Diabetes Impairs Hypothalamo-Pituitary-Adrenal (HPA) Responses to Hypoglycemia, and Insulin Treatment Normalizes HPA but not Epinephrine Responses

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We recently established that in addition to plasma adrenocorticotrophic hormone (ACTH) and corticosterone, hypothalamic corticotrophin-releasing hormone (CRH) mRNA and hippocampal type 1 glucocorticoid receptor (GR1) mRNA were also upregulated in uncontrolled streptozotocin-induced diabetes. In the current study, control, diabetic, and insulin-treated diabetic rats underwent a hyperinsulinemic-hypoglycemic glucose clamp to evaluate central mechanisms of hypothalamo-pituitary-adrenal (HPA) and counterregulatory responses to insulin-induced hypoglycemia. Increases in plasma ACTH, corticosterone, and epinephrine were significantly lower in diabetic rats versus controls. Insulin treatment restored ACTH and corticosterone but not epinephrine responses to hypoglycemia in diabetic rats. Glucagon and norepinephrine responses to hypoglycemia were not affected by diabetes or insulin treatment. In response to hypoglycemia, hypothalamic CRH mRNA and pituitary proopiomelanocortin mRNA expression increased in control and insulin-treated but not in untreated diabetic rats. Arginine vasopressin mRNA was unaltered by hypoglycemia in all groups. Interestingly, hypoglycemia decreased hippocampal GR1 mRNA expression in control and insulin-treated diabetic rats but not in diabetic rats. In contrast, type 2 glucocortoid receptor (GR2) mRNA was not altered by hypoglycemia. In conclusion, despite increased basal HPA activity, HPA responses to hypoglycemia were markedly reduced in uncontrolled diabetes. We speculate that the defect in CRH response could be related to the defective GR1 response. It is intriguing that insulin treatment restored the HPA response to hypoglycemia but, surprisingly, not the deficient epinephrine response. This is important because during severe hypoglycemia, epinephrine is an important counterregulatory hormone. *Diabetes* 51:1681–1689, 2002

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ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; CRH, corticotrophin-releasing hormone; GR1, type 1 glucocorticoid receptor; HPA, hypothalamo-pituitary-adrenal; POMC, proopiomelanocortin; PVN, paraventricular nucleus; RIA, radioimmunoassay; ROD, relative optical density; SAS, sympathoadrenal system; SSC, sodium chloride–sodium citrate; STZ, streptozotocin.

Hypoglycemia is the most serious acute complication in insulin-treated diabetic patients undergoing intensive insulin therapy. It is often the limiting factor in attempts to maintain proper glycemic control in patients with type 1 diabetes (1). Because brain glucose metabolism is limited when blood glucose levels fall below the normal physiological range of blood-to-brain glucose transport, it becomes evident that the prevention or rapid recovery of hypoglycemia is critical for protecting brain energy requirements and, eventually, for survival.

A decrease in insulin secretion, along with an increase in glucagon secretion, stands high in a hierarchy of glucose counterregulatory mechanisms that govern the recovery from hypoglycemia (2). However, in situations of impaired or deficient glucagon secretion, such as type 1 diabetes, epinephrine and, more chronically, cortisol begin to play a very important role in the recovery from hypoglycemia (3). As such, regulation of the hypothalamo-pituitary-adrenal (HPA) axis is an important factor in the pathology of defective counterregulatory mechanisms in diabetes, since the HPA axis not only regulates glucocorticoid secretion but can also affect epinephrine secretion (4). The important roles of glucocorticoids in glucoregulation are the regulation of liver sensitivity to glucagon and epinephrine and reduction of hepatic and peripheral insulin sensitivity (5).

