Intrahepatic Glucose Flux as a Mechanism for Defective Intrahepatic Islet α-Cell Response to Hypoglycemia

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OBJECTIVE—Glucagon responses to hypoglycemia from islets transplanted in the liver are defective. To determine whether this defect is related to intrahepatic glycogen, islets from inbred Lewis rats were transplanted into the hepatic sinus (H group), peritoneal cavity (P group), omentum (O group), and kidney capsule (K group) of recipient Lewis rats previously rendered diabetic with streptozotocin (STZ).

RESEARCH DESIGN AND METHODS—Glucagon responses to hypoglycemia were obtained before and after transplantation under fed conditions and after fasting for 16 h and 48 h to deplete liver glycogen.

RESULTS—Glucagon (area under the curve) responses to hypoglycemia in the H group (8,839 ± 1,988 pg/ml per 90 min) were significantly less than in normal rats (40,777 ± 8,192; $P < 0.01$). Fasting significantly decreased hepatic glycogen levels. Glucagon responses in the H group were significantly larger after fasting (fed 8,839 ± 1,988 vs. 16-h fasting 24,715 ± 5,210 and 48-h fasting 29,639 ± 4,550; $P < 0.01$). Glucagon response in the H group decreased after refeeding (48-h fasting 29,639 ± 4,550 vs. refed 10,276 ± 2,750; $P < 0.01$). There was no difference in glucagon response to hypoglycemia between the H and the normal control group after fasting for 48 h (H 29,639 ± 4,550 vs. control 37,632 ± 5,335; P = NS). No intragroup differences were observed in the P, O, and K groups, or normal control and STZ groups, when comparing fed or fasting states.

CONCLUSIONS—These data suggest that defective glucagon responses to hypoglycemia by intrahepatic islet α-cells is due to dominance of a suppressive signal caused by increased glucose flux and glucose levels within the liver secondary to increased glycogenolysis caused by systemic hypoglycemia. ***Diabetes* 57: 1567–1574, 2008**

We and others have previously reported that diabetic recipients of successful intrahepatic pancreatic islet transplantation have defective intrahepatic α-cell glucagon responses to hypoglycemia (1–6). This is an important clinical issue because patients who are chosen for islet transplantation are at especially high risk of severe hypoglycemia (7,8). Within several years after transplantation, most recipients usually return to insulin therapy and are once again at risk for hypoglycemia.

When the liver site is used, islets are infused via the hepatic portal vein and lodge in the hepatic sinusoids, where they establish connections with arterioles from the central hepatic artery (9,10). α-Cells on the periphery of the islets are in intimate contact with hepatocytes. Because the liver is a major site of glycogen storage and glucose production, we hypothesize that intrahepatic α-cells are exposed to higher glucose concentrations than what is delivered by the hepatic artery as a result of hypoglycemia. If so, it is possible that increased glucose flux within the hepatic parenchyma during hypoglycemia-induced glycogenolysis provides a strong inhibitory signal to the intrahepatic α-cells.

To examine this hypothesis, we transplanted islets isolated from inbred Lewis rats into the hepatic sinus (H group), peritoneal cavity (P group), omental pouch (O group), and left kidney capsule (K group) of recipient Lewis rats previously rendered diabetic with streptozotocin (STZ). Glucagon responses to hypoglycemia were analyzed under the conditions of fed, fasting for 16 h, and fasting for 48 h to evaluate: *1*) the degree to which α-cell glucagon responses to hypoglycemia are defective, *2*) whether glucagon responses in the H group are significantly greater after liver glycogen storage is depleted; and *3*) whether the α-cell defective response returns after refeeding.

RESEARCH DESIGN AND METHODS

Six-week-old inbred male Lewis rats were purchased from Charles River laboratories. Animals were placed in rooms with a 12-h light/dark cycle with constant temperature and given free access to food and water. Subcutaneous insulin pellets (0.5 units/24 h) were provided for STZ-administered animals to avoid excessive hyperglycemia. All experiments were approved by the Pacific Northwest Research Institute Institutional Animal Care and Use Committee.

Islets were transplanted into the hepatic sinus (H group, $n = 10$), the peritoneal cavity (P group, $n = 7$), the omental pouch (O group, $n = 5$), and the left kidney capsule (K group, $n = 5$) of STZ-induced diabetic recipients. Nontransplanted STZ-induced diabetic (S group, $n = 9$) and normal control (C group, $n = 6$) animals were also studied. Blood glucose level and body weight were measured in transplanted rats every day for 2 weeks after transplantation and once a week thereafter. Insulin pellets were removed from all the recipients within 2 weeks after transplantation. In some intraperitoneal, transplanted recipients and STZ recipients without transplantation, insulin pellets were re-implanted if the blood glucose levels were >350 mg/dl.

One month after transplantation, intravenous insulin tolerance tests (ITTs) were performed to evaluate glucagon responses to hypoglycemia. Each rat had three ITTs under fed, 16-h fasting, and 48-h fasting conditions. Each ITT was 1 week apart for the H group and 1 month apart for the other groups. Four rats from the H group had ITTs again on the 4th week after returning to the fed condition. At the end of the experiment, animals were killed and liver samples were taken for glycogen staining and measurement by a chemical method.

Transplant recipient preparation. Seven-week-old Lewis rats were given an intraperitoneal injection of 120 mg/kg STZ (Sigma, St. Louis, MO). Rats were considered diabetic when blood glucose levels exceeded 450 mg/dl on

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ITT, insulin tolerance test; STZ, streptozotocin.

two sequential measurements. Animals that did not develop diabetes by 1 day were given the same dose of STZ again. Insulin pellets (Linshin Canada, Scarborough, ON, Canada) delivering ~0.25 unit/24 h were inserted under the neck skin to partially regulate blood glucose levels. ITTs were performed in animals 2 weeks after diagnosis of diabetes. Only animals shown to have defective glucagon responses to hypoglycemia (peak δ glucagon <50 pg/ml) were accepted as recipients.

Islet preparation. Six-week-old male Lewis rats were used as donors. On the day of transplantation, pancreata were cannulated for infusion of collagenase and then removed from the animal for processing (11). After isolation, islets were hand picked and counted. A total of 1,800–2,200 islets from three to four rats were pooled for culture in RPMI-1640 (Sigma) with 11.1 mmol/l glucose for 2 h. Islets were then collected and washed with Hank's buffer three times. After the final wash, supernatant was removed as much as possible without losing islets. Using a rat serum–coated 1-ml Eppendorf pipette tip, islets were transferred to a rat serum–coated microfuge tube. The 1,800–2,200 islets were then transplanted into each recipient within 20 min thereafter.

