Altered Regulation of Insulin Signaling Components in Adipocytes of Insulin-Resistant Type II Diabetic Goto-Kakizaki Rats

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We investigated the cellular mechanism(s) of insulin resistance associated with non–insulin-dependent diabetes mellitus (NIDDM) using adipocytes isolated from non-obese, insulin-resistant type II diabetic Goto-Kakizaki (GK) rats, a well-known genetic rat model for type II diabetic humans. In adipocytes isolated from control rats, insulin (5 nmol/L) stimulated particulate serine/threonine protein phosphatase-1 (PP-1) activity (56% increase over the basal value after 5 minutes). In contrast, adipocytes from diabetic GK rats exhibited a 32% decrease in basal ($P < .05$) and a 65% decrease in insulin-stimulated PP-1 activity compared with values in control Wistar rats. Conversely, cytosolic PP-2A activity was elevated in diabetic GK rats in the basal state (twofold increase *v* controls, $P < .05$). Insulin treatment resulted in a 50% to 60% inhibition in PP-2A activity in control rats, but failed to inhibit PP-2A activity in diabetic GK rat adipocytes. The defects in PP-1/PP-2A activation/inactivation were accompanied by inhibition of insulin's effect on mitogen-activated protein kinase (MAPK) activation. In addition, insulin-stimulated tyrosine phosphorylation of insulin receptor (IR) substrate-1 (IRS-1) was decreased more than 90% compared with control values, while a twofold increase in basal IRS-1 phosphorylation status was observed in diabetic GK rats. The abnormalities in IRS-1 phosphorylation were accompanied by a severe impairment of insulin-mediated targeting of the Grb2/Sos complex to the plasma membrane. We conclude that (1) a rapid activation of PP-1 along with concomitant inhibition of cytosolic PP-2A may be important in the mechanism of insulin action in a normal cell, and (2) the resistance to insulin in terms of glucose uptake and glycogen synthesis observed in diabetic GK rats is partly due to defective regulation of PP-1, PP-2A, and MAPK caused by multiple defects in the upstream insulin signaling components (IRS-1/phosphatidylinositol-3-kinase [PI3-kinase] and Grb2/Sos) that participate in insulin-mediated activation of PP-1 and inactivation of PP-2A.

THE MOLECULAR MECHANISMS of insulin associated with non–insulin-dependent diabetes mellitus (NIDDM) and obesity remain unclear. A major defect in insulin-stimulated glucose uptake and its intracellular metabolism appears to underlie the peripheral insulin resistance commonly observed in most NIDDM subjects.[1] Although the exact mechanism by which insulin regulates intracellular glucose metabolism is unclear, it is well established that insulin uses multiple, interrelated, cross-talk signaling pathways to control cellular metabolism and growth.[2]

The activation of most insulin signaling components and the downstream substrates of insulin action are controlled by covalent modification via phosphorylation and dephosphorylation.[3] Insulin is known to increase tyrosine phosphorylation of the insulin receptor (IR) and its substrates, IR substrate-1 (IRS-1), pp60, and *Shc*.[4] Phosphorylation of IRS-1 and *Shc* creates docking sites for several *Src* homology 2 (SH2) or *Src* homology 3 (SH3) domain–containing proteins, thereby linking tyrosine kinase activation of the IR to activation of at least two major pathways, one involving a *ras*/mitogen-activated protein kinase (MAPK) cascade and the other involving phosphatidylinositol-3-kinase (PI3-kinase).[2,5-6]

Activation of the *ras*/MAPK pathway is initiated by the interaction of growth factor receptor–bound protein 2 ([Grb2] a SH2 domain–containing protein) with tyrosine-phosphorylated IRS-1 or *Shc*, leading to activation and translocation of the *ras*-GTP exchange factor, son of sevenless (Sos), to the plasma membrane.[7-8] In some cell types, the insulin-stimulated Grb2/IRS-1 complex is not associated with Sos activity and does not subsequently lead to *ras* activation,[9-11] suggesting a direct role for *Shc* in insulin signaling of *ras* bypassing IRS-1.

Tyrosine-phosphorylated IRS-1 also binds the p85 subunit of PI3-kinase, which activates the 110-kd catalytic subunit of PI3-kinase.[2,12-13] Activation of PI3-kinase results in phosphorylation and activation of its downstream target, protein kinase B (c-Akt/Rac),[14] leading to phosphorylation (inactivation) of glycogen synthase kinase-3 and phosphorylation (activation) of the 70-kd ribosomal S6 kinase,[15] as well as increased glucose transport.[16-17]

In addition to these signaling pathways, insulin also activates a phospholipid signaling system resulting in the activation of protein kinase C (PKC).[18-19] How these signaling systems operate and what processes they control are important questions to be answered to understand the control of normal metabolism, as well as the pathogenesis of insulin resistance associated with NIDDM.

Non-obese, insulin-resistant Goto-Kakizaki (GK) rats are a highly inbred strain of Wistar rats that spontaneously developed type II diabetes.[20] This genetic rat model is particularly relevant to understanding human type II diabetes, because defects in glucose-stimulated insulin secretion, peripheral insulin resistance, and hyperinsulinemia are seen as early as 2 to 4 weeks after birth.[21] Therefore, this model provides a valuable tool for dissecting the pathogenesis of insulin resistance and its cellular basis. Recent studies on this rat model have demonstrated defective insulin-stimulated intracellular glucose metabolism, especially glycogen synthesis in skeletal muscle.[22] These defects are accompanied by chronic activation of diacylglycerol-sensitive PKC.[23]

To gain insight into the mechanisms that underlie insulin resistance associated with NIDDM, we examined the effect of type II diabetes on insulin regulation of the serine/threonine

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protein phosphatase-1 (PP-1) and protein phosphatase-2A (PP-2A), two major enzymes involved in the control of cellular metabolism, MAPK, and some upstream insulin signaling components (IRS-1 Sos/Grb2 association and its translocation to plasma membranes) using adipocytes isolated from the epididymal fat pads of non-obese, insulin-resistant type II diabetic GK rats.