It has been demonstrated previously that insulin-induced hypoglycemia is a potent activator of the HPA axis (6). Activation of the HPA axis results in increased secretion of corticotrophin-releasing hormone (CRH), which in turn is secreted into the hypophyseal-portal circulation and transported to the pituitary gland, where it stimulates the synthesis and cleavage of proopiomelanocortin (POMC) into a number of peptides, including adrenocorticotrophic hormone (ACTH) in the corticotroph (7). ACTH acts at the adrenal cortex to stimulate the secretion of cortisol in humans. In rodents, the major glucocorticoid is corticosterone, since these animals lack the enzyme 11β-hydroxylase, which converts corticosterone to cortisol (8). The stress response is then terminated via glucocorticoid negative feedback through occupation of corticosteroid receptors by glucocorticoids. The brain

contains both types of corticosteroid receptors: type 1 (GR1) and type 2 (GR2) glucocorticoid receptors, the occupation of which results in termination of HPA activity (9).

The purpose of this study was to determine whether central and peripheral HPA and other counterregulatory responses to hypoglycemia are altered after 8 days of streptozotocin (STZ)-induced diabetes in rats and to determine whether the observed changes in HPA function with diabetes are the result of a lack of insulin. With insulin replacement, we sought to determine whether the alterations in HPA function could be fully reversed with insulin therapy. Although the pituitary-adrenal response to insulin-induced hypoglycemia has been studied previously, some of these protocols used a single insulin injection procedure in which insulin levels peaked and dissipated rapidly, resulting in variable insulin levels (10–12). In the present study, we used the glucose clamp technique, which provided us with two main advantages. First, it enabled us to induce hypoglycemia using comparable insulin levels in all groups, and second, it avoided the added stress of injection protocols. More importantly, in this study, peripheral parameters (plasma ACTH and corticosterone) were compared with stress hormone (CRH, arginine vasopressin [AVP], and POMC) and corticosteroid receptor (GR1 and GR2) gene expression in brain and pituitary regions involved in HPA regulation. Here, we demonstrated for the first time that defects in the diabetic HPA response to hypoglycemia are associated with impaired activation of the axis, and this in turn may be caused by defects in the hippocampal GR1 system. Moreover, insulin treatment restored both central and peripheral HPA responses to hypoglycemia in diabetic rats, but not the epinephrine counterregulatory response.

RESEARCH DESIGN AND METHODS

Experimental animals and design. Male Sprague-Dawley rats (Charles River Canada, St. Constant, Québec, Canada), initially weighing between 325 and 375 g, were individually housed in opaque cages in rooms with controlled temperature (22–23°C) and humidity. The animals were fed rat chow (Ralston Purina, St. Louis, MO) and water ad libitum and were acclimatized to a 12-h light cycle (lights on between 7:00 A.M. and 7:00 P.M.) for a period of 1 week before experimental manipulation. All experiments were approved by the animal care committee of the University of Toronto and were in accordance with regulations set by the Canadian Council for Animal Care.

Hypoglycemia studies. Three groups of rats were used: *1*) normal controls ($n = 6$), *2*) untreated STZ-induced diabetic ($n = 5$), and *3*) intraperitoneally insulin-treated diabetic rats ($n = 10$). On day 0, catheters were placed into the left carotid artery and right jugular vein, as described previously (13). In diabetic and insulin-treated diabetic rats, diabetes was induced at the end of the surgery via a single injection of STZ (65 mg/kg; Sigma, St. Louis, MO), dissolved in sterile saline, through the penile vein. Control animals received a saline injection under similar conditions. Animals treated with STZ were given 10% sucrose water for 24 h after STZ injection to prevent hypoglycemia (13). This is a model of moderate diabetes characterized by fasting hyperglycemia and normal fasting plasma insulin, but reduced fed-state plasma insulin levels (14). Insulin-treated diabetic rats received Linplants (insulin ~2.5 units/day i.p.; Linshin Canada, Scarborough, Ontario, Canada), a sustained-release bovine insulin preparation, at 4 days after the induction of diabetes under light ketamine/acepromazine/xylazine anesthesia. The rationale and technical aspects of the insulin implant have been detailed previously (15). Blood glucose was monitored twice daily with a glucometer (Glucometer Elite3903; Bayer, Etobicoke, Ontario, Canada) in all animals to ensure that fasting normoglycemia was maintained in the control and insulin-treated diabetic groups, and that adequate hyperglycemia (>15 mmol/l) was achieved in the uncontrolled diabetic group. Although every effort was made to ensure with frequent monitoring that hypoglycemia did not arise in the insulin-treated diabetic rats during the day, this does not exclude the possibility that hypoglycemia may have occurred. Because these animals fed mainly during the dark cycle, the chance of hypoglycemia occurring at night was far more remote than during the day (the time during which they were monitored). The three animals that experienced hypoglycemic episodes were excluded from the study. The contents of the catheters were aspirated and reprimed with 60% polyvinylpyrrolidone solution (wt/vol of 1,000 units/ml heparin) on days 2, 4, and 6 to maintain catheter patency and acclimatize the rats to being handled.

