Islet transplantation can eliminate severe hypoglycemic episodes in patients with type 1 diabetes; however, whether intrahepatic islets respond appropriately to hypoglycemia after transplantation has not been fully studied. We evaluated six islet transplant recipients, six type 1 diabetic subjects, and seven nondiabetic control subjects using a stepped hyperinsulinemic-hypoglycemic clamp. Also, three islet transplant recipients and the seven control subjects underwent a paired hyperinsulinemic-euglycemic clamp. In response to hypoglycemia, C-peptide was similarly suppressed in islet transplant recipients and control subjects and was not detectable in type 1 diabetic subjects. Glucagon was significantly more suppressed in type 1 diabetic subjects than in islet transplant recipients \((P < 0.01)\), although the glucagon in islet transplant recipients failed to activate as in the control subjects \((P < 0.01)\). Pancreatic polypeptide failed to activate in both type 1 diabetic subjects and islet transplant recipients compared with control subjects \((P < 0.01)\). In islet transplant recipients, glucagon was suppressed normally by hyperinsulinemia during the euglycemic clamp and was significantly greater during the paired hypoglycemic clamp \((P < 0.01)\). These results suggest that after islet transplantation and in response to insulin-induced hypoglycemia, endogenous insulin secretion is appropriately suppressed and glucagon secretion may be partially restored. Diabetes 54: 3205–3211, 2005

Type 1 diabetic patients with absolute insulin deficiency are at greatly increased risk for hypoglycemic events because of the requirement for exogenous insulin and impaired glucose counterregulatory defenses (1). Recent work from Edmonton, Alberta, clearly demonstrates that intrahepatic transplantation of isolated pancreatic islets can eliminate the development of severe hypoglycemic episodes in patients with type 1 diabetes (2). Islet transplantation may abolish hypoglycemia by reducing exogenous insulin requirements, but whether improvement in glucose counterregulatory mechanisms against hypoglycemia also contributes has not been fully studied. The primary defenses against hypoglycemia are an inhibition in endogenous insulin secretion and an activation in glucagon secretion, which together serve to increase hepatic glucose production and prevent or correct low blood glucose (1). Both responses are lost in established (i.e., C-peptide–negative) type 1 diabetes in which near total destruction of insulin-producing β-cells occurs together with an associated defect in glucagon secretion from α-cells (3). The final defenses against hypoglycemia are augmented by activation of the sympathoadrenal system and are mediated through epinephrine secretion, which contributes to hepatic glucose production and decreases peripheral glucose utilization, and symptom generation, which alerts the individual to ingest food (1). Recurrent episodes of hypoglycemia blunt these latter responses, leading to a syndrome of hypoglycemia unawareness that increases the occurrence of life-threatening hypoglycemia by sixfold in type 1 diabetic patients (4).

Previous studies of islet transplant recipients have demonstrated restored inhibition in endogenous insulin secretion during hypoglycemia (5,6) and improvement in epinephrine and symptom responses to hypoglycemia in some but not all subjects (6,7). Curiously, it has been reported that glucagon secretion is not improved by islet transplantation (5,6). In type 1 diabetes, the α-cell dysfunction is specific for hypoglycemia because type 1 diabetic patients secrete glucagon normally in response to other stimuli such as arginine (3). This specific defect in glucagon secretion in response to hypoglycemia might be explained by the loss of endogenous insulin secretion because α-cells appear to require sensing an intraislet decrease in insulin to respond to hypoglycemia (8–12). Alternatively, impaired neural activation of α-cells in type 1 diabetes might account for the defective glucagon response to hypoglycemia (13).

The persistence of an impaired glucagon response to hypoglycemia after islet transplantation has not been fully explained, and prior studies have lacked either type 1 diabetic control subjects (5) or euglycemic control experiments (6). Therefore, we studied the glucagon response during a hyperinsulinemic-hypoglycemic clamp along with the C-peptide response, a measure of endogenous insulin secretion, and the pancreatic polypeptide response, a measure of islet neural activation, in islet transplant recipients and compared them with responses in nondiabetic control and type 1 diabetic subjects. Importantly, to control for the inhibitory effect of hyperinsulinemia on
glucagon secretion (14–17), we further evaluated the glucagon response during paired hyperinsulinemic-euglycemic and -hypoglycemic clamps in islet transplant recipients and the control subjects.