The results of this study indicate that type II diabetes in GK rats results in opposing effects on adipocyte PP-1 and PP-2A activities. An elevation in basal cytosolic PP-2A activity and a lack of insulin-mediated PP-2A inactivation are accompanied by marked reductions in basal and insulin-stimulated particulate PP-1 activity and attenuation of MAPK activation by insulin. The observed defects in the regulation of these two phosphatases and MAPK may be due to a desensitization and repression of the upstream insulin signaling components, as evidenced by decreases in the tyrosine phosphorylation of IRS-1 Grb2/Sos and its association and translocation to the plasma membrane.

MATERIALS AND METHODS

Materials

Type 1 collagenase was obtained from Worthington Biochemicals (Freehold, NJ). Bovine serum albumin (radioimmunoassay grade), dithiothreitol, phenylmethylsulfonyl fluoride, leupeptin, benzamidine, pepstatin A, antipain, aprotinin, trypsin inhibitor (STI), sodium fluoride, sucrose, sodium pyrophosphate, sodium orthovanadate, Triton X-100, sodium dodecyl sulfate (SDS), myelin basic protein (MBP), and protein A Sepharose CL-6B were purchased from Sigma Chemicals (St Louis, MO). [^{32}P]orthophosphoric acid, τ-[^{32}P]adenosine triphosphate [ATP] (specific activity $>$ 3,000 Ci/mmol), and [^{125}I]-labeled protein A and protein G were purchased from New England Nuclear (Boston, MA). Phosphorylase *b* and phosphorylase kinase were from Life Technologies (GIBCO/BRL, Grand Island, NY). Okadaic acid (OA) was obtained from Moana Bioproducts (Honolulu, HI). Reagents for SDS/polyacrylamide gel electrophoresis (PAGE) and immunoblotting were purchased from Bio-Rad (Richmond, CA). Polyclonal anti–IRS-1 antibody was obtained from UBI (Lake Placid, NY). Antibodies against Grb2, Sos, and MAPK were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal anti-rabbit Glut-4 antibody (R820) was purchased from East Acres Biologicals (Southridge, MA), and porcine insulin was a kind gift from Eli Lilly (Indianapolis, IN).

Experimental Procedures

Control Wistar male rats (body weight, 160 to 170 g) were obtained from Harlan Industries (Frederick, MD). A colony of type II diabetic GK rats (originally derived by repeated inbreeding of glucose-intolerant Wistar rats[20]) was established at Winthrop Hospital, with the animals kindly supplied by Dr Robert V. Farese (VA Hospital, Tampa, FL). Seven- to 8-week-old GK rats and control Wistar rats were used throughout the study.

Isolated adipocytes were prepared from control and GK rats by collagenase digestion as detailed previously.[24] Aliquots of adipocytes (0.5 to 3 mL) were resuspended in 2 to 3 mL Krebs-HEPES buffer, pH 7.4, containing 3% bovine serum albumin and 30 mg/dL glucose and incubated with insulin (5 to 10 nmol/L) for 0 to 5 minutes at 37°C. At the end of incubation, the medium was removed and cells were processed for (1) extraction and assay of PP-1 and PP-2A activities in the particulate and cytosolic fractions, (2) isolation of plasma membranes (PMs) and intracellular membranes (IMs), (3) immunoprecipitation of the IR and IRS-1 followed by Western blot analysis of the immunoprecipitates with antiphosphotyrosine antibody, and (4) immunoprecipitation of MAPK and assay of in vitro kinase activity using τ-[^{32}P]ATP and MBP as a substrate.

Extraction and assay of PP-1 and PP-2A activities in the particulate and cytosolic fractions. Serine/threonine protein phosphatases were extracted from the adipocytes of control and GK rats before and after insulin treatment. The cell extracts were fractionated by ultracentrifugation[25] and assayed as detailed in our recent report.[26] [^{32}P]-labeled glycogen phosphorylase *a* was used as a substrate. OA at a concentration of 2 nmol/L was used to discriminate activities due to PP-1 and PP-2A.[27] As indicated in our previous studies,[26,28] at this concentration OA inhibits PP-2A, and the remaining activity represents PP-1.

Immunoprecipitation of PP-1$_G$ and assay of PP-1 catalytic activity in the immunoprecipitates. For measurement of PP-1 enzyme activity in the immunoprecipitates of control and insulin-exposed cells, immunoprecipitations were performed on ice as described previously.[26] Briefly, the particulate fractions in lysis buffer (100 μg protein) were precleared by incubation with rat immunoglobulin G (5 μg/ml, coupled to protein-A Sepharose) at 4°C for 1 hour. The supernatants were immunoprecipitated with PP-1$_G$ subunit antibody (10 μg/mL) for 1 hour, followed by treatment with 50 μL protein A Sepharose CL6B (50% vol/vol) for 1 hour. In some experiments, the antibody was preincubated with the competing peptide before adding to the cell lysates. The immunocomplexes were washed four times with ice-cold wash buffer, resuspended in the same buffer containing 15 μg/mL site-1 peptide (against which the antibody was raised), and incubated at 4°C for 1 hour to release the bound enzyme from the immunocomplex. An aliquot of the supernatant was assayed for PP-1 activity as already described.

Analysis of Sos/Grb2 association and its translocation to plasma membranes. IMs and PMs were prepared according to the protocol described by Ramlal et al.[29] PMs were separated from mitochondria and low-density microsomes (LDMs) by sucrose density centrifugation as detailed in our previous report.[30] The membranes were solubilized in lysis buffer containing protease inhibitors and phosphatase inhibitors. Equal amounts of cell lysates (100 μg protein) in a final volume of 1 mL were incubated with 10 μL polyclonal anti-Sos antibody for 3 hours at 4°C with constant mixing, followed by addition of 25 μL protein A Sepharose (50% vol/vol). The incubation was continued for 2 hours. The immunoprecipitates were washed four times with 1 mL ice-cold lysis buffer and once with ice-cold PBS. The beads were boiled for 10 minutes with 25 μL 3× Laemmli sample buffer to release the bound antigen/antibody complex. The immunoprecipitates were separated on a 10% SDS/polyacrylamide gel, followed by Western blotting with anti-Grb2 antibody[7-8] and detection with [^{125}I]-protein A.[31] Signal intensity was quantified by densitometric analysis of the bands. The blots were stripped and reprobed with a polyclonal anti-Sos antibody to assess the relative content of Sos in the immunoprecipitates. The percent association of Grb2 with Sos was calculated by dividing the values for Grb2 bands by those for Sos bands.

