Islet Transplantation Restores Normal Levels of Insulin Receptor and Substrate Tyrosine Phosphorylation and Phosphatidylinositol 3-Kinase Activity in Skeletal Muscle and Myocardium of Streptozocin-Induced Diabetic Rats

Francesco Giorgino, Francesco Logoluso, Alberto M. Davalli, Raffaele Napoli, Luigi Laviola, Michael F. Hirshman, Edward S. Horton, Gordon C. Weir, and Robert J. Smith

Insulin-dependent diabetes in rats is characterized by abnormalities of post-binding insulin signaling reactions that are not fully corrected by exogenous insulin therapy. The aim of this study was to investigate the effects of islet transplantation on insulin signaling in skeletal muscle and myocardium of streptozocin (STZ)-induced diabetic rats. Control rats, untreated diabetic rats, and diabetic rats transplanted with syngeneic islets under the kidney capsule were studied. Compared with controls, diabetic rats were characterized by multiple insulin signaling abnormalities in skeletal muscle, which included *1*) increased insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit and insulin receptor substrates IRS-1 and IRS-2, *2*) increased substrate tyrosine phosphorylation in the basal state, *3*) a decreased amount of IRS-1 protein, *4*) markedly elevated basal and insulin-stimulated phosphatidylinositol (PI) 3-kinase activity in anti-IRS-1 immunoprecipitates from total tissue extracts, and *5*) increased PI 3-kinase activity in low-density microsomes. A similar augmentation of insulin receptor and substrate tyrosine phosphorylation in response to STZ-diabetes was also found in myocardium, although with lower magnitude than that found in skeletal muscle. In addition, STZ-diabetes resulted in decreased IRS-1 and increased IRS-2 protein levels in myocardium. Islet transplantation fully corrected the diabetes-induced changes in protein tyrosine phosphorylation and PI 3-kinase activity and normalized IRS-1 and IRS-2 protein content in both skeletal muscle and myocardium. Thus, insulin delivered into the systemic circulation by pancreatic islets transplanted under the kidney capsule can adequately correct altered insulin signaling mechanisms in insulinopenic diabetes.
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From the Istituto di Clinica Medica (F.G., F.L., L.L.), Endocrinologia e Malattie Metaboliche, University of Bari School of Medicine, Bari, Italy; and the Joslin Diabetes Center (A.M.D., R.N., M.F.H., E.S.H., G.C.W., R.J.S.), Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Francesco Giorgino, MD, PhD, Istituto di Clinica Medica, Endocrinologia e Malattie Metaboliche, University of Bari School of Medicine, Piazza Giulio Cesare 11, I-70124 Bari, Italy. E-mail: giorginf@tin.it.

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anti-PY, anti-phosphotyrosine; BSA, bovine serum albumin; IRS, insulin receptor substrate; KpNPPase, K^+-stimulated *p*-nitrophenol phosphatase; NP-40, nonidet P-40; PI, phosphatidylinositol; PTP, phosphotyrosine-protein; STZ, streptozocin.

Tyrosine phosphorylation of the insulin receptor β-subunit (1) and of receptor substrates, including insulin receptor substrate (IRS)-1 (2), IRS-2 (3), and other proteins (4–6), represent the first measurable signaling reactions following insulin binding to the receptor α-subunit and subsequent activation of the protein tyrosine-kinase intrinsic to the cytoplasmic portion of the transmembrane β-subunit. These tyrosine phosphorylation reactions activate multiple branching signaling pathways that mediate insulin regulation of cellular growth and metabolism in target tissues. In skeletal muscle, insulin-stimulated tyrosine phosphorylation of the substrate IRS-1 results in rapid recruitment and activation of the enzyme phosphatidylinositol (PI) 3-kinase (7), which has been shown to represent a necessary signaling component for insulin regulation of metabolic responses, including stimulation of glucose transport (8–10), amino acid transport (11), and glycogen synthesis (12,13). In addition to exerting metabolic effects, insulin receptor–mediated tyrosine phosphorylation of cytoplasmic substrate proteins promotes changes in gene expression that ultimately control cell growth and differentiation. For example, insulin increases mRNA levels of the proto-oncogenes *c-fos* and *c-jun* in skeletal muscle cells both in vivo (14) and in vitro (15), and evidence has been provided that induction of the *c-fos* gene requires activation of the insulin receptor tyrosine kinase (16) and subsequent tyrosine phosphorylation of the Shc proteins (17).

Abnormalities of post-binding insulin signaling reactions have been reported in various experimental models of insulin-dependent and non-insulin-dependent diabetes. Rats made insulin deficient and diabetic with the β-cell toxin streptozocin (STZ) become insulin resistant and show a marked increase in insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit and IRS-1 in skeletal muscle (18,19) and liver (19). The activity of PI 3-kinase associated with IRS-1 is similarly increased in these animals, likely as a consequence of increased IRS-1 tyrosine phosphorylation (20). A previous study from our laboratory demonstrated that increased receptor and IRS-1 tyrosine phosphorylation are the consequence of insulin deficiency rather than hyperglycemia because they remained unaltered after correction of hyperglycemia by phlorizin and were promptly reduced by administration of insulin subcuta-

neously (18). However, the levels of insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation were lower in insulin-treated STZ-diabetic rats than in control animals (18). Therefore, these studies established the important modulatory role of ambient insulin concentrations on insulin-induced protein tyrosine phosphorylation in rat skeletal muscle, but also underscored the inability of insulin therapy to fully correct abnormal insulin signaling reactions in diabetic muscle. We and others have also shown that STZ-diabetic rats are characterized by impaired skeletal muscle glucose transport due to decreased GLUT4 protein content and reduced transporter translocation in response to insulin (21,22). In this model of diabetes, insulin therapy does not restore insulin-stimulated skeletal muscle glucose transport and transporter translocation to normal (23), and this is similar to what happens in human type 1 diabetes (24,25). It is possible that insulin therapy cannot normalize the glucose transport system in diabetic skeletal muscle because it cannot fully correct altered signaling mechanisms in this tissue.

The inability of insulin therapy to produce normal levels of insulin receptor and substrate tyrosine phosphorylation in insulinopenic animals could be potentially explained by several factors acting alone or in concert. First, to achieve normoglycemia, higher plasma insulin concentrations than normal are generated as a consequence of repeated insulin injections (18), and chronic hyperinsulinemia may downregulate insulin signaling reactions in skeletal muscle. Second, subcutaneous insulin therapy in rats cannot produce a completely normal glucose profile, and variably high and low glucose values may adversely affect signaling reactions in muscle, as has been shown in other experimental systems (26–28). Third, insulin given subcutaneously may not be adequate in terms of route of administration (29) because the hormone is not delivered into the portal vein, as occurs physiologically, but into the peripheral circulation. The objective of this study was to test the hypothesis that restoration of glucose-regulated insulin secretion is able to fully normalize the skeletal muscle insulin signaling system in insulin-deficient diabetic rats. For this purpose, rats were made diabetic by administration of STZ and then treated by transplantation of pancreatic islets under the kidney capsule. We show that islet transplantation fully corrected the abnormal insulin signaling system in skeletal muscle as well as in the myocardium of STZ-diabetic animals.

RESEARCH DESIGN AND METHODS

Animals. Male inbred Lewis rats weighing ~200 g (Taconic Farms, Germantown, NY) were used as both islet donors and recipients. The animals were housed at 23°C with a 12-h light/dark cycle and allowed free access to standard rat diet plus water. The rats were randomly divided into three different groups. Controls (n = 8) were left untreated and studied 10 weeks later. Untreated diabetic rats (n = 8) were left untreated for 4 weeks, then given a single intravenous STZ injection of 65 mg/kg body wt (Sigma, St. Louis, MO), and studied 6 weeks later. Transplanted diabetic rats (n = 8) were made diabetic by a single intravenous injection of STZ (65 mg/kg body wt), underwent islet transplantation 2–3 weeks later, and were studied after an additional 8 weeks. Rats were considered diabetic when the nonfasted blood glucose level was >300 mg/dl.

All animals were studied after an overnight fast between 8:30 and 10:30 A.M. Maximal insulin stimulation was achieved by intraperitoneal injection of 20 U insulin 30 min before the rats were killed. The rats were considered to be insulin stimulated when serum insulin concentration exceeded 20,000 µU/ml. Basal animals were handled, but received no intraperitoneal injection. The animals were killed by decapitation, and blood was collected for serum glucose and serum insulin determinations. Hindlimb skeletal muscles and the cardiac ventricles were rapidly excised and snap-frozen in liquid N_2.

Serum glucose was determined by glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin concentration was determined with a double antibody radioimmunoassay using guinea pig anti-rat insulin serum, a rat insulin standard, and ^{125}I-labeled insulin (Linco, St. Louis, MO).

