

Islet Auto-Transplantation into an Omental or Splenic Site Results in a Normal Beta Cell but Abnormal Alpha Cell Response to Mild Non-Insulin-Induced Hypoglycemia

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The present studies were designed to determine if totally pancreatectomized dogs that underwent islet auto-transplantation retained a functional pancreatic counterregulatory response to mild non-insulin-induced hypoglycemia. Six dogs underwent total pancreatectomy followed by islet auto-transplantation to spleen or omentum. The animals recovered and fasting plasma glucose and insulin levels were normal. Each study consisted of a 40-min control and 2-h test period. At the onset of the test period, a glycogen phosphorylase inhibitor was administered to create mild hypoglycemia. Plasma glucose in the transplanted dogs fell from 120 ± 4 to 80 ± 3 mg/dL, similar to the minimum in control dogs without islet auto-transplantation (108 ± 2 to 84 ± 5 mg/dL). The fall in plasma insulin was similar in both groups. Glucagon, however, rose in response to hypoglycemia in the control dogs ($\Delta 24 \pm 7$ pg/mL; $p < 0.05$), but failed to rise significantly in the transplanted dogs ($\Delta 9 \pm 6$ pg/mL). In fact, only 1 of 7 control dogs failed to increase plasma glucagon by at least 25%, whereas 4 of 6 transplanted dogs failed to do so. In conclusion, in conscious dogs with successfully auto-transplanted islets, the beta cell response to mild non-insulin-induced hypoglycemia was normal, whereas the alpha cell response was not.

Key words: Arginine stimulation, canine, glucagon, hypoglycemia, islet transplantation

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Introduction

The brain depends exclusively on glucose for fuel in most normal physiological conditions (1); therefore, hypoglycemia can be critical if not prevented or rapidly corrected. The initial response to a fall in glucose involves a decrease in endogenous insulin secretion from pancreatic beta cells and an increase in glucagon release from alpha cells (2–6). In people with type 1 diabetes, there is no beta cell response and, unfortunately, there is often a blunted alpha cell response to hypoglycemia, as well. Although pancreatic islet transplantation in accord with the Edmonton protocol has recently received a great deal of attention as a potential cure for type 1 diabetes (7), there are still unresolved questions concerning the pancreatic counterregulatory response to hypoglycemia following islet transplantation.

Previous studies showed that the glucagon response to insulin-induced hypoglycemia did not improve following islet allo-transplantation and was similar to that of non-transplanted type 1 diabetic subjects, in contrast to the robust rise in healthy controls (8–10). Additionally, the alpha cell response to insulin-induced hypoglycemia was blunted in humans (9) and dogs (11) that underwent pancreatectomy with islet auto-transplantation to the liver, but was normal in dogs that underwent auto-transplantation to the spleen (12) or peritoneal cavity (11). Factors, alone or in combination, which may have confounded interpretation of the above findings include the immunosuppressive therapy used, the diabetic background of the recipients, and the transplantation site. Moreover, the insulin used to create hypoglycemia may itself have inhibited the alpha cell response.

The present study was designed to eliminate some of the afore-mentioned confounding variables. The aim was to determine if totally pancreatectomized dogs that had undergone islet auto-transplantation to either the omentum or spleen, both resulting in drainage into the hepatic

portal vein, retained a normal pancreatic counterregulatory response to mild non-insulin-induced hypoglycemia as would occur with fasting. Oral administration of a glycogen phosphorylase inhibitor (BAY R3401) (13–16) was used to achieve mild hypoglycemia, without the necessity of infusing insulin. We have previously shown that administration of this compound causes a fall in plasma insulin and rise in plasma glucagon in normal conscious dogs (2). Use of the glycogen phosphorylase inhibitor, as opposed to exogenous insulin, let us directly examine not only the alpha cell response to physiological hypoglycemia, but also the changes in endogenous insulin secretion.

Materials and Methods

Animals and surgical procedures

Two groups of non-diabetic, overnight-fasted, conscious mongrel dogs were studied: six dogs underwent total pancreatectomies and subsequent islet auto-transplantation (University of Alberta) and seven control dogs were sham treated (i.e. no auto-transplantation; four from University of Alberta and three from Vanderbilt University). Of the six healthy, non-diabetic dogs that underwent total pancreatectomy and subsequent islet auto-transplantation, three had islets transplanted to a splenic site (S; three males; 23.2 ± 1.7 kg) and three had islets transplanted to an omental site (O; three females; 24.4 ± 3.0 kg). These dogs were housed in a large animal vivarium and attended in accordance with the recommendation of the Canadian Council on Animal Care (HSLAS 089/09/05). A veterinarian supervised their overall care. Surgical procedures were performed under general halothane anesthesia. Post-operation hydration was maintained with Ringer's solution. Water was offered on day 2 and food was increased to a standard full diet (2–3 cups dry food per day, Lab Diet 5006 Canine Diet, PMI Nutrition International, LLC, Brentwood MO; $\frac{1}{2}$ of 630-g can per day of Friskies Alpo Beef Dinner, Friskies Petcare, North York, ON) supplemented with Cotazym (Organon Canada Inc., Westhill, ON, Canada) from day 4. Analgesia (Buprenorphine, Schering, Toronto, ON, Canada) was given post-operatively as required. The dogs were weighed weekly and permitted unrestricted exercise. Blood samples were taken daily at 8:00 a.m. for glucose determination. The animals were allowed to recover 8–21 months prior to the study.

