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Effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in diabetic rats

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Inouye, Karen, Kathy Shum, Owen Chan, Julian Mathoo, Stephen G. Matthews, and Mladen Vranic. Effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in diabetic rats. *Am J Physiol Endocrinol Metab* 282: E1369–E1379, 2002. First published April 8, 2002; 10.1152/ajpendo.00480.2001.—To understand the mechanisms whereby recurrent hypoglycemia increases the risk of subsequent hypoglycemia, it was necessary to differentiate the effects of recurrent hyperinsulinemia from those of hyperinsulinemic hypoglycemia. We examined basal and hypoglycemic endocrine function in normal rats, streptozotocin-diabetic controls, and diabetic rats exposed to 4 days of 2 episodes/day of hyperinsulinemic hypoglycemia (DH) or hyperinsulinemic hyperglycemia (DI). DH and DI rats differentiated the effects of hyperinsulinemia from those of hypoglycemia. In diabetic controls, basal plasma ACTH tended to be increased, and plasma corticosterone, plasma somatostatin, and pancreatic prosomatostatin and proglucagon mRNA were increased ($P < 0.05$) vs. normal rats. These parameters were normalized in DH and DI rats. In diabetic controls, glucagon, epinephrine, norepinephrine, corticosterone, and peak glucose production responses to hypoglycemia were reduced ($P < 0.05$) vs. normal rats. In DI rats, epinephrine responses were normalized. Conversely, DH rats displayed marked further impairment of epinephrine and glucose production responses and increased peripheral insulin sensitivity ($P < 0.05$ vs. diabetic controls). Both insulin regimens partially normalized glucagon and fully normalized norepinephrine and corticosterone responses. In summary, recurrent hyperinsulinemia in diabetic rats normalized most pituitary-adrenal, sympathoadrenal, and pancreatic parameters. However, concurrent hypoglycemia further impaired epinephrine and glucose production responses and increased insulin sensitivity. We conclude that *1*) recurrent hypoglycemia may increase the risk of subsequent hypoglycemia by increasing insulin sensitivity, and *2*) epinephrine counterregulation is particularly sensitive to impairment by recurrent hypoglycemia.

hypoglycemic glucose clamp; epinephrine; glucose production; insulin sensitivity

ANTECEDENT EXPOSURE to hypoglycemia is a primary underlying factor in the increased risk of hypoglycemia in type 1 diabetic patients undergoing intensive insulin therapy (23). In diabetes, antecedent hypoglycemia reduces neuroendocrine and autonomic responses to subsequent hypoglycemia (15, 16, 44, 46, 48, 49), decreases glycemic thresholds for counterregulatory responses (15), and causes hypoglycemia unawareness (44, 46). These defects increase the susceptibility of intensively treated patients to hypoglycemia (14). The mechanisms underlying impaired counterregulation are not well understood. Exposure to glucocorticoids during antecedent hypoglycemia (20, 21) and increased brain glucose uptake after antecedent hypoglycemia (5) may contribute to impaired counterregulation. Recurrent hypoglycemia per se may also increase the risk of hypoglycemia by increasing muscle insulin sensitivity (45).

Data in dogs, nondiabetic humans, and type 1 diabetic patients suggest that insulin per se may influence counterregulatory responses to hypoglycemia (17, 19, 43). Antecedent exposure to insulin can also affect subsequent counterregulation (18, 27). Therefore, to understand the mechanisms by which recurrent hypoglycemia increases the risk of hypoglycemia, it is necessary to differentiate the effects of recurrent hyperinsulinemia per se from those of recurrent hyperinsulinemic hypoglycemia. During hypoglycemia, insulin has an acute effect to enhance most counterregulatory responses (17, 19, 43), and, in nondiabetic subjects, antecedent insulin can improve subsequent counterregulation (18, 27). In dogs, exposure to hyperinsulinemic euglycemia improves catecholamine responses to subsequent hypoglycemia (18). In humans, antecedent hypoglycemia induced with high doses of insulin causes less impairment of counterregulation than antecedent hypoglycemia induced with low doses of insulin (27). When we recently examined the effects of recurrent hyperinsulinemia with and without hypoglycemia on counterregulation in normal rats, we did not observe such an effect of insulin (52). Although hyperinsulinemic hypoglycemia impaired epinephrine and glucose production responses to subsequent hypoglycemia, hyperinsulinemic euglycemia also impaired these responses.

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During hyperinsulinemic euglycemia, moderate decreases in glucose occurred. These decreases may have been sufficient to impair epinephrine responses. The data suggest that the protective effect of insulin on counterregulation can be abolished by even moderate decrements in glucose.

The present study aimed to differentiate the effects of recurrent hyperinsulinemia per se from those of hyperinsulinemic hypoglycemia on counterregulatory system function in diabetic rats both at baseline and in response to hypoglycemia. In diabetes, counterregulatory systems are altered. Pancreatic function (57), liver and muscle glucose metabolism (51), and hypothalamo-pituitary-adrenal activity (8, 12, 31, 37, 50) differ, and sympathoadrenal function may differ (32), from nondiabetic subjects. Therefore, hyperinsulinemia and hypoglycemia may also have different effects. No studies, to our knowledge, have clearly differentiated the effects of hyperinsulinemia from those of hyperinsulinemic hypoglycemia in diabetes. Previous studies only compared the effects of hyperinsulinemic euglycemia (49) or hyperglycemia (15, 46) with those of hyperinsulinemic hypoglycemia and did not include control groups that were not insulin treated. Therefore, the effects of hyperinsulinemia per se could not be determined. We compared counterregulatory system function in normal rats, untreated diabetic rats, and diabetic rats exposed to recurrent hyperinsulinemic hypoglycemia or hyperinsulinemic hyperglycemia. Inclusion of untreated diabetic rats enabled us to differentiate the effects of hyperinsulinemia from those of hyperinsulinemic hypoglycemia. We demonstrated that recurrent hyperinsulinemia in diabetic rats can normalize most pituitary-adrenal, sympathoadrenal, and pancreatic parameters. However, concurrent exposure to hypoglycemia further impairs epinephrine and glucose production responses and increases peripheral insulin sensitivity.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Quebec, Canada) weighing 300–400 g were studied. Rats were individually housed in a temperature- and light-controlled environment (12:12-h light-dark schedule) with free access to rat chow (Rodent Laboratory Chow 5001; LabChows, Agribrands Canada) and water. Diabetes was induced by intraperitoneal injection of streptozotocin (STZ, 65 mg/kg; Sigma Chemical, St. Louis, MO) dissolved in saline. After STZ injection, drinking water was replaced with 10% sucrose water for 24 h. This dose of STZ produced diabetes with fed-state glucose levels mostly ranging from 18 to 25 mM. Rats with blood glucose levels <15 mM were excluded from the study. All procedures were in accordance with the Canadian Council on Animal Care standards and were approved by the Animal Care Committee of the University of Toronto.

