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RESEARCH ARTICLE | *Translational Physiology*

Co-impairment of autonomic and glucagon responses to insulin-induced hypoglycemia in dogs with naturally occurring insulin-dependent diabetes mellitus

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[1]*Department of Veterinary Medicine and Epidemiology, University of California, Davis, California;* [2]*Department of Small Animal Clinical Sciences, University of Florida, Gainesville, Florida;* [3]*Department of Medicine, University of Washington, Seattle, Washington;* [4]*Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California; and* [5]*Department of Nutrition, School of Veterinary Medicine, University of California, Davis, California*

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Gilor C, Duesberg C, Elliott DA, Feldman EC, Mundinger TO, Taborsky GJ Jr, Nelson RW, Havel PJ. Co-impairment of autonomic and glucagon responses to insulin-induced hypoglycemia in dogs with naturally occurring insulin-dependent diabetes mellitus. *Am J Physiol Endocrinol Metab* 319: E1074–E1083, 2020. First published October 12, 2020; doi:10.1152/ajpendo.00379.2020.—This study aimed to investigate the contributions of two factors potentially impairing glucagon response to insulin-induced hypoglycemia (IIH) in insulin-deficient diabetes: *1*) loss of paracrine disinhibition by intra-islet insulin and *2*) defects in the activation of the autonomic inputs to the islet. Plasma glucagon responses during hyperinsulinemic-hypoglycemic clamps (≅40 mg/dL) were assessed in dogs with spontaneous diabetes (n = 13) and in healthy nondiabetic dogs (n = 6). Plasma C-peptide responses to intravenous glucagon were measured to assess endogenous insulin secretion. Plasma pancreatic polypeptide, epinephrine, and norepinephrine were measured as indices of parasympathetic and sympathoadrenal autonomic responses to IIH. In 8 of the 13 diabetic dogs, glucagon did not increase during IIH (diabetic nonresponder [DMN]; $\Delta = -6 \pm 12$ pg/mL). In five other diabetic dogs (diabetic responder [DMR]), glucagon responses ($\Delta = +26 \pm 12$) were within the range of nondiabetic control dogs ($\Delta = +27 \pm 16$ pg/mL). C-peptide responses to intravenous glucagon were absent in diabetic dogs. Activation of all three autonomic responses were impaired in DMN dogs but remained intact in DMR dogs. Each of the three autonomic responses to IIH was positively correlated with glucagon responses across the three groups. The study conclusions are as follows: *1*) Impairment of glucagon responses in DMN dogs is not due to generalized impairment of α-cell function. *2*) Loss of tonic inhibition of glucagon secretion by insulin is not sufficient to produce loss of the glucagon response; impairment of autonomic activation is also required. *3*) In dogs with major β-cell function loss, activation of the autonomic inputs is sufficient to mediate an intact glucagon response to IIH.

NEW & NOTEWORTHY In dogs with naturally occurring, insulin-dependent (C-peptide negative) diabetes mellitus, impairment of glucagon responses is not due to generalized impairment of α-cell function. Loss of tonic inhibition of glucagon secretion by insulin is not sufficient, by itself, to produce loss of the glucagon response. Rather, impaired activation of the parasympathetic and sympathoadrenal autonomic inputs to the pancreas is also required. Activation of the autonomic inputs to the pancreas is sufficient to mediate an intact glucagon response to insulin-induced hypoglycemia in dogs with naturally occurring diabetes mellitus. These results have important implications that include leading to a greater understanding and insight into the pathophysiology, prevention, and treatment of hypoglycemia during insulin treatment of diabetes in companion dogs and in human patients.

C-peptide; epinephrine; norepinephrine; pancreatic polypeptide; sympathoadrenal

INTRODUCTION

In nondiabetic animals and humans, the induction of hypoglycemia following the administration of exogenous insulin results in glucagon secretion from pancreatic α-cells, which then has a primary and critical role in the return of plasma glucose to euglycemia, that is, glucose counterregulation (17). Two major mechanisms have been proposed to mediate the increase of glucagon secretion in response to insulin-induced hypoglycemia (IIH). The first mechanism is release of the α-cell from the tonic inhibition by a paracrine effect of endogenous insulin within the islet. As plasma glucose concentrations fall below basal levels, endogenous insulin secretion is rapidly diminished until between ∼65 and 80 mg/dL it is completely shut off (16), releasing the α-cell from tonic restraint (3). The mechanism of this restraint was originally linked to activation of the insulin receptor on α-cells that is known to inhibit glucagon secretion (35). More recent studies suggest that the activity of islet β- and δ-cells is linked and that it is islet somatostatin that tonically inhibits glucagon secretion (54). Mimicking the disinhibition of glucagon secretion (33) or blocking it (42) can clearly influence glucagon secretion in nondiabetic animals and humans.

The second mechanism is stimulation of glucagon secretion by the activation of the autonomic nervous system inputs to the islet that occurs during hypoglycemia. The autonomic inputs to the islet include the parasympathetic nerves innervating the islet, the sympathetic nerves innervating the islet, and the sympathoadrenal neurohormone, epinephrine (EPI) (27). In dogs, the sympathetic neuropeptide galanin (11) may also contribute to the stimulation of glucagon secretion (9). Parasympathetic nerves release acetylcholine, activating the M3 muscarinic receptor subtype on α-cells to stimulate glucagon release (1). The parasympathetic neuropeptide, vasoactive intestinal polypeptide (VIP), also stimulates glucagon secretion, particularly at low glucose concentrations (23, 34). Both the adrenal medulla and sympathetic nerves release

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catecholamines that activate the β-2 adrenergic receptor on α-cells to stimulate glucagon secretion (1). Each of these autonomic inputs becomes activated at different levels of hypoglycemia (27, 53), implying that their relative contributions to the glucagon response vary with the level of hypoglycemia (53). In addition, each of the three autonomic inputs is capable of stimulating glucagon secretion (27) and can do so in a redundant manner (24, 26). Surgical ablation or pharmacological blockade of autonomic signaling of all three autonomic stimuli markedly reduces the glucagon response to IIH in nondiabetic animals (including dogs and nonhuman primates) and in humans (20, 28, 30).

In type 1 diabetes, there is a progressive impairment leading to a total loss of the glucagon response to IIH (6). Loss of paracrine disinhibition of the α-cell by intra-islet insulin has been invoked to explain the impairment of glucagon secretion in diabetes (42). Likewise, defects in the activation of the autonomic inputs to the endocrine pancreas have been proposed to contribute to the impairment of the glucagon response to IIH in type 1 diabetes (51). Indeed, there is evidence in support of both hypotheses. Infusing exogenous insulin to replace the endogenous insulin lost by the destruction of islet β-cells in type 1 diabetes and then switching it off during hypoglycemia appears to restore the glucagon response to hypoglycemia in diabetic animals (56). Furthermore, the impairment of autonomic activation resulting from prior hypoglycemia known as hypoglycemia-associated autonomic failure (HAAF) results in impairment of the glucagon response to subsequent hypoglycemia (32), and reversing this impairment partially to fully restores the glucagon response early in type 1 diabetes (8, 14). Therefore, the goal of this study was to investigate the potential contributions of two factors to the impairment of the glucagon response to IIH in insulin-deficient diabetes: *1*) loss of paracrine disinhibition by intra-islet insulin and *2*) defects in the activation of the autonomic inputs to the islet.