Four control dogs (three males, one female; 22.7 ± 2.2 kg) from the University of Alberta, with general care as described above, and three control dogs from Vanderbilt University (two males, one female; 22.2 ± 1.1 kg) were also studied. The dogs at Vanderbilt University were fed once daily a diet of meat (Kal Kan, Vernon, CA) and chow (Purina Lab Canine Diet No. 5006; Purina Mills, St. Louis, MO) composed of 46% carbohydrate, 34% protein, 14% fat and 6% fiber based on dry weight. These animals were housed in a facility that met American Association for the Accreditation of Laboratory Animal Care guidelines, and the protocols were approved by the Vanderbilt University Medical Center Animal Care Committee (IACUC approval M/03/082).

We obtained arterial access in the dogs studied at Vanderbilt University in order to compare arterial hormone levels to those in deep venous (described under *Experimental Design*) samples. Approximately 7 days prior to the study, the three dogs at Vanderbilt University had a cut-down in the left inguinal region under general anesthesia (15 mg/kg body weight sodium pentothal presurgery; 1.0% isoflurane as an inhalation anesthetic during surgery). A Silastic catheter (Dow Corning Corp., Midline, MI) was inserted into the left femoral artery for blood sampling as previously described (17). The arterial catheter was filled with heparinized saline (200 U/mL; Abbott

Laboratories, North Chicago, IL) and the free end was knotted and placed in a subcutaneous pocket until the study day. On the morning of the study, the catheter was exteriorized under local anesthesia (2% lidocaine; Abbott Laboratories), flushed with saline, and subsequently used for blood sampling. The animals were studied only if the following criteria were met at least 2 days prior to the study: (1) leukocyte count $< 18\,000/\text{mm}^3$, (2) hematocrit $> 35\%$, (3) good appetite and (4) normal stools.

Preparation of transplant tissue

The pancreas was mobilized with all major vascular connections maintained until just before excision of the gland. The pancreas was excised, weighed and pancreatic ducts cannulated. Islets were isolated by perfusion of collagenase (2 mg/mL, Type V: Sigma, St. Louis, MO) via the ducts, automated dissociation and discontinuous density gradient purification with Ficoll as previously described (18). The islet mass for the pure islets was quantified according to criteria established at the 1989 International Workshop on Islet Assessment (19).

For unpurified microfragments, which were used for all transplant recipients except one splenic recipient, the pancreas was processed the same to the purification step, but no Ficoll purification was used. The islets in this preparation were more trapped and surrounded by exocrine tissue. Pancreatic microfragments were not assessed for islet mass because entrapped islets do not stain well with dithiazone, making it difficult to accurately assess islet mass.

Transplantation

Following islet isolation or preparation of the pancreatic microfragments, the tissue was immediately transplanted as an autograft into the spleen or omental pouch. For recipients of intrasplenic islet autografts, the islets were refluxed into the splenic vein as previously described (20). For omental pouch recipients, the greater omentum was mobilized and folded upon itself, creating a circular pouch of approximately 10–15 cm diameter. One-half of the tissue preparation was dispersed into the omentum, which was then folded upon itself. The margin of the omentum at the periphery was sutured together using 3–0 prolin. The remaining tissue was again dispersed on the omentum, which was once again folded onto itself and sutured at the periphery. Using this technique, a large volume of tissue can be dispersed evenly through the omentum (21). Following the transplant procedure, the incision was closed and the animals were returned to the vivarium.

Evaluation of graft function

Fasting plasma glucose was monitored daily using a glucose strip (One Touch, Lifescan Johnson & Johnson Co.). No animals had a fasting glucose level indicative of diabetes. Please see Figures 1–2 for fasting insulin and glucose levels, which were indicative of good metabolic control.

Experimental design

Each overnight fasted-(16-h) conscious dog was placed in a Pavlov harness on the morning of the study. A catheter (Intramedicut Catheter Kit; 18-gauge angiocath, 30-cm catheter; Sherwood Medical, St. Louis, MO) was placed into the right or left saphenous vein in the hindlimb and advanced into the inferior vena cava so as to obtain deep venous blood samples. An angiocath (20-gauge; Becton Dickinson, Sandy, UT) was inserted into the right or left cephalic vein for infusion of glucose (20% Dextrose; Baxter Healthcare Corporation, Deerfield, IL) as needed. Each experiment consisted of a 60-min acclimation period (–100 to –40 min), a 40-min basal period (–40 to 0 min), and a 2-h experimental period during which mild hypoglycemia was achieved (0–120 min). At the onset of the experimental period (time 0), all dogs were given the glycogen phosphorylase inhibitor, Bay R3401 (Bayer Co., Pittsburgh, PA; 8 mg/kg in a 0.5% methyl cellulose/water solution (50 mL)), orally to decrease the plasma glucose level. Because previous

