Pancreatic Response to Mild Non–Insulin-Induced Hypoglycemia Does Not Involve Extrinsic Neural Input

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Mild non–insulin-induced hypoglycemia achieved by administration of a glycogen phosphorylase inhibitor results in increased glucagon and decreased insulin secretion in conscious dogs. Our aim was to determine whether the response of the endocrine pancreas to this mild hypoglycemia can occur in the absence of neural input to the pancreas. Seven dogs underwent surgical pancreatic denervation (PDN [study group]), and seven dogs underwent sham denervation (control [CON] group). Each study consisted of a 100-min equilibration period, a 40-min control period, and a 180-min test period. At the start of the test period, Bay R3401 (10 mg/kg), a glycogen phosphorylase inhibitor, was administered orally. Arterial plasma glucose (mmol/l) fell to a similar minimum in CON (5.0 ± 0.1) and PDN (4.9 ± 0.3). Arterial plasma insulin also fell to similar minima in both groups (CON, 20 ± 6 pmol/l; PDN, 14 ± 5 pmol/l). Arterial plasma glucagon rose to a similar maximum in CON (73 ± 8 ng/l) and PDN (72 ± 9 ng/l). Insulin and glucagon secretion data support these plasma hormone results, and there were no significant differences in the responses in CON and PDN for any parameter. Pancreatic norepinephrine content in PDN was only 4% of that in CON, confirming successful sympathetic denervation. Pancreatic polypeptide levels tended to increase in CON and decrease in PDN in response to mild hypoglycemia, indicative of parasympathetic denervation. It thus can be concluded that the responses of α- and β-cells to mild non–insulin-induced hypoglycemia can occur in the absence of extrinsic neural input. *Diabetes* 50:2487–2496, 2001

The counterregulatory response to insulin-induced hypoglycemia in vivo consists of an increase in glucagon secretion and a decrease in insulin release, in addition to changes in the plasma levels of other hormones and metabolites. However, insulin is known to inhibit its own release and to reduce glucagon secretion. Therefore, it has been difficult to separate the effects of hypoglycemia per se from those of combined hyperinsulinemia and hypoglycemia on insulin and glucagon secretion.

A recent study made use of a glycogen phosphorylase inhibitor to create mild hypoglycemia in the conscious dog by reducing glucose production, thereby avoiding the confounding effects of hyperinsulinemia (1). In that study, a change in glycemia of only 0.6 mmol/l (5.8 ± 0.2 to 5.2 ± 0.3 mmol/l by 30 min) resulted in a significant fall in plasma insulin and a significant rise in plasma glucagon. A glucose level of 5.2 mmol/l is much higher than the traditional threshold of 3.8 mmol/l generally thought to be required for the α-cell response to insulin-induced hypoglycemia in humans (2) and dogs (3). It thus seems that the α-cell is more sensitive to a decline in glucose than previously thought. The β-cell, however, has already been shown to respond to small changes in glucose during insulin-induced hypoglycemia. Specifically, plasma c-peptide levels, an index of insulin secretion, fell as glucose decreased from 5.1 to 4.9 or from 4.7 to 4.4 mmol/l (4.5). Our previous data obtained using the glycogen phosphorylase inhibitor showed that the β-cell also responds sensitively to hypoglycemia in the absence of exogenous insulin administration (1).

The exact mechanism by which insulin secretion is controlled in response to hypoglycemia is controversial. Although insulin secretion in vivo seems to be very sensitive to small changes in glucose, work in isolated rodent islets has shown that insulin secretion does not change when the glucose concentration falls from ~4.5 to 0 mmol/l (6,7). This could mean that insulin secretion had already been maximally inhibited at a glucose level in the 4–5 mmol/l range. This is conceivable, especially because the threshold for a decrease in insulin secretion in rodent islets is most likely higher than for canine islets, as a result of the increased fasting plasma glucose level in rodents. However, this failure of a small fall in glucose to inhibit insulin could also mean that some factor, such as an intact nervous system or an intact circulatory system, allows for the sensitive control of the β-cell in vivo and that this control is lost in vitro. In support of neural regulation, epinephrine (released from the adrenal medulla in response to sympathetic nerve stimulation) and norepinephrine (the classical sympathetic nervous system [SNS] neurotransmitter) both have been shown to increase during hypoglycemia and to inhibit insulin secretion both in vitro and in vivo (8–10). Additional evidence to suggest that the SNS can control β-cell insulin release is that direct stimulation of sympathetic nerves results in an inhibition