To investigate these mechanisms in animals with naturally occurring diabetes, we used client-owned dogs with insulin-dependent diabetes, studied at the University of California Davis, Veterinary Medical Teaching Hospital (VMTH). We hypothesized that glucagon responses during hypoglycemia would be impaired in dogs with naturally occurring diabetes and that activation of the three autonomic inputs would be correlated with glucagon responses to IIH. Plasma C-peptide responses were measured to assess residual endogenous insulin secretion, and pancreatic polypeptide (PP), norepinephrine (NE), and epinephrine (EPI) were measured to quantify activation of the autonomic nervous system inputs to the pancreas during IIH. Then, the C-peptide and autonomic responses were analyzed in relation to the glucagon responses to IIH. Hyperinsulinemic-hypoglycemic clamps (glucose ≅ 40 mg/dL) were performed in 13 diabetic dogs and in six healthy nondiabetic control dogs. Plasma pancreatic polypeptide (PP) responses were measured as an index of the activation of the parasympathetic nerves innervating the islet, epinephrine (EPI) responses as an index of adrenomedullary input to the islet, and norepinephrine (NE) as the index of the activation of sympathetic nerves. We also measured plasma cortisol as a nonautonomically mediated counterregulatory hormone response to IIH. Finally, we measured the autonomic and glucagon responses to ingestion of a meal to determine whether any impairments observed during IIH were specific for the stimulus of hypoglycemia.

MATERIALS AND METHODS

Diabetic and control dogs and hyperinsulinemic-hypoglycemic clamps. Thirteen client-owned dogs diagnosed with naturally occurring insulin-dependent diabetes and six healthy nondiabetic control dogs were studied at the University of California Davis VMTH. The experimental protocol was approved by the UC, Davis Animal Care and Use Committee, and owners provided consent to have their animals participate in the study. All diabetic dogs were insulin-dependent since the time of their diagnosis and still treated with twice-daily insulin injections (either NPH or Lente) at the time of enrollment into the study. In the afternoon before hypoglycemic clamp experiments, a double-lumen sampling catheter was introduced into the right jugular vein using local lidocaine anesthesia. Dogs were then fasted overnight. The following morning, insulin was withheld from the diabetic dogs. After collection of two blood samples for baseline measurements, a continuous infusion of regular porcine insulin (5 mU/kg/min, Squibb-Novo, Bagsvaerd, Denmark) was initiated into the distal port of the double-lumen catheter to lower plasma glucose to euglycemia (100 mg/dL) in the diabetic dogs. Plasma glucose (PG) concentrations were then subsequently allowed to decrease to 40–45 mg/dL over ~30 min in all dogs. Plasma glucose was measured every 5–15 min. Small blood samples (<0.5 mL) were collected from the proximal port of the catheter, plasma was separated within 30 s in a microfuge, and PG was measured with a Beckman II glucose analyzer (Fullerton, CA). Dextrose (50% solution) was infused at a variable rate as required to maintain PG as close as possible to 40 mg/dL. Once PG was stabilized below 50 mg/dL (time = ~30 min from when euglycemia was maintained in all animals), PG was clamped (at means ± SD = 41.4 ± 3.5 mg/dL) for 60 min with frequent measurements of PG and adjusting the rate of dextrose infusion as necessary. Samples were collected for hormone measurement at 15-min intervals. Insulin infusion was discontinued at 90 min; however, the dextrose infusion was continued as necessary to avoid hypoglycemia below 40 mg/dL. At the time that PG returned to euglycemia (~100 mg/dL), a meal stimulation test was conducted by feeding a standardized meal [10 ± 2 g/kg (amount consumed) of Hill's prescription diet W/D] at time zero. Plasma samples for glucose and hormone concentrations were collected at 2, 5, and 15 min after the initiation of feeding.

Intravenous glucagon tolerance tests for C-peptide responses. Intravenous glucagon tolerance tests were performed in a subset of the dogs (5 of the 6 controls and 10 of the 13 diabetic animals) on a separate day from the clamps. Briefly, a baseline blood sample was collected and then glucagon (Eli Lilly, Indianapolis, IN) was administered intravenously at a dose of 1 mg/dog. Blood samples were collected for C-peptide measurement every 5 min for 20 min.

Sample collection, handling, and analysis. All blood samples were collected from the proximal port of the double-lumen catheter, and the port was flushed with heparinized saline between each sample collection. Insulin and dextrose were infused only through the distal port. Blood samples for PG measurements were placed in heparinized tubes. Samples for plasma PP, glucagon, and C-peptide determinations were placed into tubes containing EDTA and aprotinin (Sigma). Blood samples for plasma catecholamine determination were placed into tubes containing EGTA and glutathione. All samples were kept on ice until centrifugation (2,500 rpm × 20 min at 4°C). Samples were stored at −80°C until assayed. All measurements were made in duplicate.

Plasma glucose concentrations were measured by the glucose oxidase method with a Beckman glucose analyzer (Beckman Instruments, Inc., Fullerton, CA). Plasma PP and glucagon immunoreactivity were measured by the Radioimmunoassay Core of the Diabetes Center at Washington University in St. Louis, MO, with specific radioimmunoassays with reagents from Linco Research (St. Louis, MO). The antibody for the plasma glucagon assay has a high specificity for the COOH-terminus of the glucagon molecule and is therefore specific for pancreatic glucagon (19). However, it also appears to detect a high-molecular-weight interference factor (likely GI glucagon) that remains constant

for individual animals and does not increase during hypoglycemia. We therefore have expressed the glucagon responses to IIH as the increment above basal (Δ) that effectively eliminates any contribution of this interference factor to the reported glucagon responses to IIH. The intra- and interassay coefficients of variation for the plasma glucagon assay are <10% and <11%, respectively. Plasma NE and EPI concentrations were measured with a highly sensitive and specific radioenzymatic assay (13) in Dr. Taborsky's laboratory (University of Washington, Seattle). The intra- and interassay coefficients of variation for the plasma catecholamine assay are <6% and <12%, respectively. Plasma cortisol was measured in Dr. Feldman's laboratory (University of California, Davis, CA) as previously described with intra- and interassay coefficients of variation of <7.3% and <10.5%, respectively (15). Plasma C-peptide concentrations were measured with an assay targeting canine C-peptide in the laboratory of Dr. Kenneth Polonsky (University of Chicago, IL) with intra- and interassay coefficients of <5% and <6%, respectively (40).

