Insulin-dependent diabetic recipients of successful pancreas allografts achieve self-regulatory insulin secretion and discontinue exogenous insulin therapy; however, chronic hyperinsulinemia and impaired insulin sensitivity generally develop. To determine whether insulin resistance is accompanied by altered signal transduction, skeletal muscle biopsies were obtained from pancreas-kidney transplant recipients (n = 4), nondiabetic kidney transplant recipients (receiving the same immunosuppressive drugs; n = 5), and healthy subjects (n = 6) before and during a euglycemic-hyperinsulinemic clamp. Basal insulin receptor substrate (IRS)-1 Ser (312) and Ser (616) phosphorylation, IRS-1–associated phosphatidylinositol 3-kinase activity, and extracellular signal–regulated kinase (ERK)-1/2 phosphorylation were elevated in pancreas-kidney transplant recipients, coincident with fasting hyperinsulinemia. Basal IRS-1 Ser (312) and Ser (616) phosphorylation was also increased in nondiabetic kidney transplant recipients. Insulin increased phosphorylation of IRS-1 at Ser (312) but not Ser (616) in healthy subjects, with impairments noted in nondiabetic kidney and pancreas-kidney transplant recipients. Insulin action on ERK-1/2 and Akt phosphorylation was impaired in pancreas-kidney transplant recipients and was preserved in nondiabetic kidney transplant recipients. Importantly, insulin stimulation of the Akt substrate AS160 was impaired in nondiabetic kidney and pancreas-kidney transplant recipients. In conclusion, peripheral insulin sensitivity and responsiveness on peripheral glucose uptake is unknown. We have previously reported that insulin-mediated nonoxidative glucose metabolism (16). Skeletal muscle insulin resistance in this patient group can partly be attributed to immunosuppressant therapy, which is necessary to avoid organ rejection (4,8,9). However, sustained systemic insulin delivery from the transplanted pancreas also results in chronic peripheral hyperinsulinemia, which can contribute to an attenuation of insulin sensitivity (2–4,10).

The molecular mechanism by which transplant recipients develop impaired insulin sensitivity and responsiveness on peripheral glucose uptake is unknown. We have previously reported that insulin-mediated nonoxidative glucose metabolism is coupled with impaired glycogen synthase enzyme activity in skeletal muscle from pancreas-kidney transplant recipients (6). Moreover, defects in insulin receptor number and affinity, as well as protein expression of the insulin-regulated glucose transporter (GLUT4), have been observed in skeletal muscle from pancreas and pancreas-kidney transplant recipients (11–13). Thus, receptor and postreceptor defects in skeletal muscle contribute to whole-body insulin resistance in this patient group (4,8,13–15).

The present study was undertaken to characterize postreceptor insulin signal transduction in skeletal muscle from pancreas-kidney transplant recipients. Basal signaling events were of particular interest, since in vitro studies in cell culture systems provide evidence that hyperinsulinemia leads to excessive serine phosphorylation of the insulin receptor substrate (IRS)-1, thereby engaging a negative feedback mechanism to modulate insulin action along pathways important for glucose metabolism (16). Insulin action along the canonical signaling pathway was also compared between pancreas-kidney and nondiabetic kidney transplant recipients receiving similar immunosuppression therapy and nondiabetic healthy control subjects.

RESEARCH DESIGN AND METHODS
The three study groups were 1) four successful whole pancreas-kidney transplant recipients with systemic delivery of insulin with enteric drainage of the exocrine secretion, 2) five nondiabetic kidney transplant recipients with portal insulin secretion from their native pancreas, and 3) six nondiabetic healthy control subjects. All transplanted subjects received cadaveric grafts,
and these subjects received immunosuppressive medication consisting of 5–10 mg/day prednisolone, 150–300 mg/day cyclosporine A, and 50–75 mg/day azathioprine. The pretesting conditions have previously been reported (2,6).

The subjects studied in this group represent a subgroup of the original cohort previously described, for which sufficient skeletal muscle biopsy material was available for the insulin-signaling analysis (2,6). The study was approved by the local ethics committees and was in accordance with the Helsinki Declaration.