RESEARCH DESIGN AND METHODS

Subjects were recruited from the islet transplantation program at the Hospital of the University of Pennsylvania (HUP). The type 1 diabetic subjects (n = 6) had long-standing C-peptide–negative disease and were on the waiting list for islet transplantation because of frequent severe hypoglycemia complicated by unawareness. Three subjects each used multiple daily injections of lispro and tacrolimus, and either rapamycin or mycophenolate mofetil. The islet transplant recipients (n = 6) were studied a mean of 7.5 (range 6–12) months after their last transplant. The procedure for islet transplantation at HUP has been previously reported (18). In short, subjects underwent one to three intraportal islet infusions under daclizumab induction immunotherapy. Four of the six islet transplant recipients achieved insulin independence, whereas two continued a markedly reduced dose of insulin to maintain near normal glycemia. Three of these six subjects had a well-functioning kidney allograft. All received tacrolimus and either rapamycin or mycophenolate mofetil for maintenance immunosuppression, and two subjects with a kidney allograft also received 5 mg prednisone daily. All medications were withheld on the morning of study.

Healthy nondiabetic control subjects (n = 7) were age-, sex-, and BMI-matched to the islet transplant recipients. This study protocol was approved by the Institutional Review Board of the University of Pennsylvania, and all subjects gave their written informed consent to participate.

Metabolic studies. All subjects were admitted to the HUP General Clinical Research Center the afternoon before study. Subjects fasted overnight after 2000 for 12 h before testing. Islet transplant recipients who were not insulin independent held any long-acting insulin for 2000 for 12 h before testing. Type 1 diabetic subjects gave their written informed consent to participate. Subjects fasted overnight after Metabolic studies.

RESEARCH DESIGN AND METHODS

Data are means ± SE. Basal levels of glucose and islet hormones are the mean of the −30 and 0 min values prior to the start of the hypoglycemic clamp. *P < 0.05 and †P < 0.01 for comparison with control subjects. ‡P < 0.01 and §P < 0.05 for comparison with type 1 diabetic subjects. ¶Exogenous insulin requirements at the time of study. |Survey of hypoglycemia severity (7, most; 0, none) developed by Clarke et al. (23). ||Islet equivalents (IEs) transplanted per kilogram recipient body weight, where an IE approximates a standard islet diameter of 150 μm. PP, pancreatic polypeptide.

The impact of insulin per se on the glucagon response independent from hypoglycemia. Control subjects (n = 7) and islet transplant recipients (n = 3) participated in the euglycemic clamp in randomized order with the hypoglycemic clamp at least 1 week but not longer than 1 month apart. Two of the islet transplant recipients repeated the hypoglycemic clamp because the euglycemic clamp was introduced after their initial study; thus the three islet transplant recipients underwent the paired euglycemic-hypoglycemic clamps a mean of 13 (range 6–18) months after their last transplant. The hyperinsulinemic-euglycemic clamp was conducted as described for the hypoglycemic clamp above, but with a target plasma glucose of 90 mg/dl for the entire 360-min study (17,20).

Biochemical analysis. Blood samples were collected on ice into chilled tubes containing EDTA and the protease inhibitors leupeptin and aprotinin (Sigma-Aldrich, St. Louis, MO) for islet hormones and tubes containing EGT A and glutathione (Amersham, Arlington Heights, IL) for catecholamines, centrifuged at 4°C, separated, and frozen at −80°C for subsequent analysis. Plasma glucose was measured in duplicate by the glucose oxidase method using an automated glucose analyzer (YSI 2300; Yellow Springs Instruments). Plasma immunoreactive insulin, C-peptide, glucagon, and pancreatic polypeptide were measured in duplicate by double-antibody radioimmunoassay (Linco Research, St. Charles, MO, ALPCO Diagnostics, Windham, NH, for the pancreatic polypeptide assay only). Plasma epinephrine and norepinephrine were measured by high-performance liquid chromatography with electrochemical detection. Samples from paired euglycemic-hypoglycemic experiments of each subject were assayed simultaneously.

