The insulin signaling cascade was investigated in rat myocardium in vivo in the presence of streptozocin (STZ)-induced diabetes and after diabetes treatment by islet transplantation under the kidney capsule. The levels of insulin-stimulated tyrosine phosphorylation of the insulin receptor β-subunit, insulin receptor substrate (IRS)-2, and p52SHC were increased in diabetic compared with control heart, whereas tyrosine phosphorylation of IRS-1 was unchanged. The amount of the p85 subunit of phosphatidylinositol 3-kinase (PI 3-kinase) and the level of PI 3-kinase activity associated with IRS-2 were also elevated in diabetes, whereas no changes in IRS-1-associated PI 3-kinase were observed. Insulin-induced phosphorylation of Akt on Thr-308 was increased fivefold in diabetic heart, whereas Akt phosphorylation on Ser-473 was normal. In contrast with Akt phosphorylation, insulin-induced phosphorylation of glycogen synthase kinase (GSK)-3, a major cellular substrate of Akt, was markedly reduced in diabetes. In islet-transplanted rats, the majority of the alterations in insulin-signaling proteins found in diabetic rats were normalized, but insulin stimulation of IRS-2 tyrosine phosphorylation and association with PI 3-kinase was blunted. In conclusion, in the diabetic heart, 1) IRS-1, IRS-2, and p52SHC are differently altered, 2) the levels of Akt phosphorylation on Ser-473 and Thr-308, respectively, are not coordinately regulated, and 3) the increased activity of proximal-signaling proteins (i.e., IRS-2 and PI 3-kinase) is not propagated distally to GSK-3. Islet transplantation under the kidney capsule is a potentially effective therapy to correct several diabetes-induced abnormalities of insulin signaling in cardiac muscle but does not restore the responsiveness of all signaling reactions to insulin. Diabetes 50:2709–2720, 2001

Multiple changes in cardiac structure and function have been reported in the course of both experimental and human diabetes, contributing to the identification of a specific diabetic cardiomyopathy. These include increased contractility, impaired diastolic compliance, reduced cardiac output, hypertrophy, and fibrosis (1,2). Moreover, cardiomyocytes show specific diabetes-induced biochemical defects, such as diminished glucose utilization during ischemia (3), reduced insulin stimulation of glycogen synthesis (4), increased fatty acid oxidation (5), and altered expression of myosin α- and β-heavy chains (6).

Insulin plays an important role in regulating cardiac muscle metabolism by controlling the rates of glucose uptake, glycogen synthesis, glycolysis, and fatty acid oxidation (7). In the cardiomyocyte, insulin also affects transmembrane ion transport and the expression of genes involved in cell growth and differentiation (7). The biological effects of insulin in myocardium are mediated by cell-surface receptors composed of two extracellular α-subunits, containing hormone binding sites, and two membrane-spanning β-subunits, encoding an intracellular tyrosine kinase (8). Binding of insulin activates the receptor kinase, leading to receptor autophosphorylation and tyrosine phosphorylation of multiple substrates, including the IRS and Shc proteins (reviewed in Virkamaki et al. [9]). Through these initial tyrosine phosphorylation reactions, insulin signals are transduced to a complex network of intracellular lipid and serine/threonine kinases that are ultimately responsible for specific biological effects. We recently reported an increase in insulin receptor expression and tyrosine kinase activity in the heart of streptozocin (STZ)-diabetic rats (4,10). However, upregulation of insulin receptor signaling obviously cannot account for the reduced insulin bioeffects demonstrated in diabetic myocardium, including the impaired stimulation of glycogen synthase activity (4). Whether specific proteins downstream of the insulin-signaling cascade, such as Akt, glycogen synthase kinase (GSK)-3, and mitogen-activated protein kinase (MAPK), show altered expression and/or activation in the heart of STZ-diabetic animals has not been investigated.

Conventional subcutaneous insulin therapy is reportedly not always associated with restored heart function in diabetes (11–13). Moreover, excessive or inappropriate...
insulin administration has been suggested to favor the risk of cardiovascular disease, probably through an accelerated progression of coronary atherosclerosis (14–16). By contrast, diabetes therapy with islet transplantation may have beneficial effects on the progression of diabetic vascular complications (17–20) and is associated with improved contractile performance of the cardiac muscle (21). It is possible that the reported beneficial effects of islet transplantation on cardiac function involve correction of altered signaling mechanisms in the cardiac tissue.