Surgical Procedure

Surgery was performed 7 days before the hypoglycemic glucose clamp experiment. In diabetic rats, this was on *day 14* after induction of diabetes. Catheters were inserted in the left carotid artery and right jugular vein under general anesthesia (100 mg/kg ketamine chloride; MTC Pharmaceuticals, Cambridge, ON, Canada; 1 mg/ kg acepromazine maleate, Wyeth-Ayerst Canada, Montreal, PQ, Canada; 1 mg/kg xylazine, Bayer, Etobicoke, ON, Canada) for sampling of blood and infusion of test substances, respectively, as previously described (52).

Treatment Protocol

The following treatments were initiated 3 days after surgery (i.e., 17 days after induction of diabetes).

Diabetic plus recurrent hyperinsulinemic hypoglycemia (n = 14). Diabetic plus recurrent hyperinsulinemic hypoglycemia (DH) rats underwent 4 days of two episodes per day of hyperinsulinemic hypoglycemia. Insulin (100 U/ml Iletin II regular insulin injection; Eli Lilly, Indianapolis, IN) was administered two times daily by subcutaneous injection (~2 U/100 g body wt) at 9 AM and 1 PM to yield two ~90-min episodes at blood glucose levels of ~2.5 mM. For each hypoglycemic episode, blood glucose was measured from tail nick samples collected every 30 min over a 3-h period, as described previously (Glucometer Elite blood glucose meter; Bayer; range 2.1–29 mM; see Ref. 52). Morning and afternoon episodes were separated by a 1-h rest period, during which the rats were allowed to recover from hypoglycemia before the second insulin injection. During the rest period and after afternoon hypoglycemia, food and 10% sucrose water were fed to aid recovery.

Diabetic plus recurrent hyperinsulinemic hyperglycemia (n = 12). The diabetic plus recurrent hyperinsulinemic hyperglycemia (DI) group controlled for the insulin doses administered to the DH group and thus differentiated the effects of hyperinsulinemia per se from the effects of hypoglycemia. DI rats underwent identical treatment to DH rats but were maintained at a hyperglycemic level of 19.1 ± 1.1 mM throughout treatment with an intravenous infusion of 40% dextrose (Abbott Laboratories). Hyperglycemia, rather than euglycemia, was maintained so that the effects of insulin, independent of its effects to normalize glucose, could be determined.

Diabetic control rats (n = 15). Diabetic control rats underwent 4 days of sham treatment, in which insulin injections were replaced with saline injections. To control for the potential mild stress inflicted by repeated measurement of blood glucose during treatment of DI and DH rats, diabetic control rats underwent identical handling every 30 min throughout each sham treatment episode to simulate the blood sampling procedure in the DH and DI groups.

Normal rats (n = 17). Normal rats underwent the same sham treatment as diabetic control rats.

After afternoon treatment on *day 4*, all rats were fasted overnight before either undergoing glucose clamp experiments [DH (n = 8), DI (n = 7), diabetic control (n = 10), and normal (n = 12)] on *day 5* or being killed by decapitation for analysis of pancreatic proglucagon and prosomatostatin mRNA levels [DH (n = 6), DI (n = 5), diabetic control (n = 5), and normal (n = 5)].

Hyperinsulinemic Euglycemic-Hypoglycemic Glucose Clamp Experiments

On *day 5*, hyperinsulinemic euglycemic-hypoglycemic glucose clamps were performed in all groups. At 8:30 AM, overnight-fasted rats were weighed and connected to the

infusion and sampling apparatus. All procedures were carried out with minimal disturbance to the rats. After set-up, animals were allowed to rest for 2 h before experimentation. It is important to note that rats were conscious and allowed to roam freely in their cages throughout the experiment. At 10:30 AM, basal plasma glucose levels were measured, and blood samples for hormones were collected over a 20-min period. After the baseline period, a constant infusion of insulin (50 $mU \cdot kg^{-1} \cdot min^{-1}$) was begun. This high dose of insulin was required to attain hypoglycemic glucose levels of 2.5 mM in the insulin-resistant diabetic control group and has been used previously to attain similar hypoglycemia in normal rats (36). Plasma glucose was maintained at a euglycemic target of 5.7 ± 0.5 mM by means of a variable infusion of 50% dextrose. Glucose infusion rates were based on measurements of plasma glucose taken every 5 min. At the same time, a primed (4 μCi) 0.07 μCi/min infusion of HPLC-purified [3-^{3}H]glucose (New England Nuclear, Boston, MA) was begun for measurement of glucose turnover. After a period of at least 1 h of tracer equilibration, and once target euglycemia was maintained for 20 min (0–20 min), blood samples for counterregulatory hormones and glucose turnover were collected over a 20-min period (20–40 min). Plasma glucose was then allowed to drop to a hypoglycemic target of 2.5 ± 0.2 mM and was maintained within this range for 105 min (0–105 min). At the onset of hypoglycemia, the [3-^{3}H]glucose infusion rate was reduced to 0.025–0.05 μCi/min to minimize the increase in specific activity during the transition from euglycemia to hypoglycemia. This ensured accurate measurement of glucose turnover during the hypoglycemic period. Blood samples for counterregulatory hormones and glucose turnover were collected only after the 2.5 ± 0.2 mM target was attained. Blood was centrifuged immediately for collection of plasma. Packed blood cells were resuspended in 1% heparin saline and reinfused in the rats after each sampling to prevent volume depletion and anemia.

Analytical Methods

Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Fullerton, CA; see Ref. 39). [3-^{3}H]glucose specific activity was determined as previously described (scaled down for a smaller plasma volume of 50 μl; see Ref. 25). Plasma insulin was measured by RIA, as described previously (35). Plasma glucagon (Diagnostics Products, Los Angeles, CA), somatostatin (Euro-Diagnostica), adrenocorticotropic hormone (ACTH; Diasorin, Stillwater, MN), and corticosterone (Diagnostics Products) were measured by commercially available RIA kits. Plasma catecholamines were measured by the single isotope derivative radioenzymatic assay technique (53).

Proglucagon and Prosomatostatin mRNA Analysis

Pancreases were homogenized in ice-cold 4 M guanidine thiocyanate (Sigma Chemical) and were immediately frozen at −70°C until total RNA was extracted with a modified version of the guanidine thiocyanate water-saturated phenol extraction method (11). After extraction, RNasin ribonuclease inhibitor (Promega, Madison, WI) was added to the total RNA, which was stored at −70°C. For Northern blotting, total RNA (10 μg) was run on 1% agarose-formaldehyde gels. RNA was then transferred to nylon membranes and cross-linked by ultraviolet radiation. Nylon membranes were successively hybridized with ^{32}P-labeled prosomatostatin, 18S, and proglucagon cDNA probes. Probes were labeled with [^{32}P]dCTP (New England Nuclear) using an oligolabeling kit (Amersham Pharmacia Biotech). After hybridization, membranes were washed in 2× saline-sodium citrate (SSC) and then in 0.2× SSC. Membranes were then exposed to Kodak Biomax MS-1 film at −70°C (exposure time: proglucagon 1 day, prosomatostatin 2 days, 18S 10 min). Relative optical densities of the proglucagon and prosomatostatin signals were quantified with a computerized image analysis system (Imaging Research, St. Catharine's, ON, Canada) and were expressed relative to the intensity of the 18S mRNA signal.