Islet isolation and transplantation. Isolation and transplantation of rat pancreatic islets were performed as described previously (30). Briefly, animals were anesthetized with sodium amobarbital (Eli Lilly, Indianapolis, IN), a laparotomy was performed, and the pancreas was exposed. The common bile duct was ligated, and the ampulla of Vater was cannulated proximally with P-50 polyethylene tubing and injected with 10 ml of M-199 medium (Gibco BRL, Gaithersburg, MD) containing 1.5 mg/ml collagenase (type P; Boehringer Mannheim, Mannheim, Germany). The pancreas was dissected from the surrounding tissues, removed, and incubated in a stationary bath for 15–18 min at 30°C. After incubation, the digested tissue was washed with M-199 medium containing 10% newborn calf serum and filtered through a tissue collecting sieve (40 mesh; Fisher Scientific, Pittsburgh, PA). The islets were then purified by a density gradient (Histopaque-1077; Sigma) and centrifuged at 2,500 rev/min for 20 min. Islet yield was determined by analyzing 100-µl samples from the final preparation. An aliquot of 2,000 freshly isolated islet equivalents was aspirated into a 200-µl pipette tip (USA Scientific Plastics, Ocala, FL) connected to a 1-ml syringe (Hamilton, Reno, NE), and then transferred into P-50 polyethylene tubing (Becton Dickinson, Parsipanny, NJ). Under light methoxyflurane (Pitman-Moore, Mundelein, IL) anesthesia, the left kidney of the recipient rat was exposed through a lumbar incision. A capsulotomy was performed on the caudal outer surface of the kidney, the tip of the tube was advanced under the capsule, and the islets were injected. The tubing was then removed, and the capsulotomy was cauterized with a disposable low-temperature cautery pen (Surgicare, Dayville, CT).

Antibodies. Polyclonal anti-phosphotyrosine (anti-PY) antibody was prepared in rabbits by injection of phosphotyrosine polymerized by 1-ethyl-3(3-dimethylaminopropyl)carbodiimide with alanine, threonine, and keyhole limpet hemocyanin, and purified by affinity chromatography on a phosphotyramine-sepharose column as previously described (31). A polyclonal anti-insulin receptor antibody against a lys-lys-asn-gly-arg-ile-leu-thr-leu-pro-arg-ser-asn-pro-ser peptide, corresponding to the last 15 amino acids in the COOH-terminal region of the human insulin receptor sequence was kindly provided by Dr. Bentley Cheatham (Joslin Diabetes Center, Harvard Medical School, Boston, MA). A polyclonal anti-peptide antibody against a tyr-ala-ser-ile-asn-phe-gln-lys-gln-pro-glu-asp-arg-gln peptide, corresponding to the last 14 amino acids in the COOH-terminal region of rat IRS-1 (2), was generated and purified on a specific peptide affinity column as previously described (15,31). Polyclonal PI 3-kinase (anti-p85) and anti-IRS-2 antibodies were purchased from Upstate Biotechnology (Saranac Lake, NY).

Immunoblotting. The frozen skeletal and cardiac muscle tissue was powdered in a stainless steel mortar and pestle with liquid N_2 and homogenized for 30 s with an Ultra-Turrax (Janke & Kunkel, IKA-Werk, Staufen, Germany) in ice-cold lysis buffer containing 20 mmol/l Tris-HCl (pH 7.6), 137 mmol/l NaCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10% glycerol, 10 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l EDTA, 2 mmol/l phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 2 mmol/l sodium orthovanadate, and 1% Nonidet P-40 (NP-40). The tissue homogenate was incubated for 45 min at 4°C with gentle stirring and then centrifuged at 100,000g for 60 min. The resulting supernatant was collected and assayed for protein concentration using the Bradford dye binding assay kit (32) with bovine serum albumin (BSA) as a standard.

For immunoblotting studies, equal amounts of solubilized tissue proteins were resolved by 7% or 10% SDS-PAGE directly or after immunoprecipitation with specific antibodies and protein A-sepharose beads, as indicated. The resolved proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-ECL; Amersham Life Science, Arlington Heights, IL) using a transfer buffer containing 192 mmol/l glycine, 20% (vol/vol) methanol, and 0.02% SDS. The membranes were incubated in TNA buffer (10 mmol/l Tris-HCl, pH 7.8, 0.9% NaCl, 0.01% sodium azide) supplemented with 5% BSA and 0.05% NP-40 at 37°C for 2 h to reduce nonspecific binding, and then incubated overnight at 4°C with anti-PY, anti-insulin receptor, anti-IRS-1, anti-IRS-2, or anti-p85 antibodies, as indicated. The proteins were visualized by enhanced chemiluminescence (ECL) using horseradish peroxidase-labeled anti-rabbit IgG (Amersham Life Science) and quantified by densitometric analysis using Optilab image analysis software (Graftek SA, Mirmande, France).

Microsomal membrane preparation. Skeletal muscle microsomal membranes were prepared as previously described (33). Briefly, dissected muscles were weighed, minced with scissors, and homogenized sequentially with a Polytron (Brinkmann Instruments, Westbury, NY) and a Potter-Elvejhem homogenizer (A.H. Thomas, Swedesboro, NJ) in Tris-sucrose buffer (225 mmol/l sucrose, 100 mmol/l Tris-HCl, pH 7.6, 0.2 mmol/l EDTA) at 4°C. The homogenate was first centrifuged at 34,000g for 20 min, and the supernatant was recentrifuged at 227,000g for 60 min. The pellet was suspended in a small volume of buffer containing 225 mmol/l sucrose, 1 mmol/l Tris-HCl (pH 8.5), and 1 mmol/l $MgCl_2$ at 4°C, and layered on a discontinuous 0.9 mol/l sucrose gradient in 20 mmol/l Tris-HCl (pH 7.4) and 1 mmol/l EDTA at 4°C. After centrifugation at 135,000g for 60 min,

the microsomal membrane band at the gradient interface plus all buffer above the band were diluted in water and centrifuged at 227,000*g* for 60 min. The resulting pellet was resuspended in 20 mmol/l HEPES and 250 mmol/l sucrose to a final concentration of 1–2 mg/ml and stored in liquid N_2.

To assess the degree of contamination of microsomal membranes with plasma membranes, the activity of K^+-stimulated *p*-nitrophenol phosphatase (KpNPPase), a plasma membrane marker, was assayed in the absence or presence of 20 mmol/l KCl (34,35).

Immunoprecipitation of PI 3-kinase. Total skeletal and cardiac muscle extracts (1–2 mg) or solubilized skeletal muscle microsomal membranes (50 µg) were used for immunoprecipitation of PI 3-kinase. Microsomal membranes were solubilized by gentle stirring in lysis buffer for 45 min at 4°C, and insoluble material was removed by centrifugation at 12,000*g* for 10 min at 4°C. Immunoprecipitation was carried out by incubation of the lysate overnight at 4°C with anti-IRS-1 or anti-p85 antibodies, as indicated, followed by adsorption of resulting immune complexes to protein A-sepharose beads for 2 h at 4°C. The pelleted beads were washed in phosphate-buffered saline containing 1% NP-40 and 100 µmol/l sodium orthovanadate (three times), 100 mmol/l Tris-HCl (pH 7.6) containing 500 mmol/l LiCl and 100 µmol/l sodium orthovanadate (three times), and 10 mmol/l Tris-HCl (pH 7.6) containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 µmol/l sodium orthovanadate (two times).

PI 3-kinase assay. The activity of PI 3-kinase in the immunoprecipitates was determined as previously described (36). Briefly, the washed immunoprecipitates were resuspended in 50 µl of 10 mmol/l Tris-HCl (pH 7.6) containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 µmol/l sodium orthovanadate, and then combined with 10 µl of 100 mmol/l $MgCl_2$ and 10 µl of 2 µg/µl PI (Avanti, Birmingham, AL) that had been sonicated in 10 mmol/l Tris-HCl (pH 7.6) containing 1 mmol/l EGTA. The PI 3-kinase reaction was started by the addition of 10 µl of 10 mmol/l ATP containing 30 µCi [γ-^{32}P]ATP. After 10 min at 22°C with constant vortexing, the reaction was stopped by the addition of 20 µl 8 N HCl and 160 µl chloroform/methanol (1:1, vol/vol). The mixture was vigorously mixed and centrifuged to separate the phases. The lower organic phase containing the reaction products was removed, applied to a silica gel thin-layer chromatography plate (Merck, Darmstadt, Germany), and resolved by ascending chromatography in chloroform/methanol/water/ammonia (60:47:11.3:2, vol/vol). The PI 3-phosphate product was visualized by autoradiography, identified by its comigration with a PI 4-phosphate standard, and quantified by scanning densitometry.

Statistical analysis. All data are expressed as means ± SE. Statistical analyses were performed by unpaired Student's *t* tests.