Immunoprecipitation of the IR and IRS-1 from cell lysates and Western blot analysis. Adipocytes from control and insulin-treated cells were solubilized in buffer containing 1% SDS with protease and phosphatase inhibitors. The lysates were centrifuged at 16,000 × *g* for 10 minutes. Equal amounts of clear supernatants (500 μg protein) from control and diabetic rat adipocytes were incubated overnight at 4°C with a polyclonal anti-IR antibody (10 μL; Transduction Laboratories, Lexington, KY) or anti–IRS-1 antibody (2 μL; UBI). The next day, 50 μL protein A Sepharose beads (50% vol/vol) were added and the incubation was continued for an additional 2 hours. The beads were washed with lysis buffer as already described, followed by separation of the immunoprecipitates by SDS/PAGE. The proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane and probed with antiphosphotyrosine antibody (Zymed Laboratories, San. Francisco, CA), followed by detection with [^{125}I]-protein A. Tyrosine phosphorylation of IRS-1 and the IR was quantified by measuring the intensity of the

bands by densitometric analysis of the autoradiograms, and corrected for variations in the content of IR or IRS-1 in the immunoprecipitates by dividing the values for tyrosine-phosphorylated bands by those for protein bands.

In vivo phosphorylation and immunoprecipitation of IRS-1. Adipocytes (2 to 3 mL) were incubated with [^{32}P]-orthophosphate (0.3 mCi/mL) in Krebs-Ringer HEPES buffer for 2 hours, followed by insulin (10 nmol/L) for 1 to 5 minutes. The cells were rinsed four times with 1 mL ice-cold PBS containing phosphatase and protease inhibitors[26] and homogenized in buffer containing 20 mmol/L HEPES (pH 7.4), 1 mmol/L EDTA, 2 mmol/L sodium vanadate, 100 mmol/L sodium pyrophosphate, 100 mmol/L sodium fluoride, 40 mmol/L β-glycerophosphate, 1 mmol/L benzamidine, 0.1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, aprotinin, antipain, STI, and pepstatin A, 100 mmol/L NaCl, and 1% Triton X-100. Equal amounts of lysates (500 μg protein) were precleared with rat IgG (5 μg/mL, coupled to protein A Sepharose) at 4°C for 1 hour. The supernatants were immunoprecipitated overnight with a polyclonal anti–IRS-1 antibody (2 to 5 μL). The next day, 100 μL protein A Sepharose beads (50% vol/vol) were added and the incubation was continued for an additional 2 hours with shaking. The beads were washed with lysis buffer as already described, followed by separation of the immunoprecipitates by SDS/PAGE. The proteins were transferred to a PVDF membrane, and the blots were subjected to autoradiography at −70°C. The content of IRS-1 protein in the immunoprecipitates was estimated by probing the blots with an anti–IRS-1 antibody followed by incubation with horseradish peroxidase–conjugated secondary antibodies and detection by enhanced chemiluminescence (ECL).

Immunoprecipitation and assay of MAPK activity in vitro. Adipocytes were lysed with 1 mL ice-cold RIPA buffer containing 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, and 1 mmol/L sodium orthovanadate. The lysates were centrifuged at 16,000 × *g* for 10 minutes, and equal amounts of clear supernatants (100 μg protein) were immunoprecipitated for 2 hours with 5 μg anti-MAPK antibody (Zymed; prebound to protein A Sepharose) at 4°C with constant shaking. The immunoprecipitates were washed four times with ice-cold lysis buffer and once with kinase buffer. The beads were reconstituted with 25 μL kinase buffer and assayed for kinase activity[32] using MBP (final concentration, 0.2 mg/mL) and 1 μL τ-[^{32}P]ATP (specific activity, 3,000 Ci/mmol). After a 10-minute incubation at 30°C, the reaction was arrested by transferring half of the reaction mixture to peptide paper (phosphocellulose discs; GIBCO/BRL), which was then immersed in a dish containing 180 mmol/L orthophosphoric acid. The peptide paper was washed five times for 5 minutes each in phosphoric acid and dried in acetone. The radioactivity bound to the paper was counted in a scintillation counter.

Protein assay. Proteins in the cellular extracts and lysates were quantified using bicinchonic acid[33] or the Bradford technique.[34]

Statistics

The results are presented as the mean ± SEM of four to six individual experiments performed in duplicate. Student's *t* test or ANOVA was used to compare mean values among control and diabetic rats.

RESULTS

Characteristics of Diabetic GK Rats

As reported previously,[20-21] the insulin resistance of diabetic GK rats was accompanied by a twofold increase in postprandial serum glucose when compared with Wistar controls (19.5 ± 2.6 *v* 8.9 ± 2.8 mmol/L, blood samples collected at the time of death at 8 AM). In contrast, body weight was comparable between the age-matched Wistar control and GK rats (193 ± 13.7 *v* 185 ± 9.2 g). Plasma insulin levels were not measured in this study, but several other studies have reported twofold increases of plasma insulin in 8-week-old GK rats.[21]