Glucose Turnover Determinations

Data for specific activity and plasma glucose concentrations were smoothed using the optimized Optimal Segments program (6). Rates of glucose appearance and glucose utilization were calculated according to Steele's non-steady-state equations (25, 55). Endogenous glucose production was calculated by subtracting the exogenous glucose infusion rate from the total rate of glucose appearance. The metabolic clearance rate (MCR) of glucose was calculated by dividing glucose utilization by plasma glucose concentration. Glucose production, glucose utilization, and MCR during the euglycemic period were calculated as the average of three measurements taken during the steady-state period of euglycemia (20–40 min). Because glucose utilization and MCR during the hypoglycemic period were also in a steady state, these values were calculated as the average of seven measurements taken during this period (0–105 min).

Statistical Analysis

All data are presented as means ± SE. Statistical analysis was performed with Statistica software (Statsoft, Tulsa, OK). Baseline measurements were analyzed by one-way ANOVA followed by Duncan's post hoc test for multiple comparisons. During the hypoglycemic phase of the glucose clamp, data were analyzed by two-way ANOVA with a repeated-measures design, followed by Duncan's post hoc test. Significance was assumed at $P < 0.05$.

RESULTS

Glucose Levels and Insulin Doses During Recurrent Hyperinsulinemic Hypoglycemia and Hyperinsulinemic Hyperglycemia

Blood glucose levels over the 4 days of recurrent hyperinsulinemic hypoglycemia (DH) and recurrent hyperinsulinemic hyperglycemia (DI) treatment are shown in Fig. 1. During morning and afternoon hypoglycemia, blood glucose levels fell to ~3 mM by 90 min after insulin injection and remained at ~2.5 mM for 90 min thereafter. The rats were hypoglycemic for a total of 3 h/day. During recurrent hyperglycemia, mean blood glucose was maintained at 19.1 ± 1.1 mM. Mean insulin doses over the 4 days of treatment did not differ (DH: 2.1 ± 0.2 U/100 g body wt; DI: 2.0 ± 0.1 U/100 g body wt, P = not significant).

Basal Glucose and Hormone Levels After Treatment

Fasting plasma glucose and hormone levels on the morning of the glucose clamp experiment are summarized in Table 1 (ACTH and corticosterone in Fig. 2, *A* and *B*). Plasma glucose was similarly elevated ($P <$ 0.05) in all three diabetic groups compared with nor-

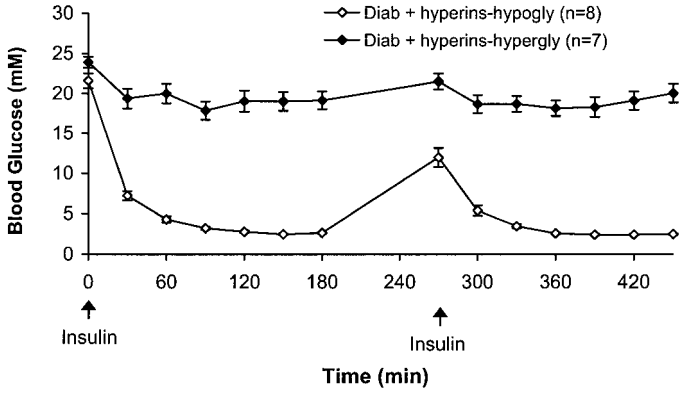

Fig. 1. Blood glucose levels during *days 1–4* of recurrent hyperinsulinemic hypoglycemia [diabetic + hyperinsulinemic hypoglycemia (DH)] and hyperinsulinemic hyperglycemia [diabetic + hyperinsulinemic hyperglycemia (DI)] in diabetic rats. Insulin was injected immediately after 0 and 270 min of sampling. Values are expressed as means ± SE.

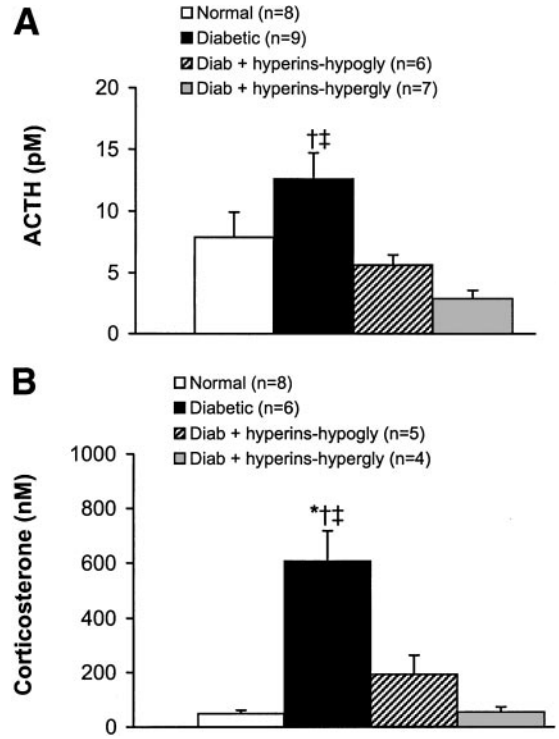

Fig. 2. Basal plasma adrenocorticotropic hormone (ACTH; *A*) and corticosterone (*B*) levels in normal, diabetic control, DH, and DI rats. ACTH was increased in diabetic controls compared with DH and DI rats. Corticosterone was increased in diabetic controls compared with all groups. Values are expressed as means ± SE. *$P < 0.05$ vs. normal. †$P < 0.05$ vs. DH. ‡$P < 0.05$ vs. DI.

mal rats. Plasma insulin tended to be decreased in diabetic control rats compared with normal rats. However, because of the variability among the groups, the difference was not statistically significant when compared by Duncan's post hoc test. DH and DI rats displayed increased ($P < 0.05$) insulin levels compared with diabetic control rats. Despite identical insulin doses during treatment, insulin levels in DH rats were increased by nearly twofold ($P < 0.05$) compared with DI rats. Basal plasma glucagon levels were similar in all groups. Plasma somatostatin levels were increased ($P < 0.05$) in diabetic controls compared with normal rats but were partially normalized in DH rats and fully normalized ($P < 0.05$ vs. diabetic control) in DI rats. Somatostatin levels in DH and DI rats did not differ from one another. Basal epinephrine levels were similar among normal, diabetic control, and DH rats but were increased ($P < 0.05$) in DI rats compared with DH rats and diabetic controls. In contrast, norepinephrine levels were increased ($P < 0.05$) in DH rats compared with DI rats and diabetic controls. Basal plasma ACTH tended to be increased ($P < 0.07$) in diabetic controls compared with normal rats (Fig. 2*A*). After both insulin regimens, ACTH was reduced ($P < 0.05$) compared with diabetic controls. ACTH levels tended to be higher in DH rats compared with DI rats. However, the difference was not statistically significant. Plasma corticosterone was markedly increased ($P < 0.05$) 12-fold in diabetic control rats compared with normal rats (Fig. 2*B*). After both insulin regimens, corticosterone levels were dramatically reduced ($P < 0.05$ vs. diabetic control). Corticosterone levels in DI rats were fully normalized, whereas corticosterone levels in DH rats tended to be increased compared with normal rats. As with ACTH, however, there were no significant differences in corticosterone levels between DH and DI rats.

