Increased Responses of Glucagon and Glucose Production to Hypoglycemia With Intraperitoneal Versus Subcutaneous Insulin Treatment

Calvin K.N. Wan, Adria Giacca, Munehide Matsuhisa, Ban El-Bahrani, Loretta Lam, Carol Rodgers, and Z. Qing Shi

The study aim was to investigate the effect of the route of insulin treatment on the glucagon and glucose production (GP) responses to hypoglycemia in the diabetic rat. Experiments were performed in 4 groups of rats: (1) streptozotocin (STZ)-induced diabetic, untreated (D, n = 7), (2) diabetic treated with subcutaneous insulin (DSC, n = 8), (3) diabetic treated with intraperitoneal insulin (DIP, n = 6), and (4) normal control (N, n = 10). Slow-release insulin implants were used in DSC and DIP rats for 10 to 14 days (3 U/d). A hyperinsulinemic (120 pmol · kg^{-1} · min^{-1} insulin)-hypoglycemic (glycemia = 2.5 ± 0.1 mmol/L) clamp following an isoglycemic basal period was performed in 5-hour fasted rats. Basal plasma glucose was normalized in both DSC and DIP rats; however, in DSC but not DIP rats, glucose normalization required peripheral hyperinsulinemia. Tracer-determined GP, which was elevated in D rats, was completely normalized in DIP but only partially corrected in DSC rats. Basal glucagon levels were similar in all groups. During hypoglycemia, GP was suppressed in D rats (Δ, −28.9 ± 5.0 μmol · kg^{-1} · min^{-1}), moderately increased in DSC rats (Δ, 6.1 ± 5.6, $P < .01$ *v* D), but markedly increased in DIP and N rats (Δ, 34.5 ± 4.5 for DIP and 16.8 ± 2.8 for N; $P < .01$ *v* D, $P < .05$ for DIP *v* DSC or N). Plasma glucagon increased 6-fold in N (945 ± 129 pg/mL), only doubled in D (424 ± 54), and tripled in DSC (588 ± 83), but increased 5-fold in DIP rats (1,031 ± 75, $P < .05$ *v* D and DSC). We conclude that in STZ-diabetic rats, (1) intraperitoneal but not subcutaneous insulin treatment normalizes basal GP, and (2) intraperitoneal insulin treatment as compared with subcutaneous treatment alleviates peripheral hyperinsulinemia and results in increased glucagon and GP responses to hypoglycemia.

HYPOGLYCEMIA is a frequent and potentially life-threatening complication in the treatment of type 1 diabetes mellitus,[1] especially under intensive insulin therapy.[2] Type 1 diabetes is associated with an impaired counterregulatory response to hypoglycemia, characterized by a decreased α-cell sensitivity to hypoglycemia[3] and a blunted glucagon release.[1,3] In addition, the increase in glucagon-mediated glucose production (GP) is impaired and delayed compared with normal individuals.[4] Impaired glucagon responses to hypoglycemia are also found in alloxan-diabetic dogs[5] and streptozotocin (STZ)-diabetic rats.[6] Recently, we found that the impaired glucagon response to hypoglycemia in the STZ diabetic rat was markedly improved by insulin-independent restoration of normoglycemia using phloridzin, which corrects hyperglycemia by increasing glycosuria, but was only minimally improved by restoration of euglycemia with subcutaneous insulin treatment.[6]

Subcutaneous insulin injection is the conventional means of insulin treatment. However, it appears to be associated with a high incidence of hypoglycemic episodes, especially with intensive insulin treatment. A number of studies have shown that intraperitoneal insulin treatment reduces the incidence of hypoglycemic episodes compared with intensive subcutaneous insulin treatment.[7-13] The mechanism for this beneficial effect is not clear. However, it has been shown that intraperitoneal insulin is almost entirely absorbed into the portal circulation[14] and results in more rapid and consistent absorption than peripheral injection.[15] This may protect against late postprandial hypoglycemic events.[10] In addition, in the depancreatized dog, we found that insulin infused peripherally produced a greater suppression of glucagon production and GP than equidose portally infused insulin.[16] It has been proposed that the suppression of GP by peripheral insulin infusion occurs via suppression of (1) free fatty acids (FFAs) and, possibly, gluconeogenic precursors; and (2) glucagon secretion.[16-18] It is likely that a relative peripheral hyperinsulinemia, frequently associated with subcutaneous (peripheral) insulin therapy as compared with the physiological portal route of insulin delivery, may exert an inhibitory effect on the counterregulatory mechanisms, especially glucagon secretion, during hypoglycemia.