Effect of Diabetes on GK Rat Adipocyte PP-1 PP-2A Activities

Our recent studies on cultured rat skeletal muscle cells and freshly isolated rat adipocytes indicated that insulin caused a rapid activation of membranous PP-1 and a concomitant inhibition of cytosolic PP-2A activities.[26,28] To understand the molecular basis of the recently reported inhibition of glycogen synthesis in GK rats,[22] we measured the activities of PP-1 and PP-2A in basal and insulin-stimulated rat adipocytes. Compared with normal controls, insulin resistance associated with the type II diabetes of GK rats was accompanied by a 32% decrease in basal PP-1 activity assayed in the particulate fraction ($P < .05$ *v* controls; Fig 1A). Exposure of adipocytes isolated from control rats to 5 nmol/L insulin for 5 minutes resulted in a rapid activation of particulate PP-1 activity (56% over basal value). In contrast, adipocytes isolated from GK diabetic rats exhibited only a 19.5% increase in PP-1 activity over basal levels, which amounts to only one third of the insulin effect on PP-1 activation when compared with normal controls ($P < .001$ *v* normal controls). Immunoprecipitation of the particulate fraction with an antibody directed against the glycogen-associated subunit of PP-1 (PP-1_G) followed by assay of PP-1 catalytic activity in the immunoprecipitates revealed that the reductions observed in PP-1 activation of GK diabetic rat adipocytes were due to inhibition of the glycogen-bound form of PP-1 (Table 1) without alteration in the other forms of PP-1 that were present in the immunodepleted supernatants. In control rat adipocytes, insulin caused a 120% increase in glycogen-associated PP-1 activity over basal values. In contrast, adipocytes from GK diabetic rats exhibited only a 40% increase in glycogen-bound phosphatase activity in response to insulin. In addition, diabetes caused a 36% decrease in the basal activity of glycogen-bound PP-1 when compared with controls (Table 1). The content of the PP-1 catalytic subunit or PP-1_G subunit was not different between control and GK diabetic rat adipocytes (Fig 1B and C).

In contrast to these reductions of basal and insulin-stimulated PP-1 activities, diabetic GK rats also exhibited a marked increase in basal PP-2A activity (73% increase over normal controls, $P < .005$) in the cytosolic fraction (Fig 2), while insulin did not decrease the elevated cytosolic PP-2A activity in GK rat adipocytes (2.3 ± 0.22 *v* 2.0 ± 0.15 nmol/mg/min; Fig 2). In adipocytes isolated from control rats, insulin did cause a significant decrease in cytosolic PP-2A activity (47% *v* basal value, $P < .05$).

Effect of GK Diabetes on Insulin-Stimulated MAPK Activation

The effect of diabetes on insulin stimulation of MAPK activation is shown in Fig 3. In adipocytes of control Wistar rats, insulin caused an approximate 100% increase in MAPK activity measured in the immunoprecipitates with MBP as the substrate. In contrast, adipocytes from diabetic GK rats exhibited only a 55% increase in MAPK activity in response to insulin. There was a small but significant decrease in basal MAPK activity in adipocytes of diabetic GK rats compared with control rats.

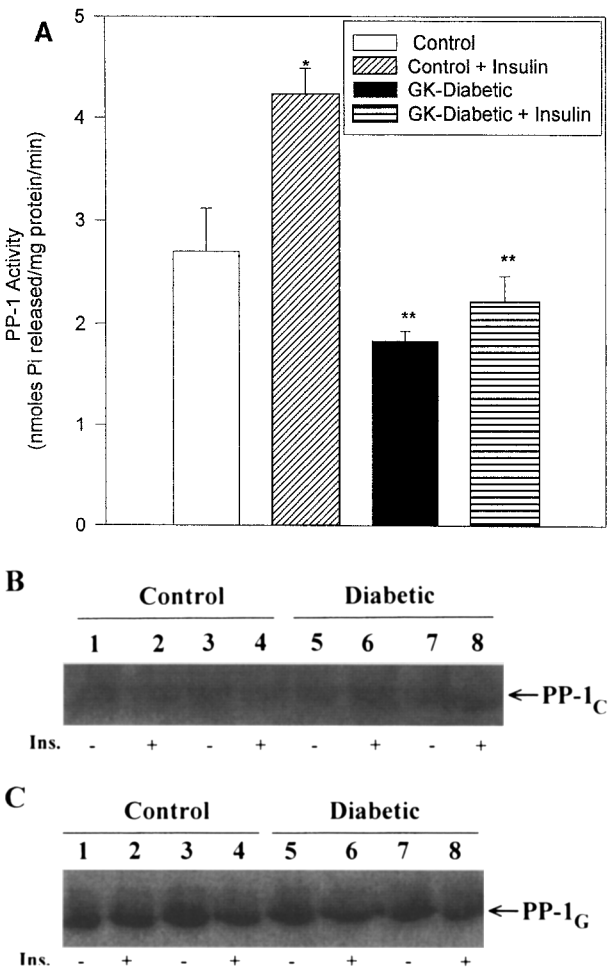


Fig 1. (A) Effect of diabetes in GK rats on basal and insulin-stimulated adipocyte PP-1 activity measured in the particulate fractions of control and insulin-treated rat adipocytes using [^{32}P]-labeled phosphorylase *a* as substrate. Results are the mean ± SEM of 5 to 6 different experiments in triplicate. *$P < .05$ *v* untreated control; **$P < .05$ *v* untreated control and insulin-treated control. Protein contents of PP-1 catalytic subunit (B) and PP-1$_G$ subunit (C) in control and diabetic GK rat adipocytes. Plasma fractions (50 μg protein) from control (−) and insulin-treated (+) rat adipocytes were separated by 7.5% SDS/PAGE followed by Western blot analysis with anti–PP-1$_G$ or –PP-1C antibody and detection with ECL. An autoradiogram from a representative experiment is shown.

Effect of Diabetes on GK Rat Adipocyte Tyrosine Phosphorylation of the IR and IRS-1

To examine whether the abnormal regulation of PP-1/PP-2A and MAPK in adipocytes of diabetic GK rats is due to defective insulin signaling through IRS-1, we measured the tyrosine phosphorylation status of IRS-1 in IRS-1 immunoprecipitates (performed on cell lysates with equal amounts of proteins) by Western blot analysis using polyclonal antiphosphotyrosine antibody. In adipocyte preparations from control rats, IRS-1 tyrosine phosphorylation was barely detectable in the basal state (Fig 4A, lane 1). Insulin rapidly increased tyrosine phosphorylation of IRS-1 within 1 minute, which was sustained during the second minute of incubation (Fig 4A, lanes 2 and 4) and returned to basal undetected levels within 5 minutes (Fig 4A, lanes 3 and 6). In contrast, IRS-1 immunoprecipitates from insulin-treated adipocytes isolated from diabetic GK rats exhibited a more than 90% decrease in tyrosine phosphorylation of IRS-1 (Fig 4A, lanes 8 and 10 *v* lanes 2 and 4). Diabetes did not significantly alter IRS-1 protein content (Fig 4B). Analysis of the tyrosine phosphorylation status of the IR in IR immunoprecipitates also showed reductions in insulin-stimulated tyrosine phosphorylation of the IR in adipocytes isolated from GK rats (Fig 4C). However, these rats also exhibited marked decreases in IR protein in the immunoprecipitates (Fig 4D). Thus, when the tyrosine phosphorylation data were normalized for the