**Blood chemistry and euglycemic-hyperinsulinemic clamp.** All investigations were performed in subjects fasted overnight. Blood samples were drawn for determination of plasma glucose, C-peptide, nonesterified free fatty acids (NEFAs), cyclosporine A, creatinine, serum insulin, and HbaA1c (A1C) by chemistry profiles, are provided (Table 1). Fasting plasma creatinine, serum insulin, and HbaA1c (A1C) were assessed directly on the protein A-Sepharose beads (19). Reaction products were resolved by thin-layer chromatography and quantified using a Phosphor Imager (Bio-Rad).

**Statistical analysis.** Data are presented as means ± SE. Statistical differences were identified using Fisher’s least significant difference post hoc analysis. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

The clinical characteristics of the study participants have been reported previously (2,6). Additional biopsy material was available from this subgroup of the original study cohort, and the clinical characteristics, including blood chemistry profiles, are provided (Table 1). Fasting plasma C-peptide levels were higher in the pancreas-kidney and non-diabetic kidney transplant groups, respectively, compared with healthy groups (\( P < 0.05 \)) because of the higher plasma creatinine in the transplant recipients (\( P < 0.05 \)). Plasma cyclosporine levels were similar between the two transplant groups. NEFA levels were unaltered between the subjects. Basal plasma NEFA concentrations tended to be higher in the pancreas-kidney recipients and in the non-diabetic kidney recipients compared with the control subjects (Table 2). After insulin stimulation, plasma NEFA concentrations (i.e., the average concentration of the last

<table>
<thead>
<tr>
<th>Table 1: Clinical characteristics of the study participants</th>
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<td>Healthy subjects</td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>Sex (M/F)</td>
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<td>Age (years)</td>
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<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>BSA (m²)</td>
</tr>
<tr>
<td>A1C (%)</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
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<tr>
<td>Plasma cyclosporine (ng/ml)</td>
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<tr>
<td>Plasma glucose (mmol/l)</td>
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<tr>
<td>Plasma C-peptide (pmol/l)</td>
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<tr>
<td>Serum insulin (pmol/l)</td>
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<tr>
<td>Serum insulin levels during the clamp (pmol/l)</td>
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<tr>
<td>Basal R₃ (mg · kg fat-free mass⁻¹·min⁻¹)</td>
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<td>Total R₃ at 40 mU</td>
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Data are means ± SE. *\( P < 0.05 \) vs. healthy subjects, †\( P < 0.05 \) vs. non-diabetic kidney transplant recipients. NA, not applicable.
two samples in the steady-state 40 mU·m⁻²·min⁻¹ clamp period) were significantly reduced in healthy subjects and nondiabetic kidney transplant recipients (Table 2) and tended to be reduced in the pancreas-kidney recipients (P = 0.07).

**Whole-body glucose uptake.** Whole-body insulin-mediated glucose uptake in this cohort is reported in Table 1. Only the results from baseline and the 40 mU·m⁻²·min⁻¹ clamp period are reported here, since this condition corresponds to the biopsy sampling period. The mean steady-state plasma glucose levels during the hyperinsulinemic clamp were similar between the three groups and were unaltered from the basal plasma glucose levels (Table 1). Fasting serum insulin levels were significantly higher between the transplanted versus healthy subjects, as well as between the pancreas-kidney and nondiabetic kidney transplant subjects (P < 0.05, Table 1). During the insulin infusion (40 mU/m² per min), the peripheral serum insulin concentrations were comparable between the groups (Table 1). Whole-body insulin-mediated glucose uptake was significantly lower in the transplanted groups than in the healthy subjects (Table 1), predominantly because of the reduced nonoxidative glucose metabolism (2.6).

**IRS-1–associated PI 3-kinase activity.** Signal transduction was determined in skeletal muscle biopsies obtained before (basal) and after the insulin infusion period (40 mU/ m² per min) during the euglycemic-hyperinsulinemic clamp (insulin-stimulated). Basal IRS-1–associated PI 3-kinase activity was similar between healthy subjects and nondiabetic kidney transplant recipients (Fig. 1). In contrast, basal IRS-1–associated PI 3-kinase activity was increased in pancreas-kidney transplant recipients compared with healthy subjects (2.0-fold, P < 0.05). Insulin infusion led to a 2.1- to 2.3-fold increase in IRS-1–associated PI 3-kinase activity in healthy subjects and in nondiabetic kidney transplant recipients (P < 0.05). In contrast, IRS-1–associated PI 3-kinase activity was not further increased after insulin infusion in pancreas-kidney transplant recipients.