Statistical analysis. Normal distribution of data was assessed by the Shapiro–Wilk normality test. When the assumption of normality was rejected, the data were log transformed and then evaluated for normality again. When appropriate, parametric tests were used for comparisons and correlations, and the data are presented as means ± SD. For data that were not normally distributed, comparisons were performed with nonparametric tests and presented as median (range).

In control dogs, baseline euglycemia (EuG_{t1}) was defined as the mean of the −10- and 0-min time points. In diabetic dogs, insulin and glucose were infused for a variable amount of time until euglycemia was achieved, and EuG_{t1} was defined either as the measurement at the time of euglycemia or the mean of two time points closest to the time of euglycemia. Because PG at the 90-min time point was more variable and occasionally exceeded 50 mg/dL, the 90-min sample was excluded from calculation of hormone responses during IIH. The mean hormone concentrations within the 45-min time period during IIH (30, 45, 60, and 75 min) were used to calculate the difference between EuG_{t1} and IIH ([IIH] − [EuG_{t1}] = "IIH hormone response") in each dog. Diabetic dogs were divided into two groups based on their glucagon responses to IIH (Fig. 2): diabetic nonresponders (DMN; $N = 8$) that had glucagon IIH response below the range of control dogs and diabetic responders (DMR; $N = 5$) with glucagon IIH responses within the range or higher than those of control dogs. Absolute hormone concentrations at EuG_{t1} and IIH hormone responses were compared between controls, DMN, and DMR using ANOVA or Kruskal–Wallis test as appropriate. Post hoc multiple comparisons were performed and adjusted P values are reported. Pearson's r (for normally distributed data) or Spearman's rho (for data not normally distributed) correlations were calculated for responses of hormone pairs. Linear regression analysis was used to evaluate the relationships between changes of plasma PP, EPI, and NE responses with the change of glucagon responses. For NE and EPI, the linear regressions were performed after log transformation of the data.

For meal tests, EuG_{t2} was defined as the average of the last two samples before the animals were fed. Plasma glucose and hormone responses to feeding were defined as mean concentrations at 2, 5, and 15 min postfeeding-[EuG_{t2}].

All tests were two-tailed and $P < 0.05$ was considered significant.

RESULTS

We studied six healthy nondiabetic control dogs and 13 dogs with naturally occurring, insulin-dependent diabetes. Age, body weight, diabetes duration, and hemoglobin A1c concentrations are presented in Table 1 (differing superscripts across a row are significantly different).

Plasma C-peptide responses. As would be expected, baseline fasting plasma C-peptide concentrations were lower in the dogs with insulin-dependent diabetes than in the healthy control dogs ($P < 0.005$). All five of the six nondiabetic control dogs in which C-peptide status was assessed exhibited robust increases of plasma C-peptide concentrations in response to intravenous glucagon administration and to feeding. Plasma C-peptide concentrations increased from a baseline of 0.216 ± 0.041 pmol/L to 0.804 ± 0.124 pmol/ L at 10–20 min postinjection ($P < 0.0001$) with a percent change of +286 ± 19% ($P < 0.0001$) and area under the curve (AUC) of 9.276 ± 0.992 pmol/L/min ($P < 0.0001$). None of the diabetic dogs exhibited an increase of plasma C-peptide over baseline concentrations in response to intravenous glucagon (see Fig. 2). Plasma C-peptide responses to food ingestion were also measured in a subset of the dogs. In the nondiabetic control dogs, plasma C-peptide concentrations after feeding increased similarly to the response to glucagon (+197 ± 42%, $P < 0.005$), whereas C-peptide responses to feeding were absent in the diabetic dogs in which C-peptide was assessed (data not shown).

Table 1. *Median (range), age (yr), body weight (kg), disease duration (months prior to the study), and hemoglobin A1c (%) concentrations in 13 diabetic dogs [5 responders (DMR) and 8 nonresponders (DMN)] and 6 control dogs (differing superscripts across a row are significantly different)*

	Controls (n = 6)	DMN (n = 8)	DMR (n = 5)
Age, yr	5.3 (2.0–9.5)^{a}	10.0 (5.0–11.0)^{b}	6.5 (5.0–9.5)^{a,b}
Sex	FS = 3, MN = 3	FS = 3, MN = 5	FS = 4, MN = 1
BW, kg	33.5 (23.8–39.0)	13.0 (4.5–51.2)	36.8 (22.0–39.5)
DM duration, mo	N/A	6.0 (2–34)	8.0 (1–24)
HbA1c, %	3.0 (2.3–3.7)^{a}	6.7 (4.1–7.8)^{b}	5.8 (4.2–9.0)^{b}

DMN, diabetic nonresponders; DMR, diabetic responders; FS, female, spayed; MN, male, neutered. Values with different superscripts are significantly different ($P < 0.05$).

Plasma glucose and glucagon responses to IIH. To determine the glucagon response to IIH, a hyperinsulinemic-hypoglycemic clamp was performed in all 19 dogs. The clamped glucose level in the six nondiabetic dogs was not significantly different from that in the 13 diabetic dogs (39.5 ± 3.3 mg/dL vs. 42.3 ± 3.4 mg/dL, $P = 0.1$). Baseline plasma glucagon concentrations were not significantly different between the six nondiabetic dogs (102 ± 33 pg/mL) and the 13 diabetic dogs (110 ± 52 pg/mL, $P = 0.7$). During IIH, circulating plasma glucagon in the nondiabetic control dogs increased by +27 ± 16 pg/mL ($P = 0.009$; see below). In contrast, there was no significant increase of circulating plasma glucagon during IIH in the 13 diabetic dogs as a group (+6 ± 20 pg/mL, $P = 0.8$). Thus, the glucagon response to IIH in all 13 diabetic dogs combined was significantly lower than that in the six nondiabetic control dogs ($P = 0.04$).

However, closer examination of the glucagon responses in the 13 diabetic dogs revealed a bimodal distribution: eight diabetic dogs exhibited no discernible increase of glucagon during IIH, whereas five diabetic dogs had glucagon responses to IIH that were within the range of the nondiabetic control dogs (Fig. 1*A*). Therefore, the 13 diabetic dogs were subcategorized into diabetic responders (DMR; $n = 5$) and diabetic nonresponders (DMN; $n = 8$), based on their glucagon responses to IIH (Fig. 1, *A* and *B*). The average glucagon response to IIH in DMR dogs (+26 ± 12 pg/mL) was very similar to that in nondiabetic control dogs (+27 ± 16 pg/mL, $P = 1.0$). In contrast, the glucagon response to IIH in DMN dogs (−6 ± 12 pg/mL) was substantially and significantly lower than the glucagon responses to IIH in either nondiabetic control dogs ($P = 0.0008$) or DMR dogs ($P = 0.0017$).