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ANOVA, analysis of variance; BOHB, β-hydroxybutyrate; CON, control group; CV, coefficient of variation; HPLC, high-pressure liquid chromatography; NEFA, nonesterified fatty acids; PDN, pancreatic denervation study group; PNS, parasympathetic nervous system; PP, pancreatic polypeptide; RIA, radioimmunoassay; SNS, sympathetic nervous system; SPDV, superior pancreaticoduodenal vein.
of insulin secretion (11). In addition to the SNS, the parasympathetic nervous system (PNS) is activated during hypoglycemia, as evidenced by an increase in pancreatic polypeptide (PP) release (8). However, parasympathetic stimulation increases insulin secretion, which goes against the role for the PNS in the counterregulatory response of the β-cell to hypoglycemia (8,12).

The increase in plasma glucagon seen in response to hypoglycemia also may be mediated, in part, by the nervous system. In support of neural regulation, epinephrine is elevated and the SNS and PNS are activated during hypoglycemia, and all are capable of stimulating glucagon secretion in vivo (11,13). Epinephrine stimulation increases insulin secretion, which goes against hypoglycemia, as evidenced by an increase in pancreatic parasympathetic nervous system (PNS) is activated during hypoglycemia. In support of neural regulation, epinephrine is stimulated by preganglionic acetylcholine release. Samples were drawn twice during the insulin infusion period and twice during the insulin and atrazine infusion period. After completion of an experiment, the animal was killed and tissue samples from three areas of the pancreas were clamped with precooled tongs, frozen in liquid nitrogen, and stored at −70°C for the later measurement of norepinephrine. In addition, samples from each of the seven lobes of the liver were taken from two CON and two PDN dogs for analysis of liver norepinephrine content. This was done to ensure that the pancreatic denervation had not affected liver innervation.

Analytical procedures. One milliliter of the collected blood was placed in a tube that contained 20 μl of 0.2 mol/l glutathione (Sigma Chemical) and 1.8 mg of EGTA (Sigma) for catecholamine measurement, and the remaining blood was placed in a tube that contained potassium EDTA (1.6 mg of EDTA/ml; Sigma, Munich, Germany). After gentle mixing, a blood sample with EDTA was pipetted into a tube that contained 5 ml of 4% perchloric acid, which was used later for metabolite measurement. The catecholamine, whole blood, and metabolite tubes were centrifuged at 3,000 rpm for 7 min. Supernatants from these tubes were placed in an ice bath during the experiment and then stored at −70°C until the assays were run. Plasma from the catecholamine tubes was used to determine epinephrine and norepinephrine levels by high-pressure liquid chromatography (HPLC) with isotope dilution techniques of variation (CVs) of 7 and 6%, respectively (23). The deproteinized supernatants from the metabolite tubes were used for whole-blood measurements of lactate, alanine, β-hydroxybutyrate (BOHB), and glycerol according to the method of Lloyd et al. (24) adapted to the Monarch 2000 centrifugal analyzer (Monarch, Lexington, MA). Enzymes and coenzymes for these metabolic assays were purchased from Sigma Chemical and Boehringer-Mannheim Biochemicals (Mannheim, Germany).