**Akt Ser (473) phosphorylation.** Basal Akt Ser (473) phosphorylation was similar between nondiabetic kidney transplant recipients, pancreas-kidney transplant recipients, and healthy subjects (Fig. 2). Insulin infusion led to a 2.0- and 2.6-fold increase in Akt Ser (473) phosphorylation in healthy subjects and nondiabetic kidney transplant recipients, respectively (P < 0.05). In contrast, in pancreas-kidney transplant recipients, Akt Ser (473) phosphorylation was unaltered after insulin infusion. Akt protein expression was similar between groups (data not shown).

**AS160 phosphorylation.** Basal AS160 phosphorylation was unaltered between nondiabetic kidney transplant recipients and healthy subjects (Fig. 3). However, basal AS160 phosphorylation was increased in the pancreas-kidney transplant recipients versus healthy subjects (1.4-fold, P < 0.05). Insulin infusion increased AS160 phosphorylation 2.1-fold in healthy subject (P < 0.05). In contrast, AS160 phosphorylation was unaltered after insulin infusion in nondiabetic kidney and pancreas-kidney transplant recipients.

**ERK-1/2 mitogen-activated protein kinase Thr (202)/Tyr (204) phosphorylation.** Basal ERK-1/2 mitogen-activated protein kinase (MAPK) Thr (202)/Tyr (204) phosphorylation was similar between healthy subjects and nondiabetic kidney transplant recipients (Fig. 4). However, basal ERK-1/2 MAPK Thr (202)/Tyr (204) phosphorylation was higher in the pancreas-kidney transplant recipients versus healthy subjects (2.4-fold, P < 0.05). Insulin infusion increased ERK-1/2 MAPK Thr (202)/Tyr

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**TABLE 2**

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<th></th>
<th>Basal</th>
<th>Insulin-stimulated</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>0.36 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Nondiabetic kidney</td>
<td>0.47 ± 0.04</td>
<td>0.05 ± 0.01*</td>
<td>0.04</td>
</tr>
<tr>
<td>Pancreas-kidney</td>
<td>0.58 ± 0.23</td>
<td>0.10 ± 0.05*</td>
<td>0.07</td>
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Data are means± SE. Plasma NEFA levels were determined under basal and insulin-stimulated conditions. Values are the average of the last two samples taken in the steady-state period of the basal and insulin-stimulated (40 mU/m² per min) clamp period. The P value indicated in the table is the differences between basal and insulin-stimulated conditions. *P < 0.05 vs. healthy subjects.

**FIG. 2.** Akt phosphorylation. Akt Ser (473) phosphorylation was measured as an estimation of Akt activation. Representative immunoblots are shown in the upper panel. The graph shows means ± SE arbitrary units. □, basal condition; ■, insulin-stimulated condition. *P < 0.05 vs. healthy subjects at basal.

**FIG. 1.** IRS-1–associated PI 3-kinase activity. Muscle biopsies were obtained from six healthy volunteers, five nondiabetic kidney transplant recipients, and four pancreas-kidney transplant recipients before (basal) and during (insulin-stimulated) a euglycemic insulin clamp. Muscles lysates were immunoprecipitated with an anti–IRS-1 antibody, and PI 3-kinase activity was measured. Results are means ± SE arbitrary units. □, basal condition; ■, insulin-stimulated condition. *P < 0.05 vs. basal of each group, PIP3, phosphatidylinositol 3,4,5-triphosphate.
creased (NS) after insulin infusion. In nondiabetic kidney transplant recipients, Ser (616) phosphorylation was slightly increased compared with basal (45% compared with healthy subjects). In nondiabetic kidney transplant recipients, insulin de-altered after insulin stimulation in the healthy subjects.