Pancreatic Proglucagon and Prosomatostatin mRNA

Pancreatic proglucagon and prosomatostatin mRNA levels were markedly increased ($P < 0.05$) in diabetic control rats compared with normal rats (Fig. 3, *A* and *B*). Both insulin regimens fully normalized ($P < 0.05$ vs. diabetic control) proglucagon mRNA levels. Prosomatostatin mRNA levels were partially normalized in

Table 1. *Effects of diabetes, diabetes + recurrent hyperinsulinemic hypoglycemia, and diabetes + recurrent hyperinsulinemic hyperglycemia on basal plasma glucose and hormone levels*

	Normal ($n = 12$)	Diabetic ($n = 10$)	DH ($n = 8$)	DI ($n = 7$)
Glucose, mM	6.3 ± 0.1	17.5 ± 2.3[a]	19.9 ± 2.4[a]	21.5 ± 3.4[a]
Insulin, pM	87 ± 15	35 ± 3	223 ± 40[a,b,d]	130 ± 32[b]
Glucagon, ng/l	68 ± 6	94 ± 8	86 ± 8	87 ± 10
Somatostatin, pM	25 ± 4	42 ± 6[a,d]	30 ± 5	27 ± 3
Epinephrine, nM	0.43 ± 0.04	0.30 ± 0.07	0.34 ± 0.07	0.58 ± 0.06[b,c]
Norepinephrine, nM	1.48 ± 0.13	0.98 ± 0.13	1.55 ± 0.28[b,d]	0.88 ± 0.09[a]

Values are means ± SE. DH, diabetic + hyperinsulinemic hypoglycemia; DI, diabetic + hyperinsulinemic hyperglycemia. $P < 0.05$ vs. normal (a), diabetic control (b), DH (c), and DI (d).

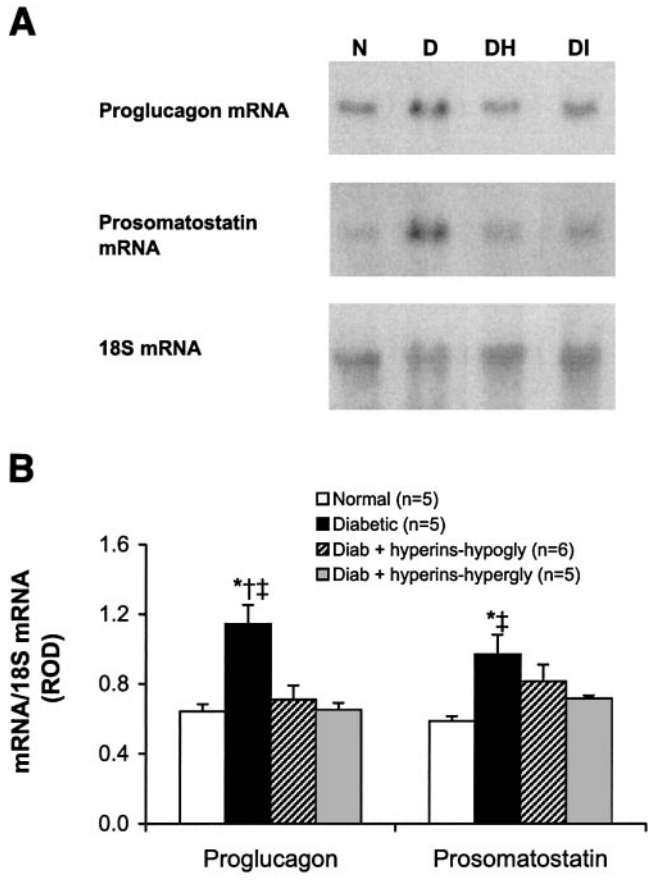

Fig. 3. Computerized images of basal pancreatic proglucagon, prosomatostatin, and 18S mRNA (*A*) and densitometric analysis of proglucagon and prosomatostatin mRNA relative to 18S mRNA (*B*) in normal (N), diabetic control (D), DH, and DI rats. Proglucagon mRNA was increased in diabetic control rats compared with all groups. Prosomatostatin mRNA was increased in diabetic control rats compared with normal and DI rats. Values are expressed as means ± SE of relative optical density (ROD). *$P < 0.05$ vs. normal. †$P < 0.05$ vs. DH. ‡$P < 0.05$ vs. DI.

DH rats and fully normalized ($P < 0.05$ vs. diabetic control) in DI rats. There were no significant differences in prosomatostatin mRNA levels between DH and DI rats.

Glucose, Insulin, and Counterregulatory Hormone Levels During Hyperinsulinemic Euglycemic-Hypoglycemic Glucose Clamps

Plasma glucose levels during hyperinsulinemic euglycemia were at similar steady-state levels for all groups (5.7 ± 0.2 mM) and dropped to similar levels during the hypoglycemic period (2.5 ± 0.1 mM; Fig. 4). Plasma insulin levels were measured at the end of the euglycemic period (40 min) and the end of the hypoglycemic period (105 min). There were no significant differences in plasma insulin in any of the groups during the euglycemic and hypoglycemic periods (Table 2).

Glucagon responses to hypoglycemia were impaired ($P < 0.05$) in all diabetic groups compared with normal rats (Fig. 5*A*). However, in DH and DI rats, glucagon levels at the end of the hypoglycemic period were increased ($P < 0.05$) compared with diabetic control rats, indicating partially improved glucagon responses. Glucagon responses in DH and DI rats did not differ from one another.

Epinephrine responses to hypoglycemia were markedly impaired ($P < 0.05$) in diabetic control rats compared with normal rats (Fig. 5*B*). In DI rats, epinephrine responses were fully normalized ($P < 0.05$ vs. diabetic control). DH rats displayed markedly reduced epinephrine responses compared with DI rats. Moreover, these responses were further reduced ($P < 0.05$ by *t*-test) by nearly 50% compared with diabetic control rats. Diabetic control rats also displayed impaired ($P < 0.05$) norepinephrine counterregulatory responses compared with normal rats (Fig. 5*C*). Unlike their effect on epinephrine, both insulin regimens fully normalized ($P < 0.05$ vs. diabetic control) norepinephrine responses.