Calculations and statistics. Incremental C-peptide, glucagon, and pancreatic polypeptide were calculated as measures of islet β-, α-, and pancreatic polypeptide–cell responses by subtracting baseline values (basal) from those obtained during the last 60 min of hypoglycemia (final). Incremental epinephrine, norepinephrine, and autonomic symptom responses were calculated similarly. All data are expressed as mean ± SE. Comparisons between groups were performed with one-way or repeated measures ANOVA as appropriate using the computer software Statistica (Tulsa, OK). Significance was considered at P < 0.05 (two-tailed).
RESULTS

Subject characteristics. All three groups were comparable in age, sex, and BMI, whereas HbA1c and basal glucose levels were significantly lower in the control subjects compared with the type 1 diabetic subjects and with the islet transplant recipients (Table 1). Basal insulin and glucagon were higher in the type 1 diabetic subjects compared with the control subjects ($P < 0.01$ for insulin) and with the islet transplant recipients ($P < 0.05$ for glucagon; Table 1), likely consequences of the exogenous insulin requirement and absent endogenous insulin secretion (which negatively regulates glucagon secretion [8,23]) in long-standing type 1 diabetes. The absent endogenous insulin secretion in the type 1 diabetic subjects is evidenced by the undetectable basal C-peptide levels, which were significantly lower than in the control subjects and islet transplant recipients ($P < 0.01$ for both comparisons; Table 1). Islet transplant recipients received $15,075 \pm 1,918$ islet equivalents (IEs) per kilogram body weight, required significantly less insulin therapy, and had negligible scores on Clarke’s survey of hypoglycemia severity (24) compared with the type 1 diabetic subjects ($P < 0.001$ for both comparisons; Table 1).

All three islet transplant recipients participating in the paired euglycemic-hypoglycemic studies required some insulin ($0.26 \pm 0.04 \text{ units} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) to maintain excellent glycemic control (HbA1c 6.0 ± 0.2%) despite preserved graft function (basal C-peptide 1.02 ± 0.34 ng/ml) and were free of hypoglycemic events (Clarke survey score 1.0 ± 0.6 of 7 [24]).

Insulin and glucose during the hypoglycemic clamp. The insulin infusion administered during the hypoglycemic clamp resulted in comparable hyperinsulinemia in all three
Importantly, the hyperinsulinemic-euglycemic clamp resulted in a notable suppression of glucagon levels by secreting glucagon to near baseline levels. These studies indicate that intrahepatic islets respond appropriately to hypoglycemia by suppressing endogenous glucagon levels, especially in type 1 diabetic subjects.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Control subjects (n = 7)</th>
<th>P (control vs. type 1 diabetic)</th>
<th>Type 1 diabetic subjects (n = 6)</th>
<th>P (islet vs. type 1 diabetic)</th>
<th>Islet recipients (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔEpinephrine (pg/ml)</td>
<td>287.3 ± 83.2</td>
<td>0.02</td>
<td>30.4 ± 14.4</td>
<td>0.09</td>
<td>103.4 ± 36.1</td>
</tr>
<tr>
<td>ΔNorepinephrine (pg/ml)</td>
<td>102.2 ± 22.3</td>
<td>0.15</td>
<td>54.6 ± 19.5</td>
<td>0.46</td>
<td>80.0 ± 26.8</td>
</tr>
<tr>
<td>ΔAutonomic symptom score</td>
<td>10.5 ± 3.7</td>
<td>0.19</td>
<td>3.9 ± 3.5</td>
<td>0.23</td>
<td>12.2 ± 5.4</td>
</tr>
</tbody>
</table>

**DISCUSSION**

These studies indicate that intrahepatic islets respond appropriately to hypoglycemia by suppressing endogenous insulin secretion and preventing a decline in glucagon levels by secreting glucagon to near baseline levels. Importantly, the hyperinsulinemic-euglycemic clamp ex-
periments demonstrate that the transplanted α-cell is inhibited by insulin to the same degree as seen in normal non-diabetic control subjects. Moreover, the magnitude of glucagon secretion is greater in response to hypoglycemia compared with euglycemia, indicating α-cell recognition of hypoglycemia by islet transplant recipients. Thus, the absent suppression of glucagon secretion in the transplant recipients compared with the type 1 diabetic subjects during hypoglycemia is likely the result of the hypoglycemia and not a lack of suppression by hyperinsulinemia.