Experimental and clinical evidence suggests that many of the current immunosuppressant treatment strategies can account for the increased risk of peripheral insulin resistance that develops after the transplantation (25). Here we provide evidence that insulin action on IRS-1–associated PI 3-kinase activity was similar between the healthy subjects and the nondiabetic kidney transplant recipients, providing evidence that immunosuppressant drugs are without effect on insulin signaling. However, in the pancreas-kidney recipients, basal IRS-1–associated PI 3-kinase activity was elevated to a level comparable with insulin-stimulated activity in healthy subjects. This elevation in basal PI 3-kinase activity may be explained by hyperinsulinemia in the pancreas-kidney recipients (26, 27).

We next assessed phosphorylation of Akt, AS160, and ERK-1/2 as markers of metabolic and mitogenic/gene regulatory cascades. Akt is linked to the regulation of glucose uptake through phosphorylation of AS160, a Rab GTPase-activating protein that regulates GLUT4 exocytosis (28). Basal Akt, AS160, and ERK-1/2 MAPK phosphorylation was similar between healthy subjects and nondiabetic kidney transplant recipients, consistent with our results for PI 3-kinase activity. However, despite the twofold increase in basal PI 3-kinase activity in pancreas-kidney recipients, basal Akt phosphorylation was comparable to healthy subjects. While the mechanism for this apparent disassociation of Akt from PI 3-kinase is unclear, basal AS160 and ERK-1/2 phosphorylation was also elevated (approximately twofold) in skeletal muscle from type 1 diabetic patients who have undergone a combined pancreas and kidney transplantation. Two control groups were studied: healthy individuals and nondiabetic kidney transplant recipients. The nondiabetic kidney transplant recipients and the pancreas-kidney transplant recipients received the same immunosuppressive treatment.

DISCUSSION

The available treatment options for discontinuing insulin treatment in type 1 diabetic patients with complete β-cell failure are limited. Pancreas transplantation constitutes one option available to surgically treat type 1 diabetes. Although pancreas transplantation increases quality of life and decreases the mortality rate for type 1 diabetic patients (20–23), hyperinsulinemia and peripheral insulin resistance often develop (8,24). The molecular mechanism for peripheral insulin resistance after pancreas transplantation is unresolved but involves defects in insulin action on skeletal muscle glucose metabolism (5). Here we determined postreceptor insulin-signaling events in skeletal muscle from type 1 diabetic patients who have undergone a combined pancreas and kidney transplantation. Two control groups were studied: healthy individuals and nondiabetic kidney transplant recipients. The nondiabetic kidney transplant recipients and the pancreas-kidney transplant recipients received the same immunosuppressive treatment.

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diabetic pancreas-kidney transplant recipients. Under insulin-stimulated conditions, Akt phosphorylation was increased in nondiabetic kidney transplant recipients and impaired in pancreas-kidney transplant recipients, whereas AS160 phosphorylation was severely blunted in both groups. Because AS160 is the most proximal step identified in the insulin-signaling cascade to GLUT4 translocation (28), this signaling defect may provide a mechanism for the impaired whole-body glucose uptake noted in nondiabetic kidney and diabetic pancreas-kidney transplant recipients. Although insulin action on AS160 phosphorylation was impaired, ERK-1/2 phosphorylation was enhanced in nondiabetic kidney and diabetic pancreas-kidney transplant recipients. Similar results have been reported in skeletal muscle from type 2 diabetic patients, where MAPK signaling is preserved, despite impaired PI 3-kinase signaling to Akt (29,30). Thus, skeletal muscle insulin resistance in pancreas-kidney transplant recipients is manifested along metabolic rather than mitogenic/gene regulatory signaling cascades.

To further explore the mechanism for peripheral insulin resistance in pancreas-kidney-transplant recipients, we assessed IRS-1 serine phosphorylation. Several lines of evidence link IRS-1 serine phosphorylation to insulin resistance in response to cytokines (31), elevations in free fatty acids (32,33), hyperinsulinemia (33), and hyperglycemia (16). Consequently, serine phosphorylation has been implicated in the development of peripheral insulin resistance in type 2 diabetes (16,27,30,34–36). Here we explored two sites of serine phosphorylation on IRS-1 [Ser (312) and Ser (616)] associated with the development of peripheral insulin resistance (16,37–39). Phosphorylation of IRS-1 at Ser (312) and Ser (616) was elevated under basal conditions in nondiabetic kidney transplant and pancreas-kidney transplant recipients versus healthy subjects. These results provide evidence that hyperinsulinemia alone is unlikely to account for the increase in IRS-1 serine phosphorylation, since insulin levels in the nondiabetic kidney transplant recipients are modestly elevated compared with the healthy subjects. Nevertheless, we cannot exclude the possibility that chronic exposure to hyperinsulinemia may affect insulin signaling in the non-diabetic kidney transplant recipients.