The plasma samples were used for all other measurements. Glucose concentrations were immediately determined from four 10-μl aliquots of plasma using the glucose oxidase method with a glucose analyzer (Beckman Instruments, Fullerton, CA). Immunoactive plasma insulin was measured using a double-antibody radioimmunoassay (RIA) with an interassay CV of 7% (Linco Research, St. Charles, MO). Immunoactive plasma glucagon and plasma PP (to each of which 500 KIU/ml Trasylol was added; FBA Pharmaceu- ticals, New York, NY) were also measured using a double-antibody RIA with interassay CVs of 7 and 9%, respectively. C-peptide (in plasma to which 500 KIU/ml Trasylol had been added) was measured at the University of Chicago (Department of Medicine, Section of Endocrinology) by an RIA. Plasma cortisol was measured using a γ-coat RIA (Diagnostic Products, Los Angeles, CA) with an interassay CV of 8%. Neutrophil fatty acids (NEFAs) were determined with an enzymatic colorimetric assay (Wako NEFA C test kit; Wako Chemicals, Richmond, VA) on a Monarch 2000 centrifugal analyzer, with an interassay CV of 3%.

Frozen tissue samples were ground into powder, and 5 mmol/l glutathione.
RESULTS

Glucose and hormone levels. Basal plasma glucose (Fig. 1) was similar in both groups (6.04 ± 0.13 mmol/l in CON, 5.93 ± 0.15 mmol/l in PDN) and reached minima of 5.00 ± 0.11 and 4.92 ± 0.28 mmol/l, respectively (P < 0.05 for both groups). Arterial plasma insulin (Fig. 2) fell from 59 ± 9 pmol/l to a minimum of 20 ± 6 pmol/l in CON and from 45 ± 6 pmol/l to a minimum of 14 ± 5 pmol/l in PDN (P < 0.05 for both groups). The fall in portal plasma insulin mirrored the fall in arterial plasma insulin. The portal-arterial insulin gradient (Fig. 3) decreased from 105 ± 34 pmol/l to a minimum of 17 ± 7 pmol/l in CON and 73 ± 23 to a minimum of 8 ± 2 pmol/l in PDN (P < 0.05 for both groups). Plasma C-peptide levels, an index of insulin secretion, were consistent with the insulin data and fell significantly upon administration of the drug. There was no significant difference between CON and PDN in glucose, insulin, or C-peptide levels.

Arterial plasma glucagon (Fig. 4) reached a similar maximum in CON and PDN (54 ± 5 to 73 ± 8 ng/l; 43 ± 4 to 72 ± 9 ng/l, respectively; P < 0.05 for both groups). The rise in portal plasma glucagon mirrored the rise in arterial plasma glucagon (P < 0.05). The portal-arterial glucagon gradient increased from 12 ± 5 to 35 ± 19 ng/l in CON and from 10 ± 2 to 35 ± 12 ng/l in PDN (P < 0.05). Cortisol, epinephrine, and norepinephrine levels remained unchanged throughout the experiment in both CON and PDN.
There were no significant differences between CON and PDN for any of these parameters.

Metabolite measurements. Arterial blood lactate and alanine levels fell significantly with time in response to the mild hypoglycemia ($P < 0.05$) (Fig. 5). There were no significant differences between CON and PDN with regard to lactate or alanine at any time. Arterial blood glycerol, BOHB, and arterial plasma NEFA levels all increased in response to mild hypoglycemia (Fig. 6). The rise in the glycerol level in CON approached significance ($P = 0.051$), whereas all other changes were significant ($P < 0.05$). There was no significant difference between CON and PDN with regard to glycerol, NEFA, and BOHB at any time, although there was a nonsignificant trend for PDN BOHB levels to increase more than CON BOHB levels.

Denervation assessment. Pancreatic norepinephrine levels were measured in freeze-clamped pancreas at the end of the study to assess sympathetic denervation ($n = 6$ per group). Tissue norepinephrine in PDN ($21,547 \pm 11,273$ pg/g tissue) was found to be only 4% of CON ($495,779 \pm 68,016$ pg/g tissue), suggesting a 96% denervation. Liver norepinephrine levels were also measured in two CON and two PDN dogs to ensure that the pancreatic denervation procedure had not decreased liver innervation. The data confirmed that the hepatic norepinephrine content in the PDN dogs was not reduced.