We therefore hypothesized that the route of insulin administration is a significant determinant of the response of glucagon, and presumably also of GP, to hypoglycemia. In this study, we compared the effects of long-term insulin delivery via the intraperitoneal versus subcutaneous route on the counterregulatory responses to hypoglycemia in the STZ-induced diabetic rat.

MATERIALS AND METHODS

Animal Models

Male Sprague-Dawley rats (Charles River, Montreal, Canada) initially weighing 300 to 400 g were used for the experiments. All rats were fed with a standard rat chow (Ralston Purina, St Louis, MO) and water ad libitum. They were randomized into 4 groups: (1) normal control (N), (2) untreated diabetic control (D), (3) diabetic, treated with subcutaneous insulin (DSC) for 10 to 14 days, and (4) diabetic, treated with intraperitoneal insulin (DIP) for 10 to 14 days. Diabetes was induced in D, DSC, and DIP rats by a single intravenous injection of

From the Department of Physiology and Faculty of Physical and Health Education, University of Toronto, Toronto, Ontario, Canada; and First Department of Medicine, Osaka University Medical School, Osaka, Japan.

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C.K.N.W. and A.G. contributed equally to this study.

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Address reprint requests to Z. Qing Shi, PhD, Department of Pharmacology, Amgen, Mail Stop 15-2-A, One Amgen Center Drive, Thousand Oaks, CA 91320.

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STZ 65 mg/kg (Sigma Chemical, St Louis, MO) in saline solution under light ether anesthesia. The rats received 10% glucose in the drinking water for the first 24 hours after STZ injection. Venous glucose levels were subsequently monitored for 2 days for evidence of fasting hyperglycemia greater than 14 mmol/L. Rats with fasting glycemia less than 14 mmol/L were not used. D and N rats were left untreated for 10 to 14 days. Slow-release insulin implants (LinShin Canada, Scarborough, Canada) were implanted subcutaneously in DSC and intraperitoneally in DIP rats under ether anesthesia. The rationale and technical aspects of the insulin implant have been detailed elsewhere.[6,19] With the implantation of 1.5 full-size insulin implants in each DSC and DIP rat, the nominal rate of insulin release was 3 U/d. Fasting blood glucose levels were monitored every 2 to 3 days by the glucose oxidase method (Glucometer Elite; Bayer, Etobicoke, Canada). The mean fasting blood glucose during treatment was 7.4 ± 1.4 mmol/L in DIP and 6.8 ± 1.4 mmol/L in DSC (NS). Insulin-treated rats with fasting glucose less than 3.5 mmol/L (2 DIP and 2 DSC) or greater than 12 mmol/L (1 DIP and 1 DSC) were excluded from the study. The fed blood glucose levels were not measured in the present study; however, in identically treated rats used for a different study, they were 15.7 ± 5.5 mmol/L in DIP and 13 ± 1.6 (NS) in DSC.

Surgical Procedures

Two to 3 days before the experiment, the left carotid artery and right jugular vein were cannulated while the rats were under pentobarbital general anesthesia (50 mg/kg body weight intraperitoneally). Polyethylene catheters (length 10 cm, PE-50; Clay Adams, Boston, MA), each extended with a segment of silastic tubing (length 2.5 cm, ID 0.020 in, OD 0.037 in; Dow Corning, Midland, MI) for a flexible and noninjurious insertion, were used for the vascular catheterization. The PE-50 catheters were then tunneled subcutaneously and exteriorized, with the aid of a 16-gauge needle, from the back of the neck. All catheters were filled with a mixture solution of 60% polyvinyl pyrrolidone and heparin (1,000 U/mL) to maintain patency. On the day of the experiment, the catheter content was aspirated and the catheters were flushed with heparinized (10 U/mL) saline. The carotid catheter was used for arterial blood sampling and the jugular catheter for infusion of [3-^{3}H]-glucose, insulin, and glucose via serial T-shaped 23-gauge needle connectors.