RESULTS

General characteristics of experimental animals. The body weight, serum glucose, and serum insulin determinations from the experimental animals are illustrated in Table 1. The rats in all three groups weighed ~200 g at the beginning of the study. After 10 weeks, at the time the animals were killed, normal control rats had gained an average of 148 g and showed normal fasting serum glucose levels. In contrast, diabetic rats were characterized by markedly lower body weight compared with controls (246 ± 9 vs. 348 ± 10 g, $P < 0.05$) and had higher serum glucose levels (456 ± 10 vs. 106 ± 5 mg/dl, $P < 0.05$). Transplanted diabetic animals gained an average of 62 g more than the untreated diabetic rats and had serum glucose values in the normal range. In the basal fasting state, serum insulin concentrations appeared to be lower in the untreated diabetic animals than in control and transplanted diabetic rats, although this difference was not statistically significant. Additional metabolic characteristics of the experimental animals analyzed in this study have been reported elsewhere (37). Diabetic animals subjected to islet transplantation were shown to have greatly improved glucose tolerance measured by intraperitoneal glucose tolerance test in comparison with the untreated diabetic rats (37). Thus, islet transplantation achieved significant restoration of glucose-induced insulin secretion.

Tyrosine phosphorylation and protein content of insulin receptors, IRS-1, and IRS-2 in skeletal muscle. To investigate the effects of insulin on protein tyrosine phosphorylation in skeletal muscle, overnight fasted animals were studied in the basal state or 30 min after intraperitoneal injection with 20 U insulin. Hindlimb muscles were rapidly removed, frozen in liquid N_2, and homogenized in buffer containing 1% NP-40 plus various protease and phosphatase inhibitors, as described in METHODS. Figure 1*A* shows a representative experiment in which the solubilized muscle proteins were resolved by SDS-PAGE and tyrosine phosphoproteins were identified by immunoblotting with anti-PY antibody. In all animals, insulin injection resulted in the stimulation of tyrosine phosphorylation of a 95 kDa protein corresponding to the insulin receptor β-subunit and two protein bands migrating at ~165–180 kDa and representing the insulin receptor substrates IRS-1 and IRS-2 (18,31,38). Injection of insulin (20 U/rat) intraperitoneally yielded maximal levels of insulin receptor and substrate tyrosine phosphorylation (data not shown), similar to the intracardiac insulin injection protocol (10 U/kg body wt) used in our previous studies (18,31). Basal tyrosine phosphorylation of the insulin receptor β-subunit was similar in control, diabetic, and transplanted rats, whereas insulin-stimulated receptor tyrosine phosphorylation was increased in the diabetic rats (Fig. 1*A*). Tyrosine phosphorylation of the insulin receptor substrates in the 165–180 kDa range was increased both in the basal state and after insulin stimulation in diabetic skeletal muscle (Fig. 1*A*). The levels of insulin receptor and substrate tyrosine phosphorylation in the experimental animals were quantified by densitometric analysis of multiple anti-PY antibody immunoblots (Fig. 1*B* and *C*). The bands in the 165–180 kDa range were considered collectively because of the difficulty in obtaining separate resolution of individual phosphoproteins in that molecular weight range. Insulin-stimulated tyrosine phosphorylation of the receptor β-subunit was increased by 65% in diabetic rats ($P < 0.05$) (Fig. 1*B*), whereas IRS-1/2 tyrosine phosphorylation was increased approximately twofold in diabetic rats both in the basal state and after stimulation with insulin ($P < 0.05$) (Fig. 1*C*). Thus, tyrosine phosphorylation of the 165–180-kDa substrate proteins was elevated in the absence of an apparent increase in tyrosine phosphorylation of the receptor β-subunit in basal diabetic animals (Fig. 1). The levels of insulin receptor and substrate

TABLE 1
Characteristics of the experimental animals

Rats	*n*	Body weight (g)	Serum glucose (mg/dl)	Serum insulin (µU/ml)
Control	8	348 ± 10	106 ± 5	38.6 ± 11.4
Diabetic	8	246 ± 9*	456 ± 10*	18.0 ± 5.9
Transplanted	8	308 ± 4*†	97 ± 3†	28.2 ± 2.8

Data are means ± SE. Control male Lewis rats (control) were left untreated and studied 10 weeks later; untreated diabetic rats (diabetic) were left untreated for 4 weeks and then received a single intravenous injection of 65 mg/kg body wt of STZ and were studied 6 weeks later; and transplanted diabetic rats (transplanted) were made diabetic by a single intravenous injection of 65 mg/kg body wt of STZ, underwent islet transplantation 2–3 weeks later, and were studied 8 weeks later. Body weight was determined and serum glucose and insulin levels were measured in the basal fasting state on the day that the animals were killed. *$P < 0.05$ vs. control rats; †$P < 0.05$ vs. diabetic rats. The data in this table have been previously reported in part (37).

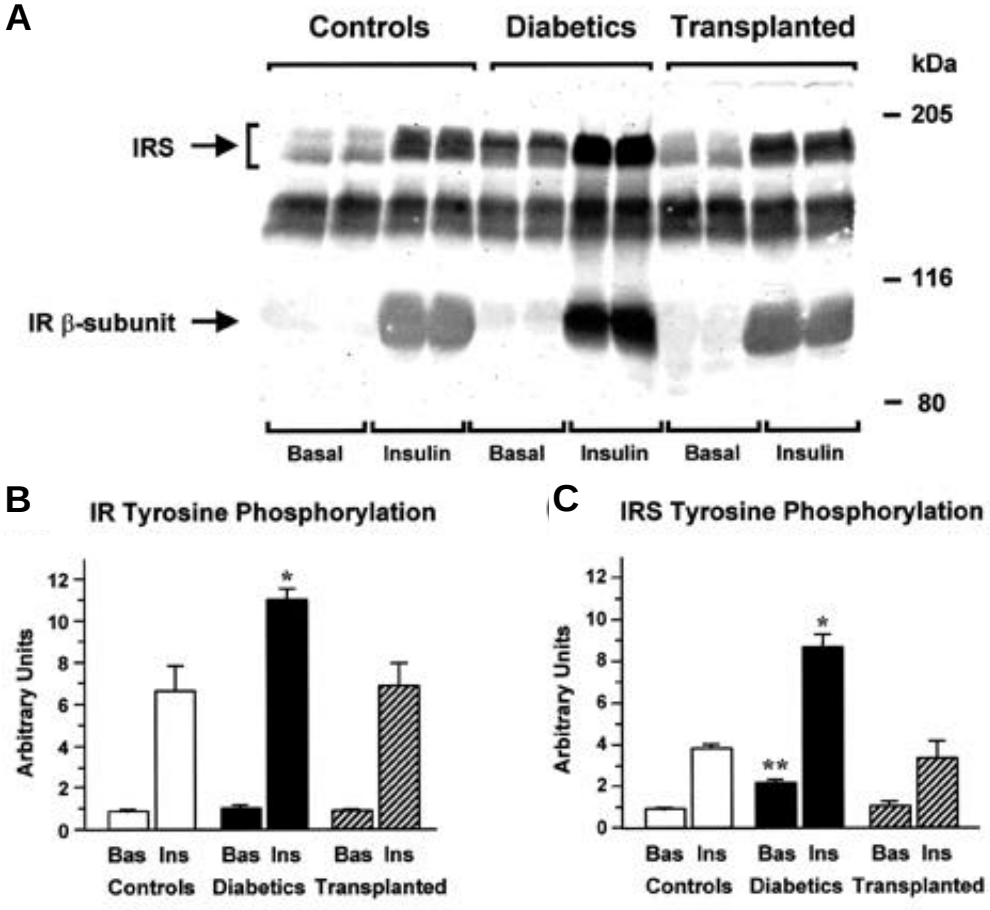

FIG. 1. Insulin receptor and substrate tyrosine phosphorylation in rat skeletal muscle. Control, untreated diabetic, and transplanted diabetic rats were studied in the basal state or 30 min after injection of 20 U of insulin intraperitoneally. Solubilized muscle proteins were resolved by 7% SDS-PAGE, and tyrosine phosphoproteins were detected by immunoblotting with anti-PY antibody, as described in METHODS. *A* shows a representative experiment in which samples from each animal were processed and analyzed in duplicate. *B* and *C* show the quantitation of basal (Bas) and insulin-stimulated (Ins) tyrosine phosphorylation of the insulin receptor β-subunit and of the receptor substrates in the 165–180 kDa range, respectively (n = 4 animals in each condition). IR, insulin receptor; IRS, insulin receptor substrates in the 165–180 kDa range. *P < 0.05 vs. insulin-stimulated control and insulin-stimulated transplanted rats; **P < 0.05 vs. basal control and basal transplanted rats.

tyrosine phosphorylation were not different from those of control rats in diabetic rats treated by islet transplantation (Fig. 1*B* and *C*), indicating that islet transplantation was fully effective in correcting abnormal protein tyrosine phosphorylation in diabetic skeletal muscle.