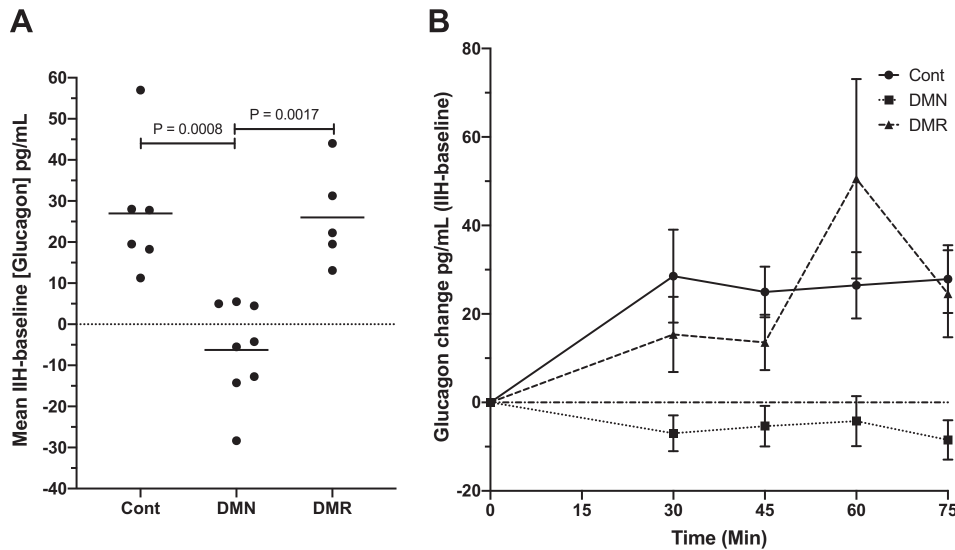

Fig. 1. Glucagon responses in 13 diabetic dogs [five responders (DMR, broken line, triangles) and eight nonresponders (DMN, dotted line, squares)] and six nondiabetic control dogs (solid line, circles). Insulin was infused at a constant rate until 90 min and dextrose was infused at a variable rate during that time to maintain plasma glucose at ∼40 mg/dL between 30 and 75 min. (*A*) plasma glucagon responses to insulin-induced hypoglycemia (IIH) (mean IIH baseline). Horizontal line within each group represents the median. (*B*) time course of Δglucagon (change compared with concentration at $t = 0$ min) (means ± SE). DMN, diabetic nonresponders; DMR, diabetic responders.

Fasting glucagon concentrations [at hyperglycemia for diabetics, plasma glucose (PG) = 269 ± 146, preclamp] were not significantly different in diabetic dogs at euglycemia compared with nondiabetic control dogs at euglycemia (PG = 128 ± 65 vs. 102 ± 33 pg/mL, respectively, $P = 0.4$), and there was no difference between DMN versus DMR ($P = 0.3$).

Plasma C-peptide and glucose responses in DMN versus DMR. There was no difference in fasting C-peptide concentrations or C-peptide responses to intravenous glucagon between the DMN and DMR dogs (Fig. 2; AUC = −0.02 ± 0.10 pmol/L/min and +0.04 ± 0.20 pmol/L/min, $P = 0.7$). Likewise, there was no significant difference in the degree of hypoglycemia achieved during IIH between the DMR dogs (41.5 ± 4.3 mg/dL) and the DMN dogs (42.8 ± 2.9 mg/dL, $P = 0.5$; Fig. 3).

Autonomic responses to IIH. To determine whether differences in the autonomic responses to IIH might be responsible for the differences in the glucagon responses between DMN and DMR dogs, we measured the PP, EPI, and NE responses to IIH.

Plasma pancreatic polypeptide responses to IIH. At baseline, there were no differences in plasma PP concentrations among the three groups ($P = 0.36$). The PP response to IIH in all diabetic dogs together was not different from that in nondiabetic control dogs ($P = 0.46$). However, median PP responses in DMN [+110 (range +23 to +630) pg/mL] were significantly lower than in DMR dogs [+1,120 (range +351 to +1,309) pg/mL, $P = 0.002$] and tended to be lower than in nondiabetic control dogs [+482 (range +310 to +841) pg/mL, $P = 0.1$; Fig. 4*A*]. Glucagon and PP responses to IIH were significantly correlated

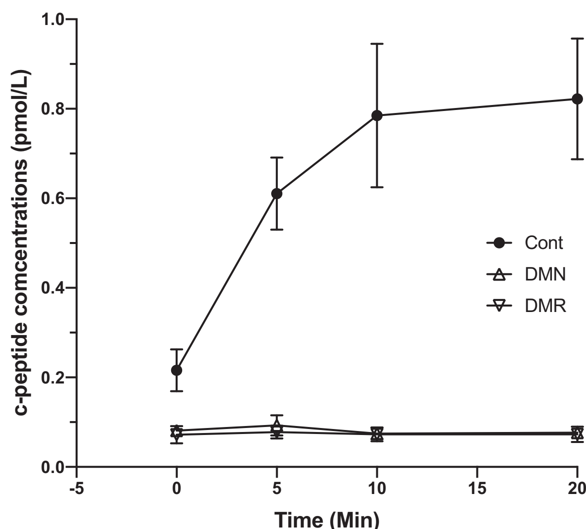

Fig. 2. Plasma C-peptide concentrations (mean ± SE) before and following intravenous administration of 1 mg of glucagon in nondiabetic. Cont, control dogs (); DMN, diabetic nonresponders; DMR, diabetic responders.

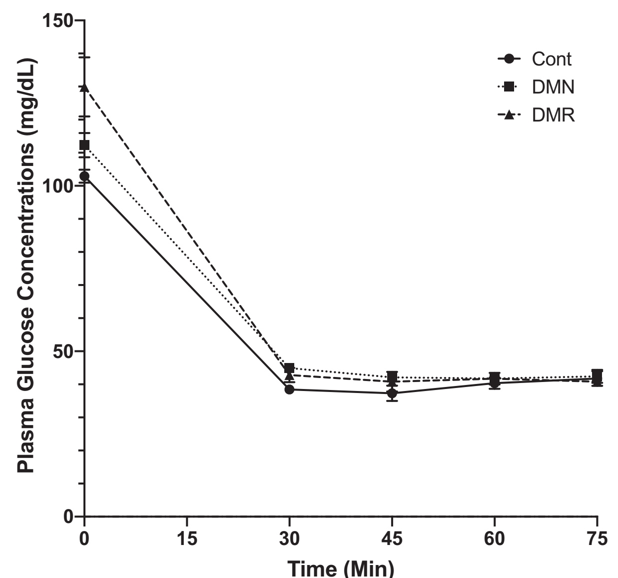

Fig. 3. Mean (±SE) plasma glucose concentrations in 13 diabetic dogs [five responders (DMR, broken line, triangles) and eight nonresponders (DMN, dotted line, squares)] and six nondiabetic control dogs (Cont, solid line, circles). Insulin was infused at a constant rate until 90 min and dextrose was infused at a variable rate during the clamps to maintain plasma glucose at ∼40 mg/dL between 30 and 75 min. DMN, diabetic nonresponders; DMR, diabetic responders.