Experimental Protocol

The rats were fasted 5 to 6 hours before the experiments. A hyperinsulinemic-hypoglycemic clamp was used to determine the counterregulatory response to hypoglycemia. To determine glucose turnover, constant [3-^{3}H]-glucose infusion at 0.07 $\mu Ci \cdot min^{-1}$ was started at −90 minutes immediately after an initial bolus priming injection of 3.2 μCi. The tracer was allowed to equilibrate for 45 minutes before the basal sampling period (P1, −45 to 0 minutes). Plasma glucose specific activity was maintained relatively stable between −45 and 0 minutes (within ± 14%), which suggests that a steady state was reached. Insulin infusion (120 $pmol \cdot kg^{-1} \cdot min^{-1}$) was initiated at 0 minutes to reduce plasma glucose to a hypoglycemic level of 2.5 ± 0.1 mmol/L. The time required for induction of hypoglycemia (plasma glucose <60 mg/dL) was variable among the groups (D, 121.5 ± 22.5 minutes; DSC, 65.0 ± 19.8 minutes; DIP, 44.8 ± 8.0 minutes; and N, 75.1 ± 12.5 minutes). The only difference was found between D and DIP rats ($P < .05$), whereas DSC and DIP rats were not different from N rats. A variable exogenous infusion of 25% glucose solution (Abbott Laboratories, Montreal, Canada) was adjusted at 7.5-minute intervals, according to instant plasma glucose measurements, to maintain stable hypoglycemia using a glucose clamp technique[20] modified for the hypoglycemic condition. Blood samples (100 μL) were drawn at −45, −30, −15, and 0 minutes during P1 and at 0, 15, 30, and 45 minutes during P2 with heparinized microtubes. After centrifugation, the plasma was transferred to microtubes containing a trace amount of sodium fluoride and stored at −20°C for assays of [3-^{3}H]-glucose and insulin. For glucagon, 250 μL blood was drawn at −30 and 0 minutes during P1 and 15 and 45 minutes during P2 in chilled tubes containing EDTA and Trasylol (FBA Pharmaceuticals, New York, NY). After removal of the plasma from centrifuged whole-blood samples, the packed blood cells were resuspended in heparinized saline (10 U/mL) to replace the plasma volume and infused back to the animal to minimize blood loss as described previously.[6] Hematocrit values at the end of the experiments were greater than 35%. To determine plasma catecholamine levels, an additional 100-μL blood sample was drawn at the end of P1 and P2, respectively, using chilled tubes containing glutathione and EGTA.

Laboratory Methods

Plasma glucose concentrations were measured by the glucose oxidase method using a Glucose Analyzer II (Beckman, Fullerton, CA). Insulin (kit from Linco, St Louis, MO) and glucagon[21] were analyzed by radioimmunoassay (coefficient of variation [CV] = 9% and 15%, respectively). FFA levels were measured by a fluorometric method.[22] To measure the radioactivity of [3-^{3}H]-glucose, plasma samples were deproteinized with zinc sulfate and barium hydroxide. The supernatant was evaporated at 70°C, redissolved in water, and counted in Ready Safe scintillation fluid (Beckman) by a liquid scintillation counter. Plasma concentrations of epinephrine and norepinephrine were measured by a radioenzymatic assay (CV = 15%).[23]

Calculations and Statistics

The rates of glucose appearance and disappearance (Rd) were calculated according to Steele's non–steady-state equations.[24] Endogenous GP was calculated as the difference between the tracer-derived rate of appearance and exogenous glucose infusion at each time point. Data were smoothed with the optimal-segments routine.[25] All data are presented as the mean ± SE. Differences between experimental groups were tested with 1-way ANOVA followed by Tukey's *t* test. Differences between experimental periods were analyzed by 2-way ANOVA.