Intrahepatic transplantation. Recipients (10- to 12-week-old male Lewis rats previously rendered diabetic with STZ) were anesthetized by inhalation of 2% isoflurane. Once anesthetized, the abdomens were shaved, disinfected, and opened. Using a rat serum–coated glass pipette, islets were transferred to another siliconized rat serum–coated fine-tip glass pipette. One end of the glass pipette was connected to a 3-ml syringe. The other tip of the pipet was pulled to close to 0.7 mm OD and connected to a silastic tubing (0.3 mm ID, 0.64 mm OD; Sigma). The other end of silastic tubing was connected to the metal part of a 23G needle. The portal vein was identified and punctured by the 23G needle. The 1,800–2,200 islets in 1 ml volume were then infused by slow injection over 1–2 min into the portal vein. Checking islets by microscope using this technique confirmed no damage to islets. The puncture site was pressed with gauze for at least 5 min to stop bleeding; the gauze was then carefully removed, and the abdomen was closed. This technique permitted quantitative transfer islets to the liver in a short time.

Kidney capsule transplantation. Each recipient was anesthetized as described above. The left kidney was exposed through a flank incision. A small hole in the kidney capsule was made near the caudal pole. PE-50 tubing was advanced into the kidney capsule. A total of 1,800–2,200 islets in <200 μl rat serum was slowly infused under the kidney capsule using PE-50 tubing (Braintree Scientific, Braintree, MA) connected to a 1-ml syringe. Then the flank incision was closed, and the animal was allowed to wake up under close observation.

Intraperitoneal transplantation. The abdomen of the anesthetized recipient was opened by a 0.5-cm incision. A total of 1,800–2,200 islets in 400 μl rat serum was transplanted into the peritoneal cavity via a rat serum–coated 1-ml Eppendorf pipette tip. The incision was then closed, and the animal was allowed to recover.

Omental transplantation. To create an omental pouch, the greater omentum was spread out, and a 7-0 suture was run along the margin of the omentum. A total of 1,800–2,200 islets in <200 μl rat serum was slowly dripped onto the exposed omentum using a 200-μl Eppendorf pipette. The suture was then pulled up and tied, and the incision was closed.

Jugular vein permanent cannulation. Two weeks after transplantation, the right jugular veins of the H and C group recipients were permanently cannulated. On the day of cannulation, anesthesia was maintained by inhalation of 2% isoflurane. The skin was incised on the top of the skull, and four self-tapping skull screws were placed. The jugular vein was cannulated with silastic tubing (0.51 mm ID, 0.94 mm OD; Sigma), and the tubing was secured to a silicone ring. The other end of the catheter was led subcutaneously toward the skin incision on the skull and connected to a piece of stainless steel tubing (1.5 cm) that was bent 90°. This "elbow" was secured by dental acrylic to the skull screws. The cannula was flashed with saline, 0.05 ml heparin (500 μl/ml), and refilled with 50 μl 50% solution of polyvinylpyrrolidone dissolved in heparin, and a port plug was inserted into the free end of the cannula. The catheters were flushed once a week. Animals in the other groups did not have permanent jugular vein catheters, and 1 month elapsed between fed, 16-h, and 48-h ITTs.

Intravenous ITT. Rats were kept anesthetized by inhalation of 1% isoflurane. Blood samples of 0.3 ml were taken from the jugular vein. After two basal samples were taken, 0.5 units/ml Humulin regular insulin was infused into rats at a rate of 50 μl/min. Once blood glucose levels were <60 mg/dl, insulin infusion was stopped. Blood was sampled for glucose, plasma glucagons, and C-peptide at the time insulin infusion was stopped and 30, 45, 60, and 90 min later.

Glycogen measurement. Liver glycogen was measured using a modified method of Lo et al. (12). In brief, at the time of death, small pieces of liver (100 mg or less) were taken, excess blood was blotted with gauze, and samples were weighed. Samples were placed into a cryovial, flash frozen with liquid nitrogen, and stored at −80 until the time of assay. Tubes were placed on ice, and 0.5 ml 30% KOH saturated with Na_2SO4 was added to each sample, making sure that the tissue was completely immersed. Samples were placed in a boiling water bath for 20 min and then cooled on ice. Cold 95% ethanol, 0.55 ml, was added to each tube to precipitate the glycogen. Tubes were left on ice for 30 min and then centrifuged at 840*g* for 20 min. Supernatants were carefully aspirated. Glycogen precipitates were then dissolved in 1 ml distilled water. Triplicate 15- to 30-μl aliquots of glycogen samples were placed in 12 × 75 mm glass tubes, and the volume was brought up to 150 μl with distilled water. One hundred fifty microliters of 5% phenol was added to all tubes. Sulfuric acid, 0.75 ml, was rapidly added to all tubes, making sure to direct the stream to the sample surface. Samples were incubated at room temperature for 20 min. Absorbance was read at 490 on a Beckman DU64 spectrophotometer. Glycogen values were corrected by the weight of the liver samples.

Glycogen staining. Pieces of liver tissues from fed, 16-h fasted, and 48-h fasted rats were fixed in 4% formaldehyde for 6 h and paraffin embedded. Tissue sections were stained for glycogen using the PAS technique. Briefly, sections were dewaxed in xylene and rehydrated through graded alcohol. They were incubated in 1% periodic acid solution for 5 min, washed in distilled water, and subsequently immersed in Schiff reagent (SurgiPath) for 15 min at room temperature. Slides were then washed in running water for 10 min, counterstained in Harris hematoxylin (Richard Allan Scientific), dehydrated, and mounted.

Assays. Plasma glucose was measured immediately using a Roche Accu-Chek glucose analyzer. Blood samples were collected into ice-chilled tubes. Trasylol, 1,000 IU/ml, was added to prevent degradation of glucagon. Plasma C-peptide and glucagon were measured using a rat radioimmunoassay (Linco Research, St. Charles, MO).

Statistics. Data are presented as the mean ± SE. Results were analyzed using Wilcoxon's matched-pair signed-rank test or ANOVA, as appropriate. A P value <0.05 was considered statistically significant.

RESULTS

Blood glucose after transplantation. Blood glucose levels in diabetic rats after STZ administration before insertion of the insulin pellets were >600 mg/dl. On the day of transplantation, the blood glucose levels in recipients were 350–550 mg/dl. The islet grafts were successful in establishing normoglycemia throughout the entire experimental period in the O, K, and H groups (Fig. 1). The mean time needed to normalize blood glucose levels after transplantation in the O, K, and H groups was 7 ± 2.9, 2.5 ± 0.4, and 2.3 ± 0.7 days, respectively. Islets transplanted into the peritoneal cavity failed to restore euglycemia. However, the P group recipients still benefited from transplantation because they required fewer insulin pellets than the STZ controls and maintained lower blood glucose levels (304 ± 57 vs. 438 ± 68 mg/dl, respectively, at day 70; $P < 0.05$). No spontaneous normalization of blood glucose levels in the S group was observed during the experiment period. The drop in blood glucose levels in the S group at day 49 in Fig. 1 was due to reinstitution of insulin pellets implants.