Hypoglycemia. On day 7, the rats were fasted for 24 h before the start of the experiment. During the fasting period, insulin-treated diabetic animals received 5% sucrose water to prevent hypoglycemia (16). On day 8, the experiments were carried out in unrestrained conscious rats. Catheters were extended outside of the cage to minimize investigator interaction and were connected to infusion pumps. The animals were allowed 2.5 h to recover from handling stress before basal hormone samples were collected (at 10:30 A.M. and 11:00 A.M.), just before the start of the insulin infusion (11:00 A.M.). The rats then underwent a hyperinsulinemic-hypoglycemic glucose clamp. A constant insulin (50 $mU \cdot kg^{-1} \cdot min^{-1}$ regular porcine insulin; Eli Lilly, Indianapolis, IN) and variable dextrose (25%; Abbott Laboratories, Montreal, Québec, Canada) infusion through the jugular vein catheter were used to maintain plasma glucose levels at 2.2 ± 0.7 mmol/l for 130 min. The concentration of glucose was determined every 5 min from a 10-μl sample of plasma using a Beckman glucose analyzer II (Palo Alto, CA). Arterial blood samples were obtained from the carotid catheter at regular time intervals throughout the glucose clamp. For ACTH, glucagon, and insulin measurements, blood was collected in chilled tubes containing EDTA (Sangon Limited Canada, Scarborough, Ontario, Canada) and Trasylol (Bayer, Etobicoke, Ontario, Canada). Blood samples for catecholamines were collected in chilled tubes containing 1.5 mg reduced glutathione (Boehringer Mannheim, Mannheim, Germany) and 5 μl of EGTA (Sigma). Serum was collected for corticosterone measurements. Plasma was aliquoted into storage tubes and stored at −20°C (or −80°C for catecholamine determination). Erythrocytes, after removal of plasma, were resuspended in heparinized saline (10 units/ml) and reinfused after each blood sampling to prevent volume depletion and anemia. Hematocrit, determined at the beginning and at the end of the experiment, was maintained >35%. At the end of the experiment, the rats were killed by decapitation. Trunk blood samples were collected, and brains and pituitary glands were removed and stored at −80°C until sectioning.

In situ hybridization. The method of in situ hybridization has been described in detail previously (17). Coronal cryosections (12 μm) were obtained through selected hypothalamic (bregma −2.00 mm) and hippocampal (bregma −3.80 mm) regions according to stereotaxic coordinates of Paxinos and Watson (18). The sections were then thaw-mounted onto (poly)-L-lysine (Sigma)–coated slides, fixed for 5 min in 4% phosphate-buffered paraformaldehyde, rinsed in PBS (2 min), dehydrated in an ethanol series (70 and 95%), and stored in 95% ethanol at 4°C until use.

The 45-mer antisense CRH (bases 536–580) (19), AVP (bases 588–632) (19), POMC (bases 572–616) (19), GR1 (bases 2942–2986) (20), and GR2 (bases 1321–1365) (21) oligonucleotide probes were synthesized by Dalton Chemical Laboratories (Toronto, Ontario, Canada). The probes were labeled using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d'Urfé, Québec, Canada) and [^{35}S]deoxyadenosine 5′-(α-thio)triphosphate (1,300 Ci/mmol, NEN; DuPont Canada, Mississauga, Ontario, Canada) to a specific activity of 1.0×10^9 cpm/μg. Labeled probe in hybridization buffer (180 μl) was applied to each slide at a concentration of 1.0×10^6 cpm/μl. Slides were incubated overnight in a moist chamber at 42.5°C. After washing in 1× sodium chloride–sodium citrate (SSC) for 20 min at room temperature and 1× SSC for 35 min at 55°C, the slides were rinsed once with 1× SSC and with 0.1× SSC at room temperature, dehydrated in 70 and 95% ethanol (1 min each), air dried, and exposed to autoradiographic film (Biomax; Eastman Kodak, Rochester, NY). The films were developed using standard procedures (exposure: CRH, 21 days; AVP, 2 days; POMC, 2 h; GR1, 14 days; and GR2, 28 days).