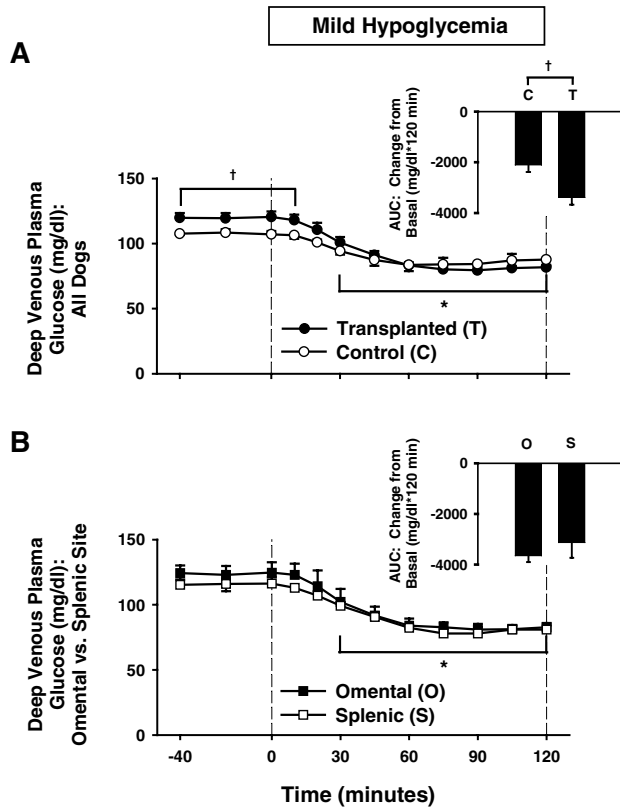


Figure 1: Deep venous plasma glucose levels in the basal period (–40 to 0 min) and after administration of the glycogen phosphorylase inhibitor (8 mg/kg orally at 0 min) in 18 h fasted conscious dogs. The inset shows the AUC of the experimental period after subtracting the time-adjusted basal period. Data are expressed as mean ± SE. For panel A, T represents dogs that underwent islet auto-transplantation (n = 6) while C represents control dogs that did not undergo islet transplantation (n = 7). * = p < 0.05 versus basal period for each group at the time points indicated. † = p < 0.05 for C versus T as indicated. For panel B, O represents the subset of T that had islets transplanted to the omental site (n = 3) while S represents the subset of T that had islets transplanted to the splenic site (n = 3). Data are expressed as mean ± SE. * = p < 0.05 versus basal period for each group at the time points indicated. There was no significant difference between the groups.

administration of BAY R3401 and maintenance of euglycemia in conscious dogs did not result in any changes in insulin or glucagon levels, we can assume that the drug does not directly influence alpha or beta cell secretion (15). We thus conclude that the observed changes were the result of hypoglycemia per se. During the experimental period, plasma glucose was measured every 5 min to avoid a decrease below 70 mg/dL and thereby avoid a catecholamine counterregulatory response. A small amount of glucose was infused peripherally to keep glucose above 70 mg/dL in two transplanted (0.8 ± 0.8 mg/kg/min; one O and one S) and two control (1.1 ± 0.4 mg/kg/min) dogs, for less than 30 min per dog. Blood was collected at 20-min intervals during the basal period and at 10–15-min intervals during the experimental period from the deep venous catheter (and the arterial catheter in the Vanderbilt control dogs) for the measurement of plasma insulin, glucagon, and cortisol.

