice treated with pioglitazone were analyzed by Western blotting. The abundance of the studied proteins was determined to be similar in liver and adipose tissue of nondiabetic control animals. In both epididymal fat and liver, the IR levels in

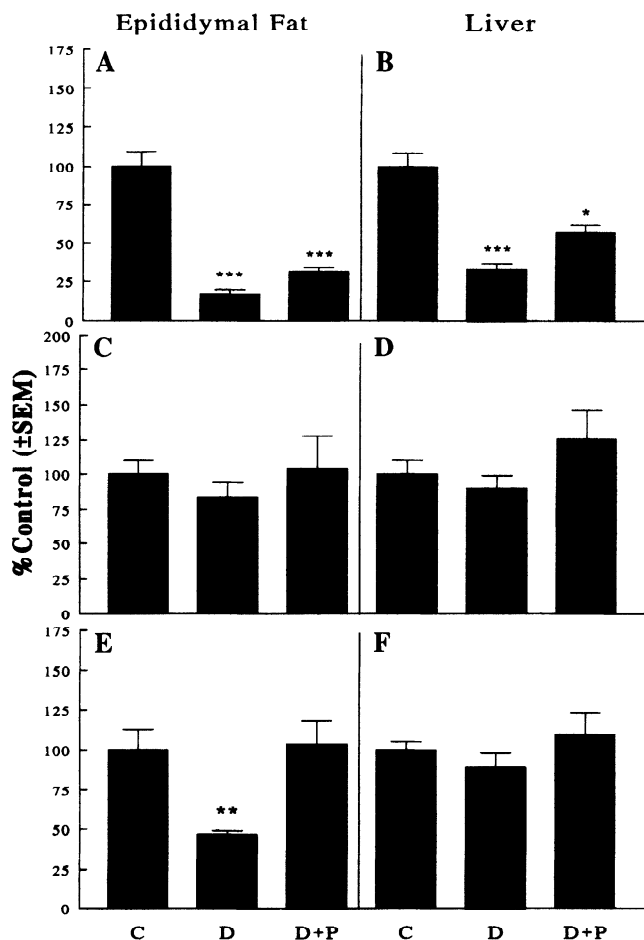


Fig. 1. Altered expression of the insulin receptor (IR, A and B), IR substrate-1 (IRS-1, C and D), and phosphatidylinositol 3-kinase (PI3K, E and F) p85 α . Equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes, and immunostained for the appropriate proteins; signals were quantitated using a video densitometer. Each graph depicts protein levels of C57 mice (C), KKA^y mice (D), and KKA^y mice treated with pioglitazone (D+P) as %Control C57, with epididymal fat data at left and liver data at right. Values are means \pm SE; $n = 12$ for all experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All statistical tests were performed using ANOVA and the Tukey-Kramer multiple comparisons test.

KKA^y mice were 17 ± 3 and $33 \pm 4\%$ of the levels in C57 mice, respectively. Treatment of KKA^y mice with pioglitazone increased IR levels to 32 ± 3 and $57 \pm 5\%$ of levels observed in the normal mice (Fig. 1, A and B). IRS-1 protein levels were not significantly different between C57 and KKA^y mice, and pioglitazone treatment did not significantly alter IRS-1 protein abundance in either tissue (Fig. 1, C and D). PI3K p85 α protein levels were significantly lower in KKA^y fat ($47 \pm 3\%$ of control) and were fully corrected by pioglitazone administration to $103 \pm 15\%$ of control levels (Fig 1E). However, p85 α levels in liver were not significantly different between C57 and KKA^y mice and were not altered by pioglitazone treatment (Fig. 1F).