To determine the molecular HPA responses to insulin-induced hypoglycemia, we used selected hypothalamic, hippocampal, and pituitary tissue sections collected from a previous study (22). These animals received the same three treatment regimens as above, but they were killed between 10:00 A.M. and 11:00 A.M. on day 8 for analysis of basal HPA function. Using in situ hybridization, CRH, AVP, POMC, and corticosteroid receptor mRNA responses to insulin-induced hypoglycemia were determined by simultaneously running selected "basal" tissue sections with tissue sections collected from animals that underwent the hypoglycemic clamp.

Plasma hormone and catecholamine determination. Plasma insulin was measured using a modified version of the insulin radioimmunoassay (RIA) by Herbert et al. (23). Plasma ACTH (Diasorin, Stillwater, MN), corticosterone (ICN Pharmaceuticals, Orangeburg, NY), and glucagon (Diagnostic Products, Los Angeles, CA) concentrations were determined using commercially available RIA kits.

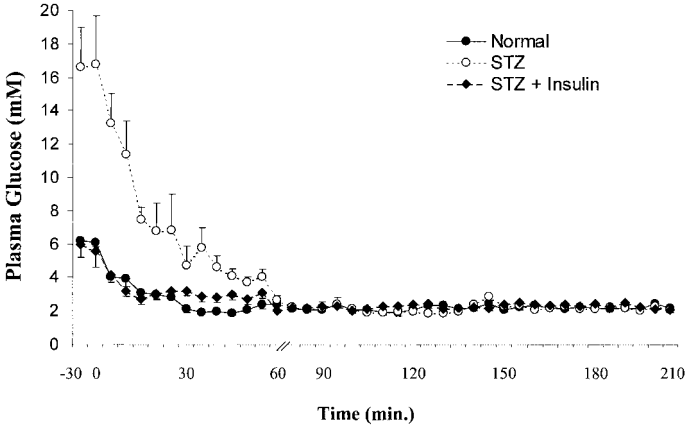

FIG. 1. Plasma glucose concentrations for normal (solid line), STZ-diabetic (dotted line), and IP insulin-treated diabetic rats (dashed line) during the hyperinsulinemic-hypoglycemic glucose clamp. Results are expressed as means ± SE.

Plasma epinephrine and norepinephrine concentrations were determined using the simultaneous single-isotope derivative radioenzymatic assay technique described previously (24).

Data analysis. For in situ hybridizations, brain sections were processed simultaneously for each probe to allow for direct comparison between treatment groups. Six to eight sections were selected from each animal for each probe, based on visual inspection of the desired region. The sections were exposed together with ^{14}C-standards (American Radiochemical, St. Louis, MO) to ensure analysis in the linear region of the autoradiographic film. The relative optical density (ROD) of the signal on autoradiographic film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research, St. Catherines, Ontario, Canada). Hormone data are presented as the means ± SE, and in situ hybridization data are expressed as ROD (means ± SE). Statistical analysis was by one- or two-way ANOVA for independent or repeated measures, as appropriate, using the Statistical Analysis System package for personal computers (SAS Institute, Cary, NC), with $P < 0.05$ as the criterion for statistical significance.