RESULTS

Baseline Values (P1)

Ten to 14 days after the induction of diabetes, the mean body weight was lower in D versus N, DIP, or DSC rats (Table 1). Basal (P1) arterial glucose levels were significantly greater in D rats (20.8 ± 1.2 mmol/L, $P < .005$ *v* all other groups; Fig 1). Both DIP (7.9 ± 1.1 mmol/L) and DSC (6.8 ± 0.6 mmol/L) showed normalized plasma glucose levels (P = NS *v* N, 7.9 ± 0.1 mmol/L). Basal arterial insulin was diminished in D rats to 50% of the level in N rats, and it was elevated in DSC rats but was not significantly different in DIP and N rats. Basal glucagon levels during P1 were not different among all groups (DSC, 184 ± 16 pg/mL; DIP, 193 ± 21; D, 188 ± 24; and N, 143 ± 16; P = NS; Fig 2). FFA levels were highest in D and lowest in DSC rats (Table 1).

Plasma Glucose, Glucose Infusion Rate, and Arterial Insulin Concentration During Hypoglycemia (P2)

Arterial plasma glucose was reduced to similar levels by insulin infusion in all groups during P2, despite the marked difference in their basal values. Arterial glucose levels were maintained in the hypoglycemic range (N, 2.5 ± 0.1 mmol/L; D, 2.7 ± 0.1; DIP, 2.3 ± 0.1; and DSC, 2.5 ± 0.1; Fig 1). Glucose infusion rates during P2 were not significantly different among groups. Arterial insulin levels were similar in all groups

Table 1. Body Weight, Glucose Infusion Rate, GP, Glucose Rd, and Epinephrine, Norepinephrine, FFA, and Insulin Levels in the Four Groups

Parameter	D	N	DSC	DIP
Body weight (g)	315 ± 14	365 ± 13*	373 ± 15*	377 ± 21*
Ginf, P2 (μmol · kg^{-1} · min^{-1})	13.3 ± 3.3	15.6 ± 1.1	17.2 ± 1.1	17.2 ± 2.2
Basal GP (μmol · kg^{-1} · min^{-1}), P1	91.1 ± 5.6	50.0 ± 1.7*†	73.9 ± 4.4*	46.7 ± 3.3*†
ΔGP (P2)	−28.9 ± 5.0	16.8 ± 2.8*‡	6.1 ± 5.6*	34.5 ± 4.5*†
Basal Rd (μmol · kg^{-1} · min^{-1}), P1	92.3 ± 5.0	50.0 ± 1.7*†	79.0 ± 3.9*	52.8 ± 3.3*†
ΔRd (P2)	−20.6 ± 5.0	30.0 ± 2.2*‡	18.9 ± 5.6*	47.3 ± 3.9*†
Epinephrine (pmol/L)				
P1	169 ± 63	131 ± 55	324 ± 137	219 ± 75
P2	2,294 ± 361§	3,295 ± 901§	1,660 ± 411§	2,278 ± 324§
Norepinephrine (pmol/L)				
P1	132 ± 28	198 ± 46	156 ± 35	83 ± 8
P2	489 ± 161§	880 ± 165§‡	754 ± 335§	410 ± 98§
FFA (μEq/L)				
P1	1,011 ± 100	729 ± 47*†	461 ± 66*	602 ± 71*
P2	491 ± 45§	667 ± 50*†‡	305 ± 27*§	404 ± 67
Insulin (pmol/L)				
P1	114 ± 18	173 ± 21†	439 ± 43*‡	208 ± 28*†
P2	5,109 ± 388§	6,487 ± 412*§	5,738 ± 479§	5,943 ± 522§

Abbreviation: Ginf, glucose infusion rate.
*$P < .05$ *v* D.
†$P < .05$ *v* DSC.
‡$P < .05$ *v* DIP.
§$P < .05$ *v* P1.

(Table 1). FFAs decreased from basal in D and DSC, tended to decrease in DIP, but did not change significantly in N rats.

Glucagon Response to Hypoglycemia

The time course of changes in arterial glucagon is plotted in Fig 1. During hypoglycemia (P2), arterial glucagon increased 6-fold in N rats (945 ± 129 pg/mL), but the increase in D rats (424 ± 54 pg/mL, 2-fold basal values) was markedly blunted and delayed, similar to previous reports.[3,26] The glucagon response to hypoglycemia in diabetic animals was significantly but moderately improved by subcutaneous insulin treatment (DSC, 588 ± 83 pg/mL, $P < .05$ *v* D). Intraperitoneal insulin treatment (DIP) resulted in a 5-fold increment in plasma glucagon (1,031 ± 75 pg/mL, $P < .05$ *v* D and DSC), which was nearly 2-fold greater versus DSC rats and not different versus N rats (P = NS).