GRB2, Nck, and Syp levels were regulated in an opposite manner from IR and p85 α levels. GRB2 protein abundance was significantly elevated in KKA^y epididymal fat ($321 \pm 25\%$ of control; Fig. 2A). In liver tissue,

the increase in GRB2 protein was not as great ($118 \pm 6\%$ of control), although the difference was significant (Fig. 2B). Pioglitazone administration did not alter fat GRB2 levels to a large extent, but it did lower the slightly elevated KKA^y liver GRB2 levels to control levels. Like GRB2, Nck levels were much higher in KKA^y fat compared with C57 mice ($186 \pm 12\%$ of control), and there was a smaller but significant elevation of Nck in KKA^y liver compared with C57 mice ($124 \pm 7\%$ of control; Fig. 2, C and D). Again, there was little effect of pioglitazone treatment on Nck protein levels in the treated KKA^y tissues. Syp protein levels were $43 \pm 11\%$ higher in KKA^y fat than in C57 (Fig. 2E) and $25 \pm 4\%$ higher in KKA^y liver compared with C57 (Fig. 2F). Both of these increases were statistically significant ($P < 0.05$). Pioglitazone treatment did not significantly change Syp levels in fat, but it did lower liver Syp levels in liver tissue toward control levels (Fig. 2, E and F).

mRNA abundance of IR, IRS-1, and p85 α transcripts. PI3K has been implicated in insulin-stimulated glucose transport (10), and KKA^y mice display a defect in insulin's ability to promote glucose uptake in fat and

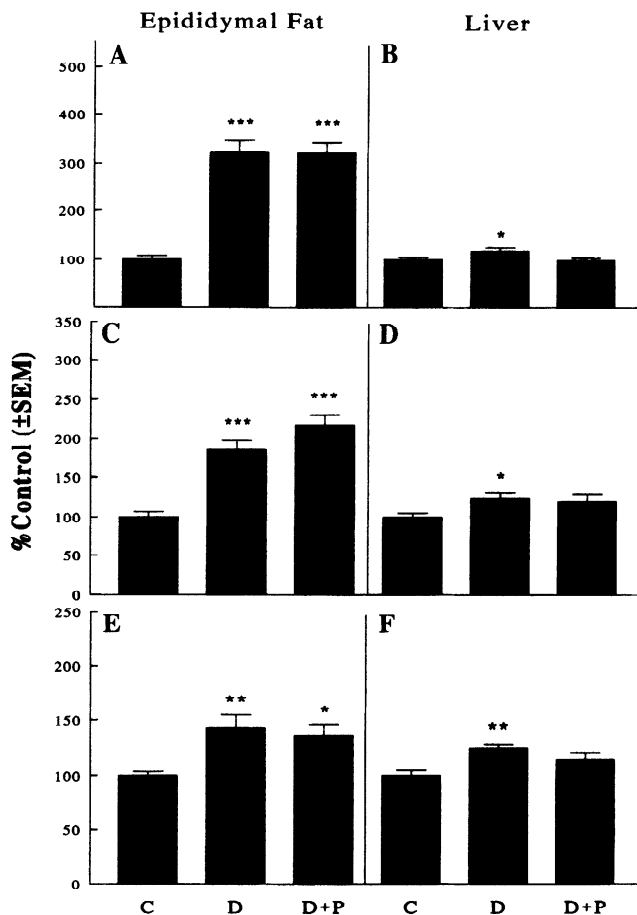


Fig. 2. Altered expression of GRB2 (A and B), Nck (C and D), and Syp (E and F). Equal amounts of total protein were analyzed as discussed in Fig. 1. Each graph depicts protein levels of C57 mice (C), KKA^y mice (D), and KKA^y mice + pioglitazone (D+P) as %Control C57, with epididymal fat data at left and liver data at right. Values are means \pm SE; $n = 12$ for all experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. All statistical tests were performed using ANOVA and the Tukey-Kramer multiple comparisons test.

Table 1. *RNA transcripts encoding IR, IRS-1, and PI3K p85 α in epididymal fat expressed as %C57 nondiabetic control*

Transcript	Animal Groups		
	C57	KKA ^y	KKA ^y + Pio
IR			
9.5 kb	100 \pm 17.7	65.7 \pm 9.3	79.2 \pm 5.3
7.5 kb	100 \pm 17.0	66.3 \pm 8.0	99.3 \pm 10.8
IRS-1	100 \pm 14.6	53.3 \pm 4.3*	53.4 \pm 13.5
PI3K	100 \pm 12.3	75.3 \pm 6.2	66.7 \pm 3.9*