Parasympathetic denervation was assessed by measuring arterial plasma PP levels. During the control period, the PP values in CON were lower than those in PDN ($24 \pm 4$ and $43 \pm 10$ pmol/l, respectively), yet this did not reach statistical significance. Although there was a trend toward a small increase in PP in CON and a trend toward a small decrease in PDN, these changes were not significantly different from the respective basal periods. However, these changes from basal in the two groups were significantly different from each other ($P < 0.05$) (Fig. 7A), indicative of decreased parasympathetic activity in PDN compared with CON.

For further activation of the PNS and thereby more thorough testing of the extent of parasympathetic denervation, insulin was infused peripherally at the end of the experimental period for 90 min in three CON and four PDN dogs to create deep hypoglycemia ($\sim 2.7$ mmol/l). After 45 min of deep hypoglycemia, blood was sampled, then atropine was infused for another 45 min. Because atropine blocks muscarinic acetylcholine receptors, the PP re-
TABLE 1
Arterial plasma epinephrine, norepinephrine, and cortisol in the control period (−40 to 0 min) and after administration of the glycogen phosphorylase inhibitor (0 to 180 min) to create hypoglycemia (∼5.0 mmol/l) in 18-h fasted conscious dogs

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>−40</th>
<th>−20</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>120</th>
<th>150</th>
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<tr>
<td>Epinephrine (pmol/l)</td>
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<tr>
<td>CON</td>
<td>717 ± 240</td>
<td>1,188 ± 209</td>
<td>1,140 ± 235</td>
<td>901 ± 202</td>
<td>1,151 ± 180</td>
<td>1,499 ± 462</td>
<td>1,463 ± 224</td>
<td>1,206 ± 326</td>
<td>1,063 ± 266</td>
<td>1,417 ± 153</td>
<td>1,162 ± 272</td>
<td>1,634 ± 505</td>
<td>1,483 ± 470</td>
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<tr>
<td>PDN</td>
<td>625 ± 180</td>
<td>905 ± 165</td>
<td>858 ± 123</td>
<td>669 ± 207</td>
<td>806 ± 205</td>
<td>581 ± 157</td>
<td>1,070 ± 317</td>
<td>687 ± 135</td>
<td>929 ± 182</td>
<td>733 ± 173</td>
<td>916 ± 304</td>
<td>763 ± 203</td>
<td>848 ± 228</td>
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<td>Norepinephrine (nmol/l)</td>
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<tr>
<td>CON</td>
<td>0.89 ± 0.20</td>
<td>1.35 ± 0.12</td>
<td>1.13 ± 0.18</td>
<td>1.20 ± 0.22</td>
<td>1.48 ± 0.33</td>
<td>1.30 ± 0.07</td>
<td>1.40 ± 0.28</td>
<td>1.09 ± 0.15</td>
<td>1.09 ± 0.16</td>
<td>1.21 ± 0.14</td>
<td>1.15 ± 0.13</td>
<td>1.25 ± 0.13</td>
<td>1.24 ± 0.23</td>
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<tr>
<td>PDN</td>
<td>0.90 ± 0.20</td>
<td>0.99 ± 0.10</td>
<td>1.00 ± 0.10</td>
<td>0.95 ± 0.12</td>
<td>1.03 ± 0.12</td>
<td>0.96 ± 0.08</td>
<td>1.15 ± 0.13</td>
<td>1.07 ± 0.11</td>
<td>1.27 ± 0.15</td>
<td>0.97 ± 0.13</td>
<td>1.08 ± 0.09</td>
<td>1.31 ± 0.28</td>
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<td>Cortisol (nmol/l)</td>
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<tr>
<td>CON</td>
<td>90 ± 30</td>
<td>101 ± 37</td>
<td>88 ± 37</td>
<td>98 ± 47</td>
<td>95 ± 46</td>
<td>103 ± 46</td>
<td>92 ± 38</td>
<td>90 ± 38</td>
<td>81 ± 33</td>
<td>85 ± 24</td>
<td>94 ± 20</td>
<td>74 ± 18</td>
<td>75 ± 25</td>
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<tr>
<td>PDN</td>
<td>48 ± 10</td>
<td>62 ± 22</td>
<td>60 ± 16</td>
<td>54 ± 9</td>
<td>56 ± 11</td>
<td>56 ± 14</td>
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<td>77 ± 7</td>
<td>78 ± 10</td>
<td>66 ± 18</td>
<td>47 ± 17</td>
<td>49 ± 15</td>
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Data are means ± SE; n = 7 for CON and PDN. Statistical comparisons were made by two-way ANOVA with repeated-measures design. There were no significant differences between groups or within groups for any of these hormones.