Table 1. Analysis of Glycogen-Bound PP-1 Activity in Immunoprecipitates of Particulate Fractions Prepared From Control and Diabetic Rat Adipocytes

Group	Treatment	PP-1 Activity	% Insulin Effect
Control	None	1.08 ± 0.150	
	Insulin	2.38 ± 0.320*	121 ± 11
Diabetic	None	0.70 ± 0.100	
	Insulin	1.00 ± 0.150†	42 ± 4

NOTE. Particulate fractions with equal amounts of protein (100 μg) were constituted to 1 mL with lysis buffer and precleared with rat IgG coupled to protein A Sepharose at 4°C for 1 hour. The supernatants were incubated with PP-1$_G$ subunit antibody (10 μg/mL) for 1 hour, followed by treatment with 50 μL protein A Sepharose for 1 hour with constant shaking. The supernatants were saved and assayed for PP-1 activity. The immune complexes were washed four times with lysis buffer, resuspended to the original volume with lysis buffer, and incubated with the antigenic peptide (15 μg/mL) at 4°C for 1 hour to release the bound enzyme from the immunocomplex. An aliquot of the supernatant was assayed for PP-1 activity. Results are the mean ± SEM of 4 experiments performed in duplicate.

*$P < .05$ *v* no treatment.

†$P > .05$ *v* insulin-treated control.

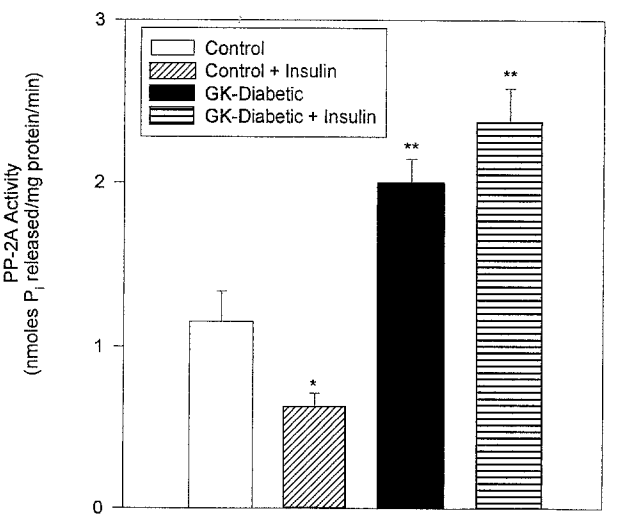


Fig 2. Diabetic GK rats exhibit elevations in basal PP-2A activity and insulin fails to suppress PP-2A. PP-2A activity was measured in the cytosolic fractions of control and insulin-treated rat adipocytes prepared from control and diabetic rats. Results are the mean ± SEM of 5 to 6 separate experiments performed in triplicate. *$P < .05$ *v* untreated control; **$P < .05$ *v* untreated control and insulin-treated control.

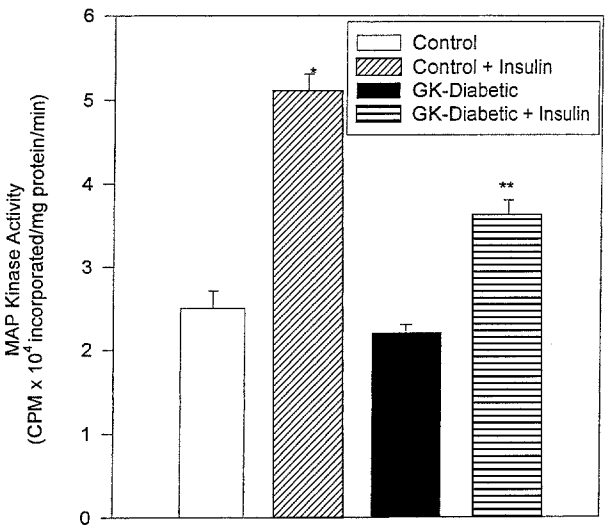


Fig 3. Effect of diabetes on basal and insulin-stimulated MAPK activities in adipocytes. Control and insulin-treated adipocyte cytosolic fractions were immunoprecipitated with MAPK antibody. MAPK activity was assayed in the immunoprecipitates using γ-[^{32}P]-labeled ATP and MBP as substrate. Radioactivity incorporated into MBP was determined by spotting an aliquot of the reaction mixture on peptide paper. Results are the mean ± SEM of 4 to 5 independent experiments. *$P < .05$ *v* untreated controls; **$P < .05$ *v* insulin-treated controls.

reductions in IR protein, there was no difference in the extent of IR tyrosine phosphorylation between normal control and GK diabetic adipocytes (Fig 4E). Similar results were reported by Bisbis et al[35] in GK diabetic rat liver.

Immunoprecipitation of IRS-1 from [^{32}P]-labeled cells followed by SDS/PAGE and autoradiography showed that IRS-1 immunoprecipitated from diabetic GK rat adipocytes was phosphorylated in the basal state when compared with control cells (Fig 5, lanes 3 and 4 *v* lane 1). Insulin did not further increase the incorporation of [^{32}P] into IRS-1 in GK rat adipocyte preparations (Fig 5, lanes 5 and 6). In contrast, insulin did increase the incorporation of [^{32}P] into IRS-1 in adipocytes isolated from control rats (Fig 5, lane 2). The observed alterations in IRS-1 phosphorylation were not due to variations in the amount of IRS-1 protein immunoprecipitated from cell lysates.