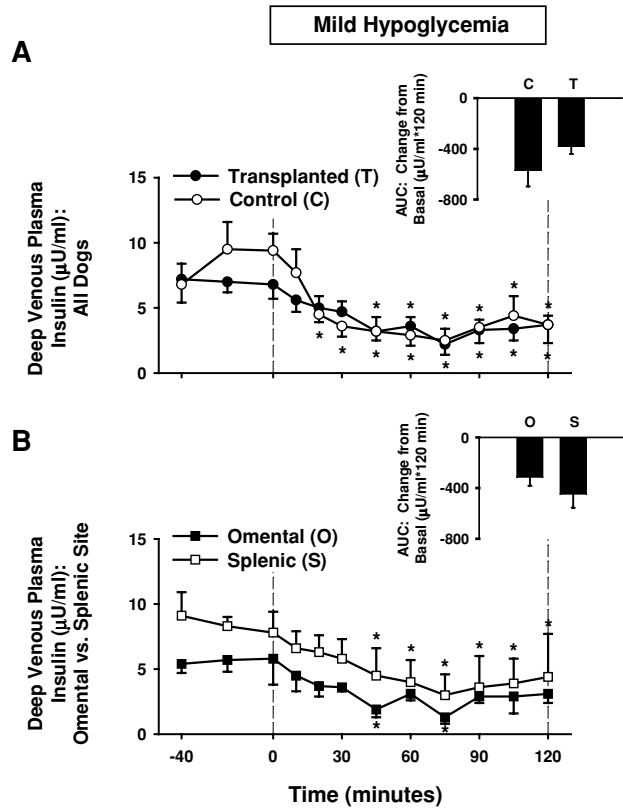


Figure 2: Deep venous plasma insulin levels in the basal period (–40 to 0 min) and after administration of the glycogen phosphorylase inhibitor (8 mg/kg orally at 0 min) in 18 h fasted conscious dogs. The inset shows the AUC of the experimental period after subtracting the time-adjusted basal period. Data are expressed as mean ± SE. For panel A, T represents dogs that underwent islet auto-transplantation (n = 6) while C represents control dogs that did not undergo islet transplantation (n = 7). * = p < 0.05 versus basal period at the time points indicated. There was no significant difference between the groups. For panel B, O represents the subset of T that had islets transplanted to the omental site (n = 3) while S represents the subset of T that had islets transplanted to the splenic site (n = 3). Data are expressed as mean ± SE. * = p < 0.05 versus basal period at the time points indicated. There was no significant difference between the groups.

At the end of the experiment the control dogs studied at Vanderbilt University were euthanized with a pentobarbital solution (Euthanasia-5; Veterinary Laboratories Inc., Lenexa, KS). The auto-transplanted and control dogs studied at the University of Alberta had their deep venous catheter and angiocath removed, and were injected with Penicillin G (600 000 U) for a few days to prevent infection. One to 6 months following the experiment, the transplanted and control dogs at the University of Alberta were given an arginine stimulation test to determine if the alpha and beta cells could increase hormone secretion normally in response to this challenge. After overnight fasting, the dog was placed in a Pavlov frame and two inter-catheters were placed in the saphenous vein, one for injections and the other for blood samples. Two baseline samples (3 mL) were taken, at –10 and 0 min. Samples were transferred to 3 mL EDTA tubes that contained 1500 KIU Trasylol and plasma determined for plasma glucose, insulin, and

glucagon. At 0 min, 2.5 g of arginine was injected over 1 min, and blood samples were taken at 2, 4, 6, 8, 10, 15, 20, 25, 30 and 40 min.

The immediate processing of the samples was described previously (22,23). Plasma levels of glucose were measured as previously described (22,23). Plasma immunoreactive levels of insulin, glucagon and cortisol were all measured as previously described at Vanderbilt University (22,23). Note that immunoreactive glucagon values include approximately 15–25 pg/mL of cross-reacting, non-glucagon material, which is equivalent and unchanging in all blood vessels and thus provides a constant background.

Calculations

Area under the curve (AUC) for each parameter in each group was calculated for the entire experimental period using the trapezoidal rule for each dog. The AUC of the basal period (time-adjusted) was subtracted from the AUC of the corresponding experimental period of each dog to calculate the Δ AUC, thus accounting for any baseline differences between the groups.

Statistical methods

The null hypothesis was that pancreatic islet auto-transplantation would not affect the alpha cell response to non-insulin-induced hypoglycemia in conscious dogs. The primary outcome measure was plasma glucagon. The study was designed to have 80% power to detect a 70% decrease in glucagon AUC in auto-transplanted dogs compared to control dogs, using an independent t-test with a 0.05 two-sided significance level. On the basis of previous studies, we anticipated an increase of ~ 1700 pg/mL over 2-h of hypoglycemia with a standard deviation of 700 pg/mL in the control group. With a sample size of 6 per group, we estimated we could significantly detect a difference of ~ 1200 pg/mL during our 2-h clamp (i.e. a rise of only 500 pg/mL or less in the transplanted group). This sample size estimation was performed using Dupont and Plummer's PS-Power and Sample Size Program (Version 2.1.31; <http://www.mc.vanderbilt.edu/prevmed/ps/index.htm>).

Data are expressed as mean \pm SE. Statistical comparisons for the time course data presented in Figures 1–4 were made by two-way analysis of variance (ANOVA) with repeated-measures design, using Tukey test post hoc analysis to determine at what times the differences existed. Independent t-tests were used to determine significance for the AUC data portrayed in the figures. The statistical software SigmaStat (SPSS Science, Chicago, IL) was used for all analyses, and statistical significance was accepted at $p < 0.05$.

Results

Fasting plasma glucose levels were in a normal range for both control and transplanted dogs, although the levels were minimally elevated in the transplanted dogs. Upon administration of the glycogen phosphorylase inhibitor, the plasma glucose level (Figure 1) reached similar minima of 84 ± 5 and 80 ± 3 mg/dL in the control (C) and transplanted (T) groups respectively ($p < 0.05$ compared to the basal period for each group; no significant difference between groups). Because of the modest although significant baseline difference between the groups the transplanted group exhibited a greater fall in glucose as evidenced by the area under the curve (AUC) insert in Figure 1. When the transplanted dogs were looked at according to transplant site, there was no significant difference at any time between those that had islet auto-transplantation to the

omental (O) site compared to the splenic (S) site, and the AUC changes were similar. The plasma cortisol levels did not increase significantly in either control dogs (4 ± 2 to 5 ± 1 μ g/dL) or dogs with transplanted islets (5 ± 1 to 6 ± 1 μ g/dL) indicative of the very mild hypoglycemic level obtained.