The objective of this study was to analyze cardiac insulin-signaling systems in STZ-diabetic rats and to evaluate the effects of restoring glucose-regulated insulin secretion. 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 the heart of insulin-deficient diabetic rats is characterized by heterogeneous alterations of multiple signaling proteins, including insulin receptor substrate (IRS)-2, Shc, phosphatidylinositol 3-kinase (PI 3-kinase), Akt, and GSK-3. Moreover, treatment of hyperglycemia by islet transplantation results in the correction of several diabetes-related changes in expression and activity of these proteins but is not able to restore the hormone responsiveness of all insulin signaling-reactions.

RESEARCH DESIGN AND METHODS

Animals. Animals have been described in detail elsewhere (10). Briefly, male inbred Lewis rats that weighed 200 g (Tacicon 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 were left untreated and were studied 10 weeks later. Untreated diabetic rats 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. Diabetic rats that received a transplantation were made diabetic by STZ, 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 between 8:30 and 10:30 a.m. after an overnight fast. Maximal insulin stimulation was achieved by intraperitoneal injection of 20 units insulin 30 min before the rats were killed. The rats were considered to be insulin-stimulated when serum insulin concentrations 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. The cardiac ventricles were rapidly frozen and stored at −80°C for 3 months.

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 125I-labeled insulin (Linco, St. Louis, MO).

Islet isolation and transplantation. Isolation and transplantation of rat pancreatic islets were performed as described previously (22). Briefly, animals were anesthetized with sodium amobarbital (Elly 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 (Becton Dickinson, Parsippany, NJ) and received an injection of 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 Chemical) centrifuged at 2,500 g for 20 min. An aliquot of 2,000 freshly isolated islet equivalents was aspirated into a 200-μl pipette tip (USA Scientific Plastic, Ocala, FL) connected to a 1-ml syringe (Hamilton, Reno, NE) and then transferred into P-50 polyethylene tubing. 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-insulin receptor β-subunit and monoclonal antiphosphotyrosine antibodies (PI99) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-Akt, anti-phyospho-Akt (Thr-208), anti-phyospho-Akt (Ser-473), anti-MAK kinase (MEK)-1/2, anti-phospho-MEK-1/2 (Ser-218/222), anti-phospho-regulated (Rho) guanosine triphosphate (GTP)-regulated kinases (ERK)-1/2 (Thr-202/Tyr-204), and anti-phospho-GSK-3 were purchased from Upstate Biotechnology (Saranac Lake, NY) and Transduction Laboratories (Lexington, KY), respectively. Polyclonal antibodies to the p85 subunit of PI 3-kinase, anti-Ir1-1, anti-Ir2-1, and anti-GSK-3 antibodies were purchased from Upstate Biotechnology. Anti-MAK (Erk-1/2) antibodies were from Zymed Laboratories (San Francisco, CA).

Immunoprecipitation and immunoblotting. The frozen cardiac muscle tissue was powdered in a stainless-steel mortar and pestle with liquid N2 and homogenized for 30 s with an Ultra-Turrax (Janke & Kunkel, IKA-Werk, Staufen, Germany) in ice-cold lysis buffer containing 50 mmol/l HEPES (pH 7.5), 150 mmol/l NaCl, 1 mmol/l MgCl2, 1 mmol/l CaCl2, 10% glycerol, 10 mmol/l sodium pyrophosphate, 10 mmol/l sodium fluoride, 2 mmol/l EDTA, 2 mmol/l β-glycerolphosphate, 2 μg/ml aprotinin, 0.001% 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 100,000 g for 60 min. The supernatant was centrifuged and assayed for protein concentration using the Bradford dye binding assay kit with bovine serum albumin (BSA) as a standard. Equal amounts of cardiac extracts (1–2 mg) were subjected to immunoprecipitation with anti-insulin receptor β-subunit, anti-Ir-1-1, anti-Ir-2-1, and anti-Shc antibodies overnight at 4°C, as indicated. The resulting immune complexes were adsorbed to Protein A-Sepharose beads for 2 h at 4°C, washed three times with lyse buffer, and then eluted with Laemmli buffer for 5 min at 100°C.

For immunoblotting studies, equal amounts of solubilized cardiac proteins were resolved by electrophoresis on 7, 10, or 12% SDS-polyacrylamide gels, as appropriate, directly or after immunoprecipitation with the specific antibodies, as indicated. The resolved proteins were electrophotgraphically 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. For reducing nonspecific binding, 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, or in phosphate-buffered saline (PBS) supplemented with 3% nonfat dry milk for 1 h at 4°C, and then incubated overnight at 4°C with the indicated antibodies. The proteins were visualized by enhanced chemiluminescence using horseradish peroxidase–labeled anti-rabbit or anti-mouse IgG (Amersham Life Science) and quantified by densitometric analysis using Optilab image analysis software (Graftek SA, Mirmande, France).