The effects of STZ-diabetes and diabetes treatment by islet transplantation on total amounts of skeletal muscle insulin receptor, IRS-1, and IRS-2 proteins are shown in Fig. 2. The total amount of skeletal muscle insulin receptor protein was increased by 36% in diabetic rats compared with controls (P< 0.05), and was indistinguishable from control in transplanted rats (P = 0.11) (Fig. 2*B*). Therefore, the ratio of receptor tyrosine phosphorylation to receptor protein was only modestly increased in diabetic rats compared with controls (117.0 ± 4.7% of control, P < 0.05), and did not differ from control in transplanted rats (94.1 ± 5.5% of control, P = 0.11). This suggests that the changes in receptor protein may be responsible, at least in part, for the observed changes in insulin-stimulated receptor tyrosine phosphorylation in the experimental animals (Fig. 1*A* and *B*). In contrast, the total amount of IRS-1 protein decreased by 40% in diabetic skeletal muscle (P < 0.05) and increased to the control level after islet transplantation (Fig. 2*D*). The total amount of IRS-2 showed a small, but statistically insignificant, decrease in diabetic rats compared with controls and a small increase that was also not statistically significant after islet transplantation (Fig. 2*F*). The reduced electrophoretic mobility of IRS-1 and IRS-2 in insulin-injected rats compared with basal rats (Fig. 2*C* and *E*) reflects an increased phosphoserine content of these proteins after insulin stimulation, as previously noted (31).

Skeletal muscle PI 3-kinase activity. Activation of the enzyme PI 3-kinase represents a key signaling event in insulin action after receptor-mediated tyrosine phosphorylation of IRS-1 and IRS-2. To determine whether increased IRS-1/2 tyrosine phosphorylation in diabetic skeletal muscle was associated with abnormal activation of PI 3-kinase and to assess the effects of islet transplantation on this signaling step, the activity of PI 3-kinase associated with the p85 regulatory subunit or with IRS-1 was analyzed in skeletal muscle extracts from control, diabetic, and transplanted rats. For this purpose, solubilized skeletal muscle from basal or insulin-injected rats was subjected to immunoprecipitation with either anti-p85 or anti-IRS-1 antibodies, and PI 3-kinase activity in the immunoprecipitates was assayed in the presence of ^{32}P-ATP and PI, as described in METHODS.

The levels of skeletal muscle PI 3-kinase activity in anti-p85 immunoprecipitates were not significantly modified by insulin stimulation in any of the experimental groups of animals (P = 0.28, 0.17, and 0.28 in control, diabetic, and transplanted rats, respectively) (Fig. 3*A*). However, p85-associated PI 3-kinase activity was increased by 45% in the skeletal muscle of untreated diabetic rats compared with controls (P < 0.05) (Fig. 3*C*), and this was associated with a coordinate 50% increase in the amount of p85 protein in diabetic muscle (P < 0.05) (Fig. 3*D*). Treatment of diabetic rats by islet transplantation reduced both p85-associated PI 3-kinase activity and p85 protein to control levels (Fig. 3*C* and *D*).

In contrast to anti-p85 immunoprecipitates, PI 3-kinase activity in anti-IRS-1 immunoprecipitates was markedly stimulated by insulin in rat skeletal muscle (Fig. 4*A*). Quantitative

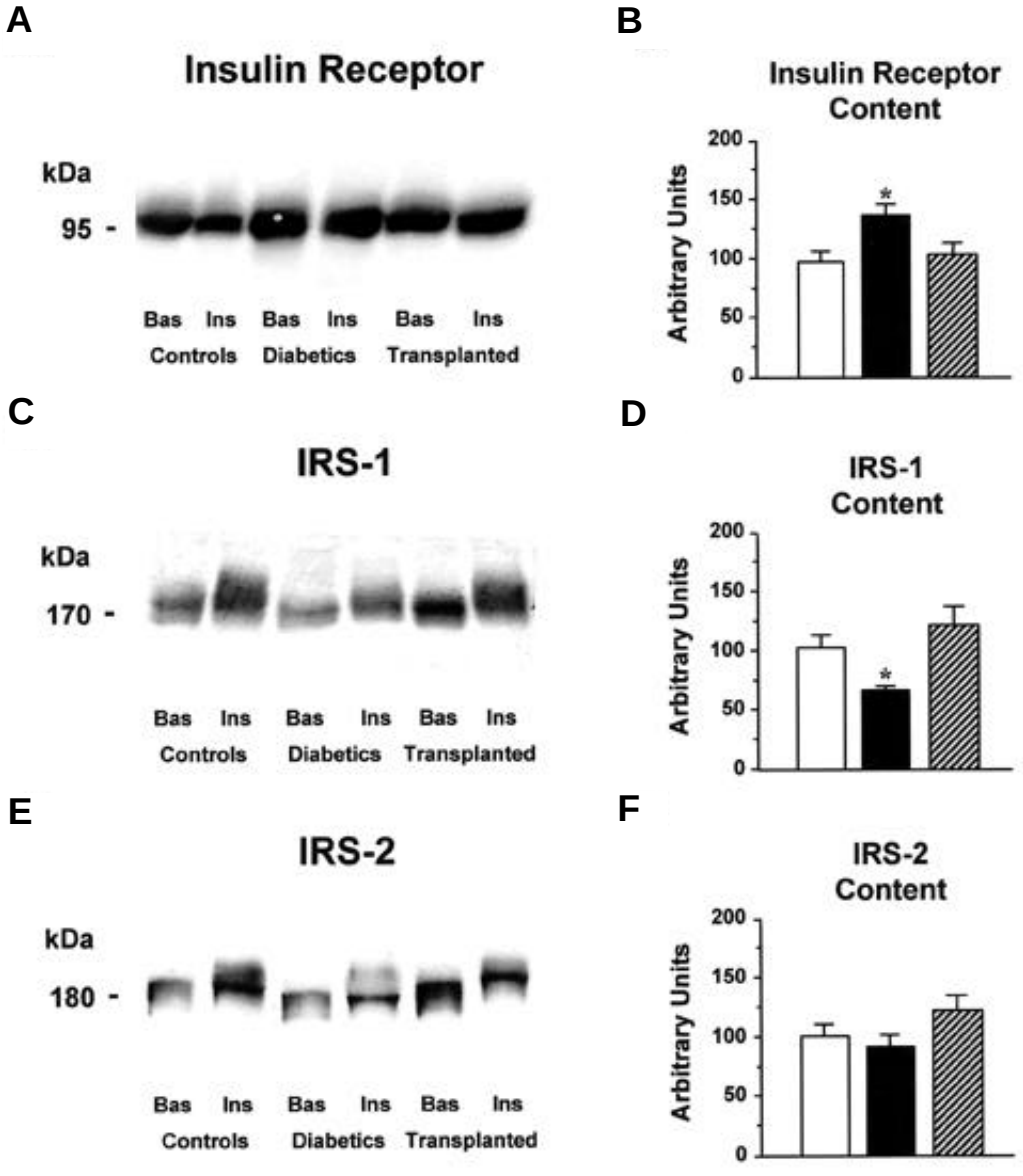

FIG. 2. Total amounts of insulin receptors, IRS-1, and IRS-2 in rat skeletal muscle. Control (□), untreated diabetic (■), and transplanted diabetic (▨) rats were studied in the basal state (Bas) or 30 min after injection of 20 U of insulin (Ins) intraperitoneally. The total amounts of insulin receptors, IRS-1, and IRS-2 were determined by direct immunoblotting with specific anti-peptide antibodies. Representative immunoblots of insulin receptors, IRS-1, and IRS-2 are presented in *A*, *C*, and *E*, respectively. The quantitation of the amounts of insulin receptors, IRS-1, and IRS-2 in the skeletal muscle of multiple experimental animals is shown in *B*, *D*, and *F*, respectively (n = 8 animals in each condition; values of basal and insulin-stimulated rats pooled together). *P < 0.05 vs. control and transplanted rats.

analysis of multiple experiments demonstrated that insulin increased IRS-1–associated PI 3-kinase activity by 500, 245, and 295% in control, untreated diabetic, and transplanted diabetic rats, respectively (P < 0.05) (Fig. 4*B*). In the skeletal muscle of diabetic rats, the levels of IRS-1–associated PI 3-kinase activity were markedly elevated in the basal state (430% of control basal, P < 0.05) to a level indistinguishable from insulin-stimulated PI 3-kinase activity in control rats (P = 0.37) (Fig. 4*A* and *B*). Insulin-stimulated PI 3-kinase activity associated with IRS-1 was also considerably higher in diabetic rats compared with controls (265% of control insulin-stimulated; P < 0.05) (Fig. 4*A* and *B*). As shown in Fig. 4*C*, the levels of basal and insulin-stimulated PI 3-kinase activity appeared to be more markedly augmented in the diabetic rats when related to the total amount of IRS-1 protein (570 and 438% of control, respectively; P < 0.05). However, both basal and insulin-stimulated levels of PI 3-kinase activity and the ratio of IRS-1–associated PI 3-kinase activity to total IRS-1 protein were not different from control in transplanted rats (P = 0.16 and 0.2 for basal and insulin-stimulated activity, respectively; P = 0.2 and 0.25 for basal and insulin-stimulated ratios of PI 3-kinase activity to IRS-1 protein, respectively) (Fig. 4). The elevated levels of IRS-1–associated PI 3-kinase activity in diabetic skeletal muscle were associated with increased IRS-1 tyrosine phosphorylation determined by immunoprecipitation with anti–IRS-1 antibody and subsequent immunoblotting with anti-PY antibody (data not shown). A trend toward increased levels of basal and insulin-stimulated PI 3-kinase activity associated with IRS-2 was also observed in the diabetic animals, although this difference was not statistically significant (data not shown).