The basal deep venous plasma insulin level (Figure 2) was not significantly different in the control dogs or dogs with transplanted islets. Insulin fell significantly in both groups in response to the mild hypoglycemia, with no significant difference between the groups. The change in the AUCs for plasma insulin was not different between control and transplanted dogs, and the transplantation site did not affect the insulin response to mild non-insulin-induced hypoglycemia. Deep venous plasma C-peptide levels also fell significantly and similarly in both groups (0.47 ± 0.05 to 0.37 ± 0.02 and 0.40 ± 0.03 to 0.31 ± 0.02 data not shown).

No significant difference in plasma glucagon was observed (Figure 3) during the basal or experimental period in the two groups. However, glucagon only rose significantly in response to mild hypoglycemia in the control group. Likewise, the change with AUC for plasma glucagon was significantly higher in control versus transplanted dogs. Additionally, it should also be noted that only one out of seven control dogs failed to increase plasma glucagon by a maximum of at least 25%, whereas four out of six transplanted dogs (two in the omental and two in the spleen site) failed to do so. Neither transplantation site resulted in an appreciable alpha cell response to hypoglycemia. It should be remembered that we induced only a mild non-insulin-dependent hypoglycemia, thus explaining the relatively small glucagon response in control animals.

In response to the arginine stimulation test, insulin levels rose in both the control dogs and those with transplanted islets (non-significant between groups; Figure 4). However, insulin only increased in response to arginine in the splenic group. Glucagon rose briefly (2 min) in response to arginine in both the control and the transplanted group (Figure 5). There was no statistical difference between the two groups at any specific time point after administration of arginine, but the glucagon AUC did not increase from basal in the group with the transplanted islets but did in the control group, with a strong tendency for a difference between the groups ($p = 0.08$). Although there was no statistical difference between the splenic and omental groups, there was an absolute rise in the splenic group of ~ 20 pg/mL, but no rise in the omental group.

The three dogs studied at Vanderbilt University had catheters in both the femoral artery and the inferior vena cava to allow comparison of arterial and deep venous blood. No significant differences between deep venous versus arterial insulin or glucagon levels were observed (data not shown). Although deep venous and arterial plasma glucose levels were not different for the majority of time points,

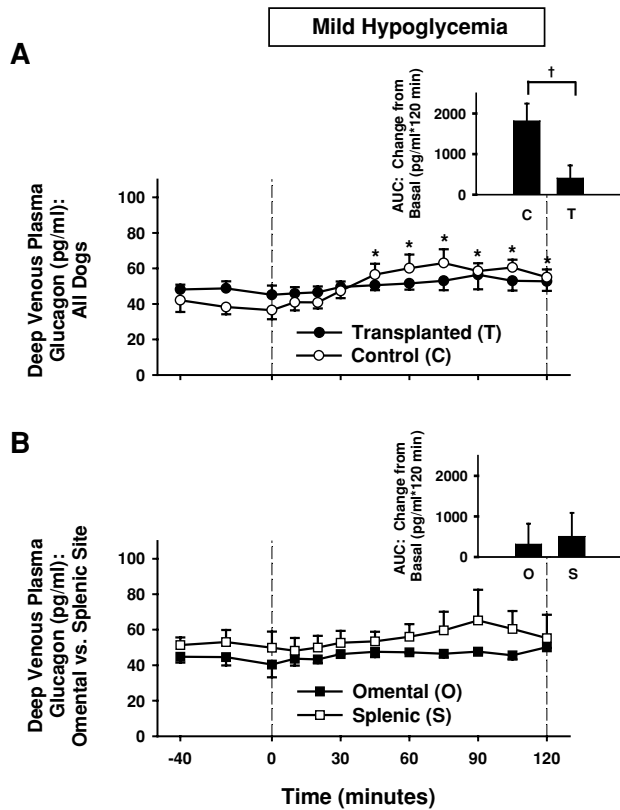


Figure 3: Deep venous plasma glucagon levels in the basal period (–40 to 0 min) and after administration of the glycogen phosphorylase inhibitor (8 mg/kg orally at 0 min) in 18 h fasted conscious dogs. The inset shows the AUC of the experimental period after subtracting the time-adjusted basal period. Data are expressed as mean ± SE. For panel A, T represents dogs that underwent islet auto-transplantation (n = 6) while C represents control dogs that did not undergo islet transplantation (n = 7). * = p < 0.05 versus basal period at the time points indicated for the control group. † = p < 0.05 for C versus T for the AUC data. For panel B, O represents the subset of T that had islets transplanted to the omental site (n = 3) while S represents the subset of T that had islets transplanted to the splenic site (n = 3). There was no significant difference between the two groups, and neither group changed significantly from basal.

approximately 40% of the samples taken were statistically lower although not clinically significant (~2–4 mg/dL) in the deep venous sample compared to the arterial sample. Because hormone levels were not different, and because glucose levels were minimally different, we conclude values in deep venous blood in this study accurately reflected those in arterial blood.