Except for diabetic control rats, corticosterone levels during hyperinsulinemic euglycemia were increased ($P < 0.05$) compared with baseline (Table 3). Absolute plasma corticosterone levels during hypoglycemia were decreased ($P < 0.05$) in diabetic control rats compared with normal rats. In response to hypoglycemia per se (i.e., from euglycemia to hypoglycemia), normal rats

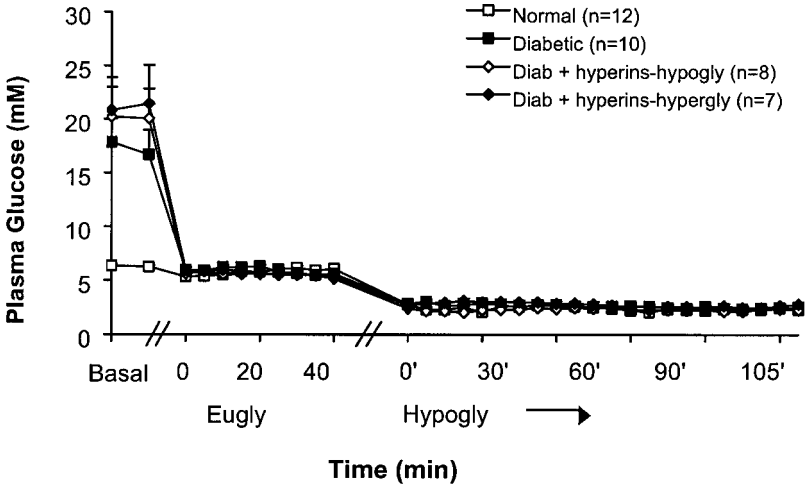

Fig. 4. Plasma glucose levels during *day 5* hyperinsulinemic euglycemic-hypoglycemic glucose clamp experiments in normal, diabetic control, DH, and DI rats.

Table 2. *Plasma insulin levels during day 5 hyperinsulinemic euglycemic-hypoglycemic clamps in normal rats, diabetic control rats, and diabetic rats exposed to recurrent hyperinsulinemic hypoglycemia or hyperinsulinemic hyperglycemia*

	n	Euglycemic Period	Hypoglycemic Period
Normal	12	19,331 ± 4,203	22,374 ± 3,970
Diabetic	8	10,784 ± 993	17,814 ± 2,220
DH	8	13,912 ± 1,213	14,668 ± 2,538
DI	7	12,946 ± 619	15,025 ± 2,330

Values are means ± SE; *n*, no. of rats.

displayed further increases ($P < 0.05$ vs. euglycemic period) in corticosterone (Fig. 6). In diabetic control rats, corticosterone responses to hypoglycemia were nearly absent ($P < 0.05$ vs. normal). Both insulin regimens normalized corticosterone responses to hypoglycemia ($P < 0.05$ vs. diabetic control).

Glucose Turnover

Glucose specific activity was near steady state throughout the entire clamp for all groups (Fig. 7*A*). Glucose production during hyperinsulinemic euglycemia was elevated ($P < 0.05$) in DH rats compared with normal rats, suggesting impaired hepatic insulin sensitivity (Table 4). In response to hypoglycemia, normal, diabetic control, and DI rats displayed similar overall glucose production responses (Fig. 7*B*). Peak glucose production at the onset of hypoglycemia, however, was reduced ($P < 0.05$) in diabetic control rats compared with normal rats and tended to be reduced ($P < 0.06$) in DH and DI rats. In DH rats, glucose production responses to hypoglycemia were markedly impaired ($P < 0.05$) compared with all groups.

Glucose utilization during hyperinsulinemic euglycemia was reduced ($P < 0.05$) in all diabetic groups compared with normal rats (Table 4). In response to hypoglycemia, glucose utilization was markedly decreased ($P < 0.05$) in all groups. Hypoglycemic glucose utilization was similar in normal and diabetic control rats but was increased ($P < 0.05$) in DH and DI rats compared with diabetic controls. MCR during hyperinsulinemic euglycemia was reduced ($P < 0.05$) in diabetic controls compared with normal rats, indicating reduced peripheral insulin sensitivity. In DH rats, but not DI rats, euglycemic and hypoglycemic MCR was increased ($P < 0.05$) compared with diabetic controls, indicating that recurrent hypoglycemia increased peripheral insulin sensitivity.

DISCUSSION

This study aimed to differentiate the effects of recurrent hyperinsulinemia per se from those of recurrent hyperinsulinemic hypoglycemia on counterregulatory system function in STZ-diabetic rats at baseline and in response to hypoglycemia. Adaptations in pituitary-adrenal and sympathoadrenal activity, pancreatic function, and glucose turnover were examined. Recurrent hyperinsulinemia, independent of glycemia, normalized basal pituitary-adrenal and pancreatic hormones, partially improved glucagon responses to subsequent hypoglycemia, and fully normalized norepinephrine and corticosterone