Glycogen after fasting. Fasting for 16 and 48 h significantly decreased glycogen storage in the liver in intrahepatic transplanted rats (fed 55.3 ± 6.4, 16-h fasted 2.23 ± 0.84, 48-h fasted 0.04 ± 0.10 μg/mg liver; $P <$ 0.0001). There were no significant differences in liver glycogen levels between normal rats and intrahepatic transplanted rats under fed, 16-h fasting, or 48-h fasting conditions (Fig. 2).

Although chemical measurements did not show significant difference in liver glycogen levels between 16- and 48-h fasting, glycogen histochemistry staining using paraffin sections demonstrated the presence of glycogen in some but not all hepatocytes after 16-h fasting (Fig. 3). Islets were observed to be in intimate contact with hepatocyctes (Fig. 3*D*). Liver glycogen was completely depleted after 48-h fasting in all rats.

Glucagon responses to hypoglycemia in the H group under fed, 16-h fasting, 48-h fasting, and refed con-

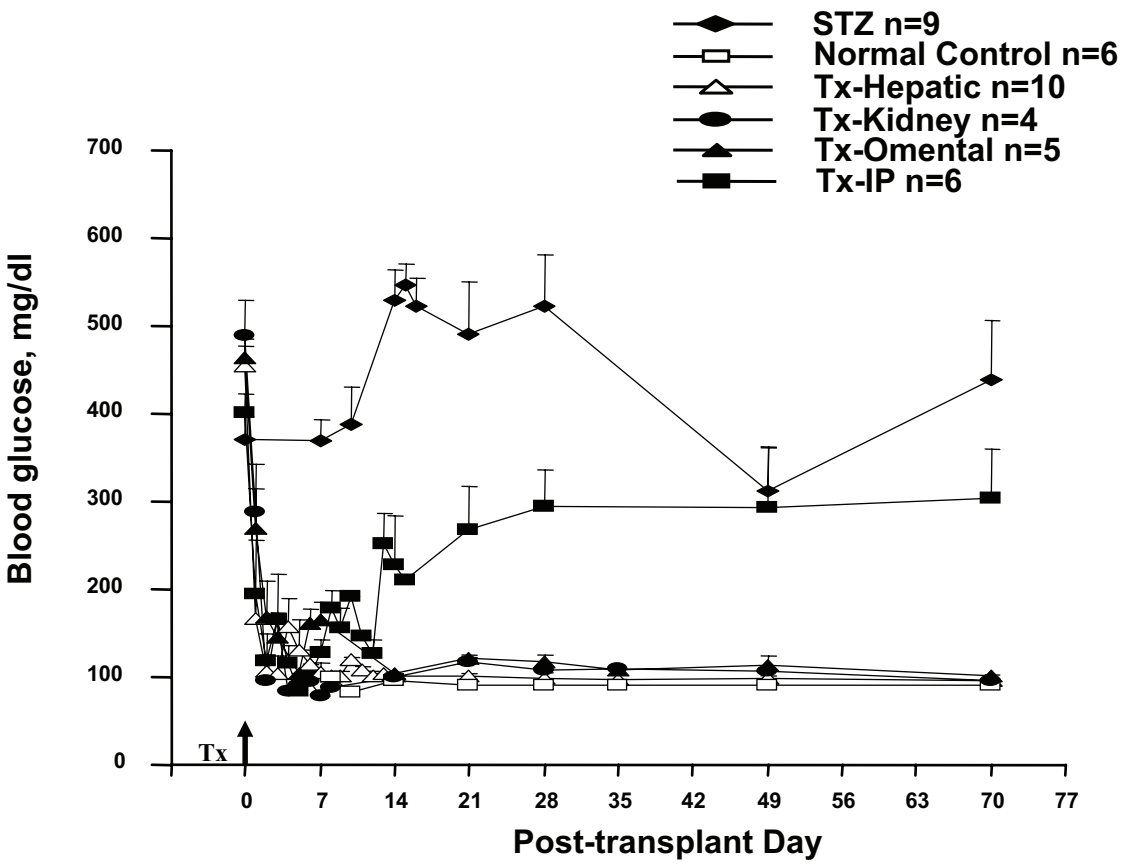

FIG. 1. Blood glucose levels posttransplantation. A total of 1,800–2,200 islets from inbred mates were transplanted to diabetic rats at day 0. Islets transplanted into the liver, kidney capsule, and omentum maintained euglycemia in recipients, whereas islets transplanted into the peritoneal cavity did not.

ditions. The basal blood glucose levels were significantly higher when animals were fed rather than fasted (fed 170 ± 11, 16-h fast 110 ± 6, 48-h fast 96 ± 4 mg/dl; $P < 0.05$) (Fig. 4). After refeeding, the basal blood glucose levels returned to the levels of their original fed state 3 weeks prior (fed 170 ± 11, refed 180 ± 19 mg/dl; P = NS). After intravenous insulin infusion, all groups achieved hypoglycemia. There was no significant difference in blood glucose levels among the fed, 16-h fasted, 48-h fasted, and refed rats when insulin infusion was stopped (22 ± 3, 21 ± 3, 17 ± 3, and 26 ± 3 mg/dl, respectively; P = NS).

A significant decline of basal C-peptide levels was observed after fasting (fed 681 ± 77, 16-h fast 388 ± 80, 48-h fast 294 ± 50 pmol/l; $P < 0.01$). After refeeding, basal C-peptide levels were increased to the levels of the fed state. During hypoglycemic challenge, C-peptide decreased to immeasurable levels in all groups.

Significantly higher glucagon responses to hypoglycemia were observed under fasting conditions (fed 8,777 ± 2,231, 16-h fast 24,217 ± 5,892, 48-h fast 30,790 ± 5,062 pg/ml per 90-min AUC; $P < 0.05$–0.01). After refeeding, glucagon responses to hypoglycemia were significantly lower than the responses observed under fasting conditions (refed 10,276 ± 2,750; $P < 0.05$), and no difference was found when comparing glucagon responses under fed and refed conditions.