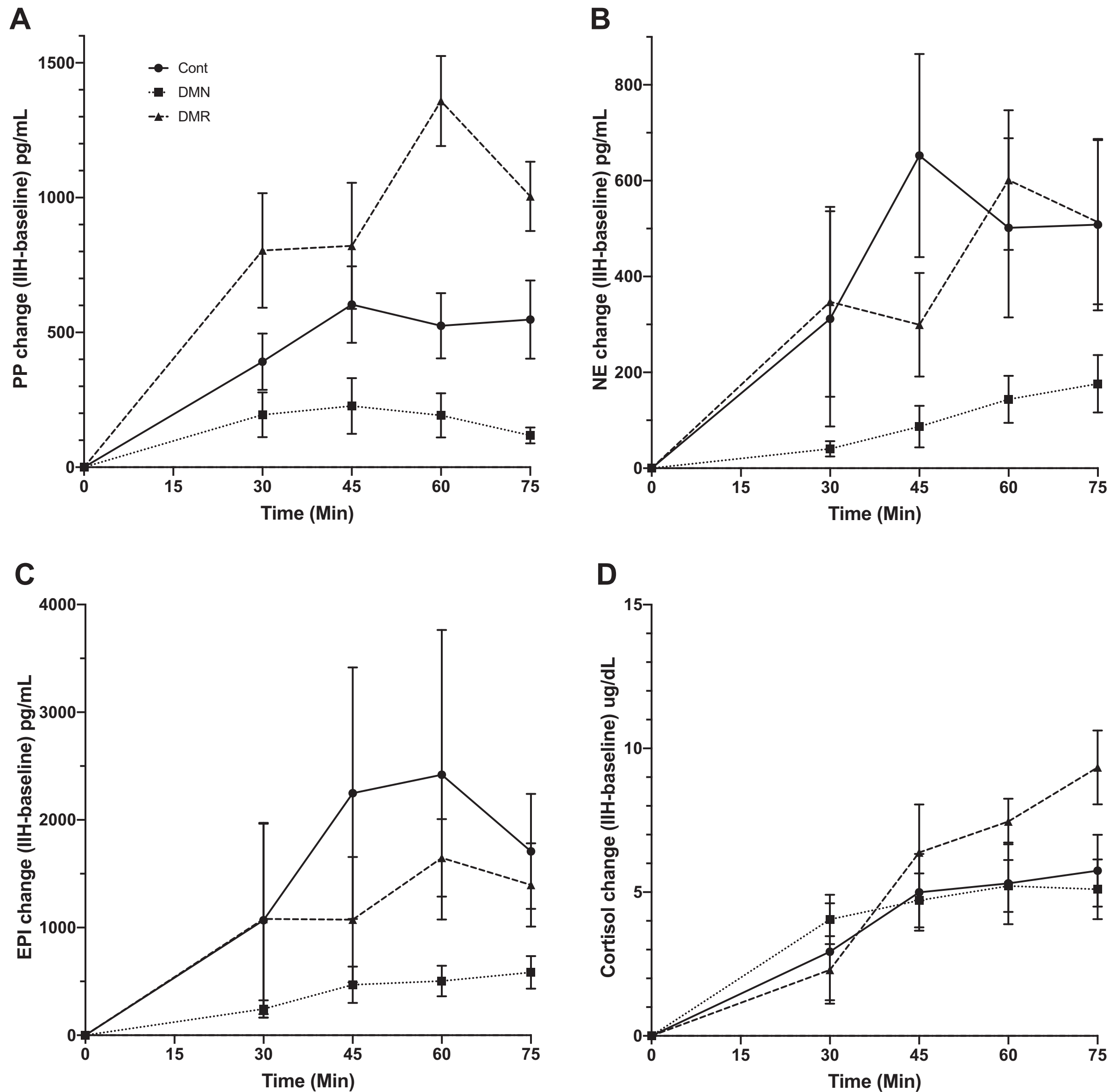

Fig. 4. Pancreatic polypeptide (*A*), norepinephrine (*B*), epinephrine (*C*), and cortisol (*D*) responses to insulin-induced hypoglycemia in 13 diabetic dogs [five responders (DMR, broken line, triangles) and eight nonresponders (DMN, dotted line, squares)] and six nondiabetic control dogs (Cont, solid line, circles). Insulin was infused at a constant rate until 90 min, and dextrose was infused at a variable rate during that time to maintain plasma glucose at ~40 mg/dL between 30 and 75 min. For each hormone, the changes (means ± SE) compared with concentration at t = 0 min) are presented over time. DMN, diabetic nonresponders; DMR, diabetic responders; EPI, epinephrine; NE, norepinephrine; PP, pancreatic polypeptide.

(r = +0.53, P = 0.02) across all three groups (Fig. 5*A*), with ~28% of the change of plasma glucagon concentrations attributable to the change of PP responses. The correlation between glucagon and PP responses to IIH was stronger when examined within the 13 diabetic dogs alone (DMN and DMR combined: r = +0.65, P = 0.016).

Plasma epinephrine and norepinephrine responses to IIH. Baseline plasma EPI concentration concentrations were not different among the three groups of dogs (P = 0.9). There was no significant difference in EPI responses to IIH between all diabetic and control dogs. However, the median EPI response to IIH in DMN dogs [+373 (range +80 to +1,095) pg/mL] was lower than the median EPI response in nondiabetic control dogs [+922 (range +445 to +6,604) pg/mL, P = 0.046, Fig. 4*C*]. The median EPI response in DMR dogs [+1,090 (range +373 to +3,088)] was not significantly different from that of nondiabetic control dogs (P = 0.1; Fig. 4*C*). There was a significant correlation between glucagon responses and LogEPI responses across all 3 groups (rho = 0.50, P = 0.026; Fig. 5*C*), with ~16% of the change of glucagon responses attributable to the change of LogEPI responses.

Median baseline plasma NE concentrations in DMN dogs [220 (range 130–300) pg/mL] were significantly higher than in nondiabetic control dogs [85 (range 60–160) pg/mL, P = 0.02] but not different from those in DMR dogs [180 (20–193) pg/mL, P = 0.99]. However, the median NE response to IIH (Fig. 4*B*) was significantly lower in DMN dogs (+108 [range −58 to +215 pg/mL) versus that in nondiabetic