DISCUSSION
There was a significant rise in the plasma glucagon levels and a significant fall in the plasma glucose levels by 120 min after atropine and insulin administration was initiated during PNS stimulation and a non–insulin-induced hypoglycemia was performed with use of the univariate test when appropriate. *F < 0.05 versus basal; n = 4 for CON and n = 7 for PDN.

**FIG. 5.** Arterial blood alanine (μmol/l) and after administration of the glycogen phosphorylase inhibitor (0 to 180 min) to create hypoglycemia (∼5.0 mmol/l) in 18-h fasted conscious dogs. Data are expressed as mean ± SE. Statistical comparisons were made by two-way ANOVA with repeated-measures design, and post hoc analysis was performed with use of the univariate test when appropriate. *F < 0.05 versus basal; n = 4 for CON and n = 7 for PDN.
The arterial and portal plasma insulin levels decreased by ~70% in both groups, whereas the arterial and portal plasma glucagon levels increased by ~50% in both groups. The changes that occurred in insulin and glucagon levels were paralleled by changes in insulin and glucagon secretion. Insulin secretion decreased to a similar minimum in both groups (from 1.76 ± 0.50 to 0.34 ± 0.12 pmol · kg⁻¹ · min⁻¹ and from 1.04 ± 0.16 to 0.17 ± 0.03 pmol · kg⁻¹ · min⁻¹ in CON and PDN, respectively). The plasma c-peptide level fell by ~60% in both groups, thus confirming the fall in insulin secretion. Glucagon secretion increased to a similar maximum in both groups (from 0.25 ± 0.08 to 0.68 ± 0.23 ng · kg⁻¹ · min⁻¹ and from 0.16 ± 0.03 to 0.53 ± 0.17 ng · kg⁻¹ · min⁻¹ in CON and PDN, respectively). It should be noted that the P-A glucagon gradient and glucagon secretion increased ~threefold, whereas the arterial and portal glucagon levels increased only ~50%.

The majority of this discrepancy can be accounted for by the fact that the plasma glucagon values include ~15–20 ng/l of cross-reacting, nonglucagon material. The level of this cross reactant is equivalent and unchanging in all vessels, thereby providing a constant background in the artery and the portal vein. If the cross-reacting material is subtracted from the portal glucagon values, then the rise in portal glucagon is ~2.5-fold, a change similar to that seen with the secretion data.

Evidence exists to support the hypothesis that the SNS plays a role in regulating β-cell secretion during hypoglycemia, including the fact that epinephrine, norepinephrine, and sympathetic activation can inhibit insulin release (8–11), but clearly other factors are also involved. For instance, glucose itself is known to affect β-cell secretion. In vitro, however, insulin secretion does not change when the glucose concentration falls from ~4.5 to 0 mmol/l (6,7). This would be explained if the nervous system were involved in decreasing insulin secretion in vivo or if insulin secretion were already maximally inhibited at a glucose level of 4.5 mmol/l. Indeed, the latter explanation seems very likely, because by the time arterial plasma glucose had reached 5 mmol/l in our studies, insulin secretion was markedly reduced.