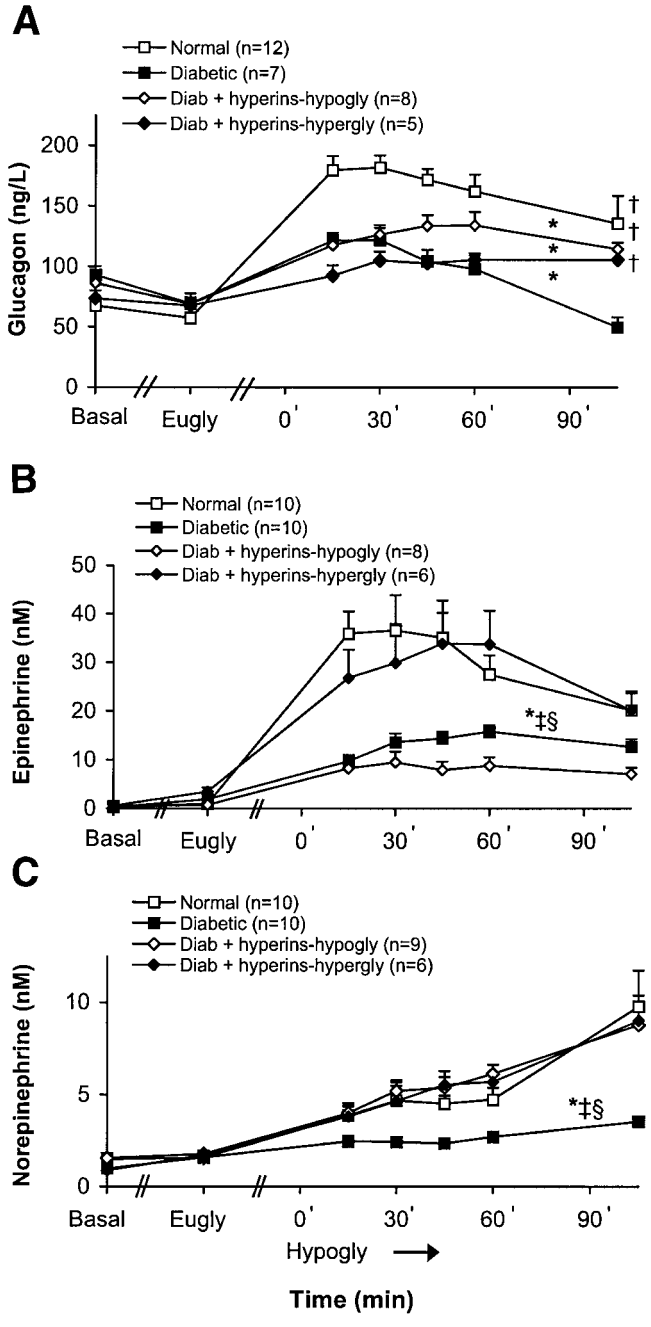

Fig. 5. Plasma glucagon (*A*), epinephrine (*B*), and norepinephrine (*C*) levels during *day 5* hyperinsulinemic euglycemic-hypoglycemic clamp experiments in normal, diabetic control, DH, and DI rats. Glucagon responses were decreased in all diabetic groups compared with normal rats. Glucagon levels at the end of the hypoglycemic period were increased in DH and DI rats compared with diabetic controls. Epinephrine responses in diabetic control rats were decreased compared with normal and DI rats and were further decreased in DH rats. Norepinephrine responses in diabetic control rats were decreased compared with normal rats but were fully normalized in DH and DI rats. Values are expressed as means ± SE. *$P < 0.05$ vs. normal. †$P < 0.05$ vs. diabetic control. ‡$P < 0.05$ vs. DH. §$P < 0.05$ vs. DI.

Table 3. *Plasma corticosterone levels during day 5 hyperinsulinemic euglycemic-hypoglycemic clamps in normal, diabetic control, DH, and DI rats*

				Hypoglycemia, min						
	n	Basal	Euglycemia	0	15	30	45	85	95	105
Normal	7	53 ± 13	817 ± 127[d]	1,071 ± 92	1,222 ± 77[e]	1,272 ± 87[e]	1,248 ± 72[e]	1,263 ± 81[e]	1,222 ± 55[e]	1,181 ± 55[e]
Diabetic	6	606 ± 111[a,b,c]	925 ± 122	989 ± 110	998 ± 98	940 ± 111	894 ± 118	859 ± 109	816 ± 107[a]	712 ± 83[a,b,c]
DH	5	193 ± 71	843 ± 208[d]	925 ± 149	1,044 ± 147	1,084 ± 148	1,133 ± 134	1,182 ± 177	1,087 ± 120	1,168 ± 197
DI	4	55 ± 18	649 ± 117[d]	918 ± 35	966 ± 40[e]	1,054 ± 74[e]	1,089 ± 80[e]	1,040 ± 107[e]	1,040 ± 121	1,066 ± 137

Values are means ± SE; *n*, no. of rats. Units are nM. $P < 0.05$ vs. normal (a), DH (b), DI (c), basal (d), and euglycemic period (e).

responses. In contrast, recurrent hypoglycemia markedly impaired epinephrine and glucose production responses. The epinephrine impairment occurred despite a protective effect of insulin treatment, since epinephrine responses were fully normalized after recurrent hyperinsulinemia. Recurrent hypoglycemia also increased muscle clearance of glucose, suggesting that recurrent hypoglycemia may in part increase the risk of hypoglycemia by increasing peripheral insulin sensitivity.

Recently, we examined the effects of recurrent hyperinsulinemia and hypoglycemia on counterregulation in normal rats (52). It should be noted that normal rats underwent recurrent hyperinsulinemic euglycemia instead of hyperinsulinemic hyperglycemia. Diabetic rats were maintained at hyperglycemia so that the effects of hyperinsulinemia, rather than those of normalized glucose, could be determined. Also, during treatment of diabetic rats, hyperinsulinemia was induced with 2 U insulin/100 g body wt compared with 0.2 U insulin/100 g in normal rats. The higher dose was necessitated by the insulin resistance in diabetic rats, since some rats did not consistently attain glucose levels <3 mM with this dose. The differentiation of the effects of insulin and hypoglycemia could only be accomplished by comparing rats exposed to hyperinsulinemic hyperglycemia or hyperinsulinemic hypoglycemia with untreated diabetic rats. Previous studies only used subjects exposed to hyperinsulinemia as controls (15, 46, 49) and thus could not identify the effects of insulin.

Pituitary-adrenal function was dysregulated in diabetic control rats (Figs. 2, *A* and *B*, and 6). Basal plasma ACTH levels tended to be increased, and corticosterone levels were markedly increased 12-fold com-

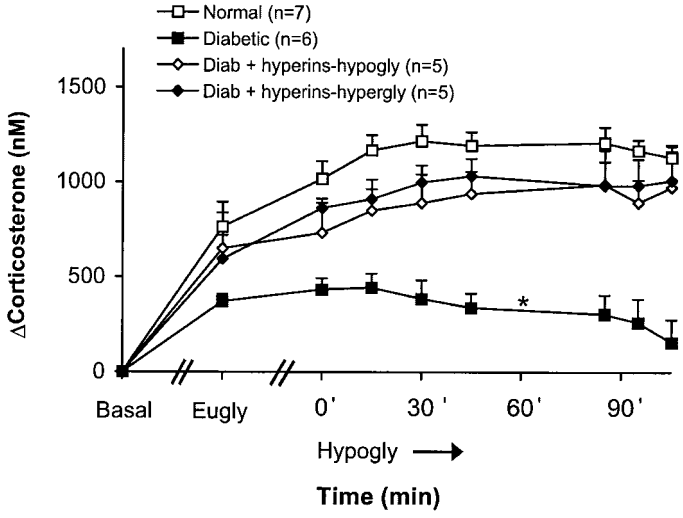

Fig. 6. Incremental corticosterone responses from baseline during *day 5* euglycemic hypoglycemic clamp experiments in normal, diabetic control, DH, and DI rats. Corticosterone responses from baseline and from euglycemia were decreased in diabetic control rats compared with all groups. Values are expressed as means ± SE. *$P < 0.05$ vs. normal, DH, and DI.