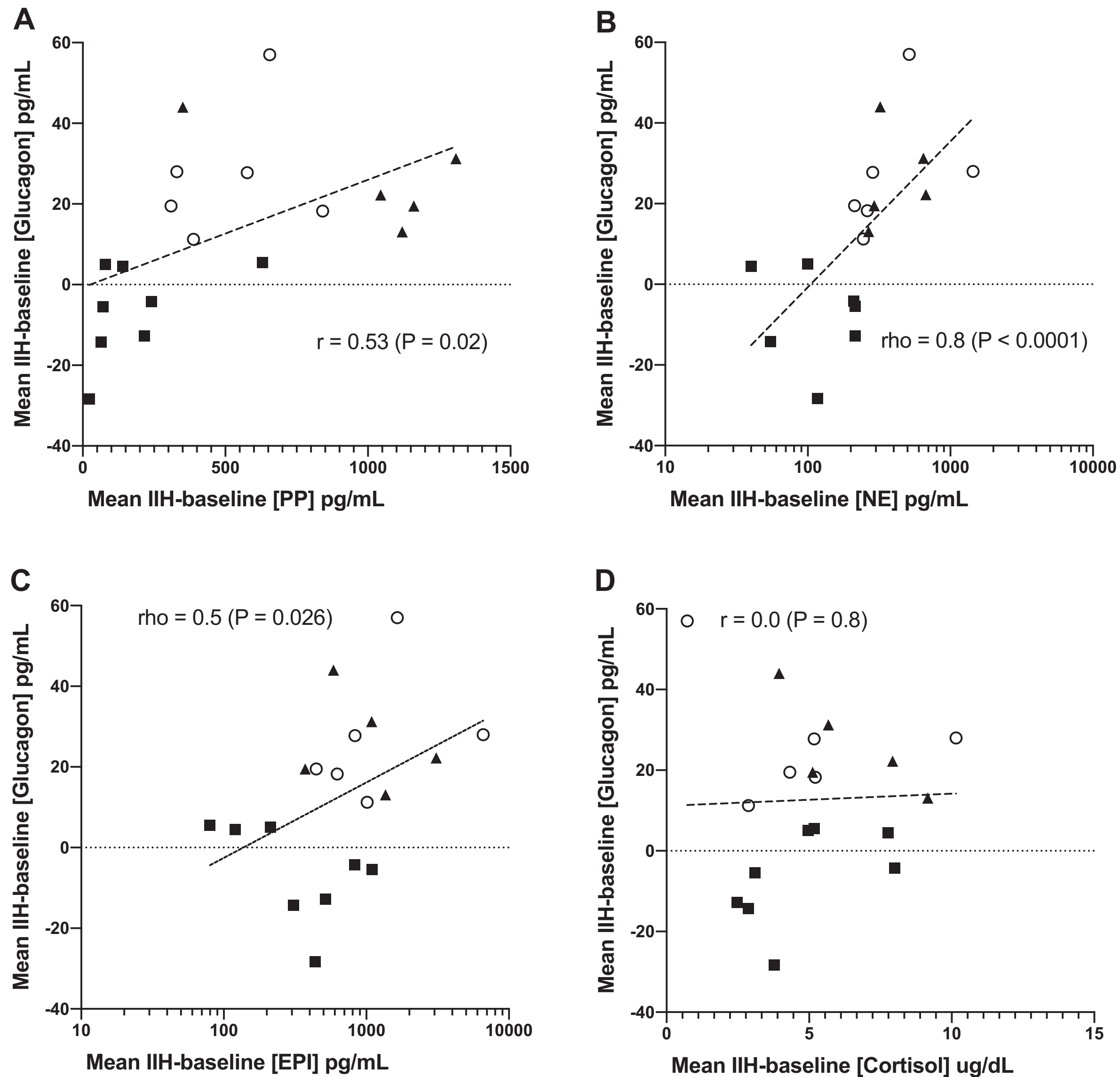

Fig. 5. Correlations between pancreatic polypeptide (*A*), norepinephrine (*B*), epinephrine (*C*), and cortisol (*D*) responses to insulin-induced hypoglycemia (IIH) with glucagon responses to IIH (Pearson's *r* or Spearman's rho when applicable) in 13 diabetic dogs [five responders (DMR, black triangles) and eight nonresponders (DMN), black squares] and six control dogs (open circles). Dashed line represents the best-fit regression line. EPI, epinephrine; NE, norepinephrine; PP, pancreatic polypeptide.

controls [+273 (range +212 to +1,443) pg/mL, $P = 0.01$] and in DMR [+322 (range +267 to +672) pg/mL, $P = 0.01$]. There was a strong correlation between glucagon responses and LogNE responses to IIH (rho = +0.80, $P < 0.0001$, Fig. 5*B*), with ∼40% of the change of glucagon responses attributable to the change of LogNE responses.

Plasma cortisol responses to IIH. Plasma cortisol concentrations at baseline [diabetes mellitus (DM): 1.9 ± 0.7 μg/dL, Cont: 1.7 ± 0.7 μg/dL] and cortisol responses to IIH (DM: +5.4 ± 2.2 μg/dL, Cont: +4.7 ± 3.1 μg/dL) did not differ significantly between groups, including between DMN and DMR (Fig. 4*D*; Table 2). There was no significant correlation between cortisol responses to IIH and glucagon responses to IIH (Fig. 5*D*).

Glucagon and autonomic responses to feeding. To determine whether the impairment in the autonomic responses to IIH observed in DMN dogs was specific for the stimulus of hypoglycemia, we measured glucagon and autonomic responses to feeding. Before the meal EuGt2, there were no significant differences in plasma glucose, glucagon, PP, NE, EPI, or cortisol concentrations between the three groups. In nondiabetic control dogs, plasma glucose, glucagon, and PP concentrations increased after feeding, whereas plasma NE, EPI, and cortisol concentrations tended to decrease (see Table 2). As would be expected, glucose tended to increase more in the diabetic dogs than in the nondiabetic control dogs. In contrast to the impaired PP and glucagon responses to IIH in DMN dogs, PP and glucagon responses to feeding in DMN dogs were not significantly lower than those in the nondiabetic controls dogs (Table 2). Changes of plasma glucagon and cortisol responses to feeding were not different across the three groups of dogs.

Table 2. *Changes of plasma glucose, glucagon, PP, NE, EPI, and cortisol concentrations after feeding (mean [hormone]$_{t2\text{-}t15}$ − EuG$_{t2}$) in 6 control dogs and 13 diabetic dogs [5 responders (DMR) and 8 nonresponders (DMN)]*

	Controls	DMN	DMR
Glucose, mg/dL	+10 ± 6	+29 ± 19	+23 ± 14
Glucagon, pg/mL	+27 ± 25	+25 ± 19	+31 ± 14
PP, pg/mL	+349 ± 118[a,b]	+190 ± 118[b]	+611 ± 330[a]
NE, pg/mL	−27 (−115 to +41)[b]	+81 (−17 to +135)[a]	+47 (−73 to +69)[a,b]
EPI, pg/mL	−184 ± 150[b]	−8 ± 68[a]	−89 ± 68[a,b]
Cortisol, µg/dL	−0.4 ± 0.7	0.3 ± 1.3	−1.1 ± 1.2

Normally distributed data are presented as means ± SD. Otherwise, data are presented as median (range). Values with different superscripts are significantly different ($P < 0.05$). DMN, diabetic nonresponders; DMR, diabetic responders; EPI, epinephrine; FS, female, spayed; MN, male, neutered; NE, norepinephrine; PP, pancreatic polypeptide.