Discussion

In non-diabetic, pancreatectomized, conscious dogs with successfully auto-transplanted islets, the beta cell response to mild non-insulin-induced hypoglycemia was nor-

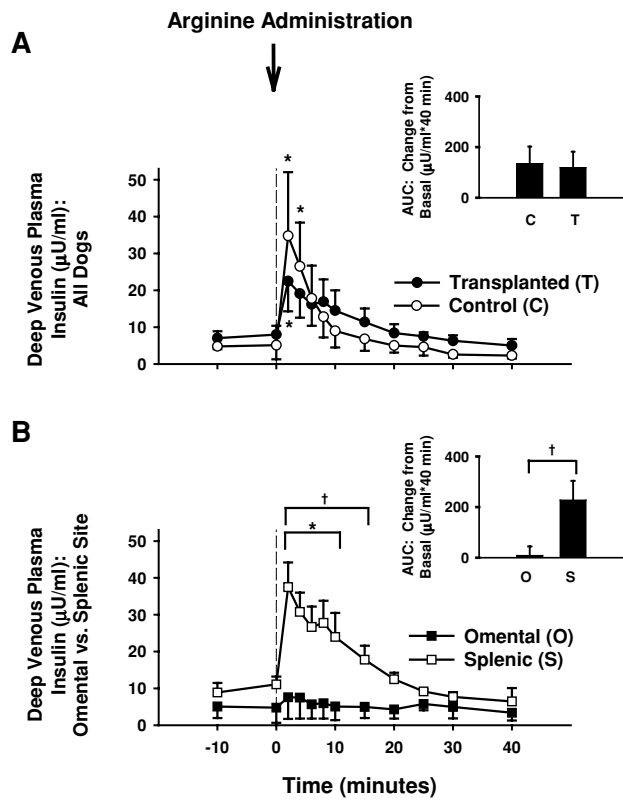


Figure 4: Deep venous plasma insulin levels in the basal period (–10 to 0 min) and after intravenous administration of arginine (2.5 g of arginine was injected over 1 min at 0 min) in 18 h fasted conscious dogs. The inset shows the AUC of the experimental period after subtracting the time-adjusted basal period. Data are expressed as mean ± SE. For panel A, T represents dogs that underwent islet auto-transplantation (n = 6) while C represents control dogs that did not undergo islet transplantation (n = 4). * = p < 0.05 versus basal period for each group at the time points indicated. There was no significant difference between the groups during the time course or for the AUC data. For panel B, O represents the subset of T that had islets transplanted to the omental site (n = 3) while S represents the subset of T that had islets transplanted to the splenic site (n = 3). * = p < 0.05 versus basal period within group S at the time points indicated. † = p < 0.05 for O versus S as indicated.

mal. However, there was no alpha cell response to the mild hypoglycemia, despite the greater fall in plasma glucose in transplanted dogs compared to controls. These findings correlate well with the reduced glucagon response to insulin-induced hypoglycemia observed in type 1 diabetic islet transplant recipients (8–10). The human studies show that simply correcting the diabetic background (hyperglycemia and lack of endogenous insulin) by islet transplantation does not result in hypoglycemic responsiveness of either native or transplanted alpha cells (9), and our study further revealed that neither a prior hyperglycemic background nor immunosuppressive therapy is required for transplanted alpha cell dysfunction. The use of

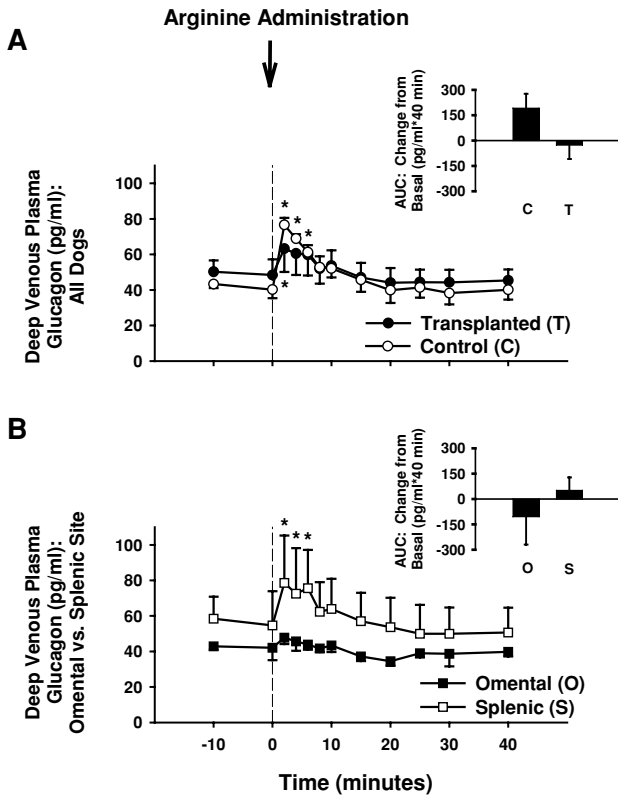


Figure 5: Deep venous plasma glucagon levels in the basal period (–10 to 0 min) and after intravenous administration of arginine (2.5 g of arginine was injected over 1 min at 0 min) in 18 h fasted conscious dogs. The inset shows the AUC of the experimental period after subtracting the time-adjusted basal period. Data are expressed as mean ± SE. For panel A, T represents dogs that underwent islet auto-transplantation (n = 6) while C represents control dogs that did not undergo islet transplantation (n = 4). * = p < 0.05 versus basal period for each group at the time points indicated. There was no significant difference between the groups during the time course, although p = 0.08 for the AUC data. For panel B, O represents the subset of T that had islets transplanted to the omental site (n = 3) while S represents the subset of T that had islets transplanted to the splenic site (n = 3). * = p < 0.05 versus basal period within group S at the time points indicated. There was no significant difference between the groups.