Effect of Diabetes on GK Rat Adipocyte Insulin-Mediated Grb2/Sos Association and Plasma Membrane Targeting of the Grb2/Sos Complex

Recent studies indicate that IR signaling acutely stimulates GTP loading of p21^{ras} by mobilizing complexes of Grb2 and the guanylnucleotide-exchange factor, Sos, to associate with the tyrosine-phosphorylated proteins IRS-1 and *Shc* in the plasma membrane.[7-8] To examine whether diabetes in GK rats is associated with alterations in the targeting of the Grb2/Sos complex, adipocyte plasma membranes (PMs) and intracellular membranes (IMs) (100 μg protein) were immunoprecipitated with Sos antibody. The immunoprecipitates were separated by SDS/PAGE followed by Western blot analysis with anti-Grb2 antibody. Figure 6A and C shows that in adipocytes isolated from control rats, 5 nmol/L insulin treatment for 3 minutes resulted in a rapid translocation of Sos to the PM (45% increase in PM Grb2 associated with Sos over the basal value; Fig 6A, lane 4 *v* lane 3). In contrast, in GK diabetic rat adipocytes, insulin did not cause translocation of the Sos/Grb2 complex to the PM (Fig 5A, lane 8 *v* lane 7, and Fig 6C). The content of Sos in PM fractions was decreased by more than 50% in GK diabetic adipocytes compared with normal controls (Fig 6B, lanes 1 to 4 *v* lanes 5 to 8). The reduction in Sos content was accompanied by a decrease in the mobility of Sos in adipocyte preparations from GK diabetic rats (Fig 6B, lanes 5 to 8 *v* lanes 1 to 4).

Quantitative analysis of the percent Grb2 associated with Sos in the IM and PM is shown in Fig 6C, after correction for variations in Sos proteins in Fig 6B. Insulin signaling resulted in translocation of the Sos complex to the PM in control adipocytes, but not in adipocytes isolated from diabetic GK rats.

Effect of Diabetes on Insulin-Stimulated Glut-4 Translocation

To examine whether the insulin resistance associated with diabetic GK rats affected the cellular content of Glut-4 and its translocation to the PM in response to insulin, immunoblot analysis of Glut-4 was performed on low-density microsomes (LDMs) and PM isolated from adipocytes before and after insulin treatment. Glut-4 content in LDMs was comparable between control and diabetic rats in the basal state (Fig 7, lane 1 *v* lane 3). Insulin did not increase the content of Glut-4 in the PM of adipocytes isolated from diabetic GK rats (lane 8 *v* lane 7).

DISCUSSION

The results of this study indicate that the insulin resistance and hyperinsulinemia of diabetic GK rats are accompanied by marked alterations in upstream and downstream insulin signaling components that participate in insulin-stimulated glucose transport and its storage as glycogen.

One of the dominant features of the insulin resistance associated with diabetic GK rat adipocytes is the impaired activation of PP-1 and inhibition of PP-2A by insulin. These observations, together with our recent findings that experimental insulin resistance induced by incubation of skeletal muscle cells with cAMP agonists and tumor necrosis factor-α ([TNF-α] a potential mediator of insulin resistance in obese/NIDDM rat models and human subjects) abrogated insulin-stimulated PP-1 activation, increased PP-2A activity, and blocked insulin-stimulated activation of the MAPK pathway,[32,36-37] suggest that a rapid activation of a membranous PP-1 along with concomitant inhibition of cytosolic PP-2A may constitute integral parts of the mechanism of insulin action. The impairment in insulin-stimulated activation of PP-1 may be responsible for the previously reported inhibition of insulin-mediated glycogen synthase activation and glycogen synthesis in diabetic GK rats. The observed inhibition in basal and insulin-stimulated PP-1 activities is not due to reductions in the content of the PP-1 catalytic subunit, but rather to defects in the activation of PP-1 via its regulatory subunit, PP-1_G. An increase in insulin-stimulated phosphorylation of PP-1_G in adipocytes and skeletal muscle preparations has recently been reported by this laboratory.[26,28]

The observed elevations in basal PP-2A activity together with

the lack of an insulin effect on PP-2A inactivation in diabetic GK rats may be due to tyrosine dephosphorylation of the PP-2A catalytic subunit via an activated tyrosine phosphatase. In support of this observation, several recent studies indicate that PP-2A activity is also regulated by tyrosine and/or threonine phosphorylation of the catalytic subunit.[38-40] We have also recently shown that insulin deactivates PP-2A in skeletal muscle cells by increasing tyrosine phosphorylation of the catalytic subunit, and cAMP agonists counterregulate insulin's inhibitory effects on PP-2A activity by decreasing tyrosine phosphorylation of PP-2A via an activated tyrosine phosphatase.[37]

Accompanying the alterations in PP-1 and PP-2A activities,

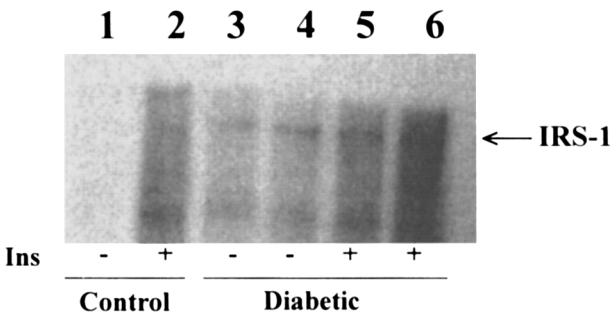
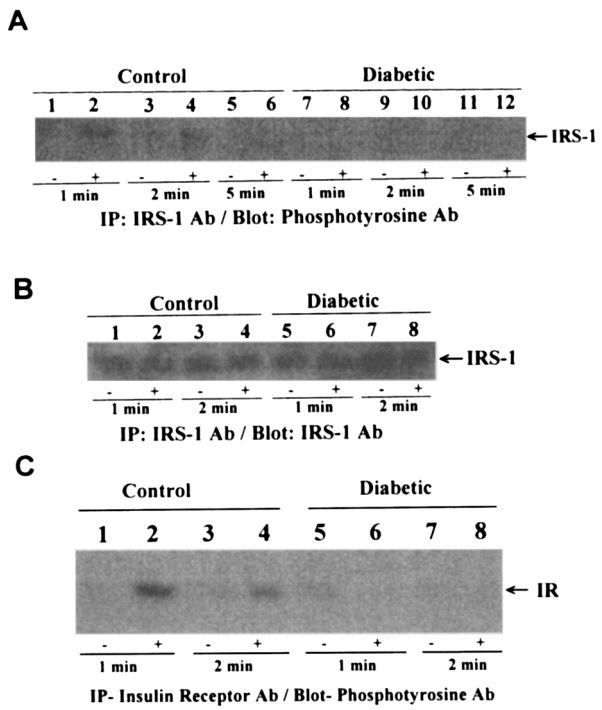