Measurement of PI 3-kinase activity. Total cardiac muscle extracts (1–2 mg) were subjected to immunoprecipitation with anti-Ir-1-1, anti-Ir-2-1, or anti-phospho antibodies overnight at 4°C, as indicated. The resulting immune complexes were adsorbed to Protein A-Sepharose beads for 2 h at 4°C, and the pelleted beads were washed in PBS 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 100 mmol/l Tris-HCl (pH 7.6) containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 μmol/l sodium orthovanadate (twice). The activity of PI 3-kinase in the immunoprecipitates was determined in the presence of 2 μmol/l phosphatidylinositol (Avanti, Birmingham, AL) and 1 mmol/l ATP containing 30 μCi [γ-32P]ATP at 37°C for 10 min. The reaction products were subjected to a silica gel TLC plate (Merck, Darmstadt, Germany) and resolved by ascending chromatography in chloroform/methanol/water/ammonia (60:47:11.3:2, vol/vol). The PI 3-P product was visualized by autoradiography, identified by its co-migration with a PI 4-P standard, and quantified by scanning densitometry.

Measurement of Akt kinase activity. Akt activity was assessed in vitro using a nonradioactive kinase assay (Cell Signaling Technology, Beverly, MA) according to the manufacturer’s instructions. Briefly, total cardiac muscle extracts (2 mg) were subjected to immunoprecipitation with an agarose-conjugated anti-Akt antibody for 2 h at 4°C. The resulting immune complexes were washed in lysis buffer (twice) and in kinase buffer containing 25 mmol/l Tris-HCl (pH 7.5), 5 mmol/l β-glycerolphosphate, 10 mmol/l MgCl2, 2 mmol/l DTT, and 100 μmol/l sodium orthovanadate (twice). The activity of Akt in the immunoprecipitates was determined in the presence of a synthetic

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RESULTS

Metabolic characteristics of the experimental animals. The general characteristics of the experimental animals analyzed in this study are shown in Table 1. 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. By contrast, diabetic rats were characterized by markedly lower body weight compared with controls (P < 0.05) and higher serum glucose levels (P < 0.05). Diabetic animals that received a transplant gained an average of 62 g compared with the untreated diabetic rats and had serum glucose values in the normal range. In the basal fasting state, serum insulin concentrations seemed to be lower in the untreated diabetic animals than in controls and diabetic rats that had received a transplant, although this difference was not statistically significant. Diabetic animals that were subjected to islet transplantation were also characterized by greatly improved glucose tolerance measured by oral glucose tolerance test in comparison with the untreated diabetic rats (22 and data not shown), indicating significant restoration of glucose-induced insulin secretion.

Insulin receptor. For evaluating the expression and activation of cardiac insulin-signaling proteins in the experimental rats, insulin was administered by intraperitoneal injection, and 30 min later cardiac ventricles were rapidly removed, frozen in liquid N₂, and homogenized in buffer containing 1% NP-40 plus various protease and phosphatase inhibitors (described in RESEARCH DESIGN AND METHODS). Tyrosine phosphorylation of the insulin receptor was studied by immunoprecipitation with anti-insulin receptor antibody followed by immunoblotting with anti-phosphotyrosine antibody. Insulin induced a marked increase in tyrosine phosphorylation of the insulin receptor β-subunit in all groups (Fig. 1A; P < 0.05 versus basal). Insulin-stimulated tyrosine phosphorylation of the insulin receptor was modestly but significantly increased in diabetic compared with control rats (P < 0.05) and returned to normal levels in the animals that had received a transplant (Fig. 1A). The total amount of insulin receptor protein, determined by immunoprecipitation and immunoblotting with anti-insulin receptor antibody, was increased 50% in the diabetic heart compared with control (P < 0.05) and was restored to control levels after islet transplantation (Fig. 1B). The ratio of receptor tyrosine phosphorylation to receptor protein did not differ significantly in control and diabetic rats and rats that had received a transplant (3.7 ± 0.7, 2.8 ± 0.3, and 2.6 ± 0.3, respectively).