Total epididymal fat RNA (10 μ g) was separated on 1.1% agarose gels, transferred to nylon membranes, probed for the appropriate mRNA, and quantitated. Values are means \pm SE; $n = 12$. The 2 insulin receptor (IR) transcripts (7.5 and 9.5 kb) were compared separately in the 3 groups. There was no difference in the ratio of 9.5- to 7.5-kb transcripts between groups (data not shown). IRS-1, IR substrate-1; PI3K, phosphatidylinositol 3-kinase; KKA^y + Pio, KKA^y mice treated with pioglitazone. * $P < 0.05$.

muscle (8). Therefore potential mechanisms for lowered levels of IR, IRS-1, and p85 α proteins were determined by analyzing the mRNA abundance of these transcripts in tissues of nondiabetic C57 mice, obese KKA^y insulin-resistant mice, and KKA^y mice treated with pioglitazone. A Northern blot for the IR showed transcripts of two lengths: 9.5 kb and 7.5 kb. The difference in size between these two transcripts results from differential poly-A site selection, and there is no known functional difference between the 9.5- and 7.5-kb transcripts (16). The IRS-1 mRNA transcript is 9.5 kb long, and the primary p85 α mRNA size is 7.5 kb. The abundance of these transcripts was determined as described in EXPERIMENTAL PROCEDURES. Data are summarized in Tables 1 and 2. In epididymal fat and liver, receptor mRNA levels were slightly less in the KKA^y compared with C57 mice, although the only significant difference was in liver tissue with the 7.5-kb transcript. Pioglitazone administration did not have a significant effect on receptor mRNA levels. There was no detectable difference in the 9.5- to 7.5-kb ratio between any of the groups. IRS-1 mRNA levels were significantly lower in KKA^y fat and liver compared with the C57 controls, with no correction by pioglitazone treatment. However, this difference in IRS-1 mRNA abundance was not associated with a significant lowering of IRS-1 protein levels (Fig. 1, C and

Table 2. *RNA abundance of IR and IRS-1 transcripts in liver*

Transcript	Animal Groups		
	C57	KKA ^y	KKA ^y + Pio
IR			
9.5 kb	100 \pm 9.1	70.2 \pm 11.7	79.8 \pm 9.5
7.5 kb	100 \pm 8.8	66.8 \pm 8.7*	70.6 \pm 8.0
IRS-1	100 \pm 7.2	50.7 \pm 6.2*	71.7 \pm 7.1

Total liver RNA (10 μ g) was analyzed by Northern blotting. Values are means \pm SE expressed as %Control C57; $n = 12$ for IR and 22 for IRS-1. The 2 IR transcripts (7.5 and 9.5 kb) were compared separately in the 3 groups. There was no difference in the ratio of 9.5- to 7.5-kb transcripts between groups (data not shown). PI3K p85 α mRNA levels were not determined in liver because there was no measurable difference in p85 α protein levels in this tissue. * $P < 0.05$.

D). In epididymal fat, p85 α mRNA abundance was not significantly different between the C57 and KKA^y mice, and pioglitazone treatment did not significantly alter the p85 α mRNA levels in the treated compared with the untreated KKA^y group. The mRNA levels of p85 α were not determined in liver tissue, because there was no difference in the level of p85 α protein in this tissue.

Protein and mRNA abundance of GLUT-1. Because overexpression of *ras* or *raf-1* has been shown to cause increased expression and activity of GLUT-1 in 3T3-L1 adipocytes (6, 14), and because GRB2 has been linked to *ras* activation through Son of Sevenless (SOS) (27), we sought to determine whether the high expression of GRB2 in epididymal fat from KKA^y mice relative to control mice was associated with a concordant increase in the expression of GLUT-1. As shown in Fig. 3A, GLUT-1 protein was in fact elevated to 234 \pm 22.3% of control in fat of KKA^y mice and was corrected to 106.4 \pm 26%, the level of nondiabetic mice, by pioglitazone treatment of such animals. The GLUT-1 mRNA levels were likewise elevated in the KKA^y mice to 338.1 \pm 59.5% of control but were not corrected by pioglitazone administration (329.1 \pm 67.2% control).