Fig 5. Diabetes causes increased [^{32}P] incorporation in IRS-1. Cell lysates from control and insulin-treated [^{32}P]-labeled adipocytes were immunoprecipitated with IRS-1 antibody, and the immunoprecipitates were separated on SDS/PAGE followed by autoradiography. Lane 1, untreated control; lane 2, insulin-treated control; lanes 3 and 4, untreated GK diabetic; lanes 5 and 6, insulin-treated (1 to 2 minutes) GK diabetic. Similar results were obtained in 3 separate experiments.



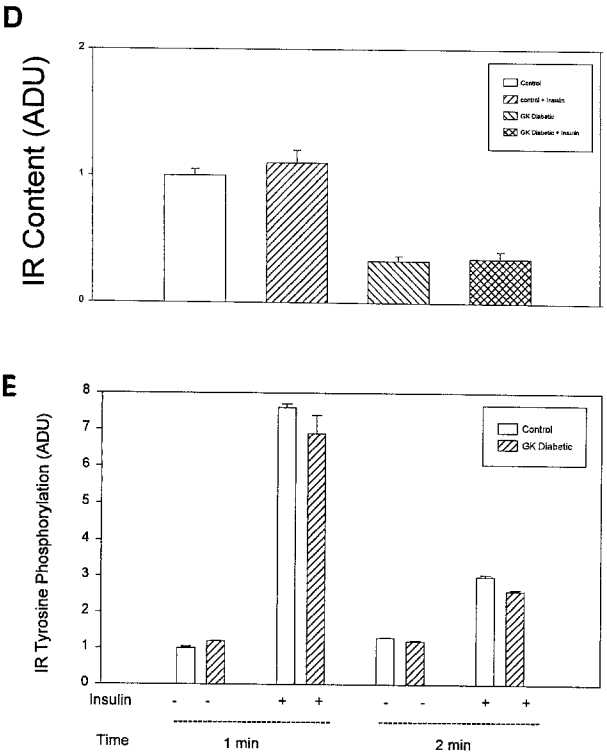


Fig 4. (A) Insulin resistance of diabetic GK rats results in decreased IRS-1 tyrosine phosphorylation. Equal amounts of cell lysates (500 μg protein) from control and diabetic GK adipocytes without and with insulin treatment (10 nmol/L × 1 to 2 minutes) were immunoprecipitated with IRS-1 antibody. The immunoprecipitates were separated by SDS/PAGE followed by Western blot analysis with phosphotyrosine antibody. A representative autoradiogram is shown. Similar results were obtained in 3 separate experiments. Lanes 1, 3, and 5, untreated controls; lanes 2, 4, and 6, insulin-treated controls; lanes 7, 9, and 11, untreated diabetic; and lanes 8, 10, and 12, insulin-treated diabetic. (B) Content of IRS-1 protein in the immunoprecipitates. Cell lysates were immunoprecipitated with anti–IRS-1 antibody, and the immunoprecipitates were separated by SDS/PAGE followed by Western blot analysis with IRS-1 antibody. Lane order is similar to A except that 5-minute time points are not included. (C) Effect of diabetes on IR tyrosine phosphorylation. Equal amounts of cell lysate proteins were immunoprecipitated with anti-IR antibody followed by Western blot analysis of the immunoprecipitates with antiphosphotyrosine antibody. A representative autoradiogram is shown. (D) Effect of diabetes on IR content in the immunoprecipitates. The immunoprecipitated IR from control and diabetic adipocytes was subjected to Western blot analysis with anti-IR antibody and detection with [^{125}I]-protein A. The intensity of 95-kd bands was quantified by densitometric analysis of the autoradiograms. For comparison of results between groups, control IR protein content was assigned a value of 1 and the rest of the data were calculated relative to basal values of the control. Results are expressed as arbitrary densitometric units from 3 experiments. (E) Quantitation of IR tyrosine phosphorylation in control and diabetic GK rats by densitometric analysis. Autoradiograms of IR tyrosine phosphorylation and IR protein were scanned for optical density, and areas beneath the peaks were determined. The magnitude of IR tyrosine phosphorylation was quantified by dividing the densitometric values for the antiphosphotyrosine blot by the values for the IR protein blot. IR tyrosine phosphorylation is expressed as ADU. To compare results from different experiments, basal IR tyrosine phosphorylation in control cells was assigned a value of 1 and the rest of the data were calculated relative to the basal value in control cells.