The significantly greater suppression of the incremental glucagon response during hypoglycemia in the type 1 diabetic subjects compared with the islet transplant recipients has not been previously demonstrated. In the study by Paty et al. (6), there was no difference in incremental glucagon during hypoglycemia between seven type 1 diabetic subjects and seven islet transplant recipients; however, the two groups were studied at different times and at different institutions, and so their results may not be directly comparable. Furthermore, the type 1 diabetic subjects in the present study were selected from our waiting list for islet transplantation and so would be expected to share a similar severity of defective glucose counter-regulation as the islet recipients had experienced before transplantation. Moreover, glucagon suppression, and not simply absence of activation, is the characteristic response to hyperinsulinemic hypoglycemia in type 1 diabetic subjects (25–27). Thus, the significantly attenuated inhibition of glucagon in the islet recipients is most likely the result of transplantation.

Insulin negatively regulates glucagon secretion from the native pancreas (14–17), but whether the same is true for intrahepatic islets has not previously been known. It is conceivable that local extraction of insulin by hepatocytes might shield intrahepatic islets from hyperinsulinemia. However, the hyperinsulinemic-euglycemic clamp experiment demonstrates that insulin-mediated suppression of glucagon is normal in islet transplant recipients. Importantly, this suppression of glucagon by hyperinsulinemia was significantly greater during euglycemia compared with hypoglycemia, further providing evidence that hypoglycemia stimulated glucagon secretion from intrahepatic islets.

One mechanism by which the α-cells contained in the intrahepatic islets may respond to hypoglycemia is by the restored inhibition in endogenous insulin secretion, as indicated by the normal suppression of C-peptide during hypoglycemia in the islet transplant recipients. In contrast to the inhibitory effect of insulin per se on glucagon secretion, increasing evidence suggests that an intraislet decrement in insulin may be necessary for glucagon secretion in response to hypoglycemia (8–12). Here, the idea is that whereas basal insulin secretion restrains glucagon secretion, the decrease in insulin secreted by the β-cells within the islet during hypoglycemia functions as a required signal to the α-cells to secrete glucagon. In our type 1 diabetic subjects, an absent decrease in insulin secretion can be inferred from the undetectable C-peptide levels in that group, whereas the islet transplant recipients likely experience an intraislet insulin decrement based on their C-peptide suppression in response to hypoglycemia that is comparable with normal.

Although intrahepatic islet transplantation appears to improve the defective glucagon secretion during hypoglycemia seen in established type 1 diabetes, the glucagon response remains significantly impaired when compared with normal. This impairment might be explained by a low engrafted islet mass. We have previously demonstrated that the β-cell secretory capacity, an estimate of the functional β-cell mass, is only 22% of normal in insulin-independent islet transplant recipients, suggesting that only a fraction of intraportally transplanted islets achieve engraftment (28). Prior studies of human donors of 50% pancreas segments (29) and human recipients of 60% pancreas segments (30) demonstrated incremental glucagon responses to hypoglycemia that were lower than normal by arithmetic and statistical comparison, respectively. Therefore, an intrahepatic islet mass ~25% of normal should not be expected to result in a quantitatively “normal” glucagon response to hypoglycemia.

Nevertheless, there may be something inherent to the intrahepatic site that mitigates an increase in peripheral glucagon levels in response to hypoglycemia after islet transplantation. Because the liver extracts ~20–30% of secreted glucagon during its first pass in the portal circulation (31,32), hepatic extraction of the glucagon secreted from the limited number of intrahepatic α-cells might result in accelerated local clearance before its appearance in the systemic circulation. This idea is consistent with results from a dog auto-islet transplantation model in which the glucagon response to hypoglycemia was significantly greater after intraperitoneal versus intrahepatic transplantation (33). There, the authors speculate that glucose production from hepatocytes may result in local glucose levels that are higher than the peripherally measured hypoglycemia and may possibly account for the impaired glucagon response in the intrahepatically transplanted animals (33). The peripheral location of α-cells in the islet may expose them to greater glucose flux from neighboring hepatocytes than seen by the β-cells and so might reconcile the normal suppression of C-peptide during hypoglycemia in the islet transplant recipients reported here and elsewhere (5,6) that suggests that intrahepatic β-cells do appropriately sense and respond to the degree of peripheral hypoglycemia and so argues against islet exposure to locally elevated glucose levels.