DISCUSSION

Insulin resistance in NIDDM is thought to result in many cases from a postreceptor defect in insulin signaling in peripheral tissues (9). Such a defect could attenuate insulin's signal, resulting in an increased demand for insulin to lower blood glucose levels. The results presented in this study show that alterations in the expression of several early components in insulin signaling are apparent in an in vivo model of insulin resistance. These alterations can be separated into two categories: proteins that are expressed at a lower level in insulin resistance compared with insulin-sensitive control animals, and proteins that are present at higher levels in insulin resistance compared with control.

Proteins that show a lowered expression in insulin-resistant diabetes are the IR and the p85 α subunit of PI3K, as shown in Fig. 1, A, B, and E. The difference in IR protein levels (Fig. 1, A and B) was much greater than the change in receptor mRNA abundance between C57 and KKA^y mice (Tables 1 and 2), and therefore the lowered expression of receptor in KKA^y tissues likely includes differences in posttranslational processes. In the case of the IR, it is known that high levels of circulating insulin will cause internalization and degradation of the receptor (13). KKA^y mice also have very high circulating insulin levels that are partially corrected by pioglitazone treatment (8). Such insulin levels could account for the lower IR protein in KKA^y mice and the partial correction of this deficit in liver tissue of the KKA^y group treated with pioglitazone (Fig. 1B).

PI3K p85 α protein levels were much lower in epididymal fat of diabetic KKA^y mice than in that of nondiabetic C57 mice but did not differ in liver tissue between these groups. The reason for this tissue-specific difference is not known; however, it appears that PI3K p85 α expression is differentially regulated in various tissues. Another possible interpretation is that there is a specific

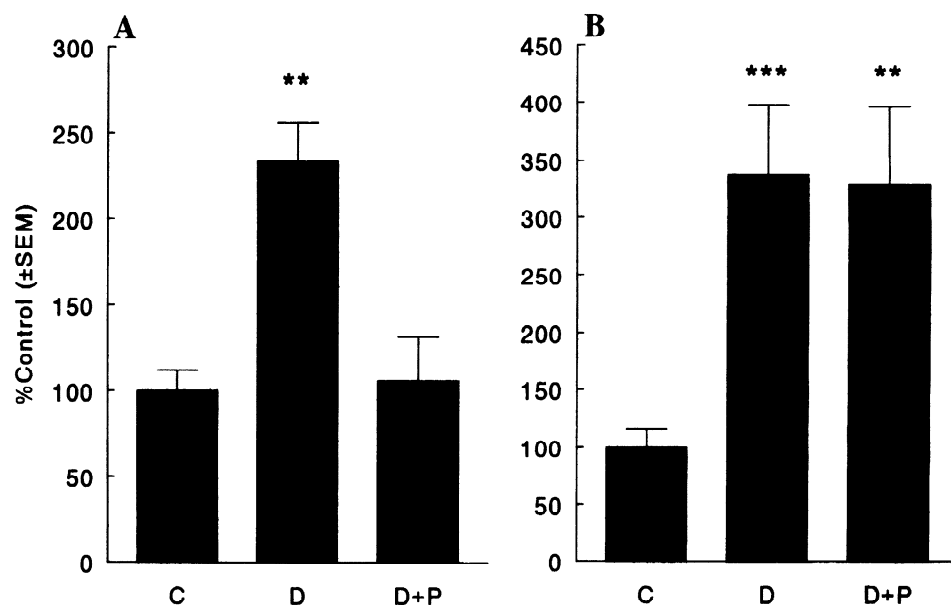


Fig. 3. Altered expression of GLUT-1 in epididymal fat. Equal amounts of protein (A) or RNA (B) were analyzed. Each graph depicts protein levels of C57 mice (C), KKA^y mice (D), and KKA^y mice + pioglitazone (D+P) as %Control C57. Values are means \pm SE; $n = 6$ for protein levels and $n = 12$ for RNA levels. All statistical tests were performed using ANOVA and the Tukey-Kramer multiple comparisons test. ** $P < 0.01$; *** $P < 0.001$ vs. control.