IRS proteins and PI 3-kinase. To investigate postreceptor insulin signaling proteins, we next evaluated tyrosine phosphorylation and expression levels of IRS-1 and IRS-2, the major insulin receptor substrates known to be expressed in the heart (24). Tyrosine phosphorylation was determined by immunoprecipitation with anti–IRS-1 or anti–IRS-2 antibodies followed by antiphosphotyrosine immunoblotting (Fig. 2A and B). In control experiments, these antibodies were found to immunoprecipitate >90% of IRS-1 or IRS-2, respectively (data not shown). This was assessed by comparing the amount of IRS-1 or IRS-2 (measured by immunoblotting an aliquot of the protein extracts with IRS-1 or IRS-2 antibodies) before and after immunoprecipitation with the specific antibodies. Insulin administration resulted in a significant increase in tyrosine phosphorylation of IRS-1 in all three experimental groups (Fig. 2A; P < 0.05 versus basal). The levels of insulin-stimulated IRS-1 tyrosine phosphorylation were not different in control and diabetic rats and rats that had received a transplant, but a modest 16% increase in basal IRS-1 tyrosine phosphorylation was found in the rats that had received a transplant (Fig. 2A; P < 0.05 versus basal). The ratio of IRS-1 tyrosine phosphorylation to IRS-1 protein after insulin stimulation, however, was increased in diabetic myocardium compared with control (4.4 ± 1.1 vs. 2.1 ± 0.2 in diabetic and control animals, respectively) and normalized after islet transplantation (2.2 ± 0.2). By contrast, tyrosine phosphorylation of IRS-2 was markedly augmented in diabetic heart, both in the basal (170% of control; P < 0.05) and insulin-stimulated (180% of control; P < 0.05) conditions (Fig. 2B). The changes in IRS-2 tyrosine phosphorylation were not corrected after islet transplantation because basal tyrosine phosphorylation remained elevated (149% of control; P < 0.05) and insulin-stimulated tyrosine phosphorylation was reduced below control levels (70% of control; P < 0.05). Therefore, insulin stimulation of IRS-2 tyrosine phosphorylation, which occurred in control and diabetic rats, was not detectable in the rats that had received a transplant (Fig. 2B). The ratio

TABLE 1

Experimental animals

<table>
<thead>
<tr>
<th>Rats</th>
<th>Body weight (g)</th>
<th>Serum glucose (mg/dl)</th>
<th>Serum insulin (μU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 8)</td>
<td>348 ± 10</td>
<td>106 ± 5</td>
<td>38.6 ± 11.4</td>
</tr>
<tr>
<td>Diabetic (n = 8)</td>
<td>246 ± 9*</td>
<td>456 ± 10*</td>
<td>18.0 ± 5.9</td>
</tr>
<tr>
<td>Transplant (n = 8)</td>
<td>308 ± 44†</td>
<td>97 ± 3*</td>
<td>28.2 ± 2.8</td>
</tr>
</tbody>
</table>

Data are means ± SE. Control male Lewis rats were left untreated and studied 10 weeks later; untreated diabetic rats 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; diabetic rats that had received a transplant 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 on the day that the animal was killed. Serum glucose and insulin levels were measured in the basal fasting state on the day that the animal was killed. The data in this table have been previously reported (10). *P < 0.05 versus controls; †P < 0.05 versus diabetic rats.
of IRS-2 tyrosine phosphorylation to IRS-2 protein in the insulin-stimulated state did not differ significantly in the experimental groups (data not shown).

The enzyme PI 3-kinase is recruited to IRS signaling complexes through binding of the two SH2 domains contained in its 85-kDa regulatory subunit to specific phosphotyrosine residues in IRS-1 and IRS-2 (9). To assess whether the changes in tyrosine phosphorylation of the IRS proteins were associated with coordinate changes in their association with the PI 3-kinase regulatory subunit p85, we analyzed IRS-1 and IRS-2 immunoprecipitates by immunoblotting with anti-p85 antibodies. Insulin injection significantly stimulated p85 association with IRS-1 in control and diabetic rats (Fig. 2C; *P < 0.05 versus basal). However, in the transplant group, a slightly higher amount of p85 protein than control was associated with IRS-1 in the basal condition (Fig. 2C; §P < 0.05 versus insulin-stimulated control). Diabetes differently affected the total amount of IRS proteins in the heart. IRS-1 protein levels were reduced in diabetic rats (57% of control; **P < 0.05; Fig. 2E), whereas IRS-2 levels were slightly but not significantly increased (Fig. 2F). Importantly, IRS-1 and IRS-2 protein levels were not different in control rats and rats that had received a transplant (Fig. 2E and F). No differences in total p85 protein levels were observed in the three experimental groups (data not shown).