Recent experimental work has demonstrated that insulin stimulation results in targeting of PI 3-kinase to low-density microsomes and GLUT4 vesicles in adipocytes (39–41), and it has been suggested that the specific localization of the enzyme in these subcellular compartments may be important for insulin regulation of GLUT4 trafficking. Since both p85- and IRS-1–associated pools of PI 3-kinase activity measured in total skeletal muscle extracts were increased in diabetes and returned to control levels after islet transplantation, we investigated whether PI 3-kinase activity in low-density microsomes was regulated in a similar manner. The activity of

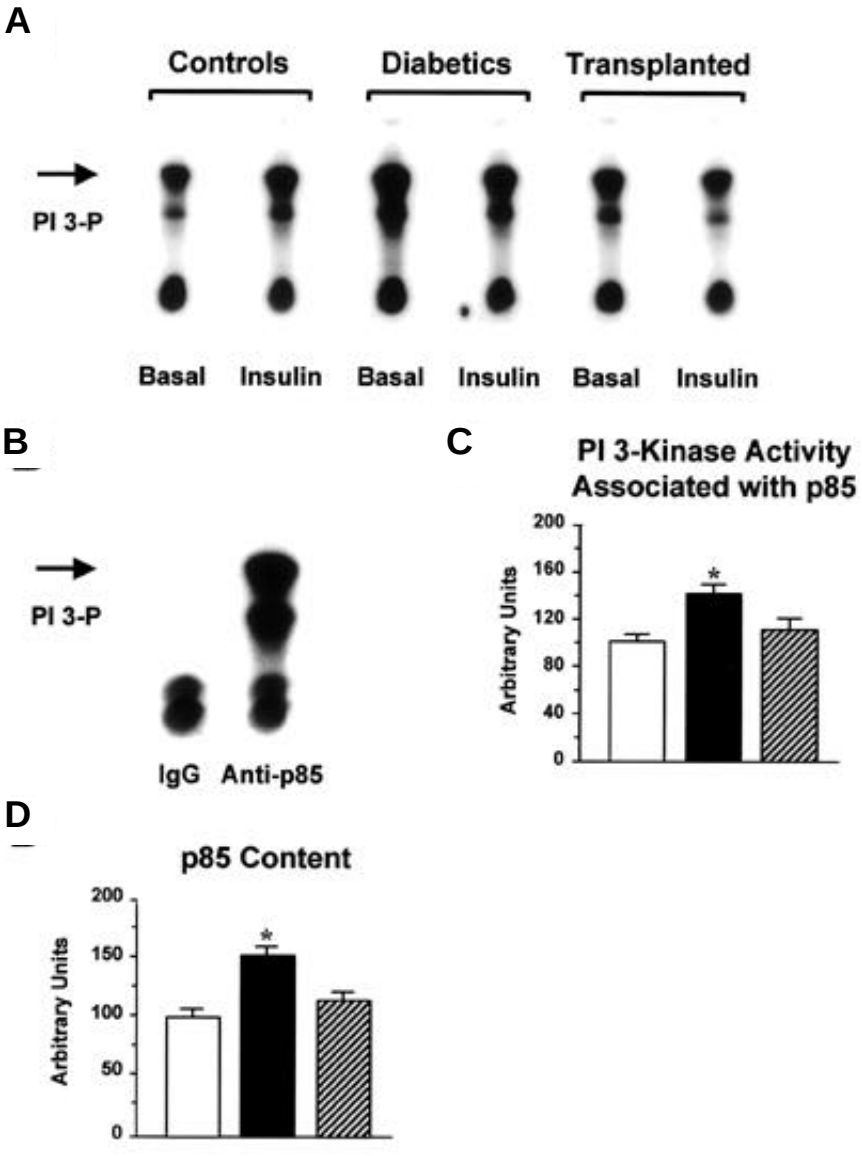

FIG. 3. Skeletal muscle PI 3-kinase activity associated with the p85 subunit. Control (□), untreated diabetic (■), and transplanted diabetic (▨) rats were studied in the basal state or 30 min after injection of 20 U insulin intraperitoneally. To measure the levels of PI 3-kinase activity associated with the p85 subunit of PI 3-kinase, solubilized skeletal muscle proteins were subjected to immunoprecipitation with anti-p85 antibody, and PI 3-kinase activity in anti-p85 immunoprecipitates was measured by an in vitro assay with ^{32}P-ATP and PI, as described in METHODS. The autoradiogram in *A* shows a representative experiment. The position of PI 3-phosphate (designated PI 3-P) is indicated. In *B*, equal amounts of solubilized skeletal muscle proteins from a control rat were subjected to immunoprecipitation with either anti-p85 antibody or control preimmune rabbit IgG in parallel. The PI 3-kinase activity precipitated by the anti-p85 antibody is specifically associated with the p85 subunit of PI 3-kinase because a large amount of activity is precipitated by the anti-p85 antibody, whereas no activity is precipitated by the pre-immune IgG. *C* shows the quantitation of PI 3-kinase activity in the experimental groups (n = 4 animals in each group; values of basal and insulin-stimulated rats pooled together). *D* illustrates the skeletal muscle amount of p85 protein measured by immunoblotting with anti-p85 antibody (n = 8 animals in each group; values of basal and insulin-stimulated rats pooled together). *P < 0.05 vs. control and transplanted rats.

KpNPPase in the microsomal membranes obtained from rat skeletal muscle was approximately twofold higher than in the starting homogenate, indicating a low degree of contamination with plasma membranes in this fraction. No differences were found in KpNPPase activity, recovery, or enrichment in the microsomal membranes from the different groups of experimental animals, as reported previously (37). Microsomal membranes were solubilized in detergent-containing buffer and subjected to immunoprecipitation with either anti-p85 or anti-IRS-1 antibodies before assaying PI 3-kinase activity in the resulting immunoprecipitates, as described in METHODS. Figure 5*A* illustrates a representative experiment in which PI 3-kinase activity was measured in anti-p85 immunoprecipitates. The activity of PI 3-kinase was not modified by insulin in any of the experimental groups of animals (P = 0.40, 0.48, and 0.35 in control, diabetic, and transplanted rats, respectively) (Fig. 5*A*). The levels of PI 3-kinase activity in anti-IRS-1 immunoprecipitates were very low and also did not show any change in response to insulin stimulation (data not shown). Thus, in contrast to the results obtained by other investigators in cultured adipocytes, we could not demonstrate insulin-regulated targeting of PI 3-kinase activity to low-density microsomes in rat skeletal muscle. PI 3-kinase activity in microsomes was modestly, but significantly, increased (27%) in diabetic rats compared with controls (P < 0.05) (Fig. 5*B*), whereas it was not different in transplanted rats compared with controls (P = 0.07) (Fig. 5*B*). Therefore, the skeletal muscle of untreated diabetic rats was characterized by increased levels of PI 3-kinase activity both in total tissue extracts and, to a lesser extent, in low-density microsomes, and this was corrected by islet transplantation.

Insulin signaling in myocardium. The abnormal tyrosine phosphorylation and content of insulin signaling proteins found in diabetic skeletal muscle was corrected by treatment of hyperglycemia with islet transplantation. To determine whether islet transplantation was capable of normalizing signaling reactions in another insulin target tissue, insulin receptor and substrate tyrosine phosphorylation was investigated in the myocardium of the experimental animals. For these studies, rats were studied in the basal state or after intraperitoneal injection of 20 U of insulin. After 30 min, cardiac ventricles were removed and homogenized in buffer containing 1% NP-40 and various protease and phosphatase inhibitors