the glycogen phosphorylase inhibitor in the present study was unique in that it allowed for the generation of a mild hypoglycemic stimulus, in the absence of confounding hyperinsulinemia generally used to induce hypoglycemia experimentally. Therefore, the absent glucagon response to mild non-insulin-induced hypoglycemia observed in this study suggests that type 1 diabetic islet transplant recipients may not respond appropriately to physiological situations such as fasting and exercise (although to date this has not been observed clinically).

The reason for the dysfunctional alpha cell secretion during hypoglycemia following islet transplantation in our studies is unclear, but the results of the arginine test imply it may not simply be related to a decreased islet cell mass or a generalized alpha cell defect caused by the islet isolation procedure (24). The fact that glucagon and insulin rose significantly in the splenic transplanted dogs after arginine administration implies that at least in that group there was an adequate islet mass to mount a response and that the alpha cells were functional. Although the response of the transplanted group tended to be blunted (p = 0.08) compared to controls, which was also observed in previous human and dog studies (9,11), a reduced response to arginine does not necessarily preclude a normal glucagon response to hypoglycemia. For instance, hemipancreatectomy blunted the alpha cell response to arginine (25) but not hypoglycemia (26). The insulin response to arginine in our study sheds further light on the issue. The relatively normal insulin response to the amino acid in the splenic group also implies islet mass was adequate at least in this group, as the acute insulin response to intravenous arginine has been shown to be strongly predictive of beta cell mass and insulin reserve following whole and segmental pancreas transplantation (27). Additionally, although the auto-transplanted dogs had significantly higher baseline glucose levels, with a tendency (NS) for decreased insulin, no dogs were diabetic. Normal beta cell function suggests that generalized cell damage and insufficient alpha cell mass were unlikely to have occurred, since in humans and dogs alpha cells are interspersed throughout the islet with no structured beta cell core (28,29). It thus seems more probable that the alpha cell defect in the transplanted dogs during hypoglycemia may be stimulus-specific, similar to the glucagon defect in long-standing type 1 diabetes. Further evidence for a stimulus-specific defect is provided by the observation that glucagon rose during exercise in auto-transplanted dogs (30) and some (31) although not all (31) allo-transplanted rats.

Possible explanations for the apparently stimulus-specific alpha cell dysfunction during hypoglycemia could be repeated unrecognized episodes of hypoglycemia, improperly reinnervated islets (32–36), or improperly revascularized islets (37–43). A novel theory to explain the absent glucagon response to hypoglycemia may relate to the increased anaerobic glucose metabolism that occurs in transplanted islets (44), likely due to low blood perfusion (45,46) and decreased tissue oxygen tension (38,46,47). A shift from oxidative to non-oxidative metabolism in transplanted islet cells may explain why a mild fall in blood sugar did not stimulate glucagon secretion.

Two previous studies described a full glucagon response to insulin-induced hypoglycemia in dogs that had undergone pancreatectomy and islet auto-transplantation to the spleen (12) or peritoneal cavity (11), but not the liver (11). We studied islets transplanted to similar sites, so the reason glucagon increased in the previous studies but not in

ours is unclear. Potential explanations for the discrepancy include the fact anesthesia was used during hypoglycemia for one study (11), the hypoglycemia was greater in the earlier studies, hyperinsulinemia was present and there may have been differences in islet mass. However, the previous studies used ~9000–13 000 islet equivalents/kg, which should be comparable to our study and well above the previously defined thresholds of 5000 (48,49) or 6000 (50) for adequate long-term function. The most likely explanation for the discrepant results is the depth of hypoglycemia generated. The previous studies involved insulin-induced hypoglycemia of ~40–50 mg/dL, which resulted in a robust rise in epinephrine that was at least 4–5 times above baseline (11,12) and approximately 2-fold greater (although not significantly) in the peritoneal compared to hepatic group (11). Our study in contrast involved non-insulin-induced hypoglycemia of ~80 mg/dL, which does not stimulate epinephrine secretion (2,51,52). The alpha cell might no longer respond to a mild decrease in glucose per se, but may still respond to the added stimulus of a rise in epinephrine, activation of the sympathetic or parasympathetic nervous system, or a greater fall in plasma glucose. Note that stimulation of the alpha cell by epinephrine might also explain the rise in glucagon during exercise in auto-transplanted dogs (30). Interestingly, whole pancreas transplantation can result in normal alpha cell responses to hypoglycemia (53–55) even 19 years after transplantation (56), although not all studies support this (57,58).