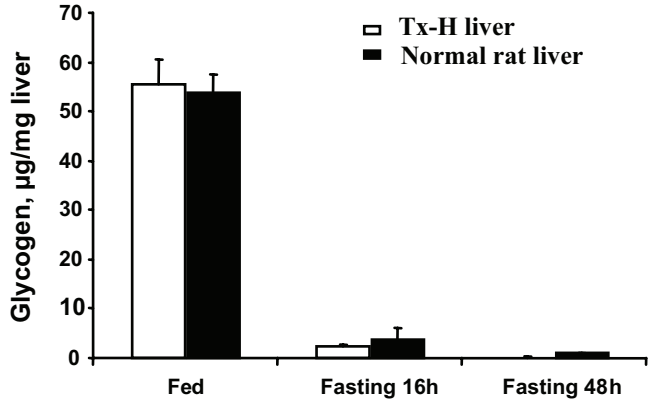

FIG. 2. Liver glycogen measurement in hepatic islet-transplanted rats and normal control rats under fed and 16- and 48-h fasting conditions.

Glucagon responses to hypoglycemia in the C and S groups under fed, 16-h fasting, and 48-h fasting conditions. The fed basal blood glucose levels in the C group after anesthetia were lower than those in the H group (C 132 ± 10, H 170 ± 11 mg/dl; $P = 0.05$). The basal blood glucose levels decreased significantly from fed levels after 16- and 48-h fasting (132 ± 10, 86 ± 3, and 85 ± 3 mg/dl, respectively; $P < 0.05$) (Fig. 5). The fed basal C-peptide levels were significantly higher in the C than in the H groups (C 1,101 ± 295, H 681 ± 77 pmol/l; $P < 0.01$). Fasting 16 and 48 h significantly decreased fed basal C-peptide levels (457 ± 76 and 303 ± 75 pmol/l, respectively; $P < 0.01$). Fasting and fed conditions did not change the magnitude of the glucagon responses to hypoglycemia in normal control group (fed 40,777 ± 8,192, 16-h fast 48,810 ± 13,785, 48-h fast 37,632 ± 5,335; P = NS). A comparison of glucagon responses in the hepatic trans-

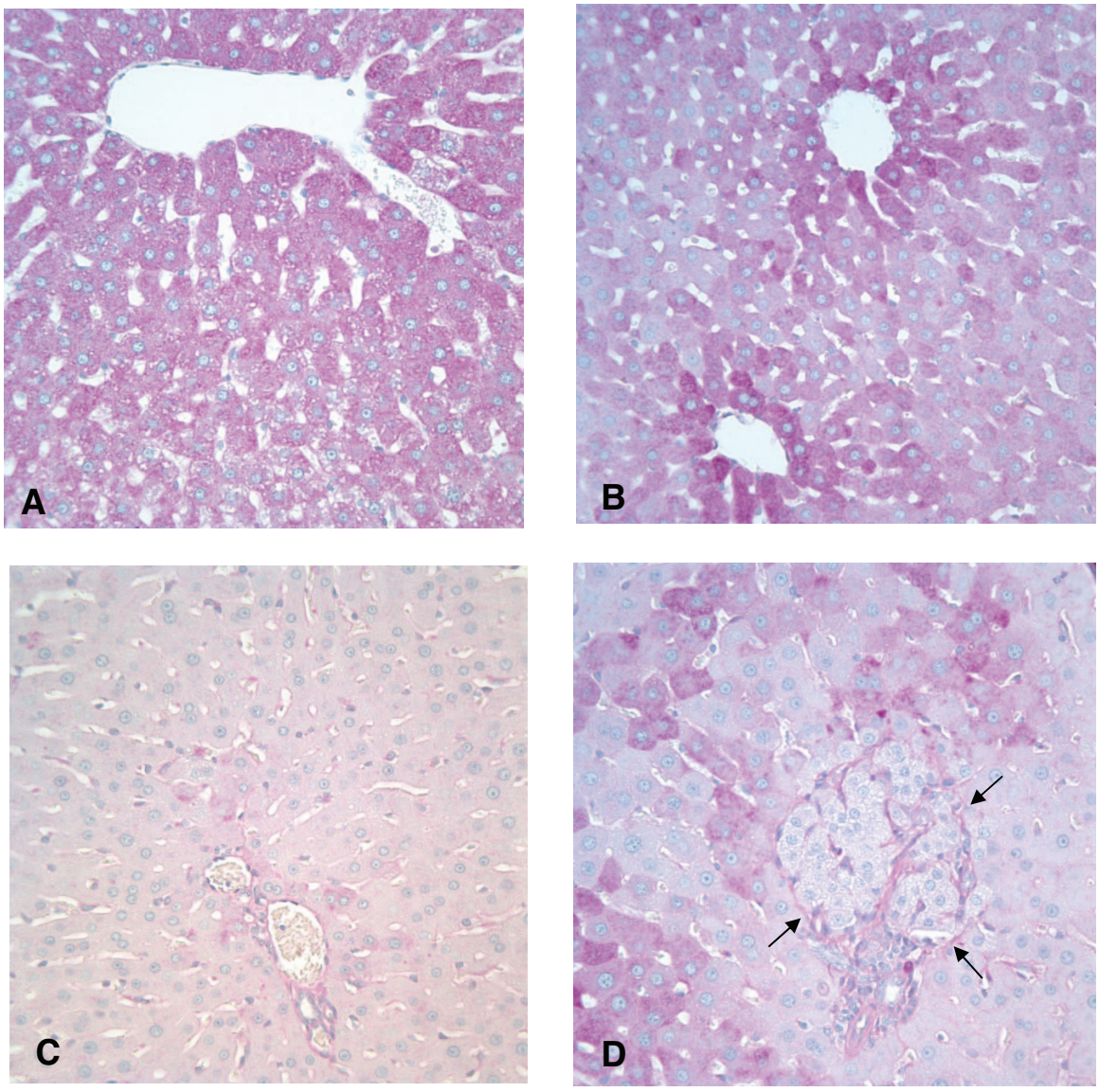

FIG. 3. Sections of rat liver with the periodic acid Schiff reaction and counterstained with hematoxylin. Glycogen is abundant in the liver of fed animals (*A*). Cellular stores glycogen are partially depleted after overnight fasting (*B* and *D*). Hepatocytes are almost entirely devoid of glycogen after 48 h fasting (*C*). One transplanted islet is shown in contact with glycogen-containing hepatocytes (*D*, *arrows*) after 16-h fasting. Original magnification ×40.

plant and the nondiabetic control group is provided in Fig. 6.