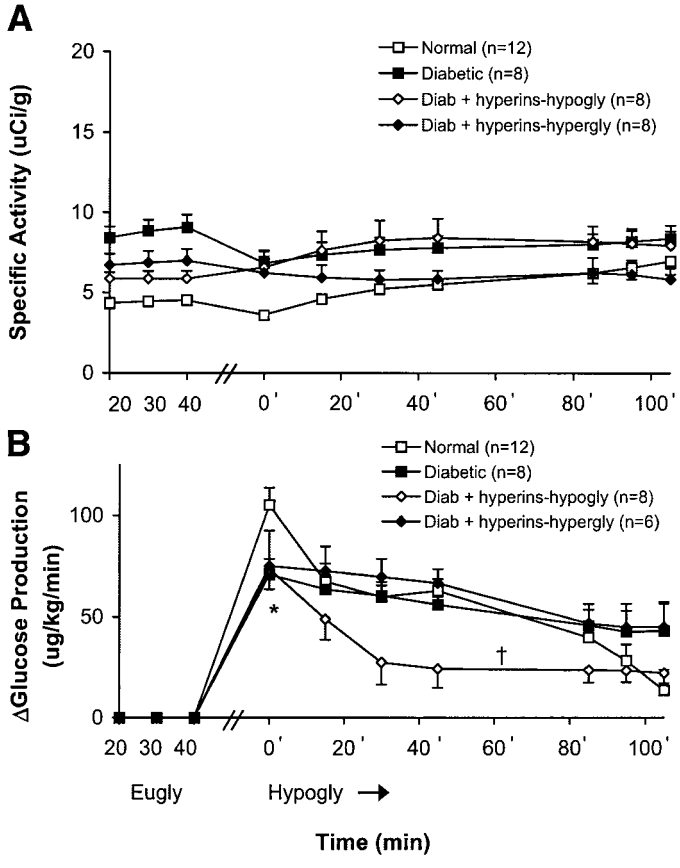

Fig. 7. Glucose specific activity (*A*) and incremental glucose production responses from hyperinsulinemic euglycemia (*B*) during *day 5* hyperinsulinemic euglycemic-hypoglycemic clamp experiments in normal, diabetic control, DH, and DI rats. Peak glucose production responses were decreased in diabetic control rats compared with normal rats. Overall glucose production responses in DH rats were decreased compared with all groups. Values are expressed as means ± SE. *$P < 0.05$ vs. normal. †$P < 0.05$ vs. normal, diabetic control, and DI.

Table 4. *Effects of diabetes, diabetes + recurrent hyperinsulinemic hypoglycemia, and diabetes + recurrent hyperinsulinemic hyperglycemia on glucose turnover during day 5 hyperinsulinemic euglycemic-hypoglycemic clamps*

	Normal (n = 12)	Diabetic (n = 10)	DH (n = 8)	DI (n = 7)
Glucose production, $\mu mol \cdot kg^{-1} \cdot min^{-1}$				
Euglycemia	14 ± 5	27 ± 4	37 ± 10[a]	33 ± 9
Glucose utilization, $\mu mol \cdot kg^{-1} \cdot min^{-1}$				
Euglycemia	226 ± 10	155 ± 12[a]	182 ± 10[a]	177 ± 16[a]
Hypoglycemia	91 ± 4[c]	82 ± 3[c]	100 ± 2[b,c]	100 ± 8[b,c]
MCR, $ml \cdot kg^{-1} \cdot min^{-1}$				
Euglycemia	38 ± 2	26 ± 2[a]	33 ± 2[b]	32 ± 3
Hypoglycemia	37 ± 2	30 ± 2	42 ± 2[b,c]	35 ± 3

Values are means ± SE; n, no. of rats. MCR, metabolic clearance rate. $P < 0.05$ vs. normal (a), diabetic (b), and euglycemic period (c).

pared with normal rats. In addition, corticosterone responses to hypoglycemia per se were nearly absent. We observed similar basal ACTH and corticosterone levels (10) and decreased responses to hypoglycemia (9) in 8-day diabetic rats. Increased basal ACTH and glucocorticoid levels have also been reported in diabetic WBN/Kob rats (58), *db*/*db* mice (13), and type 1 diabetic patients (8, 31, 50). The altered pituitary-adrenal activity was presumably the result of a lack of insulin, rather than hyperglycemia, since both insulin regimens reduced basal ACTH and corticosterone levels and restored corticosterone responses to hypoglycemia. This effect of insulin contrasts with the acute effect of high doses of insulin to stimulate ACTH and glucocorticoid secretion (26). Our data indicate that differences in pituitary-adrenal hormones between diabetic control rats and insulin-treated diabetic rats were because of insulin treatment, independent of glycemic manipulation. Interestingly, in rats exposed to recurrent hypoglycemia, ACTH and corticosterone levels tended to be elevated compared with those in rats exposed to recurrent hyperinsulinemia. This suggests that recurrent hypoglycemia per se led to elevated basal ACTH and corticosterone. Indeed, in rats exposed to repeated stress, basal ACTH and corticosterone levels can be increased (1). As we observed previously in normal rats (52), recurrent hypoglycemia per se did not affect corticosterone counterregulation. Cortisol responses are often unaltered after antecedent hypoglycemia (15, 22, 38, 49) and can be unaltered after antecedent exercise (28). These and our data suggest that recurrent hypoglycemia may have only a small effect on glucocorticoid counterregulation.

Diabetes, recurrent hypoglycemia, and recurrent hyperinsulinemia also altered sympathoadrenal activity (Table 1 and Fig. 5, *A* and *B*). Basal epinephrine levels were increased nearly twofold after recurrent hyperinsulinemia but not after recurrent hypoglycemia. Thus recurrent hypoglycemia per se may have decreased basal epinephrine levels. In contrast, norepinephrine levels were increased after recurrent hypoglycemia, but not after recurrent hyperinsulinemia, possibly because of decreased adrenal conversion of norepinephrine to epinephrine. Epinephrine responses to hypoglycemia were markedly reduced in diabetic control rats compared with normal rats. Recurrent hypoglycemia further reduced the response by nearly 50%. This occurred despite a protective effect of insulin, since epinephrine responses were fully normalized after recurrent hyperinsulinemia. In our normal rats, epinephrine responses were also impaired after recurrent hypoglycemia (52). Surprisingly, however, hyperinsulinemic euglycemia in our normal rats also reduced epinephrine responses. During hyperinsulinemic euglycemia, glucose levels dropped from 6.5 to ~4.5 mM. This moderate decrement in glucose may have been sufficient to impair epinephrine counterregulation. In nondiabetic (22, 29, 34, 38, 59, 61) and diabetic (15, 16, 44, 46, 48, 49) subjects, epinephrine counterregulation is consistently impaired after antecedent hypoglycemia. These data, along with our observations of impaired epinephrine responses after moderate decrements in glucose and defective responses despite a normalizing effect of insulin, indicate that epinephrine counterregulation is particularly sensitive to impairment by antecedent hypoglycemia. Norepinephrine counterregulation, in contrast, is often unaltered after antecedent hypoglycemia (15, 16, 29, 46, 61). In our diabetic rats, recurrent hypoglycemia per se did not impair norepinephrine responses. Rather, both insulin regimens normalized the responses.