defect in PI3K p85 α expression in fat of the KKA^y mice that does not exist in liver tissue. It is interesting to note that there is not as large a reduction in the p85 α mRNA as in the protein levels (25 vs. 50%, respectively) in the KKA^y epididymal fat. Also pioglitazone treatment of KKA^y mice resulted in increased p85 α protein levels without a similar rise in p85 α mRNA abundance. This observation suggested that the regulation of p85 α protein levels by pioglitazone may lie in a translational or posttranslational process. Recent reports have implicated PI3K in insulin-stimulated glucose transport (10), and because pioglitazone has been shown to correct glucose transport deficiencies in diabetic animals (8), it is possible that pioglitazone is acting to correct glucose levels through altering the availability of PI3K p85 α .

Unlike the levels of the IR and p85 α , IRS-1 levels were not significantly different between diabetic KKA^y mice and nondiabetic C57 control animals. Although IRS-1 mRNA abundance was lower in tissues of KKA^y mice than in C57 mice (Tables 1 and 2), and the protein levels tended to be somewhat lower in KKA^y mice compared with control mice (Fig. 1, C and D), the difference in IRS-1 protein expression did not achieve statistical significance in fat or liver tissue. This differs slightly from a previous report, in which IRS-1 protein levels were significantly decreased in diabetic *ob/ob* mouse liver (and muscle) compared with normal *ob/+* controls (23), and from other reports in which treatment of 3T3-L1 cells with high insulin concentrations led to degradation of IRS-1 (22). On the basis of these data indicating the relatively labile nature of IRS-1 in the presence of high insulin levels, one would expect to see a decreased level of IRS-1 protein in hyperinsulinemic animals expressing less IRS-1 mRNA. However, this was not the case in this model.

The present study also showed that several proteins involved in insulin signaling are elevated in the obese KKA^y mouse model of insulin resistance. GRB2, Nck, and Syp levels were all significantly higher in epididymal fat from KKA^y than in the same tissue from C57 mice.

The levels of these proteins were also higher in liver tissue of KKA^y mice compared with C57 mice, although the differences were not as great as those in epididymal fat. All three of these proteins have been implicated in mitogenesis, DNA synthesis, and cell growth (17, 27, 28). Although the significance of the elevated protein levels for these growth-related insulin-signaling mediators in obese KKA^y adipose tissue is unknown, it is possible that the increase may occur in compensation for reduced signaling in the insulin-resistant cells. The hypothesis for a compensatory mechanism of signaling in KKA^y mice is supported by observations of the expression of glucose transporters. Adipose tissue contains both GLUT-1 and GLUT-4 glucose transporters, although GLUT-4 is the predominant species (12). GLUT-1 is present in many tissues and is believed to be responsible for basal glucose uptake (12), whereas GLUT-4 is known to be the transporter responsible for the majority of insulin-stimulated glucose uptake in fat and muscle tissue (12). In prior studies, the expression of GLUT-4 was shown to be significantly lower (58% control) in epididymal fat from KKA^y mice than in control C57 mice, and KKA^y mice were also nearly unresponsive to insulin stimulation of glucose uptake (8). The lower levels of GLUT-4 and PI3K p85 α (Fig. 1) in adipose tissue could contribute to the hyperglycemia of insulin-resistant KKA^y mice, because both have been implicated in insulin-stimulated glucose uptake (10, 12).

In contrast to this insulin-stimulated pathway for glucose transport, the levels of GRB2 and GLUT-1 in adipose tissue from this model of insulin resistance were much higher than levels in nondiabetic mice (Figs. 2 and 3, respectively). GRB2 binding to phosphorylated IRS-1 causes it to bind to and activate the guanine nucleotide exchange factor SOS, leading to activation of ras (27). Activated ras binds to and activates raf-1, which then phosphorylates MAP kinase kinase. Overexpression of constitutively active forms of either ras or raf-1 in 3T3-L1 adipocytes has been shown to increase the expression and activity of GLUT-1, resulting in in-

creased basal glucose uptake (6, 14). Thus the elevated levels of GRB2 and GLUT-1 may be causally related and give rise to the higher basal glucose uptake in adipocytes from these animals compared with those from nondiabetic mice (8).