In the S control group, blood glucose levels were significantly lower after 48-h fasting because insulin pellets were implanted (Table 1). After insulin infusion, blood glucose levels reached hypoglycemic levels in the fed and 16- and 48-h fasting groups, and there was no significant difference between them (fed 43 ± 8, 16-h fast 36 ± 5, 48-h fast 29 ± 6 mg/dl; P = NS) (Table 1). No glucagon responses to hypoglycemia were observed under any condition (Fig. 5*D*). C-peptide levels were immeasurable throughout the experiment.

Comparison of different transplantation sites and the C and S groups. Blood glucose levels of fed mice before the ITT were normal in the H, K, and O groups but higher in the P group (Table 1). Fasting significantly decreased blood glucose levels in all groups. In the P group, insulin pellets were still in, so animals had mild hypoglycemia after the 48-h fast. ITTs were done under anesthesia. Blood glucose increased after anesthesia in all experimental animals. Rats in the H group had jugular vein permanent cannulation, whereas rats in the K, O, and P groups underwent surgery to cannulate the jugular vein before the ITT; therefore, blood glucose postsurgery in the K and O groups was significantly higher than that in the H group due to surgical stimulation. Fasting significantly decreased basal blood glucose levels postanesthesia. After insulin infusion, all animals achieved hypoglycemia (<60 mg/dl). There was no significant intragroup difference in the glucose nadir reached among the fed and 16- and 48-h fasting conditions in the H, K, O, and P groups, respectively. The mean glucose nadirs in the H group were significantly lower than those observed in the K, O, and P groups.

Although the H, K, and O groups maintained euglycemia throughout the experiment, C-peptide levels in both fed and fasting rats in the H group were higher than those in the K and O groups. C-peptide levels in the K and O groups may have been elevated because of surgical stimulation of glucose levels during cannulation of the jugular vein.

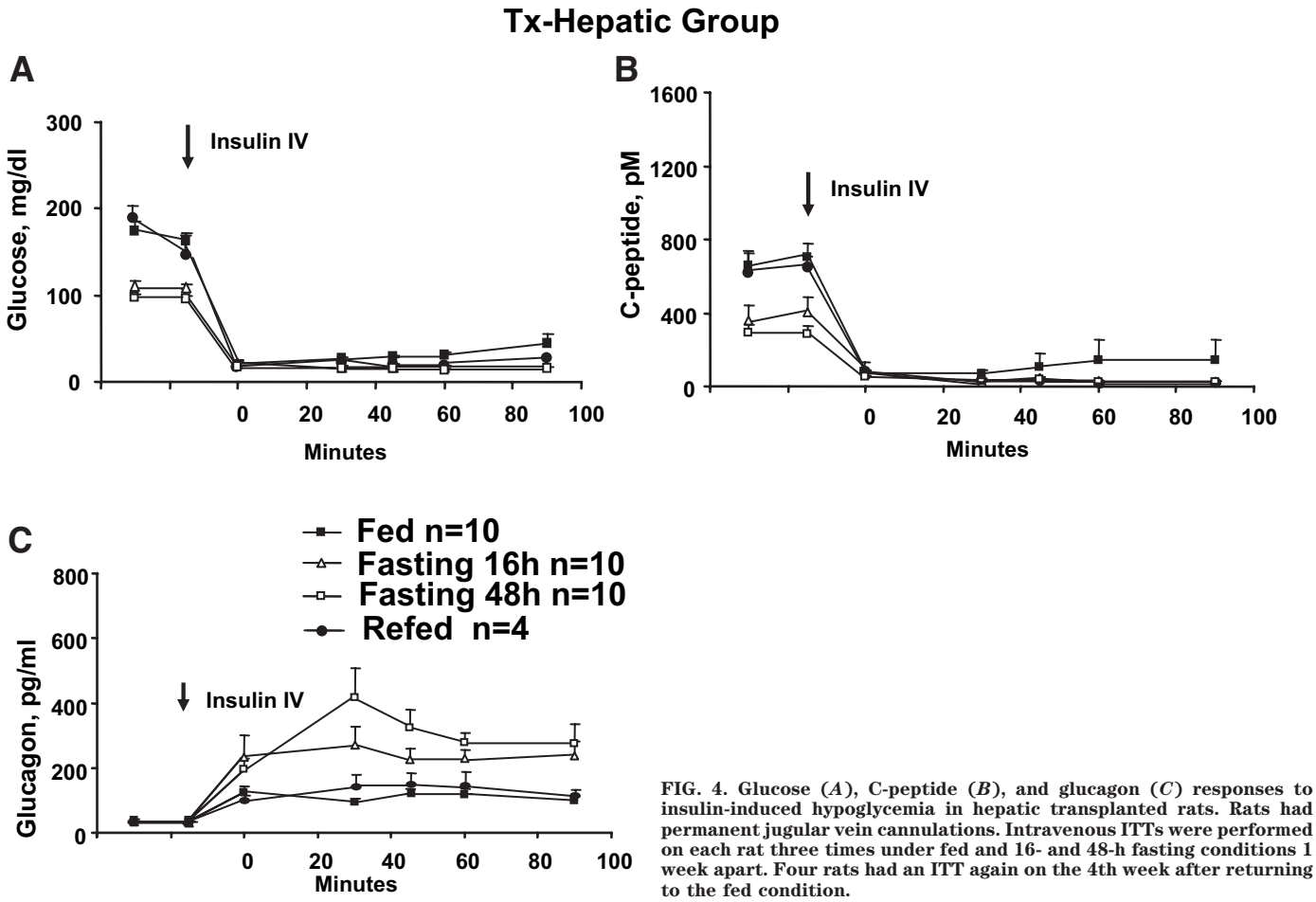

FIG. 4. Glucose (*A*), C-peptide (*B*), and glucagon (*C*) responses to insulin-induced hypoglycemia in hepatic transplanted rats. Rats had permanent jugular vein cannulations. Intravenous ITTs were performed on each rat three times under fed and 16- and 48-h fasting conditions 1 week apart. Four rats had an ITT again on the 4th week after returning to the fed condition.

C-peptide levels also showed a decline after fasting in the K and O groups. Animals in the P group failed to achieve euglycemia, and their C-peptide levels were significantly lower than those of the H, K, and O groups. After insulin infusion, C-peptide levels decreased to immeasurable levels in all groups.

No significant differences in basal plasma glucagon levels were observed among the different transplantation groups. Basal glucagon levels were not elevated after fasting for 16 or 48 h in all groups. Glucagon responses to hypoglycemia were observed in all transplantation groups. Unlike the H group, there were no intragroup differences in the K, O, and P groups when comparing glucagon responses to hypoglycemia among fed and 16- and 48-h fasting conditions.