The activity of PI 3-kinase was next measured in IRS-1 and IRS-2 immunoprecipitates from the cardiac tissue of the experimental rats. Insulin increased IRS-1-associated PI 3-kinase activity to a similar extent, ranging 2.2- to 3.3-fold over basal, in control and diabetic rats and rats that had received a transplant (Fig. 2A; **P < 0.05 versus control). By contrast, insulin stimulation of IRS-2-associated
PI 3-kinase activity was markedly increased in diabetic as compared with control rats (4.9- vs. 1.8-fold over basal; \( P < 0.05 \); Fig. 3B). In the rats that had received a transplant, the level of insulin-stimulated PI 3-kinase activity associated with IRS-2 was normalized (\( P < 0.05 \) versus diabetics and \( P < 0.05 \) versus controls), but basal PI 3-kinase activity was higher than control (144% of control; \( P < 0.05 \)), and the increase after insulin stimulation was not statistically significant (\( P < 0.05 \)). Therefore, insulin stimulation of IRS-2-associated PI 3-kinase activity was enhanced in diabetes and blunted after islet transplantation, correlating with the insulin effects on IRS-2 tyrosine phosphorylation and association with p85 (Fig. 2B and D).

Akt and GSK-3. The protein expression and activation levels of Akt, a serine/threonine kinase that lies downstream of PI 3-kinase, were next investigated in the heart of the experimental animals. Because sequential phosphorylation of Akt on Ser-473 and Thr-308 is required for full activation of the enzyme (25), the levels of Akt phosphorylation on each amino acid residue were determined by immunoblotting with phospho-specific anti-Akt antibodies. Insulin significantly increased Akt phosphorylation on Ser-473 to a similar level in the three experimental groups (\( P < 0.05 \) versus basal; Fig. 4A). A significant decrease of basal Akt phosphorylation on Ser-473 was noted in diabetic heart (33% of control; \( P < 0.05 \)), and this was corrected after islet transplantation (Fig. 4A). Akt phosphorylation on Thr-308 was also enhanced in response to insulin administration in the three experimental groups (\( P < 0.05 \) versus basal; Fig. 4B). However, in contrast with phosphorylation on Ser-473, the level of insulin-stimulated Akt phosphorylation on Thr-308 was increased fivefold in diabetic as compared with control heart (\( P < 0.05 \) versus control; Fig. 4B). Akt phosphorylation on Thr-308 was lower in rats that had received a transplant as compared with diabetic rats (\( P < 0.05 \) versus diabetic), even though it did not return to control levels (\( P < 0.05 \) versus control; Fig. 4B). Akt protein levels were not different in the experimental groups (Fig. 4C).
INSULIN SIGNALING IN DIABETIC HEART

Fig. 4

D

DESIGN AND METHODS. The autoradiograms in basal and insulin-stimulated animals with speci-plantation.

The capacity of Akt to phosphorylate the endogenous GSK-3 substrate protein was also examined. The levels of GSK-3 phosphorylation were determined by immunoblotting with phospho-specific anti-GSK-3 antibodies. Insulin administration resulted in increased serine phosphorylation of both GSK-3 isoforms in the heart of control rats (P < 0.05 versus basal; Fig. 5A). By contrast, in diabetic heart, insulin was not able to efficiently stimulate the phosphorylation of either GSK-3α or GSK-3β (P = 0.979 and P = 0.059 versus basal for GSK-3α and GSK-3β, respectively), and the level of insulin-stimulated GSK-3 phosphorylation was significantly lower than control (P < 0.05 versus control; Fig. 5A). Therefore, insulin stimulation of endogenous GSK-3 phosphorylation by Akt in vivo was markedly impaired in the diabetic heart (Fig. 5A), even though stimulation of Akt activity toward a GSK-3 peptide in vitro occurred normally (Fig. 4D). Diabetes treatment by islet transplantation restored the ability of insulin to stimulate GSK-3α and GSK-3β serine phosphorylation to control levels (P < 0.05 versus basal; Fig. 5A). The observed changes in GSK-3 phosphorylation could not be explained by different GSK-3 expression levels because the total GSK-3α and GSK-3β protein content, determined by immunoblotting with anti-GSK-3 antibody, was not different in the three experimental groups (Fig. 5B).