Evidence also exists to support the hypothesis that the nervous system influences α-cell secretion, including the fact that epinephrine, SNS stimulation, and PNS stimulation all increase glucagon release (11,13–15). It is clear, however, that other factors control glucagon secretion as well, including glucose and insulin levels. Insulin was shown recently to inhibit in vitro glucagon secretion, most likely by activating phosphatidylinositol 3-kinase in the α-cell (27). Also, because blood is known to travel from the β-cells to the α-cells, it is possible that β-cell products can influence the α-cell (28). In that regard, a study that involved retrograde perfusion of the pancreas suggested that the α-cell cannot be directly suppressed by glucose in the absence of insulin (29). It therefore remains to be determined whether it was the fall in insulin, another β-cell product, or the actual hypoglycemia that caused the increase in glucagon secretion seen in the present study.

In vivo data regarding the effects of the nervous system on α-cell secretion are seemingly discordant. It has been shown in humans that total pharmacological blockade of the autonomic nervous system does not affect glucagon secretion in response to insulin-induced hypoglycemia (30). In addition, when pancreas transplantsations were performed on patients with type 1 diabetes (who thus lack an intact extrinsic PNS), the glucagon response to hypoglycemia was significantly greater than that in nontransplanted patients with diabetes and was similar to the response in control subjects (31,32). Furthermore, it has been shown that pancreatic islet autotransplantation into the peritoneal cavity of dogs, after total pancreatectomy, results in a glucagon response to hypoglycemia that is not different from the control dogs (33). These data are in contrast with other in vivo studies that showed that the nervous system controls α-cell release (16–19,34). For instance, infusion of glucose to the head to create cerebral euglycemia at a time when the periphery was hypoglycemic resulted in an inhibition of the glucagon response by >75% (34). Another study showed that insulin-induced hypoglycemia in conscious rats elicited an increase in glucagon with or without pharmacological blockade of either muscarinic or adrenergic receptors (17). However, when both muscarinic and adrenergic receptors were blocked simultaneously, the glucagon response to the
insulin-induced hypoglycemia was significantly lower than in the other groups (17). This led to the proposal that the discrepant results between investigators is because the parasympathetic and sympathoadrenal nervous systems act redundantly in controlling glucagon release (17). Another possible explanation for the discrepant results is that the experimental designs (anesthetized versus conscious animals; insulin-induced versus non-insulin-induced hypoglycemia) and methods used (pharmacological blockade versus mechanical blockade) were different among groups. Finally, the studies involving pancreas transplantations are difficult to interpret correctly, as the individuals have not only a new pancreas with normal islets but also an endogenous pancreas with functional α-cells. Because of this, it is impossible to determine whether the transplanted or endogenous α-cells are responding to the hypoglycemia.

Substrate measurements. The blood concentrations of lactate and alanine, both of which are gluconeogetic precursors, fell similarly in CON and PDN dogs. Because glycogenolysis was inhibited by drug administration, hepatic glycogenogenesis would be expected to increase in an attempt to maintain euglycemia. The liver therefore would be expected to take up more gluconeogetic substrates, causing arterial lactate and alanine levels to fall. In support of this, we showed previously that administration of a glycogen phosphorylase inhibitor does indeed enhance gluconeogetic precursor uptake by the liver (35).

In contrast, glycerol rose during the study in CON and PDN dogs. This suggests increased lipolysis, which is supported by the fact that the plasma NEFA level also rose. Increased lipolysis was expected because glucose and insulin, both inhibitors of lipolysis, fell after drug administration (36,37). It was shown previously that the increase in lipolysis was the result of the hypoglycemia, not a side effect of the drug, because maintaining euglycemia prevented lipolysis from increasing (1). The rise in ketone production, represented by increased BOHB levels, most likely was due to increased NEFA uptake by the liver, resulting from increased plasma NEFA levels.