DISCUSSION

The purpose of this study was to determine whether defective glucagon secretion from intrahepatically transplanted islets during hypoglycemia in Lewis rats is related to intrahepatic glycogen stores. Glycogen predictably undergoes glycogenolysis and increases free glucose levels in the liver during hypoglycemia. The study was designed to examine glucagon responses to hypoglycemia caused by intravenous insulin administration. Comparisons were made among results from hepatic and nonhepatic transplantation sites, as well as nontransplanted normal animals and STZ-administered diabetic animals. The results demonstrated diminished glucagon responses to hypoglycemia from islets transplanted into the hepatic site. Fasting for 16 and 48 h depleted glycogen from the liver and improved glucagon responses from intrahepatic islets but did not change the magnitude of the responses from islets in the nonhepatic sites. These data suggest that the defective glucagon response from the intrahepatic islets is due to ongoing glycogenolysis and increased glucose flux within the microenvironment of α-cells in the liver.

This conclusion is consistent with what is known about the blood circulation of intrahepatic islets. It is known that systemically circulating blood reaches intrahepatically transplanted islets via distribution of arteriolar flow from the central hepatic artery to the core of the islet that contains β-cells (9). This accounts for the decrease in C-peptide levels observed during hypoglycemia in hepatically transplanted animals. Thereafter, blood flow via the periportal islet circulation goes from β- to α- and δ-cells on the periphery of the islet. In the situation of the intrahepatic islet, this means that its α-cells are in intimate contact with hepatocytes, from which glucose is released during glycogenolysis. We suggest that passive diffusion of glucose from these neighboring hepatocytes provides a dominant signal of high glucose concentration in the microenvironment of the α-cell, so that it overwhelms a weaker hypoglycemic signal reaching the α-cells from hepatic arterioles. Consequently, glucagon secretion is relatively suppressed rather than fully stimulated. Full restoration of glucagon response by fasting and liver glycogen depletion is consistent with this formulation.

In the current study, we observed a significant albeit much attenuated glucagon response to hypoglycemia in fed hepatic transplanted rats, a phenomenon that was not seen in human diabetic recipients of islet allografts

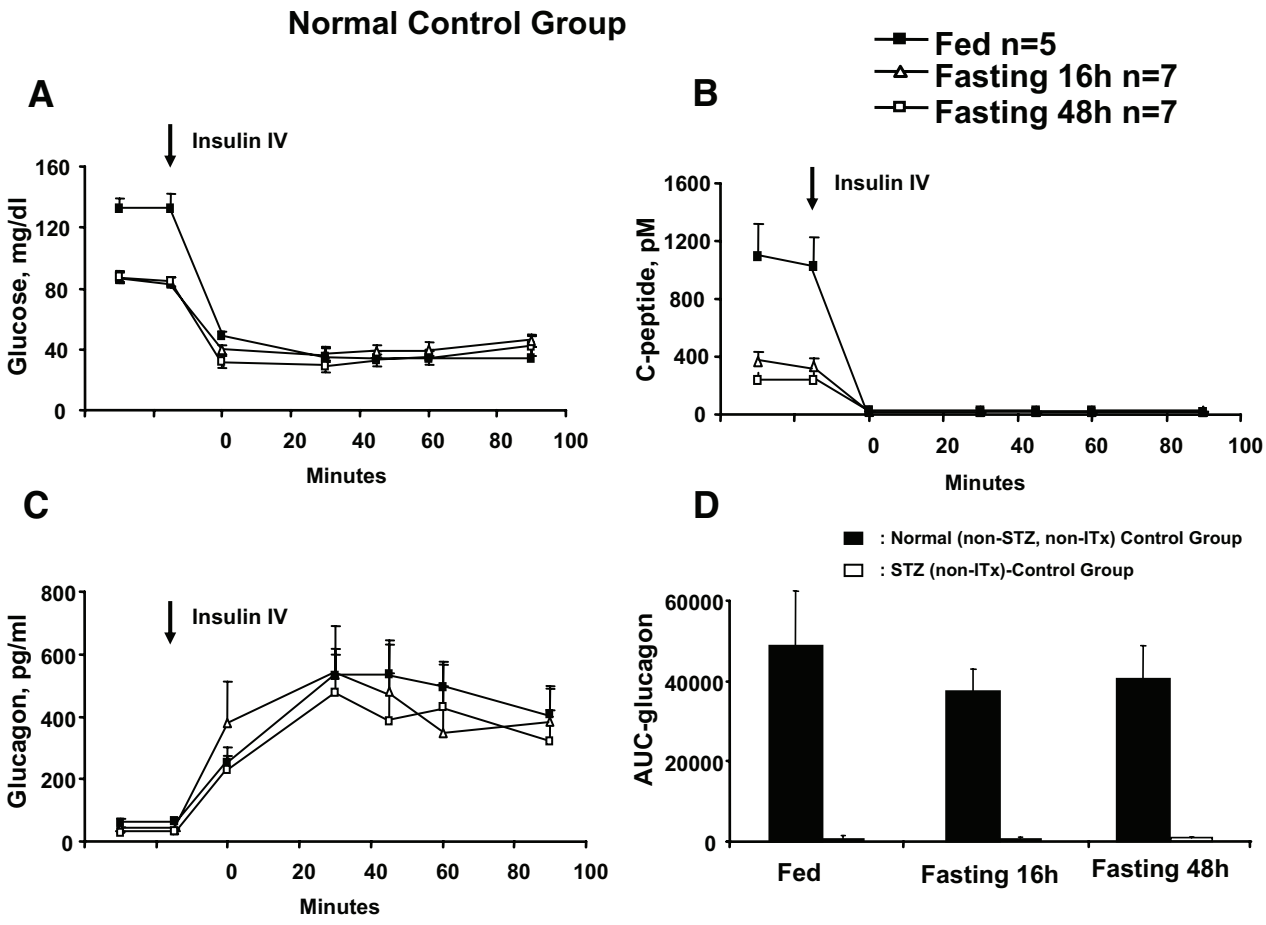

FIG. 5. Glucose (*A*), C-peptide (*B*), and glucagon (*C*) responses to insulin-induced hypoglycemia in non-STZ, nontransplanted normal control rats. Intravenous ITTs were performed three times 1 week apart in each rat under fed and 16- and 48-h fasting conditions. *D*: Comparison of area under the curve (AUC) for glucagon responses to insulin-induced hypoglycemia in normal control rats and STZ-administered diabetic rats.

(1,2,4,5). One explanation may be that the experimental period we used lasted 5 months after STZ administration; thus, β-cell regeneration in native pancreas may have occurred. If so, this would have allowed a return to normal intraislet regulation of α-cells by upsteam β-cells such that a cessation of β-cell secretion during hypoglycemia would have triggered glucagon release during hypoglycemia (13,14).