Tracer-Derived Systemic Glucose Turnover

Tracer-derived basal GP was elevated in D rats compared with N rats. The elevated basal GP was partially corrected in DSC rats but normalized in DIP rats. During hypoglycemia (P2), GP increased by 35% in N rats but was suppressed by 30% in D rats. DSC rats showed only a moderate increase (10%) and DIP rats a marked increase (70%, $P < .05$ *v* all groups) in GP (Table 1 and Fig 2). The basal glucose Rd was approximately equal to GP (steady state) and was therefore high in D rats (due to glycosuria) and lower in the euglycemic groups. In DSC rats, the basal Rd was higher versus DIP or N rats. During hypoglycemia, the Rd decreased from basal in D rats but increased in the euglycemic groups. In DSC rats, the increase from basal tended to be less versus N rats and was less versus DIP rats (Table 1).

Epinephrine and Norepinephrine Concentrations

There were no significant differences in the basal (P1) plasma levels of epinephrine or norepinephrine (Table 1). During hypoglycemia (P2), epinephrine increased to a similar extent in all groups. Norepinephrine levels were lower during hypoglycemia in DIP versus N rats ($P < .05$).

DISCUSSION

Glucagon plays a primary role in the counterregulation of glucose metabolism during hypoglycemia.[1,26] In type 1 diabetes, the glucagon response to hypoglycemia is impaired, presumably due to an intrinsic defect in the α cells of the pancreatic islets.[3] Our previous study has shown that the impairment in the glucagon response in the diabetic rat is markedly improved by an insulin-independent correction of hyperglycemia using phloridzin, but is only marginally improved by subcutaneous insulin treatment.[6]

In the present study, the impaired glucagon response to hypoglycemia in the diabetic rat was completely normalized by intraperitoneal insulin treatment for 10 to 14 days, while subcutaneous insulin treatment for the same duration only resulted in a moderate, although significant, improvement. The improvement in the glucagon response with both routes of insulin treatment could result from partial correction of chronic hyperglycemia (fed glucose levels are reduced but not normalized by continuous insulin treatment by either route), as well as normalization of glycemia just prior to the hypoglycemic challenge. Due to basal hyperglycemia, the time required to induce hypoglycemia in D rats was slightly longer than in the other groups, and we cannot exclude that the longer duration of insulin infusion in addition to basal and chronic hyperglycemia might have further reduced the glucagon response in the D group.