It is of interest to consider the possible role of the changes in substrate levels in altering insulin and glucagon secretion. NEFA and ketone concentrations increased and amino acids (represented in this study by alanine) decreased in our study. NEFA and ketones stimulate insulin secretion; thus, changes in their levels cannot explain the fall in insulin levels (7). Amino acids also stimulate insulin secretion, such that a decline in their levels could have increased insulin secretion (7). However, it seems unlikely that this is the explanation for our data, considering that the blood alanine level did not fall significantly until long after insulin and glucose had fallen. Furthermore, any effect of the fall in alanine on insulin secretion most likely would have been counteracted by the changes in the levels of ketones and NEFA. Changes in blood substrate levels are also unlikely to have caused the rise in plasma glucagon levels, as glucagon secretion is stimulated by amino acids, which fell during the study, and inhibited by NEFA and ketones, which rose during the study (7,38). These substrate changes should, if anything, have opposed the rise in glucagon seen in response to hypoglycemia.

Denervation assessment. The pancreatic SNS was successfully denervated. The pancreatic norepinephrine content in PDN was only 4% that in CON, suggesting a virtually complete sympathetic denervation. However, the extent of parasympathetic denervation was less clear. Because acetylcholine, the classic parasympathetic neurotransmitter, cannot be easily measured directly, an indirect method to assess parasympathetic innervation had to be used.

PP is a 36-amino acid peptide secreted from pancreatic islet F-cells in response to parasympathetic stimulation (39). It thus provides an indirect measure of PNS activity and has been shown to increase in response to insulin-induced hypoglycemia in conscious dogs (21). Mild hypoglycemia in our studies caused an insignificant rise in plasma PP levels in CON and an insignificant fall in PP in PDN. The modest, nonsignificant rise in CON suggests little involvement of the PNS in the pancreatic response to mild
non–insulin-induced hypoglycemia. However, because the hypoglycemia-induced change in the PP level was small, it was difficult to determine the extent of parasympathetic denervation. For this reason, we modified the experimental design in the last seven experiments to include deep, insulin-induced hypoglycemia followed by atropine administration. The deep hypoglycemia was used to generate a large PP response, and the atropine was used to block the PNS (by blocking muscarinic acetylcholine receptors). The PP released during atropine infusion was not under PNS control and was therefore subtracted from the PP released during the deep hypoglycemia to obtain the PNS-controlled PP release. The average PP released under PNS control in the denervated group was 47% that of the control group, suggesting about a halving of the PNS response.

The question of why the PP response to deep hypoglycemia in the denervated group was larger than expected thus arises. One possibility is that the parasympathetic nerves were not completely severed during the denervation procedure. This is the most straightforward interpretation of the data, yet it seems surprising that almost half of the parasympathetic neural supply was left intact, considering that the entire pancreas was stripped of all surrounding tissues and the major blood vessels were skeletonized, and considering that SNS denervation was 90% complete.

Another possibility is that the PP released during the deep hypoglycemia, that was inhibited by atropine, is not specifically indicative of PNS activity. PP release is influenced not only by the PNS but also by short entero-pancreatic reflexes, intrinsic cholinergic activity of the pancreas, and hormones such as cholecystokinin (40). Other studies revealed that total extrinsic pancreatic denervation in dogs, achieved by autotransplantation, did not change the normal rise in PP levels in response to a meal (40–42), and atropine administration blocked this increase in PP (42). Those studies suggest that atropine was inhibiting something other than the PNS that controls PP release. A likely candidate is the intrinsic intrapancreatic cholinergic network, which is independent of the spinal cord and brain. It has been shown that axons of the intrapancreatic cholinergic nerves seem to terminate in islet tissue, suggesting that they influence islet hormone secretion (43).

Finally, it is always possible that some of the rise in PP that we observed after deep hypoglycemia was released from a site other than the pancreas. PP-immunoreactive cells have been found throughout the stomach, small and large intestines, and gastric mucosa of dogs (44,45); colon and rectum of rats (46); mucosa of colon and rectum of humans (47); and mucosa of antropyloric and duodenal regions of pigs (48). Furthermore, extrapancreatic islets, including PP-containing cells, have been found in the duodenal wall of the rat (49). Thus, at least part of the PP released in CON and PDN dogs may have been released from sites other than the pancreas, thereby causing an underestimation of pancreatic denervation.