In addition to hyperinsulinemia, elevated NEFA levels may also participate in the development of insulin resistance in these patients by potentially modulating IRS-1 serine phosphorylation (32,33). Indeed, the NEFA levels in pancreas-kidney transplant recipients under basal conditions in these patients by potentially modulating IRS-1 may also participate in the development of insulin resistance in pancreas-kidney transplant recipients under basal conditions in nondiabetic kidney transplant and diabetic pancreas-kidney transplant recipients. Therefore, NEFA levels were higher in the pancreas-kidney transplanted patients. Thus, the combination of hyperinsulinemia and elevated NEFAs offer a potential mechanism for the elevated serine phosphorylation of IRS-1 in pancreas-kidney transplanted patients.

Immunosuppressive treatment has been proposed to contribute to the development of peripheral insulin resistance in pancreas-kidney and kidney transplant recipients (2,6). The immunosuppression used in pancreas-kidney transplantation, particularly prednisolone and cyclosporine, induces insulin resistance (9,10) and situations with cortisol excess; insulin resistance is due to a decrease in hepatic and peripheral insulin sensitivity (2,5,6,8–10). Whereas it is impossible to segregate the direct effect of the individual immunosuppressants in these study participants, prednisone is likely to have a major effect on insulin resistance (9). Thus, any change in the metabolic and insulin-signaling events in the nondiabetic kidney transplant recipients likely reflects deleterious effects of the immunosuppressive treatment. Although the mechanism is incompletely resolved, our data provide evidence that immunosuppressive therapy appears to influence
IRS-1 serine phosphorylation and the subsequent regulation of peripheral insulin action and metabolism, since insulin action was also suppressed in the nondiabetic kidney transplant recipients. The increase in basal IRS-1 serine phosphorylation was suppressed upon insulin infusion in the nondiabetic kidney transplant recipient and maintained in the pancreas-kidney transplant recipients. The suppression of IRS-1 Ser (616) phosphorylation correlated with changes in NEFAs upon insulin infusion, further implicating elevated NEFA levels as a negative regulator of IRS-1 through serine phosphorylation. Excessive phosphorylation of IRS-1 at Ser (616) has been proposed to act as a negative regulator of insulin signaling, thereby providing a potential mechanism for insulin resistance (34). Our clinical results provide evidence that hyperinsulinemia, elevated NEFAs, and immunosuppressive therapy are selective for serine phosphorylation of IRS-1 in type 1 diabetes as a negative regulator. Prevention of excessive serine phosphorylation of IRS-1 may preserve insulin action on glucose metabolism after pancreas transplant surgery.

ACKNOWLEDGMENTS
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Pancreas transplant therapy is a surgical option to treat late-stage type 1 diabetes. While the patients display normal diurnal glucose profiles, in many cases, peripheral insulin resistance occurs. Here we provide evidence that immunosuppressive treatment and hyperinsulinemia contribute to peripheral insulin resistance in this patient group through differential effects on IRS-1 phosphorylation at Ser (312) and Ser (616) (Table 3). Moreover, we provide evidence that phosphorylation of IRS-1 on Ser (616) is an important regulatory axis for insulin action. These signaling defects are accompanied by impaired insulin action on Akt and AS160. Defects in insulin action on the level of AS160, a functional Rab GTPase-activating protein important for GLUT4 exocytosis (28,42–45), may account for the impairment in skeletal muscle glucose uptake in this cohort. Further studies to determine the acute versus chronic effect of kidney and pancreas-kidney transplant on insulin signaling and glucose metabolism are warranted to directly link excessive serine phosphorylation with insulin resistance. Prevention of excessive serine phosphorylation of IRS-1 may preserve insulin action on glucose metabolism after pancreas transplant surgery.

Shaded areas represent the differences between kidney recipients and pancreas recipients; bold arrows represent the differences in comparison to healthy subjects.
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