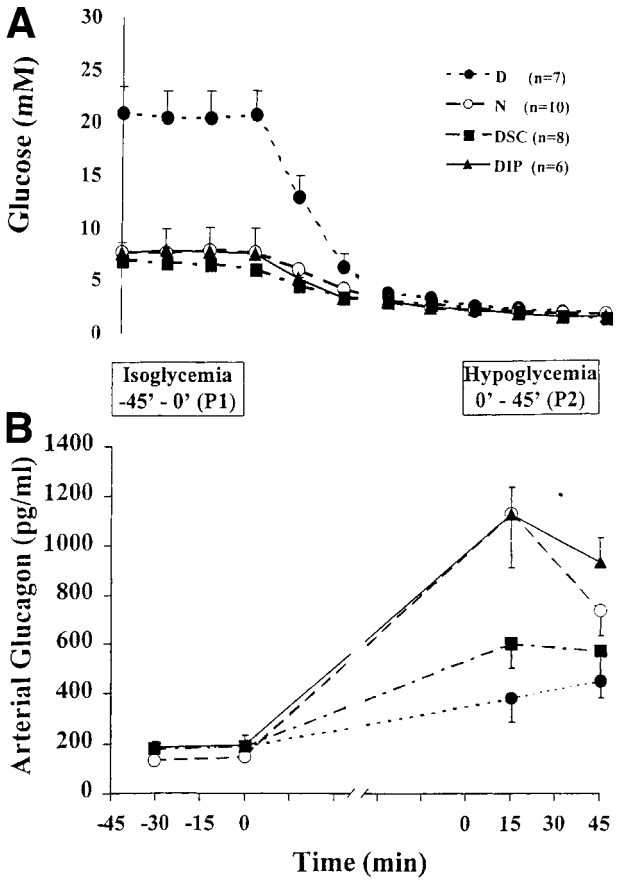

Fig 1. (A) Plasma glucose D, N, DSC, and DIP rats. Insulin infusion was initiated immediately after the basal isoglycemic period (P1) and was maintained constant throughout the experiments. Comparable hypoglycemia was attained in all groups during the hypoglycemic period (P2), despite the differences in basal plasma glucose. (B) Arterial glucagon. Glucagon levels were similar in P1 among all groups. In P2, glucagon levels in DIP and N rats were similar and were significantly higher *v* DSC and D rats ($P < .05$). Glucagon levels were higher in DSC *v* D ($P < .05$).

Glucose infusion rates were similar between DIP and DSC rats during the hypoglycemic clamp period, presumably because the greater increase in GP in DIP versus DSC occurred in the face of a greater increase in the Rd. Although insulin sensitivity was not measured with the gold-standard hyperinsulinemic-euglycemic clamp procedure, from the results of the hypoglycemic clamp, it would appear that DSC rats were insulin-resistant compared with DIP rats (lesser increase in Rd), presumably due to chronic peripheral hyperinsulinemia. Insulin resistance apparently offset the reduced GP response in DSC rats under our hypoglycemic clamp conditions, so the overall efficiency of counterregulation (measured by the glucose infusion rate) was the same in DIP and DSC rats. However, insulin resistance in DSC rats may not offset an impaired increase of GP under other conditions, eg, after bolus injection of insulin, when a rapid GP response is needed.

With both intraperitoneal and subcutaneous treatment, glucose levels were variable from rat to rat but not significantly different between DSC and DIP rats. The rats that showed fasting glucose less than 3.5 mmol/L were excluded from the study and were equally distributed between the 2 groups. Therefore, we believe it is unlikely that antecedent hypoglycemia[27] could be the cause of the impaired counterregulatory responses in DSC versus DIP rats.

Instead, we believe that the impaired counterregulatory response to hypoglycemia in the D group was only partially improved by correction of hyperglycemia in DSC rats, because of the nonphysiological (subcutaneous) route of insulin delivery which results in peripheral hyperinsulinemia. Insulin inhibits glucagon secretion and synthesis,[28,29] and the α-cell sensitivity to the inhibitory effect of insulin seems to be retained in diabetes.[30] Although basal glucagon levels were similar in DIP and DSC rats, the acute hypoglycemic challenge might have revealed an impairment in glucagon secretion and/or synthesis due to the excess basal peripheral insulin levels in DSC versus DIP. Previous studies have shown that the glucagon response to subsequent hypoglycemia can be blunted by prior hyperinsulin-