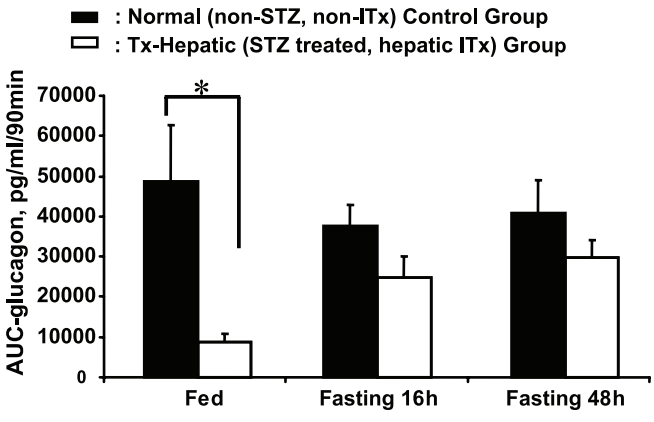

FIG. 6. Comparison of glucagon responses (calculated as area under the curve [AUC]) to insulin-induced hypoglycemia in normal control rats and intrahepatically transplanted rats under fed and 16- and 48-h fasting conditions. Compared with the control group, glucagon responses to hypoglycemia in the H group were impaired in the fed state. Depletion of liver glycogen by fasting was accompanied by restored glucagon responses to hypoglycemia in the H group. *$P < 0.05$–0.01.

It is notable that glucagon responses to hypoglycemia in rats in the fasting state, compared with those in normal control rats, were reduced in the animals with islets transplanted under the kidney capsule, in the omentum, and in the peritoneum, as well as in the intrahepatic site (Table 1); therefore, one might question whether location of the islets next to glycogen is necessary to explain the diminished function of intraheptatic islets during hypoglycemia. However, given the number of islets transplanted (1,800–2,200) in the various sites and the likely demise of some of the transplanted islets, it is almost certain that the intact pancreas in the normal rats contained many more α-cells and thus had larger glucagon responses to hypoglycemia. The important comparisons are within transplant groups, i.e., fasting and glycogen depletion improved glucagon responses to hypoglycemia only in the intrahepatic transplant group, as shown on Table 1. We have normalized the glucagon responses in each group by dividing the increase in glucagon levels during hypoglycemia by the basal C-peptide level (data not shown), making the assumption that C-peptide might reflect β-cell, and therefore by implication, α-cell mass. When we do so, the defective glucagon response before fasting in the intrahepatic transplanted animal is even more evident, whereas there are no differences in glucagon responses among all sites, including normal rat pancreases, in the fasted state.

TABLE 1
Levels of glucose, C-peptide, and glucagon in the H, K, O, P, C, and S groups

	n	Blood glucose pre-surgery (mg/dl)	Blood glucose post-surgery (mg/dl)*	Blood glucose ITT nadir (mg/dl)	Plasma C-peptide post-surgey (pmol/l)	Plasma C-peptide ITT nadir (pmol/l)	Plasma glucagon post-surgery (pg/ml)	Plasma glucagon ITT post-surgery (AUC) (pg/ml per 90 min)
Hepatic								
Fed	10	102 ± 3	170 ± 11	22 ± 3	681 ± 77	ND	38 ± 4	8,839 ± 1,988†
16-h fast	10	97 ± 2	110 ± 6	22 ± 3	388 ± 80	ND	32 ± 3	24,715 ± 5,210
48-h fast	10	81 ± 2	96 ± 4	17 ± 3	294 ± 50	ND	30 ± 4	29,639 ± 4,550
Kidney								
Fed	4	132 ± 32	280 ± 59	44 ± 4	555 ± 171	ND	50 ± 2	6,185 ± 1,612
16-h fast	5	71 ± 3	101 ± 8	32 ± 1	133 ± 22	ND	33 ± 6	6,078 ± 1,286
48-h fast	4	67 ± 2	102 ± 4	40 ± 2	93 ± 34	ND	34 ± 5	4,160 ± 1,183
Omental								
Fed	3	113 ± 20	361 ± 60	56 ± 6	284 ± 119	ND	83 ± 21	3,517 ± 628
16-h fast	5	78 ± 7	132 ± 26	41 ± 5	112 ± 6	ND	40 ± 5	3,129 ± 1,301
48-h fast	4	61 ± 2	89 ± 5	29 ± 6	177 ± 23	ND	46 ± 5	4,596 ± 472
Peritoneal								
Fed	3	195 ± 65	209 ± 98	36 ± 11	68 ± 13	ND	43 ± 6	4,420 ± 124
16-h fast	6	151 ± 72	113 ± 13	34 ± 5	82 ± 29	ND	22 ± 2	5,364 ± 2,815
48-h fast	4	56 ± 2	86 ± 15	35 ± 2	64 ± 13	ND	22 ± 4	4,235 ± 2,018
Control								
Fed	5	92 ± 2	132 ± 8	49 ± 3	1,101 ± 223	ND	62 ± 10	40,777 ± 8,192
16-h fast	7	69 ± 7	86 ± 3	40 ± 3	457 ± 76	ND	35 ± 4	48,810 ± 13,785
48-h fast	7	62 ± 5	86 ± 4	32 ± 4	303 ± 75	ND	27 ± 4	37,632 ± 5,335
STZ								
Fed	4	232 ± 52	262 ± 53	43 ± 6	ND	ND	43 ± 11	385 ± 773
16-h fast	6	203 ± 67	181 ± 53	36 ± 6	ND	ND	29 ± 4	340 ± 433
48-h fast	8	68 ± 15	81 ± 11	29 ± 5	ND	ND	21 ± 4	916 ± 329

Data are means ± SE. *H and C groups had permanent jugular vein cannulation and therefore no intrajugular vein surgery at the time of the studies. Post-surgical blood glucose levels were drawn under anesthesia, whereas pre-surgical glucose levels were drawn with no anesthesia. †P = 0.05–0.01 for fed vs. fasting. AUC, area under the curve; ND, not determined.

Thus, we conclude that the glucagon responses from all transplanted sites are smaller because of less α-cell mass than that found in normal pancreas and that the defective glucagon response to hypoglycemia is only present in the intrahepatic site, which can be corrected by fasting and glycogen depletion. The only caveat is that the defective α-cell responses in the STZ animals are due to β-cell death and not to decreased α-cell mass, i.e., absent β-cells in STZ animals render the insulin switch-off signal during hypoglycemia to α-cells unavailable, so the glucagon response to hypoglycemia is defective.