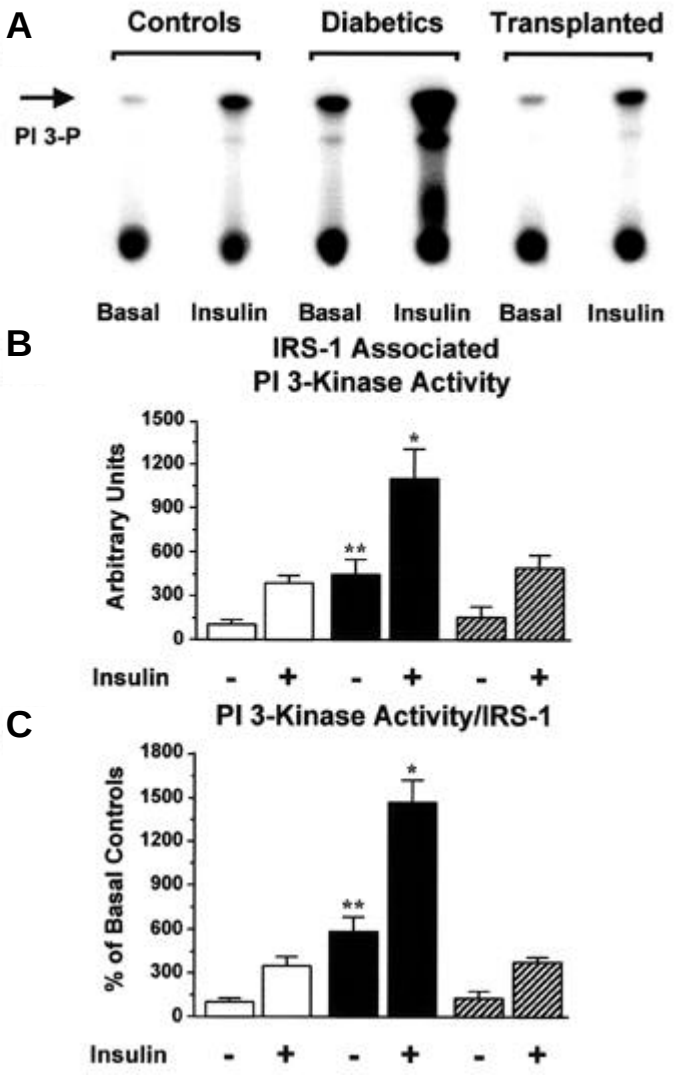

FIG. 4. Skeletal muscle PI 3-kinase activity associated with IRS-1. Control (□), untreated diabetic (■), and transplanted diabetic (▨) rats were studied in the basal state or 30 min after injection of 20 U insulin intraperitoneally. To measure the levels of IRS-1–associated PI 3-kinase activity, solubilized muscle proteins were subjected to immunoprecipitation with anti-IRS-1 antibody, and PI 3-kinase activity in the IRS-1 immunoprecipitates was assayed as described in METHODS. The autoradiogram in *A* shows a representative experiment. The position of PI 3-phosphate (PI 3-P) is indicated. The quantitation of IRS-1–associated PI 3-kinase activity in basal and insulin-stimulated rats is illustrated in *B* (*n* = 4 animals in each condition). *C* illustrates the ratio of IRS-1–associated PI 3-kinase activity to total IRS-1 protein in the experimental animals. **P* < 0.05 vs. insulin-stimulated control and insulin-stimulated transplanted rats; ***P* < 0.05 vs. basal control and basal transplanted rats.

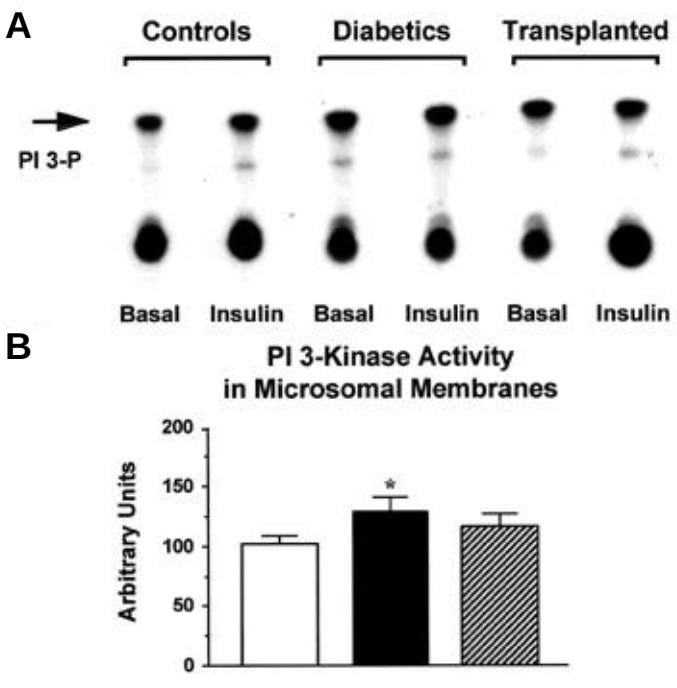

FIG. 5. PI 3-kinase activity in skeletal muscle microsomal membranes. Control (□), untreated diabetic (■), and transplanted diabetic (▨) rats were studied in the basal state or 30 min after injection of 20 U insulin intraperitoneally. Skeletal muscle microsomal membranes were prepared as described in METHODS, then solubilized and subjected to immunoprecipitation with anti-p85 antibody. PI 3-kinase activity in the immunoprecipitates was assayed as described in METHODS. A representative thin layer chromatogram is shown in *A*. The position of PI 3-phosphate (PI 3-P) is indicated. The quantitation of PI 3-kinase activity in the microsomal membranes in the experimental rats is illustrated in *B* (*n* = 8 animals in each group; values of basal and insulin-stimulated rats pooled together). **P* < 0.05 vs. controls.

before immunoblotting with anti-PY antibody, as described for skeletal muscle. The pattern of myocardial tyrosine phosphoproteins from control, diabetic, and transplanted rats is presented in Fig. 6*A*. Three distinct phosphoprotein bands in the 160–180 kDa range were found in myocardium, whereas one or two phosphoproteins of similar molecular weight were observed in skeletal muscle (Fig. 1). Densitometric analysis of multiple experiments demonstrated that insulin-stimulated tyrosine phosphorylation of the receptor β-subunit was increased by 45% in diabetic compared with control myocardium ($P < 0.05$) and was reduced to control levels in the myocardium of transplanted rats (Fig. 6*B*). The levels of tyrosine phosphorylation of the insulin receptor substrates in the 160–180 kDa range, collectively quantified, appeared to be higher in diabetic than in control or transplanted rats, although this difference was not statistically significant ($P = 0.07$) (Fig. 6*C*). IRS-1–associated PI 3-kinase activity was also slightly higher, although not significantly, in myocardium of diabetic compared with control or transplanted rats (data not shown). Interestingly, a 66-kDa tyrosine phosphoprotein (pp66) was consistently observed in diabetic myocardium, irrespective of insulin stimulation, but not in the myocardium of control or transplanted animals (Fig. 6*A*).

The changes in the amount of insulin receptors in the myocardium of the experimental rats were similar to those observed in skeletal muscle. The total amount of insulin receptor protein in myocardium was increased by 35% in diabetic rats compared with controls ($P < 0.05$) and did not differ between control and transplanted rats ($P = 0.21$) (Fig. 7*A*). The ratio of receptor tyrosine phosphorylation to receptor protein was not different from control in diabetic and transplanted rats (92.7 ± 6.1% of control, $P = 0.2$, and 98.2 ± 3.4% of control, $P = 0.33$, in diabetic and transplanted rats, respectively). Compared with skeletal muscle, a lower amount of IRS-1 and a greater amount of IRS-2 were found to be expressed in myocardium (Fig. 7*B*). The amount of IRS-1 was decreased by 41% in diabetic myocardium ($P < 0.05$) and increased after islet transplantation, approximating the