Shc, MEK, and MAPK. The Shc proteins (i.e., p66Shc, p52Shc, and p46Shc) are substrates for the insulin receptor tyrosine kinase and important signaling intermediates that lead to activation of the MEK-MAPK cascade. To examine potential effects of diabetes on the Shc-MEK-MAPK pathway in the heart, we studied tyrosine phosphorylation of the Shc proteins by immunoprecipitation with anti-Shc antibody followed by antiphosphotyrosine antibody immunoblotting. p52Shc was the predominant Shc protein found to be tyrosine phosphorylated in the basal state (Fig. 6A) and showed a significant increase in its phosphotyrosine content after insulin stimulation in all experimental animals (P < 0.05 versus basal; Fig. 6A). However, the level of insulin-stimulated tyrosine phosphorylation of p52Shc was higher in diabetic as compared with control heart (P < 0.05 versus control) and returned to normal levels after islet transplantation (Fig. 6A). Moreover, basal p52Shc tyrosine phosphorylation was slightly higher in rats that had received a transplant as compared with control rats (P < 0.05 versus control) and modestly augmented after insulin stimulation (Fig. 6A). The analysis of the Shc immunoprecipitates with anti-Shc antibodies demonstrated equal amounts of p52Shc protein in control and diabetic rats and rats that had received a transplant (Fig. 6B). Insulin significantly increased the level of MEK serine phosphorylation, evaluated by immunoblotting with phospho-specific anti-MEK antibody, in control and diabetic rats (P < 0.05 versus basal) but not in the rats that had received a transplant (P = 0.345 versus basal; Fig. 6C). MEK protein content did not differ in control and diabetic rats and rats that had received a transplant (Fig. 6D). Therefore, the stimulatory effect of insulin on p52Shc tyrosine phosphorylation and MEK serine phosphorylation was either reduced or absent after diabetes treatment by islet transplantation.

In contrast with p52Shc and MEK, the activation state of MAPK, studied by immunoblotting with antibodies to the phosphorylated forms of Erk-1 and Erk-2, seemed to be unaffected by insulin treatment in control animals (Fig. 7A) and was not different in the three experimental groups (Fig. 7B). Erk-1 and Erk-2 protein levels were also not different in control and diabetic rats and rats that had received a transplant (Fig. 7C). Therefore, insulin- and diabetes-induced changes in p52Shc tyrosine phosphorylation and MEK serine phosphorylation were apparently not associated with coordinate changes in MAPK activation.

DISCUSSION

The STZ-diabetic rat represents a well-established experimental model of diabetes, characterized by insulin deficiency associated with insulin resistance (28). Metabolic and functional abnormalities in the heart have been dem-
onstrated in STZ-diabetic animals, including derangement of the glucose transport system (29), impaired synthesis of myosin heavy chains (6), and ventricular dysfunction (1). In the present study, we analyzed potential insulin signaling abnormalities in the heart of STZ-diabetic rats after in vivo administration of insulin.

In diabetic heart, the major insulin receptor substrates, IRS-1, IRS-2, and Shc, showed a distinct pattern of dysregulation because the levels of IRS-2 and Shc tyrosine phosphorylation were augmented compared with control, whereas IRS-1 tyrosine phosphorylation was not altered. Moreover, increased IRS-2 tyrosine phosphorylation was associated with elevated levels of p85 protein and PI 3-kinase activity in IRS-2 signaling complexes, and augmented Shc tyrosine phosphorylation was associated with slightly higher, although not statistically significant, levels of MEK phosphorylation. MAPK phosphorylation was not stimulated by insulin and did not show differences in the experimental groups. The lack of insulin stimulation of MAPK is in agreement with a previous study, showing that in vivo injection of adult rats with insulin activates protein kinase B, p70S6K, and casein kinase 2 but not MAPK in the heart (30).

The levels of IRS-2, Shc, and IRS-1 tyrosine phosphorylation in diabetic heart may be the consequence of an increased amount of tyrosine phosphorylated insulin receptors (Fig. 1A) in the presence of normal levels of IRS-2 (Fig. 2F) and Shc (Fig. 6B) but reduced levels of IRS-1 (Fig. 2E) in this tissue. This may favor the interaction of IRS-2 and Shc with the activated insulin receptor kinase, leading to increased tyrosine phosphorylation of these substrates but not IRS-1. Alternatively, elevation in IRS-2 and Shc but not IRS-1 tyrosine phosphorylation could be explained by divergent regulation of substrate-specific tyrosine phosphatases in diabetes. An increased activity of PI 3-kinase, as it was found in diabetic heart in this study (Fig. 3), could selectively reduce IRS-1 protein levels. Relevant to this concept, a wortmannin-sensitive, proteasome-depen-