DISCUSSION

The goal of this study was to gain insight into the contributions of two potential mechanisms underlying the defective glucagon response to hypoglycemia that is present in type 1 diabetes: *1*) the loss of intra-islet paracrine disinhibition of glucagon secretion from α-cells by endogenous insulin, and *2*) the impairment of stimulation by activation of the autonomic inputs to the islets. Canine diabetes is invariably associated with extreme β-cell deficiency and is generally considered type 1 (39, 50). Still, to evaluate the degree of endogenous insulin secretion remaining in dogs with naturally occurring, insulin-requiring diabetes, circulating C-peptide responses to intravenous glucagon administration (a potent stimulus for insulin and C-peptide secretion from β-cells) was assessed. We observed that none of the diabetic dogs had a measurable increase of plasma C-peptide in response to intravenous glucagon. These data indicate that in these animals, no further decrease of endogenous intra-islet insulin during hypoglycemia was possible, and therefore, these diabetic dogs had totally lost the ability for paracrine disinhibition of glucagon secretion during hypoglycemia. At first consideration, it might appear that this loss of disinhibition could likely be responsible for the observed impairment of glucagon response to IIH in the diabetic dogs studied. Indeed, in eight of the 13 diabetic dogs with an inability for disinhibition (i.e., C-peptide negative), there was no significant increase of glucagon in response to IIH (DMN). However, five of the 13 diabetic dogs studied exhibited glucagon responses to IIH within the range of the nondiabetic control dogs, and yet, these five animals were similarly C-peptide negative. Other investigators have also reported that there is no correlation between residual C-peptide responses to a meal and glucagon responses to IIH in patients with type 1 diabetes (49). Together, these data imply that loss of disinhibition of the α-cell is not sufficient, by itself, to produce the loss of the glucagon response to IIH at a glucose concentration of 40 mg/dL. The results therefore implicate other mechanisms, rather than disinhibition, that are involved in mediating the normal glucagon response to IIH observed in the DMR dogs and by extension the lack of a glucagon response in the DMN dogs.

Potential autonomic mechanisms that can stimulate glucagon secretion include acetylcholine and VIP released from parasympathetic nerves innervating the islet (23), the catecholamines, EPI released from the adrenal medulla, and NE and galanin (52) released from sympathetic nerves directly innervating the islet. In nondiabetic dogs, each of these autonomic inputs to the islet is known to be activated by hypoglycemia (27), and each is capable of stimulating glucagon secretion in vivo (27). Blocking the actions of all these autonomic agents to prove that the autonomic inputs to the islet mediate the glucagon response to hypoglycemia is difficult since both classical autonomic and neuropeptide antagonists would be required in the right combinations. However, since all three autonomic pathways (parasympathetic nerves, sympathetic nerves, and the adrenal medulla) involve neurotransmission via nicotinic receptors on either postganglionic neurons or entrochromaffin cells, the ganglionic antagonists hexamethonium in rodents (22) and dogs (30) and trimethaphan in nonhuman primates (28) and humans (20) have been used to demonstrate a marked reduction in the glucagon response to hypoglycemia, establishing the autonomic nervous system as an important mediator of this response.

Because it is not technically feasible to measure acetylcholine released from islet parasympathetic nerves in vivo, we used the secretion of pancreatic polypeptide (PP) released from F-cells in the pancreatic islet, which has been widely used as an alternative index of the activation of parasympathetic nerves that innervate the islets (46). The PP response to IIH is abolished by either ganglionic (41) or muscarinic (47) blockade, consistent with the neurotransmission via cholinergic nicotinic receptors known to be present in parasympathetic ganglia (27) and activation of cholinergic muscarinic receptors present on islet F-cells (27), as well as on islet α-cells (27). Activation of the parasympathetic nerves innervating the pancreas stimulates glucagon secretion (5) in a glucose-dependent manner such that there is a larger glucagon response to parasympathetic activation when circulating glucose concentrations are low. This glucose dependence is clearly illustrated by the glucagon response to the parasympathetic neuropeptide vasoactive intestinal polypeptide (23, 29). It is noteworthy then that DMR dogs had substantially larger PP responses to IIH than DMN dogs, consistent with greater activation of the parasympathetic nerves innervating the islet. Thus, it is likely that the greater islet parasympathetic activation measured during IIH in DMR dogs contributes to their intact glucagon response to IIH.

Diabetic dogs with normal EPI response to IIH also had a normal glucagon response to IIH (DMR). Thus, it appears that the larger EPI response seen in DMR dogs, in addition to being correlated with the glucagon response to IIH, contributes to the larger glucagon response measured in DMR dogs. However, interventional experiments (e.g., administration of adrenergic receptor antagonists during IIH in dogs with naturally occurring diabetes) would be required to provide additional support for this mechanism.

Finally, the plasma NE response to IIH is also larger in the DMR dogs than in DMN dogs, and the plasma NE response to IIH is highly correlated with the glucagon response to IIH across the three groups of dogs in the study. Although the plasma NE response to IIH is indicative of a greater activation of systemic sympathetic nerves in the DMR group, these data do not definitively demonstrate a greater activation of the sympathetic nerves innervating the pancreas per se in DMR versus DMN dogs because the spillover of NE from the pancreas makes a negligible contribution to the NE measured in systemic plasma (10). However, the sympathetic nerves of the pancreas are known to be activated by neuroglucopenia or hypoglycemia in

nondiabetic dogs (12, 29). This activation of pancreatic sympathetic nerves is specific for neuroglucopenia, because it does not occur during other types of physiological stress, that is, hypotension or hypoxia (29). During IIH, both catecholamines and the canine sympathetic neurotransmitter galanin (25) are released from pancreatic sympathetic nerves, and both NE and galanin stimulate glucagon secretion in dogs (27, 52). Therefore, if the islet sympathetic nerves are activated in proportion to the increase of systemic NE, then the DMR dogs are likely to have experienced a larger degree of sympathetic stimulation of glucagon during IIH than DMN dogs. Alternatively, the low basal C-peptide levels in both DMN and DMR groups suggest a major loss of β-cell function in all of the dogs with naturally occurring diabetes, and this β-cell loss has been shown to be associated with a marked loss of islet sympathetic nerves in a number of rodent models of autoimmune diabetes (38), as well as in human subjects with type 1 diabetes (37). Therefore, if both DMN and DMR dogs lack stimulation by islet sympathetic nerves during IIH, then the intact glucagon response seen only in DMR dogs can be attributed to the preservation, only in this one group, of the other two autonomic inputs to the islet, that is, the activation of parasympathetic nerves innervating the islet and circulating adrenal EPI. In support of this possibility for redundant autonomic activation of the α-cell during IIH, previous studies have demonstrated redundancy in the autonomic mediation of the glucagon response to IIH in rats (26) and, importantly, in dogs (24). Thus, all three branches of the autonomic nervous system must be blocked before an impaired glucagon response to IIH is revealed (51).