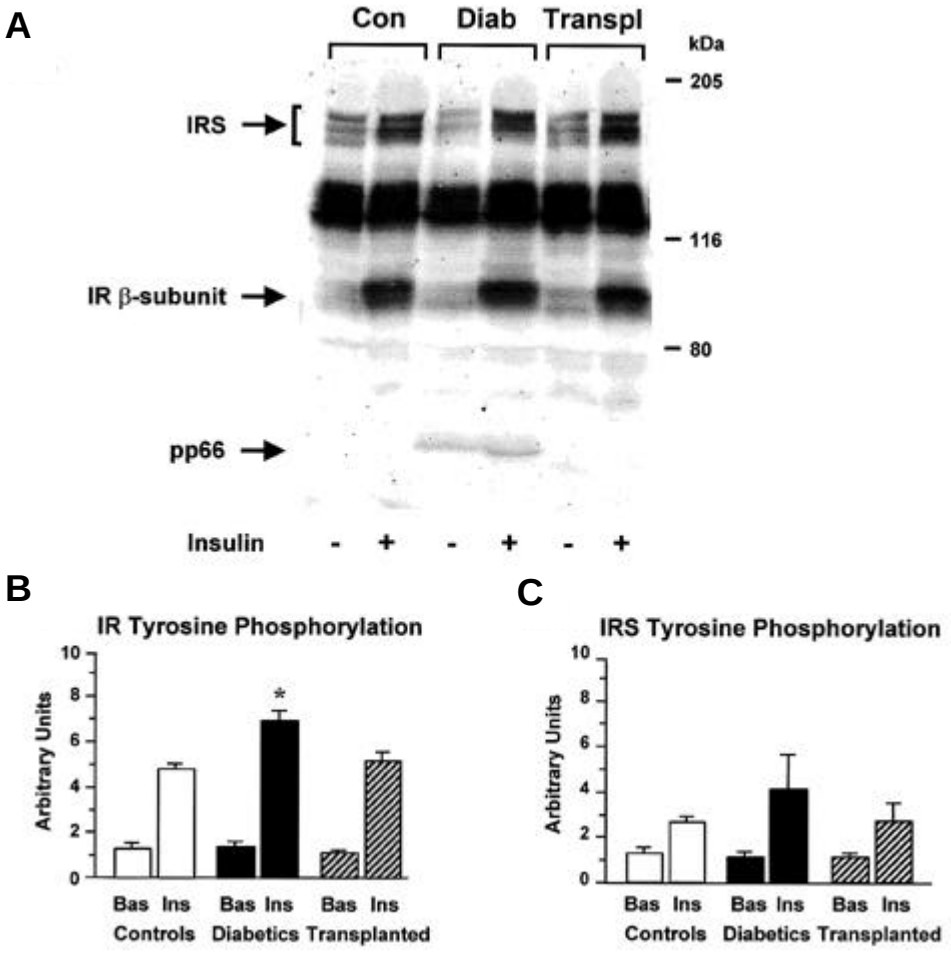

FIG. 6. Insulin receptor and substrate tyrosine phosphorylation in myocardium of control (Con), untreated diabetic (Diab), and transplanted diabetic (Transpl) rats. After overnight fasting, cardiac ventricles from basal or insulin-stimulated rats were removed and processed as described in METHODS. Myocardial proteins were resolved by 7% SDS-PAGE, and tyrosine phosphoproteins were detected by immunoblotting with anti-PY antibody. *A* shows a representative experiment. *B* and *C* show the quantitation of basal (Bas) and insulin-stimulated (Ins) tyrosine phosphorylation of the insulin receptor β-subunit and of receptor substrates, respectively (n = 4 animals in each condition). IR, insulin receptor; IRS, insulin receptor substrates in the 160–180 kDa range. *P < 0.05 vs. insulin-stimulated control and insulin-stimulated transplanted rats.

control levels (Fig. 7*C*). In contrast to IRS-1, the total amount of IRS-2 protein in myocardium was augmented in STZ-diabetes (P < 0.05), and this increase was reversed in transplanted animals (P < 0.05) (Fig. 7*D* and *E*).

DISCUSSION

The effects of diabetes treatment by islet transplantation on the initial steps of the insulin signaling cascade were investigated in the skeletal muscle and myocardium of STZ-diabetic rats. To study insulin signaling in vivo, we used an established methodology that involves insulin injection of intact animals, rapid harvesting and homogenization of muscle tissue, and immunoblotting and other biochemical methods to determine the levels of insulin receptor and substrate tyrosine phosphorylation and PI 3-kinase activity (18,31). We have shown that the insulin replacement provided by islet transplantation was fully effective in correcting multiple signaling abnormalities, which developed in the skeletal muscle and myocardium of STZ-diabetic rats as a consequence of hypoinsulinemia and/or hyperglycemia. This response occurred after restoration of glucose-regulated insulin secretion by the transplanted islets, even though insulin was delivered into the systemic rather than the portal circulation as a consequence of islet implantation under the kidney capsule.

Islet transplantation fully corrected the abnormal content and activation of various proteins involved in the mechanism of action of insulin, such as the insulin receptor, the receptor substrates IRS-1 and IRS-2, and PI 3-kinase. We observed an increase in basal as well as insulin-stimulated tyrosine phosphorylation of the substrates in the 165–180 kDa range (comprising IRS-1 and IRS-2) and IRS-1–associated PI 3-kinase activity in diabetic skeletal muscle. An increase in insulin-stimulated insulin receptor and IRS-1 tyrosine phosphorylation in STZ-diabetes has been reported previously (18–20). In this study, however, IRS-1/2 tyrosine phosphorylation and IRS-1–associated PI 3-kinase activity were also found to be considerably higher in diabetic than in control skeletal muscle in the basal state, under conditions in which differences in the level of insulin receptor tyrosine phosphorylation could not be detected. In addition, this occurred selectively in skeletal muscle and not in myocardium, even though the amount of insulin receptor protein was similarly augmented by STZ-diabetes in both of these tissues. Thus, hyperphosphorylation of IRS-1/2 and sustained activation of PI 3-kinase in diabetic skeletal muscle cannot be solely explained by increased number and overall tyrosine kinase activity of insulin receptors. Several interleukins and interferons can stimulate IRS-1 and IRS-2 tyrosine phosphorylation independently of the insulin receptor kinase in intact cells (42,43), and high glucose concentrations have been found to increase the synthesis of IL-1 and IL-8 by cultured endothelial cells (44,45). It is possible, therefore, that elevated in situ concentrations of cytokines, secreted by endothelial or muscle cells, could persistently stimulate tyrosine phosphorylation of IRS-1/2 in the skeletal muscle of markedly hyperglycemic rats. Alternatively, upreg-

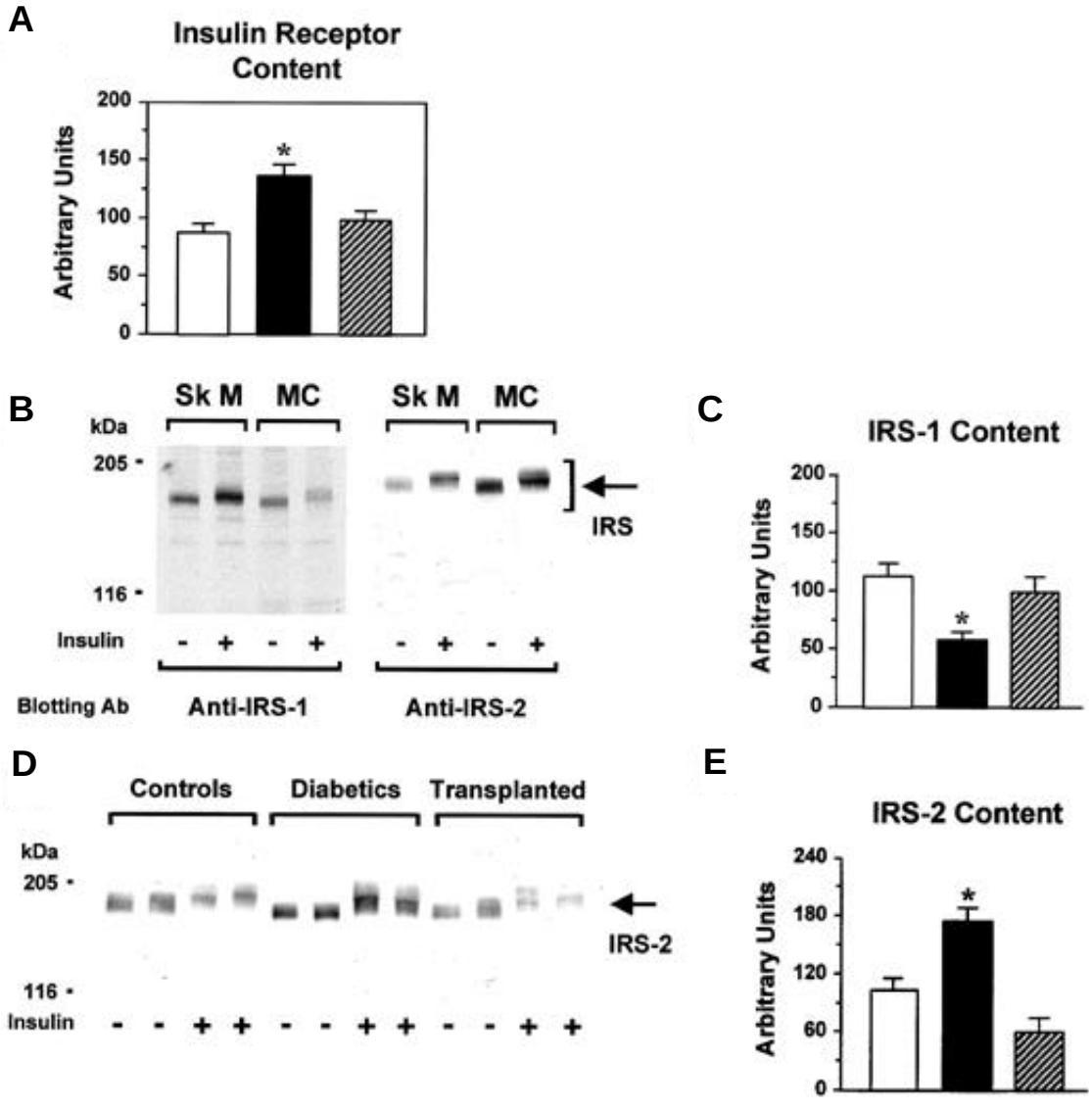

FIG. 7. Total amounts of insulin receptors, IRS-1, and IRS-2 in myocardium. The amounts of insulin receptor, IRS-1, and IRS-2 were determined by immunoblotting of solubilized myocardial proteins with specific antibodies, as described in METHODS. □, control rats; ■, untreated diabetic rats; ▨, transplanted diabetic rats. The quantitation of insulin receptors, IRS-1, and IRS-2 in the myocardium of the experimental animals is shown in *A*, *C*, and *E*, respectively (n = 4 animals in each group; values of basal and insulin-stimulated rats pooled together). *P < 0.05 vs. control and transplanted rats. *B* shows IRS-1 and IRS-2 protein levels in skeletal muscle and myocardium of basal and insulin-stimulated control rats. Equal amounts of muscle proteins were resolved by 7% SDS-PAGE and subjected to immunoblotting with either anti-IRS-1 or anti-IRS-2 antibodies, as indicated. *D* shows a representative anti-IRS-2 immunoblot of solubilized myocardial proteins from basal and insulin-stimulated control, diabetic, and transplanted rats. Sk M, skeletal muscle; MC, myocardium; IRS, insulin receptor substrates in the 160–180 kDa range.