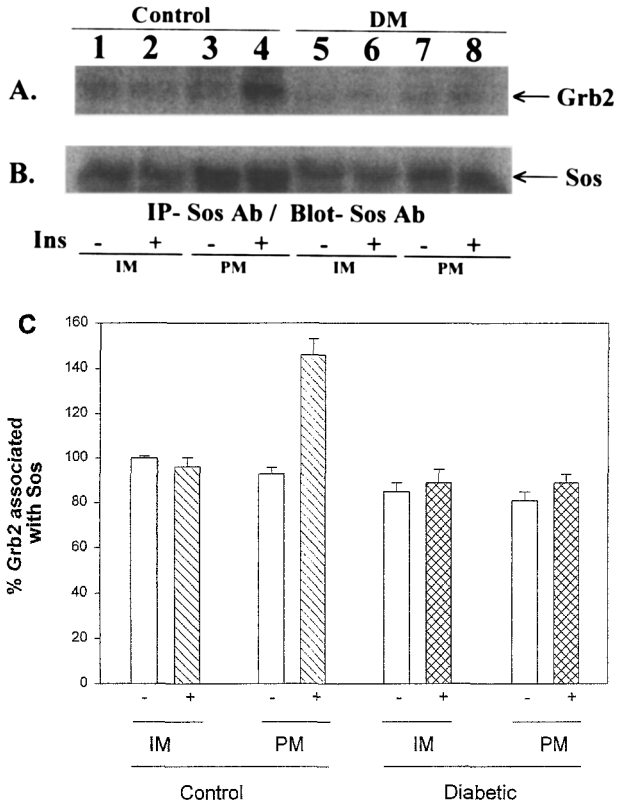


Fig 6. GK diabetes results in a decreased association of Grb2/Sos and its translocation to PMs. PMs and IMs were prepared from control and insulin-treated adipocytes. Equal amounts of proteins were immunoprecipitated with Sos antibody. The immunoprecipitates were separated by SDS/PAGE followed by Western blot analysis with Grb2 antibody (A). Lanes 1 and 2, control IM; lanes 3 and 4, control PM; lanes 5 and 6, diabetic IM; lanes 7 and 8, diabetic PM. (B) Western blot analysis of Sos immunoprecipitates with anti-Sos antibody. The upper half of the blot in A was probed with anti-Sos antibody, and the content of Sos measured by densitometry. (C) Quantitative analysis of the amount of Grb2 associated with Sos. Percent Grb2 associated with Sos was calculated by dividing the Grb2 peaks in A by the Sos peaks in B.

there is considerable inhibition (50% *v* controls) of insulin's effect on MAPK activation in diabetic GK rat adipocytes. This may be due to impaired *ras*-GTP loading because of reductions in PM targeting of the Grb2/Sos complex, and/or inactivation of MAPK and its upstream activator, MEK, by elevated PP-2A activity, as we have reported in TNF-α–induced insulin-resistant rat skeletal muscle cells.[32] However, the magnitude of inhibition of MAPK is less when compared with the alterations observed in upstream signaling components. This may be related to the fact that the MAPK pathway can be stimulated by other kinases bypassing the *ras*-GTP activation. For example, PKC can activate the MAPK pathway in skeletal muscle cells and in adipocytes via direct activation of *Raf*-1 kinase, the upstream activator of MEK.[41] Increased activation of diacylglycerol-sensitive PKC isoforms has been observed in skeletal muscle and adipocytes of diabetic GK rats.[23] We have also reported a blockade of PP-1 activation by insulin with chronic exposure of rat skeletal muscle cells to phorbol esters.[42]

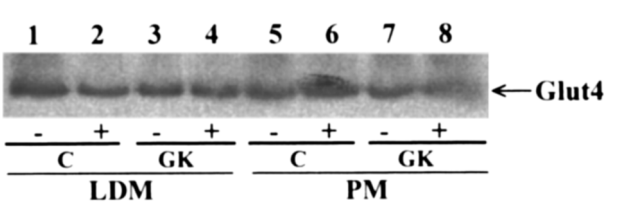


Fig 7. Adipocytes from diabetic GK rats exhibit defective translocation of Glut-4 to the PM. Proteins (10 μg) from the adipocyte LDM and PM isolated from control and diabetic GK rats were subjected to Western blot analysis with anti–Glut-4 antibody followed by detection with ECL. A representative autoradiogram is shown.

The alterations in PP-1/PP-2A and MAPK regulation in diabetic GK rat adipocytes may be due to defective insulin signaling because of desensitization and/or downregulation of the upstream insulin signaling components. In support of this conclusion, we have shown that diabetes in GK rats is accompanied by a marked reduction in insulin-stimulated tyrosine phosphorylation of IRS-1, a marked reduction in IR protein content, a decreased association of Grb2 with Sos, and an attenuation of insulin-mediated targeting of the Grb2/Sos complex to the PM.

The reduction seen in IRS-1 tyrosine phosphorylation in diabetic GK rats was not due to a generalized reduction in the phosphorylation status of IRS-1 for the following reasons. Metabolic labeling of adipocytes with [^{32}P]-orthophosphate showed increased incorporation of [^{32}P] in IRS-1 in the basal state. Insulin did not cause a further increase in IRS-1 phosphorylation. In contrast, in control adipocytes, insulin treatment did result in a considerable increase in [^{32}P] incorporation into IRS-1. The observed increase in basal IRS-1 phosphorylation in adipocytes isolated from diabetic GK rats may be due to an increase in serine phosphorylation of IRS-1, as reported by others in TNF-α–induced insulin resistance and insulin resistance associated with polycystic ovarian syndrome.[43-44]

Contrary to the reductions seen in IRS-1 tyrosine phosphorylation, insulin-mediated tyrosine phosphorylation of *Shc* protein was unaltered in adipocytes of diabetic GK rats (results not shown). Thus, IRS-1 tyrosine phosphorylation appears to play a predominant role in adipocyte insulin signal transduction. A decrease in tyrosine phosphorylation of IRS-1 as seen in the present study may lead to an impairment in PI3-kinase activation, thereby resulting in a decrease in PI3-kinase–generated signals that may mediate insulin-stimulated PP-1 activation, PP-2A inactivation,[37] and translocation of insulin-responsive glucose transporters to the PM. Future studies will examine the impact of diabetes in GK rats on the kinetics of insulin activation of PI3-kinase and its downstream target, c-*Akt* (also known as *Rac* or protein kinase B).

The reduction in tyrosine phosphorylation of IRS-1 in adipocytes isolated from diabetic GK rats is accompanied by a marked inhibition of insulin-mediated targeting of the Grb2/Sos complex to the PM. This may be due to the phosphorylation of Sos causing a dissociation of the Grb2/Sos complex due to hyperinsulinemia. In support of this observation, recent studies

by several groups have shown that insulin itself causes desensitization of *Ras* activation by a feedback phosphorylation (by MAPK) and dissociation of the Sos/Grb2 complex.[7,8] Further studies are needed to examine in detail the kinetics of Sos phosphorylation in diabetic GK rats and to identify the potential role of Sos dephosphorylation by a phosphatase in its association with Grb2 and PM targeting of the Grb2/Sos complex.

In summary, the results of the present study indicate that alterations in the IR, IRS-1, and Grb2/Sos may account for the observed insulin signaling defects in GK rats.

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