In humans, antecedent hypoglycemia impairs glucagon counterregulation in nondiabetic subjects (22, 34, 38, 59, 61), but not in type 1 diabetic subjects (15, 16), since glucagon responses are already impaired (30). In our diabetic rats, recurrent hypoglycemia per se did not impair glucagon responses. Surprisingly, both insulin regimens partially improved glucagon responses. This was unexpected given that insulin inhibits glucagon synthesis and secretion (47, 54). Correspondingly, both insulin regimens decreased (normalized) pancreatic proglucagon mRNA levels (Fig. 3). Plasma somatostatin and pancreatic prosomatostatin mRNA levels were also decreased (normalized) after both insulin regimens (Table 1 and Fig. 3). These alterations were independent of glycemic levels during treatment. Because somatostatin inhibits glucagon secretion (7, 56), decreased basal somatostatin levels in the insulin-treated rats may have contributed to the improved glucagon responses. The improved norepinephrine and corticosterone responses in these rats may have also

contributed, since both hormones stimulate glucagon secretion (2, 4).

In diabetic control rats, MCR during the euglycemic period of the glucose clamp was decreased compared with normal rats, indicating decreased peripheral insulin sensitivity. After recurrent hypoglycemia, but not recurrent hyperinsulinemia, MCR was increased. We therefore hypothesize that recurrent hypoglycemia may in part increase the risk of subsequent hypoglycemia by increasing peripheral insulin sensitivity. The increased insulin sensitivity may have been because of increased glucose transport into muscle resulting from increased muscle plasma membrane expression of GLUT4 glucose transporters, since we have shown that hypoglycemia per se can elicit these changes in muscle (45). Although overall glucose production responses to hypoglycemia were not impaired in diabetic control rats, peak responses were reduced compared with normal rats. Moreover, as we observed previously in normal rats (52), recurrent hypoglycemia resulted in marked overall impairment of glucose production responses.

The improved corticosterone and norepinephrine responses after both insulin regimens and the improved epinephrine responses after selective hyperinsulinemia may in part have been the result of decreased basal corticosterone levels. Glucocorticoids suppress sympathetic (42) and hypothalamic-pituitary-adrenal (24) activity. Therefore, the lowered basal corticosterone levels in these rats may have contributed to the above improvements in counterregulation. Recurrent hyperinsulinemia may also have contributed, since, in nondiabetic dogs, antecedent hyperinsulinemic euglycemia can enhance catecholamine responses to subsequent hypoglycemia (18). Moreover, in humans, antecedent hypoglycemia induced with high doses of insulin has been shown to cause less impairment of norepinephrine and cortisol responses than antecedent hypoglycemia induced with low doses of insulin (27). Interestingly, epinephrine responses were similarly blunted after both insulin doses. Therefore, as in the present study, epinephrine responses were not protected by insulin. Exposure to elevated glucocorticoids (20, 21), lactate (60), and/or ketones (60) during antecedent hypoglycemia, and increased brain glucose uptake after antecedent hypoglycemia (5), have been postulated to contribute to the impairment of counterregulation after antecedent hypoglycemia. Epinephrine counterregulation may be more sensitive to such mechanisms than norepinephrine and glucocorticoid counterregulation. This might explain the effect of recurrent hypoglycemia in our diabetic rats to selectively impair epinephrine responses while allowing norepinephrine and corticosterone responses to be improved by concurrent hyperinsulinemia.

Basal insulin levels after both insulin regimens were increased compared with diabetic controls (Table 1). In addition, despite identical insulin doses during treatment, insulin levels after hyperinsulinemic hypoglycemia were nearly twofold greater than those after hyperinsulinemic hyperglycemia. We cannot exclude the possibility that the differences in basal and hypoglycemic counterregulatory hormone levels between insulin-treated rats and diabetic controls were at least in part the result of, or modulated by, the higher basal insulin levels in the insulin-treated groups. Similarly, the differences in counterregulatory hormones between rats exposed to recurrent hypoglycemia and rats exposed to recurrent hyperinsulinemia may have also at least in part been due to, or modulated by, the different basal insulin levels in these two groups. Nonetheless, there is evidence to suggest that the variations in basal insulin levels may not have been responsible for the differences in the counterregulatory hormones. First, the basal insulin levels in the insulin-treated rats were below the fed-state levels of 360 (3) and 650 (33) pM seen in nondiabetic rats and do not affect catecholamine, ACTH, and glucocorticoid secretion in humans and rats (26, 41). Second, because hyperinsulinemia can stimulate the secretion of ACTH and corticosterone (26), the higher basal insulin levels in the insulin-treated rats would have been expected to cause increased, rather than decreased, basal ACTH and corticosterone levels compared with diabetic controls. Similarly, the higher basal insulin levels in diabetic rats exposed to recurrent hypoglycemia would have been expected to lead to increased epinephrine responses to hypoglycemia compared with the other groups, since insulin can enhance counterregulatory responses to hypoglycemia (17, 19, 43). Finally, during the hypoglycemic clamp, before the onset of hypoglycemia, all rats underwent a period of at least 40 min of hyperinsulinemic euglycemia where insulin levels were increased at least 60-fold above basal levels. We believe that the effects of hyperinsulinemia during the clamp would have overridden any potential effects of the differing basal insulin levels on counterregulation.

Our observations of improved glucagon, norepinephrine, and corticosterone responses in diabetic rats exposed to recurrent hypoglycemia contrast with the effect of antecedent hypoglycemia to cause generalized impairment of counterregulation in humans with type 1 diabetes (15, 16, 44, 46, 49). This difference may have been because, unlike in human studies, diabetic control rats were not insulin treated. The lack of insulin treatment likely contributed to the markedly increased basal corticosterone levels and impaired counterregulation. In insulin-treated type 1 diabetes, cortisol levels are only moderately increased (8, 31, 50) and norepinephrine and cortisol counterregulation are usually intact (15, 16, 40). In this sense, diabetic rats exposed to recurrent hyperinsulinemia were comparable to type 1 diabetic subjects. Comparison of the responses of rats exposed to recurrent hypoglycemia with those of rats exposed to recurrent hyperinsulinemia shows that recurrent hypoglycemia decreased epinephrine but did not affect glucagon, norepinephrine, and corticosterone responses. These results are comparable to those seen in human studies (15, 16, 44, 46, 49).

In summary, recurrent hyperinsulinemia in diabetic rats, independent of glycemic levels during treatment, normalized most parameters of pituitary-adrenal, sympathoadrenal, and pancreatic function, both at baseline and during hypoglycemia. Peripheral insulin sensitivity, however, was increased after recurrent hypoglycemia but not after recurrent hyperinsulinemia, suggesting that recurrent hypoglycemia may in part increase the risk of subsequent hypoglycemia by increasing insulin sensitivity. Most notably, recurrent hypoglycemia further impaired epinephrine and glucose production responses. The epinephrine defect occurred in spite of an effect of insulin treatment to normalize counterregulatory responses, indicating that epinephrine counterregulation is particularly sensitive to impairment by recurrent hypoglycemia. The specific impairment of the epinephrine response suggests that the mechanisms underlying defective counterregulation may be better understood by examining alterations in loci that regulate epinephrine secretion.

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