A fall in endogenous insulin secretion is a critical component of the normal counterregulatory response to hypoglycemia. The glycogen phosphorylase inhibitor allowed us to determine for the first time the effect of islet transplantation on the beta cell response to hypoglycemia without confounding hyperinsulinemia. Insulin and C-peptide levels fell significantly and similarly in the transplantation and control groups, with no difference between the transplantation sites, showing that the transplanted beta cell response to hypoglycemia was normal. Not only is this normal beta cell response important to the recovery from hypoglycemia, it also implies that the dysfunctional alpha cell response in our studies was not due to a failure of endogenous insulin to decrease. In contrast, a recent study found that rats with allo-transplanted islets to several different sites exhibited exercise-induced hypoglycemia owing to a failure to appropriately suppress insulin secretion, as indicated by C-peptide levels (31). The reason for this defective beta cell response is unclear, but could be due to immunosuppressive therapy used, the type of stimulus utilized, or species-related differences in islet structure. For instance, in rats blood normally flows from the central core of the islet, composed of beta cells, to the periphery of the islet where the alpha cells are found, but in the human and dog islets alpha and delta cells are interspersed throughout the islet with no central beta cell core (see discussion in (29)).

Normal islet structure and blood flow are thus an obvious important consideration for transplantation proce-

dures. Because alpha and beta cells can exert paracrine effects (insulin inhibits alpha and beta cells and glucagon can stimulate beta cells) (59), it is clear that disruption of normal islet structure or blood flow due to transplantation procedures could significantly impact islet cell function, potentially more so in rats than in dogs or humans. In fact, a recent article postulated that isolated rat islets fail to initiate a glucagon response to hypoglycemia because blood flow disruptions in the isolated islets result in an absence of the typical intra-islet insulin reduction that normally signals the alpha cell response (60). Finally, it must be remembered that disruption of the ordinarily rich islet innervation, which affects both insulin and glucagon release (for a detailed review see (61,62)), could also affect alpha or beta cell secretion following transplantation.

Because the liver may not represent the optimal transplantation site (11,49), our studies focused on islet auto-transplantation to either the spleen or omental pouch. Previous studies in auto-transplanted dogs revealed that the spleen is an optimal site for islet survival (49) and reversal of diabetes (63). It also allows for portal delivery of insulin and glucagon, and provides more effective long-term function than transplantation to the liver (64,65). The omentum has the added advantages of being easily accessible and can accommodate a large tissue volume (21,66). We previously compared islet auto-transplantation of highly purified islets into the spleen versus omental pouch of dogs (21). The islet mass required for normoglycemia was ~2.5-fold greater and the insulin levels were significantly lower in the dogs with omental transplants (21). Although the present studies tend to support these results, the higher glucose and lower insulin levels in the omental group were not statistically significant. Also, Kin et al. have recently shown that the omentum is a better site for the transplantation of rat islets than the kidney capsule (66). Although the insulin response to arginine stimulation was not different between the control and transplanted groups, in the present study insulin did not rise in the omental group ($\Delta\text{AUC} = 9 \pm 29 \mu\text{U}/\text{mL} \times 40 \text{ min}$) but increased robustly in the splenic group ($\Delta\text{AUC} = 228 \pm 62 \mu\text{U}/\text{mL} \times 40$; $p < 0.05$). The glucagon response to arginine was mathematically but not statistically ($p = 0.35$) greater in the splenic group ($\Delta\text{AUC} = 51 \pm 62 \text{ pg}/\text{mL} \times 40 \text{ min}$) compared to the omental group ($\Delta\text{AUC} = -105 \pm 135 \text{ pg}/\text{mL} \times 40 \text{ min}$). The above data suggest that the splenic site may be preferable in terms of controlling daily blood sugar and in terms of islet cell secretion although the power of our study does not permit definitive conclusions on this point. On the other hand the counterregulatory response to mild hypoglycemia was not altered by the transplantation etc.

The fact that all dogs with splenic islet transplants were male and all dogs with omental transplants were female may confound the transplantation site comparison. Unfortunately only one of the four control dogs that underwent the arginine test was female, making statistical comparisons problematic. However, although estradiol directly

inhibits the alpha cell response to arginine, and may explain the tendency for decreased glucagon in the omental group, it has no direct effect on arginine-stimulated insulin release (67). Furthermore, estrogens indirectly increase arginine-stimulated insulin secretion in women (68), but insulin only rose in our male dogs. The difference between groups during the arginine stimulation test thus appears to be site, not gender, related. We know of no evidence to suggest that gender differences could have caused the non-significant baseline variation in the groups. Moreover, because both transplantation groups exhibited similar overall glucose homeostasis and hypoglycemic counterregulation, the gender difference may be irrelevant.

In conclusion beta cells responded normally to mild non-insulin-induced hypoglycemia after islet auto-transplantation to either the spleen or omentum but alpha cells did not. The reason glucagon did not rise is unclear, but could be related to a hypoglycemia-specific alpha cell defect, unrecognized nocturnal hypoglycemia, improper reinnervation, improper revascularization, or the increase in non-oxidative glucose metabolism characteristic of transplanted islets. The mild non-insulin-induced hypoglycemia used in this study represents decreases in plasma glucose such as would occur after fasting and exercise. The failure of glucagon to rise under such circumstances following islet transplantation may put islet transplant recipients at risk for mild hypoglycemic episodes associated with food deprivation or exercise.

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