Although the extent of parasympathetic denervation remains somewhat unclear according to the data obtained during the deep hypoglycemia and atropine infusion, other evidence from our studies indicates that the PNS is not important in the counterregulatory response. First, it should be noted that the two dogs with the smallest PP response to insulin-induced hypoglycemia, suggesting the best PNS denervation (80 and 83%), had insulin and glucagon profiles similar to the other denervated dogs. Furthermore, we performed one additional experiment in which we denervated the pancreas of a dog and performed the same experiment as previously described. However, in this instance, we also cooled the vagus nerves (by means of cooling coils placed around the vagus nerves in the neck 2 weeks earlier [50]) to ensure PNS inactivation. In this case, the magnitudes of the fall in insulin and the rise in glucagon were similar to CON and PDN dogs, despite the fact that arterial plasma glucose did not fall to quite the same extent as in the two larger groups of dogs (Table 2). Thus, cooling the vagus nerves in addition to extrinsic pancreatic denervation had no effect on the responses of insulin and glucagon levels to mild non–insulin-induced hypoglycemia, further indicating that the PNS does not play a role in counterregulation during mild hypoglycemia.

Taken together, our data suggest that the PNS is not important for the normal counterregulatory response to mild, non–insulin-induced hypoglycemia. First, the PP response to mild hypoglycemia was not significantly different from basal in either group, suggesting that the PNS was minimally involved in the response to mild hypoglycemia. Second, the dogs with the most complete PNS denervation had normal pancreatic responses to mild hypoglycemia, and vagal cooling did not change these responses. Our data strongly suggest, therefore, that the PNS is not responsible for the changes in insulin and glucagon secretion that we observed.

In conclusion, sympathetic denervation of the pancreas had no effect on pancreatic counterregulation in response to mild, non–insulin-induced hypoglycemia. Although the extent of parasympathetic denervation was difficult to assess, our data suggest that the PNS was not involved in the pancreatic counterregulation caused by mild, non–insulin-induced hypoglycemia. We conclude, therefore, that α- and β-cell secretion can change in response to mild,

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**TABLE 2**

Arterial plasma glucose, insulin, and glucagon in the control period (−40 to 0 min) and after administration of the glycogen phosphorylase inhibitor to create hypoglycemia (~5.0 mmol/l) during vagal cooling (0 to 180 min) in an 18-h fasted conscious dog with a denervated pancreas (n = 1)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>−40</th>
<th>−20</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>75</th>
<th>90</th>
<th>120</th>
<th>150</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.05</td>
<td>6.27</td>
<td>6.16</td>
<td>6.11</td>
<td>6.05</td>
<td>6.90</td>
<td>5.94</td>
<td>5.61</td>
<td>5.66</td>
<td>5.66</td>
<td>5.61</td>
<td>5.77</td>
<td>5.88</td>
</tr>
<tr>
<td>Insulin (pmol/l)</td>
<td>69.6</td>
<td>86.4</td>
<td>72.0</td>
<td>64.8</td>
<td>43.2</td>
<td>29.4</td>
<td>45.0</td>
<td>28.8</td>
<td>25.2</td>
<td>19.8</td>
<td>21.0</td>
<td>40.8</td>
<td>40.2</td>
</tr>
<tr>
<td>Glucagon (ng/l)</td>
<td>53.4</td>
<td>50.3</td>
<td>53.3</td>
<td>58.1</td>
<td>65.4</td>
<td>67.2</td>
<td>73.8</td>
<td>80.2</td>
<td>84.4</td>
<td>83.4</td>
<td>91.7</td>
<td>83.0</td>
<td>74.9</td>
</tr>
</tbody>
</table>

Statistical comparisons were made by one-way ANOVA with repeated-measures design.
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