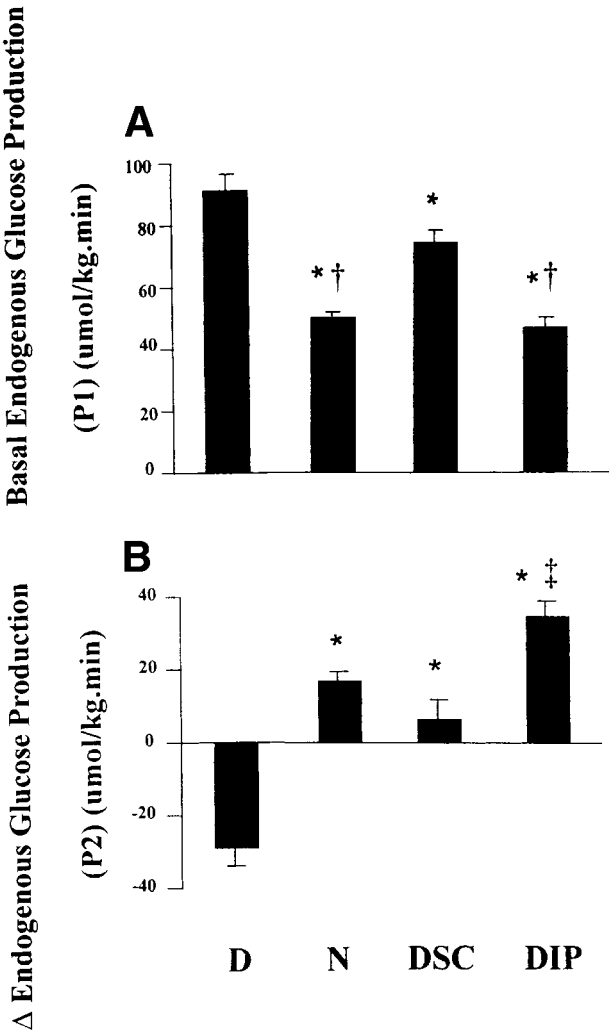

Fig 2. (A) GP in P1. Basal GP was elevated in D, intermediate in DSC, and normalized in DIP rats. (B) Incremental GP. Incremental GP was negative in D rats (GP was suppressed from basal values). In N, DIP, and DSC rats, GP during hypoglycemia increased. The increase in DIP rats was greater *v* DSC or N. *$P < .05$ *v* D, †$P < .05$ *v* DSC, ‡$P < .05$ *v* DSC or N.

emia.[31,32] However, to our knowledge, this is the first study to examine this question in the context of the route of insulin delivery.

Basal glucose levels were normalized by equidose subcutaneous or intraperitoneal treatment, despite the marked differences in basal peripheral insulin levels. This suggests that the presumably higher portal insulin levels with intraperitoneal insulin treatment had a suppressive effect on GP that offset the stimulatory effect on glucose utilization of the higher peripheral insulin levels with subcutaneous insulin. Basal GP was high in D rats. It was moderately improved by subcutaneous insulin treatment, but normalized only by intraperitoneal insulin treatment. This is in contrast to a number of recent studies evaluating the acute effect of equidose portal or peripheral insulin infusion on GP in dogs and humans, which show a greater suppression of GP with peripheral insulin,[33-35] mainly due to suppression of FFAs.[36] This observation may indicate that the direct inhibitory effect of insulin on the liver is more important for the long-term regulation of GP, whereas the indirect (peripheral) effect of insulin might be more important in the acute regulation of GP.

During hypoglycemia in normal controls, GP is stimulated by a robust glucagon secretory response that overrides the inhibitory effect of hyperinsulinemia. Long-term subcutaneous insulin treatment was associated with an attenuated increase in GP during hypoglycemia, attributable to an impaired increase in glucagon secretion. In DIP rats, glucagon values were similar to control levels and epinephrine values tended to be lower versus controls; however, the GP increment was corrected to an even greater level than normal. At present, we have no adequate explanation for this interesting finding. However, we speculate that a certain degree of hepatic insulin resistance in DIP rats may have potentiated the effects of counterregulatory hormones on GP. The possibility should also be considered that portal hyperinsulinemia in DIP rats might have increased hepatic glycogen levels. Fasting glycogen levels tended to be higher in DIP versus DSC or N rats in our studies in identically treated rats; however, the difference was not significant.[37]

An additional factor contributing to the greater increase of GP in DIP versus DSC rats is probably a greater hepatic uptake of gluconeogenic precursors. Compared with peripheral insulin delivery, equidose portal insulin delivery is associated with higher levels of circulating gluconeogenic precursors,[16] which is conducive to an increase in GP in response to acute metabolic challenges such as hypoglycemia. FFAs, which are also important for the increase in GP during hypoglycemia, were only marginally higher (NS) in DIP versus DSC rats.

The results of the present study provide an explanation for the inability of conventional or intensive subcutaneous insulin therapy to normalize the counterregulatory response of glucagon to hypoglycemia,[38,39] and perhaps also for the lower incidence of clinical hypoglycemia with intraperitoneal versus subcutaneous insulin delivery.[7-13]

In conclusion, intraperitoneal insulin treatment, as compared with subcutaneous treatment, improves basal GP, the impaired glucagon secretion, and hepatic GP in response to acute hypoglycemia in STZ-induced diabetes in the rat. The mechanism by which intraperitoneal insulin results in greater glucagon and GP responses to hypoglycemia may be related, in part, to the alleviation of peripheral hyperinsulinemia by the physiological route of insulin delivery. Therefore, alleviation of peripheral hyperinsulinemia may be beneficial not only in reducing the long-term complications of diabetes (insulin may be an atherogenic factor) but also in improving the hepatic counterregulation to hypoglycemia.

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