Another consideration for the impaired glucagon response to hypoglycemia is the uncertain neural innervation of intrahepatic islets. Both parasympathetic and sympathetic innervations contribute in a redundant fashion to α-cell activation during hypoglycemia (13,34). The absent pancreatic polypeptide response to hypoglycemia demonstrated here suggests a lack of parasympathetic innervation, although sympathetic innervation of intrahepatic islets might occur along vascular channels (35). Although innervation does not appear necessary for an intact α-cell response to hypoglycemia after transplantation of a denervated whole pancreas (36–38), neural activation may be more important when the mass of cells is reduced as after islet transplantation. Because pancreatic polypeptide (39), like C-peptide (40), is cleared by the kidney without undergoing any hepatic metabolism, local extraction of pancreatic polypeptide from intrahepatic islets cannot explain its significantly reduced response to hypoglycemia in islet transplant recipients.

Pancreatic polypeptide secretion from native islets is unlikely to improve after transplantation in subjects with long-standing type 1 diabetes, as suggested by the results reported here. The impaired pancreatic polypeptide response to hypoglycemia in type 1 diabetes develops with the impaired epinephrine and symptom responses, suggesting an underlying failure of autonomic activation (41).
Hypoglycemic avoidance does not restore epinephrine and pancreatic polypeptide responses in type 1 diabetic subjects with >10 years of insulin-dependent disease (26). Thus, some component of irreversible autonomic neuropathy may limit autonomic responses to hypoglycemia, such as the parasympathetic pancreatic polypeptide response. Interestingly, sympathoadrenal epinephrine and symptom responses following islet transplantation have been reported to be normal in one of three subjects from one study (7) and two of seven subjects from another (6), suggesting recovery of at least some autonomic function may be possible after islet transplantation. Our results are consistent with those findings since two of six islet transplant recipients studied here had both epinephrine and autonomic symptom responses that overlapped with the range of control values (data not shown), and more importantly, there was a statistical trend for greater epinephrine secretion in islet transplant recipients compared with type 1 diabetic subjects. Because of the marked variability in catecholamine and symptom measures between individuals, prospective study of a larger group of type 1 diabetic subjects both before and after islet transplantation will be necessary to best evaluate sympathoadrenal responses to hypoglycemia.

In conclusion, these studies demonstrate appropriate suppression of endogenous insulin secretion and lack of suppression of glucagon secretion in response to insulin-induced hypoglycemia after intrahepatic islet transplantation. That glucagon secretion is appropriately suppressed during hyperinsulinemic euglycemia in islet transplant recipients argues that intrahepatic α-cells can respond to hypoglycemia by maintaining glucagon secretion, albeit at levels measured peripherally that are significantly less than normal. These improvements in glucose counterregulatory mechanisms likely contribute to the improvement in clinical hypoglycemia that occurs after islet transplantation and might be improved further if present efforts to increase the mass of islets that engraft after transplantation are successful. Because insulin inhibits α-cell function and peripheral glucagon levels may under-estimate the secretion of glucagon by intrahepatic islets, future studies of the effect of various transplant sites on glucose counterregulation should include euglycemic control experiments and directly assess hepatic glucose production in addition to glucagon concentrations. Greater understanding of the mechanisms of the attenuated glucagon response to hypoglycemia will help guide future strategies for islet transplantation in which alternative sites for islet engraftment may be considered.

ACKNOWLEDGMENTS
This work was supported by the Juvenile Diabetes Research Foundation and by the Public Health Services Research Grants M01-RR-00040 (HUP General Clinical Research Center), P30-DK-19525 (University of Pennsylvania Diabetes Endocrinology Research Center), and U42-RR-016600 (HUP Islet Cell Resource Center) from the National Institutes of Health. M.R.R. has received Public Health Services Research Grant K12-RR017625 from the National Institutes of Health.

We are indebted to the islet transplant recipients and type 1 diabetic subjects for their participation, to the nursing staff of the HUP General Clinical Research Center for their subject care and technical assistance, to Dr. Heather Collins of the University of Pennsylvania Diabetes Endocrinology Research Center for performance of the radioimmunoassays, to Dr. Shiv Kapoor of the HUP General Clinical Research Center for performance of the high-performance liquid chromatography, and to Huong-Lan Nguyen for laboratory assistance.

REFERENCES
20. Mitra K, Ryan C, Veneman T, Mokan M, Jnnsen T, Kiss I, Durrant J,