Autonomic dysfunction of both branches of the ANS is a common feature of diabetes in both humans and rodent models (53). It is multifactorial including both classical diabetic neuropathy and hypoglycemia-associated autonomic failure (HAAF). The diminished PP, NE, and EPI responses to IIH in DMN dogs suggest a generalized autonomic impairment in these dogs consistent with autonomic neuropathy or HAAF instigated by prior episodes of hypoglycemia in these insulin-treated dogs. Although the diabetic dogs in this study were not fitted with continuous glucose monitors, it is quite possible that some dogs experienced hypoglycemic events that were not detected before the study. Hypoglycemia was documented in the medical records in four of the five DMR and five of the eight DMN dogs in the days and weeks before enrolling in the study. Since prior hypoglycemia was documented in dogs with both intact (DMR) and impaired (DMN) glucagon responses to IIH, it appears unlikely that HAAF contributed to either the impairment of autonomic activation or the deficient glucagon responses to IIH observed in the DMN dogs studied here. In addition, the intact cortisol response to IIH in DMN dogs argues against HAAF as the major underlying cause of the autonomic impairment, since cortisol responses are also at least partially reduced in HAAF (36, 48). Nonetheless, the significant associations of all three indices of autonomic inputs with impaired glucagon secretion during IIH are clear and suggest that the impaired autonomic responses to IIH are highly likely to have an important contribution to the loss of the glucagon response to IIH in DMN dogs in this study. Furthermore, the marked PP, NE, and EPI responses to IIH in DMR dogs, coupled with intact glucagon responses to IIH, despite absent C-peptide responses, suggest that loss of disinhibition is insufficient to impair the glucagon response to IIH in dogs with naturally occurring, insulin-requiring diabetes.

Although the absence of a glucagon response to IIH in patients with type 1 diabetes could be explained by a combination of loss of disinhibition and impaired autonomic responses to IIH seen in this study, it is likely that other factors also contribute to the loss of the glucagon response to hypoglycemia in type 1 diabetes. For example, others have demonstrated apparently normal EPI and PP responses to IIH in patients with type 1 diabetes who have lost the glucagon response to IIH (2, 55). It is unlikely that a generalized impairment of the α-cell is involved because previous studies have demonstrated a normal glucagon response to intravenous administration of arginine in patients with long-standing type 1 diabetes (18). Likewise, we found a normal glucagon response to meal ingestion in DMN dogs that had lost the glucagon response to IIH. One additional factor may be that with increased duration of type 1 diabetes, there is an increased sensitivity of the α-cell to the inhibitory effect of exogenous insulin. Early studies had demonstrated that in patients with long-standing type 1 diabetes, the glucagon response to arginine is impaired during a hyperinsulinemic-euglycemic clamp in a dose-dependent fashion (3), supporting the idea that when endogenous insulin secretion is lost, exogenous insulin may also impair the glucagon response to hypoglycemia. We believe it is likely that during IIH, multiple mechanisms are engaged to stimulate glucagon secretion, and these are dependent on the degree/severity of hypoglycemia that is being studied. During mild hypoglycemia [PG ~55–65 mg/dL (as in most clinical studies in humans)], disinhibition from the paracrine effects of endogenous insulin is more likely to be involved. In contrast, when PG falls lower than this threshold at which endogenous insulin (and C-peptide) secretion is already absent, then the autonomic mediation of the glucagon response to IIH becomes predominate and takes over (e.g., when PG = 40 mg/dL, as in this study). Indeed, in studies in nondiabetic humans with stepped hypoglycemic clamps, when glucose is lowered to ~65–70 mg/dL, there is mild autonomic activation, as assessed by increases of plasma PP and EPI responses and a small increase of plasma glucagon. When glucose is allowed to fall further to 50–55 mg/dL, more marked PP and EPI responses are observed and plasma glucagon increases further (4, 44, 45). We reported even larger increases of PP, EPI, NE, and glucagon in humans during IIH of 45 mg/dL (20) than reported at the less marked levels of hypoglycemia (50–55 mg/dL). It should be noted that it is the more severe degrees of hypoglycemia that are most clinically relevant and lead to increasingly adverse outcomes, including seizures, accidents, and hospitalizations.

The small number of subjects in subgroups limited our ability to examine the effect of breed, body weight, and sex on hormonal responses. Although there is currently no evidence in previous literature that these factors lead to differences in counterregulation of hypoglycemia dogs, especially in dogs that are spayed/neutered, this should be examined in future studies.

In summary, we have previously demonstrated that activation of islet parasympathetic nerves, the adrenal medulla, and islet sympathetic nerves all contribute to the glucagon response to IIH in a number of species, including nondiabetic dogs (30) rhesus macaques (28), and humans (20). Others have provided evidence that disinhibition of α-cells by endogenous insulin contributes to the glucagon response to IIH in nondiabetic animals (33) and humans (42, 43). We have suggested that each of these factors can contribute at different levels of hypoglycemia to the

increasing stimulation of glucagon secretion as blood glucose levels continue to fall (53). Based on the results of the present study, we propose that loss of disinhibition and impaired activation of the autonomic inputs to the islet can both contribute to the loss of the glucagon response to mild and marked hypoglycemia, respectively, in type 1 diabetes; however, the role of the autonomic impairment is dominant during more clinically significant degrees of hypoglycemia.

CONCLUSIONS

Glucagon responses to insulin-induced hypoglycemia are impaired in a subset comprising over half of the dogs with naturally occurring diabetes mellitus that we studied. The animals with impaired glucagon responses also exhibit impairments of parasympathetic (PP), adrenal medullary (EPI), and sympathetic (NE) autonomic responses to IIH. In contrast, glucagon and PP responses to feeding in the diabetic dogs with impaired glucagon responses to IIH are similar to those in diabetic dogs with a normal glucagon response to IIH and to those in healthy nondiabetic dogs, indicating that islet α-cells and F-cells are intact and capable of secreting glucagon and PP, respectively. Impaired glucagon responses to IIH are therefore likely a consequence of impaired activation of the autonomic inputs to the islets. Dogs with impaired autonomic and consequentially defective glucagon response to IIH are at increased risk for episodes of severe hypoglycemia during insulin treatment. Therefore, new approaches are needed to prevent or reverse impairments of autonomic activation to restore the glucagon responses that serve as a first line of defense in the prevention and recovery from hypoglycemia during insulin treatment of diabetes in both canine and human patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.W.N. and P.J.H. conceived and designed research; C.D., D.A.E., E.C.F., R.W.N., and P.J.H. performed experiments; C.G., R.W.N., and P.J.H. analyzed data; C.G., T.O.M., G.J.T., R.W.N., and P.J.H. interpreted results of experiments; C.G. and P.J.H. prepared figures; C.G. drafted manuscript; C.G., E.C.F., T.O.M., G.J.T., and P.J.H. edited and revised manuscript; C.G., C.D., D.A.E., E.C.F., T.O.M., G.J.T., R.W.N., and P.J.H. approved final version of manuscript.

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