ulation of substrate tyrosine phosphorylation in the presence of hypoinsulinemia could potentially result from the reduced activity of an IRS-1/2-specific phosphotyrosine-protein (PTP) phosphatase. Consistent with this hypothesis, membrane-associated PTP phosphatase activity was found to be decreased in the skeletal muscle of STZ-diabetic rats (46,47), although it is not known whether the PTP phosphatase activity measured in these previous studies selectively dephosphorylates IRS-1 and -2 versus the insulin receptor. In this scenario, it can be hypothesized that the transplantation-mediated normalization of basal and insulin-stimulated signaling reactions in rat skeletal muscle may have involved multiple mechanisms, including the restoration of normal levels of insulin receptor kinase as well as yet uncharacterized cytokines and/or PTP phosphatases controlling tyrosine phosphorylation of the substrates IRS-1 and IRS-2.

Defects in insulin-regulated glucose metabolism, including reduced glucose uptake and utilization, have been reported in the myocardium of STZ-diabetic rats (48–50). However, potential abnormalities in content and/or activity of insulin signaling proteins in this tissue or their correction by islet transplantation have not been investigated. In this study, we observed increased receptor tyrosine phosphorylation and a trend toward increased substrate tyrosine phosphorylation in the myocardium of insulin-stimulated STZ-diabetic animals. These changes were concordant with those detected in the skeletal muscle and were similarly normalized by islet transplantation. Other diabetes-associated signaling abnormalities specific to the myocardial tissue included the selective

augmentation of IRS-2 protein and the appearance of a 66-kDa tyrosine phosphoprotein, and these were also corrected by islet transplantation. Since IRS-2 protein levels were not altered in skeletal muscle and IRS-1 protein levels were reduced in both myocardium and skeletal muscle, it can be concluded that the regulation of IRS-2 by STZ-diabetes is tissue specific and distinct from that of IRS-1. In the Zucker fatty rat and the *ob/ob* mouse, two experimental models of non-insulin-dependent diabetes, IRS-1 and IRS-2 were both decreased in skeletal muscle and liver compared with controls (51,52). On the other hand, IRS-1 was markedly reduced, whereas IRS-2 was unchanged, in adipocytes from non-insulin-dependent diabetic patients (53). Potential changes in myocardial content of these two substrate proteins in response to diabetes have not been investigated in any of these previous studies, and it is not known, therefore, whether the divergent regulation of IRS-1 and IRS-2 observed in the myocardium of STZ-diabetic rats is characteristic of all diabetic states or is distinctive of insulinopenic diabetes. Treatment of insulin-deficient diabetic rats with insulin therapy or islet transplantation has recently been shown to ameliorate metabolic and functional derangements in the heart (54,55). Although the pathogenic impact of the increased IRS-2/IRS-1 ratio on diabetic myocardium has not been established, the correction of this, as well as of other signaling abnormalities by islet transplantation, may represent a potential mechanism for the known favorable effects of an adequate insulin delivery on ventricular function and cardiac performance in diabetic animals (55–57).

The complete normalization of altered protein tyrosine phosphorylation by islet transplantation could not be obtained with the administration of insulin subcutaneously. As reported in a previous study (18), intermittent subcutaneous insulin resulted in subnormal levels of insulin-stimulated receptor and substrate tyrosine phosphorylation. In addition, the levels of the IRS-1 protein, which are lower in diabetic than control skeletal muscle, were further reduced after subcutaneous insulin therapy (F. Giorgino, R.J. Smith, unpublished observations). A similar reduction of IRS-1 protein to levels lower than control has recently been demonstrated in the myocardium of insulin-treated STZ-diabetic rats in an independent study from our group (58). Exogenously administered insulin in the treatment of insulinopenic diabetes has the potential to generate peripheral hyperinsulinemia. In a previous study (18), the therapeutic regimen necessary to normalize blood glucose concentrations in STZ-diabetic rats consisted of Ultralente insulin 5 U/rat twice daily, and this resulted in fasting plasma insulin concentrations fivefold higher than control levels (79.6 ± 33.1 vs. 14.1 ± 3.5 μU/ml in insulin-treated STZ-diabetic vs. control rats). Since prolonged exposure of cells to high insulin concentrations can reportedly downregulate IRS-1 protein content and intracellular signaling (59,60), the inability of subcutaneous insulin therapy to restore normal levels of IRS-1 protein and IRS-1 tyrosine phosphorylation in diabetic muscle may be explained by the excessive increase in peripheral insulinemia associated with this treatment. It is conceivable that IRS-1 downregulation did not occur in islet-transplanted diabetic rats because insulin concentrations were closer to control values in these animals than they were in insulin-treated diabetic rats. In transplanted rats, islets were implanted under the kidney capsule, and, therefore, insulin was delivered into the peripheral circulation. It can be concluded that physiological delivery of insulin into the portal circulation is not necessary for the favorable effects of islet transplantation on insulin signaling. The improved response to islet transplantation could reflect the correction of hyperglycemia without the generation of marked hyperinsulinemia. However, it should be noted that islets release C-peptide in addition to insulin, but C-peptide is not administered with exogenous insulin therapy. The concentrations of C-peptide are very low in STZ-diabetic rats (61) and likely were augmented after pancreatic islet transplantation. It is of interest to speculate that C-peptide released by the transplanted islets may have contributed to the normalization of the insulin signaling system in diabetic muscle. In this regard, C-peptide has been shown to exert direct and specific effects on rat skeletal muscle, as demonstrated by its ability to stimulate glucose transport in isolated skeletal muscle strips (62,63), and it reportedly can prevent diabetes-induced dysfunction in rat tissues (61).

STZ-diabetic rats have decreased basal and insulin-stimulated rates of glucose transport in skeletal muscle. We have demonstrated that these defects are caused by a reduced amount of GLUT4 protein and impaired insulin-induced transporter translocation to the plasma membrane (37). Upon reexamination of the same animals, we have shown that there is a marked elevation in basal and insulin-stimulated PI 3-kinase activity associated with IRS-1. Since activation of PI 3-kinase is believed to be both necessary and sufficient for glucose transporter translocation in response to insulin (8–10,64–66), the relationship between the increased PI 3-kinase activity and the impaired glucose transport system in the skeletal muscle of STZ-diabetic animals is not clear. In addition, the PI 3-kinase and glucose transport systems were oppositely regulated by correction of hypoinsulinemia and hyperglycemia because the abnormally elevated levels of PI 3-kinase activity were reduced in transplanted animals, whereas insulin-dependent transporter translocation was increased (37). Thus, the impaired GLUT4 translocation in STZ-diabetes may result either from a defective signaling component downstream of PI 3-kinase or from a qualitative alteration of this enzyme, such as its inability to localize to low-density microsomes and GLUT4 vesicles (39–41). PI 3-kinase activity was also moderately increased, however, in low-density microsomes in diabetes, although less than in total muscle extracts, suggesting that defective GLUT4 translocation cannot be explained by reduced levels of PI 3-kinase in this cellular compartment. On the other hand, the sustained increase in PI 3-kinase activity may transduce signals unrelated to regulation of GLUT4 trafficking. For example, persistent PI 3-kinase activation may have contributed to the reduced levels of GLUT4 protein that were observed in the skeletal muscle of diabetic rats (37,67). This concept is supported by the observation that GLUT4 downregulation in response to chronic insulin stimulation of adipocytes could be prevented by inhibition of PI 3-kinase with wortmannin (60). It is possible that islet transplantation led to higher GLUT4 levels in diabetic muscle through correction of the increased PI 3-kinase activity that characterizes the diabetic state.

In summary, we have demonstrated that treatment of STZ-diabetes by islet transplantation can produce a full correction of multiple abnormalities in various proteins involved in upstream events of the insulin signaling pathway. We suggest that the exposure of skeletal muscle and myocardium to ade-

quate insulin concentrations in the course of insulinopenic diabetes is critical for the preservation of physiological insulin signaling reactions in these peripheral tissues.

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