FIG. 4. Akt phosphorylation, total protein content, and activity in rat heart. Solubilized cardiac proteins from control (□) and untreated diabetic rats (■) and diabetic rats that had received a transplant (□□) were resolved by 10% SDS-PAGE and analyzed by immunoblotting with anti–phospho-Akt (Ser-473), anti–phospho-Akt (Thr-308), or anti-Akt antibodies, as described in RESEARCH DESIGN AND METHODS. A and B: Representative immunoblots (top) and the quantitation of basal (B) and insulin-stimulated (I) Akt phosphorylation on Ser-473 and Thr-308, respectively (bottom). C: A representative immunoblot (top) and the quantitation of total Akt protein content (bottom; values of basal and insulin-stimulated rats pooled together). For assessing the level of Akt activity in vitro, solubilized cardiac proteins were subjected to immunoprecipitation with anti-Akt antibody, and Akt activity in the immunoprecipitates was assayed in the presence of ATP and a synthetic substrate, corresponding to the GSK-3α/β phosphorylation sites, followed by SDS-PAGE and immunoblotting with anti–phospho-GSK-3α/β antibody. D: A representative anti–phospho-GSK-3α/β immunoblot (top) and the quantitation of Akt activity from basal and insulin-stimulated rats (bottom), respectively. *P < 0.05 versus basal; †P < 0.05 versus basal control; §P < 0.05 versus insulin-stimulated control; ‡P < 0.05 versus insulin-stimulated diabetic.
dent degradation pathway that is specific for IRS-1 was recently described in 3T3-L1 adipocytes (31). Differential regulation of IRS-1 and IRS-2 has been observed in experimental and human diabetes. IRS-1 is reduced, whereas IRS-2 is unchanged in the skeletal muscle of STZ-diabetic rats (10) and in adipocytes from patients with type 2 diabetes (32). Increased expression of IRS-2, with no change in IRS-1, has been described in rat insulinoma cells (33,34). Different physiologic functions for IRS-1 and IRS-2 have been proposed in various cell types, including L6 myoblasts (35), brown adipocytes (36), exercised skeletal muscle (37), and transgenic animals with selective ablation of the IRS-1 or IRS-2 genes (38–40). Therefore, upregulation of IRS-2 signaling activity in diabetic heart may result in specific yet uncharacterized effects on cardiac structure and function.

Coordinate phosphorylation of Thr-308 and Ser-473 is required for full activation of the Akt kinase (25,41), whereas dissociated phosphorylation of Thr-308 and Ser-473 has been shown to result in impaired Akt activity (42). Phosphorylation on Thr-308 is mediated by phosphoinositide-dependent kinase (PDK)-1 (43), whereas the identity of the Ser-473 kinase, tentatively named PDK-2, remains undetermined. Under certain circumstances, however, PDK-1 or Akt itself may mediate Ser-473 phosphorylation (44,45). In diabetic heart, marked augmentation of Akt phosphorylation on Thr-308 but no change of Akt phosphorylation on Ser-473 was demonstrated (Fig. 4). Several lines of evidence suggest that phosphorylation of Thr-308 and Ser-473, respectively, can be individually regulated: 1) ceramide induces Ser-473 dephosphorylation without affecting Thr-308 (46); 2) osmotic stress inactivates Akt by inducing rapid dephosphorylation of Thr-308 but not of Ser-473 (47); and 3) thrombin induces Thr-308 and Ser-473 phosphorylation via partially distinct mechanisms in human platelets (42). Therefore, the selective increase of Akt phosphorylation on Thr-308 in diabetic heart may be the consequence of preferential upregulation of PDK-1 versus the hypothetical PDK-2 or, alternatively, different expression and/or activity of specific Thr-308 or Ser-473 phosphatases (46). A recent report showed that the activity of protein kinase Cζ, another direct substrate of PDK-1 (48), is also increased in diabetic heart (49), supporting the concept of PDK-1 upregulation.

Insulin stimulation of glycogen synthase activity is reduced in the myocardium of STZ-diabetic rats (4). Insulin stimulates glycogen synthase activity through activation of protein phosphatase 1 (PP1) (50) and inactivation (by phosphorylation) of GSK-3 (26,51,52). Reduced insulino-
stimulated PP1 activity was observed in cardiomyocytes from insulin-deficient diabetic rats (53), partially accounting for the defect in glycogen synthase activation. In this study, even though phosphorylation and activation of proximal insulin signaling steps (i.e., insulin receptor, IRS proteins, and PI 3-kinase) were either unchanged or upregulated in diabetic heart, insulin-stimulated GSK-3 phosphorylation was found to be profoundly diminished (Fig. 5A). It is interesting that insulin stimulation of endogenous GSK-3 phosphorylation in vivo was markedly impaired, even though stimulation of Akt activity toward a GSK-3 peptide in vitro occurred normally (Fig. 4D). Potential mechanisms for these findings include changes in the subcellular distribution of Akt, which may inhibit interaction with and phosphorylation of endogenous GSK-3, covalent or noncovalent modifications of GSK-3, making this a poor Akt substrate in vivo, or higher GSK-3 phosphatase activity in diabetic myocardium. The defective phosphorylation and inactivation of GSK-3 may contribute to impaired insulin-mediated activation of glycogen synthase in diabetic heart. An increased activity of GSK-3 has been proposed as a potential mechanism of insulin resistance through serine phosphorylation of IRS-1 (54). In addition, GSK-3 has been implicated in insulin-mediated protection from apoptosis (55) and modulation of protein synthesis through phosphorylation of the translation initi-