We also observed a higher glucagon response in the H group compared with the O, P, and K groups. Although the H, O, and K groups achieved euglycemia in recipients, liver transplantation probably resulted in a higher number of engrafted islets compared with the other transplantation sites, which was reflected by the higher basal C-peptide levels in fed rats. It is worth noting that the O, P, and K groups did not have permanent jugular vein cannulation. Thus, the three separate jugular vein surgeries just before the insulin infusion might have stimulated higher glucose and therefore higher C-peptide levels in the fed groups. Also due to postsurgical higher basal blood glucose, it was much more difficult to achieve hypoglycemia in those groups compared with the H group. The glucose nadirs were not as low in the non-H transplantation groups, which may not have fully activated the transplanted α-cells. ITTs were performed 1 week apart in the H group but 1 month apart in the other transplantation groups, making animals in the other transplantation groups 1–2 months older than those in the H group. This age issue might have also influenced the glucagon response. Nonetheless, the central experiment in this study was to examine glucagon responses to hypoglycemia after fasting. Differences were found only in the hepatic group and not in the groups using other transplantation sites.

These observations are clearly clinically relevant. If islet transplantation normalized glucose levels and patients no longer needed to use exogenous insulin therapy, then no danger of hypoglycemia would exist, and intrahepatic α-cell responses to hypoglycemia would not be needed. However, currently published information indicates that many if not most human diabetic recipients of alloislet transplantation return to insulin therapy within several years posttransplantation and therefore are once again at risk for hypoglycemia. This argues for the importance of finding an alternate nonhepatic site that will not interfere with full glucagon responses to hypoglycemia. Recently, additional metabolic defects in intrahepatically transplanted islets involving insulin synthesis, insulin secretion, and glucose oxidation have been reported (15). One study reported that α-cell responses to mild non–insulin-induced hypoglycemia (80 ± 3 mg/dl) in dogs were subnormal (16).

In conclusion, these studies strongly suggest that the mechanism of defective glucagon responses to hypoglycemia in recipients of intrahepatic islet transplantation involves the intimate relationship between the α-cells in the islets and the hepatocytes in the liver that undergo glycogenolysis during hypoglycemia. Alternate nonhepatic sites for islet transplantation will circumvent this loss of full α-cell function, which is an important consideration in view of the likelihood that many recipients of intrahepatic islets will return to exogenous insulin treatment.

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REFERENCES

1. Pyzdrowski KL, Kendall DM, Halter JB, Nakhleh RE, Sutherland DE, Robertson RP: Preserved insulin secretion and insulin independence in recipients of islet autografts. *N Engl J Med* 327:220–226, 1992
2. Kendall DM, Teuscher AU, Robertson RP: Defective glucagon secretion during sustained hypoglycemia following successful islet allo- and autotransplantation in humans. *Diabetes* 46:23–27, 1997
3. Gupta V, Wahoff DC, Rooney DP, Poitout V, Sutherland DE, Kendall DM, Robertson RP: The defective glucagon response from transplanted intrahepatic pancreatic islets during hypoglycemia is transplantation site-determined. *Diabetes*: 46:28–33, 1997
4. Paty BW, Ryan EA, Shapiro AM, Lakey JR, Robertson RP: Intrahepatic islet transplantation in type 1 diabetic patients does not restore hypoglycemic hormonal counterregulation or symptom recognition after insulin independence. *Diabetes* 51:3428–3434, 2000
5. Rickels MR, Schutta MH, Mueller R, Markmann JF, Barker CF, Naji A, Teff KL: Islet cell hormonal responses to hypoglycemia after human islet transplantation for type 1 diabetes. *Diabetes* 54:3205–3211, 2005
6. Ansara MF, Saudek F, Newton M, Raynor AC, Cryer PE, Scharp DW: Pancreatic islet transplantation prevents defective glucose counterregulation in diabetic dogs. *Transplant Proc* 26:664–665, 1994
7. Shapiro AM, Ricordi C, Hering BJ, Auchincloss H, Lindblad R, Robertson RP, Secchi A, Brendel MD, Berney T, Brennan DC, Cagliero E, Alejandro R, Ryan EA, DiMercurio B, Morel P, Polonsky KS, Reems JA, Bretzel RG, Bertuzzi F, Froud T, Kandaswamy R, Sutherland DE, Eisenbarth G, Segal M, Preiksaitis J, Korbutt GS, Barton FB, Viviano L, Seyfert-Margolis V, Bluestone J, Lakey JR: International trial of the Edmonton protocol for islet transplantation. *N Engl J Med* 355:1318–1330, 2006
8. Vantyghem MC, Hazzan M, Tourvieille S, Provost F, Perimenis P, Declerck N, Sergent G, Kerr-Conte J, Noel C, Pattou F: Selection of diabetic patients for islet transplantation: a single-center experience. *Diabete Metab* 30:417–423, 2004
9. Andersson A, Korsgren O, Jansson L: Intraportally transplanted pancreatic islets revascularized from hepatic arterial system. *Diabetes* 38 (Suppl. 1):92–195, 1989
10. Lau J, Jansson L, Carlsson PO: Islets transplanted intraportally into the liver are stimulated to insulin and glucagon release exclusively through the hepatic artery. *Am J Transplant* 6:967–975, 2006
11. Shibata A, Ludvigsen CW Jr, Naber SP, McDaniel ML, Lacy PE: Standardization of a digestion-filtration method for isolation of pancreatic islets. *Diabetes* 25:667–672, 1976
12. Lo S, Russell JC, Taylor AW: Determination of glycogen in small tissue samples. *J Appl Physiol* 28:234–236, 1970
13. Hope KM, Tran PO, Zhou H, Oseid E, Leroy E, Robertson RP: Regulation of α-cell function by the β-cell in isolated human and rat islets deprived of glucose: the "switch-off" hypothesis. *Diabetes* 53:1488–1495, 2004
14. Zhou H, Tran PO, Yang S, Zhang T, LeRoy E, Oseid E, Robertson RP: Regulation of α-cell function by the β-cell during hypoglycemia in Wistar rats: the "switch-off" hypothesis. *Diabetes* 53:1482–1487, 2004
15. Lau J, Mattsson G, Carlsson C, Nyqvist D, Köhler M, Berggren PO, Jansson L, Carlsson PO: Implantation site-dependent dysfunction of transplanted pancreatic islets. *Diabetes* 56:1544–1550, 2007
16. Gustavson SM, Rajotte RV, Hunkeler D, Lakey JRT, Edgerton DS, Neal DW, Snead WL, Penaloza AR, Cheerinton AD: Islet auto-islet transplantation into an omental or splenic site results in a normal beta cell but abnormal alpha-cell response to mild non-insulin induced hypoglycemia. *Am J Transplant* 5:2368–2377, 2004