Further evidence for a role of GLUT-1 upregulation as a compensatory response comes from studies in which 3T3-L1 adipocytes were deprived of glucose for 24 h. Such treatment resulted in an increase in GLUT-1 and a decrease in GLUT-4 expression (11). During glucose deprivation, GLUT-1 was found primarily in the plasma membrane, and the increase in GLUT-1 was attributed to an increase in the rate of synthesis and stability of the protein. One potential role of GLUT-1 is to act as a stress response protein, and it is thought that the upregulation of GLUT-1 in this condition was a compensatory response to meet the glucose requirement of the cells (12, 20). In the case of KKA^y mice, it is thus possible that the elevated levels of GRB2 and GLUT-1 are a compensatory response to the deficient glucose uptake resulting from insulin resistance and the lowered levels of IR, PI3K p85 α , and GLUT-4 in these animals.

Alternatively, GRB2 expression could be increased in response to the hyperinsulinemic condition of the animals. The link of GRB2 with dynamin (1) and the potential role of dynamin in receptor internalization suggest that increased GRB2 expression may be a compensatory response involved in meeting the increased demand for receptor internalization in hyperinsulinemia.

The altered levels of Nck and Syp in KKA^y mice may also affect cellular signaling. Nck overexpression has previously been shown to lead to cell transformation (17), and it was hypothesized that an increase in Nck protein could alter its affinity or specificity for downstream signaling components (5). Further study is needed to reveal how such a change might be related to insulin resistance and obesity. Syp protein levels were elevated to a lesser extent than either GRB2 or Nck in KKA^y mice, but it is quite possible that an alteration in expression of a protein with tyrosine phosphatase activity could affect a pathway that is activated by a tyrosine kinase. In fact, Syp was reported earlier to bind and dephosphorylate the IR in vitro (19).

Treatment of KKA^y mice with the insulin sensitizer pioglitazone corrected the expression of some of these proteins toward control levels. Although pioglitazone administration did not significantly alter the levels of GRB2, Nck, or Syp in KKA^y epididymal fat, the levels of GLUT-4 (8) and p85 α in KKA^y mice were increased from 58 to 107% of control and 47 to 103% of control, respectively, by such treatment. Likewise, pioglitazone treatment of KKA^y mice decreased GLUT-1 expression to control levels. Like p85 α , GLUT-1 protein was corrected to control levels in the absence of a correlative change in mRNA levels. Such observations suggest that the mechanism of pioglitazone action is to affect translation or stability of these proteins, or possibly that the correction of the diabetic condition in these animals by

pioglitazone results in activation of cellular processes that alter the translation or stability of these proteins.

Therefore there are differential alterations in the expression of several proteins involved in early insulin-signaling events. It is not clear whether these alterations are partly responsible for the insulin-resistant diabetes in KKA^y mice or whether they are a result of the diabetic condition itself. However, the link of elevated GRB2 and GLUT-1 expression with elevated basal glucose uptake in adipocytes from these animals suggests a compensatory mechanism for glucose entry in a condition in which normal insulin-stimulated glucose uptake is attenuated by lowered levels of GLUT-4 (8), p85 α , and/or IRs. The corrective action of pioglitazone treatment on p85 α and GLUT-4 expression may therefore lower GLUT-1 levels by reducing the need for compensation. Further studies are required to determine whether factors such as hyperinsulinemia or hyperglycemia associated with the diabetic condition are responsible for some of the observed differences between nondiabetic and diabetic animals. In addition, the expression of signaling molecules such as Shc, GAP, ras, raf, and other more distal signaling components should be studied in this model to further understand the regulation of insulin signaling in insulin resistance.

These findings suggest that components of the insulin-signaling pathway are differentially regulated in insulin-resistant diabetes and may indicate that a compensatory mechanism exists by which the expression of some signaling proteins is increased in the presence of a diminished expression of others. Also, partial correction of the expression of these insulin-signaling components was observed in insulin-resistant mice treated with pioglitazone, indicating that this antihyperglycemic agent may sensitize cells to insulin by altering postreceptor signaling.

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