FIG. 6. Phosphorylation and protein content of Shc and MEK in rat heart. For Shc analyses, solubilized cardiac proteins from control (□) and untreated diabetic rats (■) and diabetic rats that had received a transplant (□) were subjected to immunoprecipitation with anti-Shc antibodies. The resulting immune complexes were resolved by 10% SDS-PAGE and analyzed by immunoblotting with antiphosphotyrosine or anti-Shc antibodies, as described in RESEARCH DESIGN AND METHODS. Phosphorylation and protein content of MEK were determined by direct immunoblotting of cardiac proteins with anti-phospho-MEK or anti-MEK antibodies, respectively. Each panel shows a representative immunoblot on the left and the quantitation of multiple experiments on the right. A: Basal (B) and insulin-stimulated (I) p52Shc tyrosine phosphorylation. B: Total p52Shc protein content (values of basal and insulin-stimulated rats pooled together). C: MEK phosphorylation on Ser-217/221. D: total MEK protein content (values of basal and insulin-stimulated rats pooled together). *P < 0.05 versus basal; #P < 0.05 versus basal control; §P < 0.05 versus insulin-stimulated control.
It is tempting to speculate, therefore, that resistance of GSK-3 phosphorylation by insulin in cardiomyocytes may contribute not only to impaired glycogen synthesis but also to increased rates of apoptosis and reduced protein synthesis, leading to the development of diabetic cardiomyopathy.

Islet transplantation has been shown to improve metabolic abnormalities and slow the progression of chronic complications in experimental diabetes (17–22,57,58). Metabolic alterations and contractile dysfunction are also reportedly reversed in the myocardium of islet-transplanted diabetic animals (21). Although diabetes therapy by islet transplantation resulted in complete correction of insulin signaling abnormalities in the skeletal muscle (10), it did not normalize all changes in insulin signaling proteins in the heart (this study). Akt and GSK-3 phosphorylation was largely restored (Figs. 4 and 5), but IRS-2 tyrosine phosphorylation and associated PI 3-kinase were increased over control levels and became poorly responsive to insulin stimulation (Figs. 2 and 3). Although fasting plasma glucose and insulin levels of rats that had received a transplant were indistinguishable from controls, impaired glucose tolerance can be demonstrated by an oral glucose challenge in these animals (22), suggesting hyperglycemia and potential hyperinsulinemia in the fed state. In addition, substitution of pancreatic function with islet transplantation under the kidney capsule is not completely physiologic, because in this model insulin is delivered into the general circulation and not into the portal system. It is possible that IRS-2 regulation was not completely restored in this experimental model of transplantation because it is more sensitive than other signaling intermediates to near-normal fluctuations in plasma glucose and/or insulin levels. When evaluating the effects of transplanting islets in various anatomical sites or using different amounts of islets, the IRS-2 signaling pathway may eventually prove useful as a sensor of adequate tissue exposure to insulin and/or effective control of glucose spikes.

In conclusion, multiple insulin-signaling abnormalities can be demonstrated in the heart of STZ-diabetic animals. These include exaggerated tyrosine phosphorylation of IRS-2 and p52Shc and threonine phosphorylation of Akt. However, the ability of insulin to phosphorylate (and thus inactivate) GSK-3 is markedly defective in diabetic heart, and this may contribute to insulin-resistant glycogen synthesis in this tissue. Islet transplantation under the kidney capsule is a potentially effective approach to treat the metabolic dysfunction in diabetic tissues but seems to

**FIG. 7.** Erk phosphorylation and total protein content in the heart of control (■) and untreated diabetic rats (■) and diabetic rats that had received a transplant (□). Cardiac ventricles from basal (B) or insulin-stimulated (I) rats were removed and processed as described in RESEARCH DESIGN AND METHODS, and solubilized proteins were resolved by 10% SDS-PAGE. Erk-1/2 phosphorylation and protein content were determined by immunoblotting with anti–phospho-Erk or anti-Erk antibodies, respectively. A: Different amounts of heart protein (5–50 μg) from control rats analyzed by immunoblotting with anti–phospho-Erk antibody. B: A representative immunoblot (top) and the quantitation of basal (B) and insulin-stimulated (I) phosphorylation of Erk-1 and Erk-2 (bottom) in the experimental animals. C: A representative immunoblot (top) and the quantitation of total Erk-1 and Erk-2 protein content (bottom; values of basal and insulin-stimulated rats pooled together) in the experimental animals.
incompletely restore specific signaling steps in diabetic heart, such as IRS-2 tyrosine phosphorylation.

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