RESULTS

Body weight, plasma hormone, and catecholamine concentrations. Although body weights did not differ significantly between treatment groups either before surgery or 8 days after treatment, the decrease in body weight of STZ-induced diabetic rats after treatment was significant ($P < 0.05$) (day 0: 382.2 ± 14.1, 377.8 ± 19.1, and 344.6 ± 11.1 g for normal, STZ, and STZ + insulin, respectively; day 8: 357.3 ± 14.5, 324.2 ± 18.8, and 326.9 ± 8.6 g, respectively). Although hematocrit levels dropped significantly ($P < 0.05$) by the end of the clamp in all three groups, they were maintained >35% (basal: 48.3 ± 1.5, 50.2 ± 1.3, and 46.9 ± 1.7% for normal, STZ, and STZ + insulin, respectively; end of clamp: 39.7 ± 1.4, 38.7 ± 2.1, and 41.0 ± 1.3%, respectively).

Basal plasma glucose concentrations were increased ($P < 0.05$) by nearly threefold in diabetic rats compared with normal and insulin-treated diabetic animals (6.2 ± 0.3, 16.7 ± 2.6, and 5.6 ± 0.8 mmol/l for normal, STZ, and STZ + insulin, respectively). Plasma glucose levels during the hypoglycemic clamp are shown in Fig. 1. Basal plasma glucose levels of diabetic rats were almost three times higher ($P < 0.05$) than control and insulin-treated diabetic animals. The break in Fig. 1 indicates the period during which we ensured plasma glucose levels were matched between treatment groups before blood sampling was continued. Although diabetic animals started with higher basal plasma glucose concentrations, plasma glucose in all three groups was maintained at similar levels during the hypoglycemic period from 80 to 210 min.

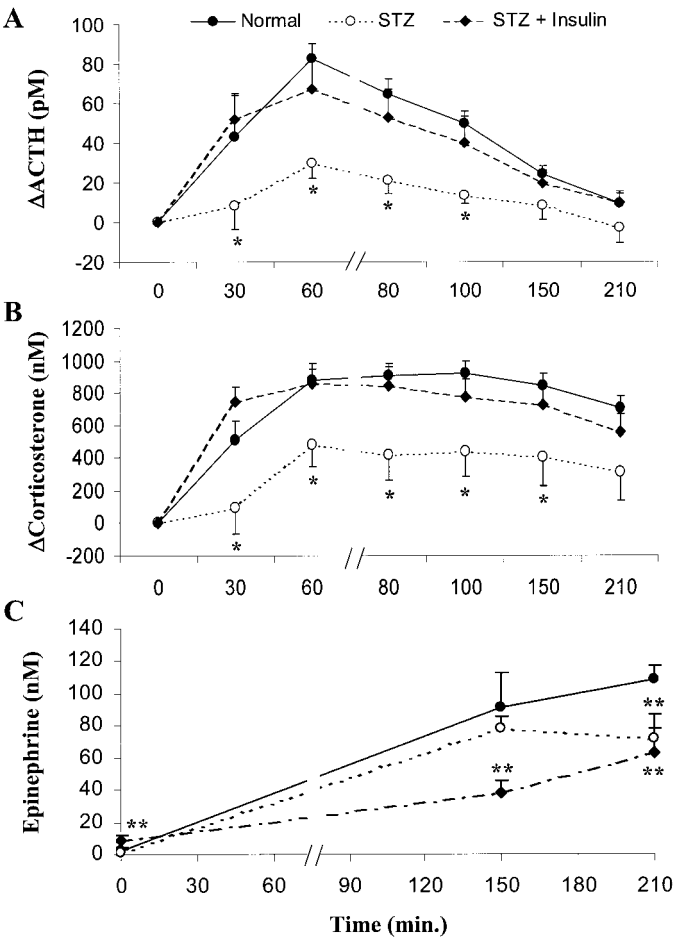

FIG. 2. Changes in plasma ACTH (*A*) and corticosterone concentrations (*B*) from basal in normal (solid line), STZ-inducd diabetic (dotted line), and insulin-treated diabetic rats (dashed line) during the hyperinsulinemic-hypoglycemic glucose clamp. *C*: Plasma epinephrine concentrations during the hyperinsulinemic-hypoglycemic glucose clamp. Results are expressed as means ± SE. *P <0.01 vs. normal and insulin-treated diabetic rats; **P <0.05 vs. normal rats.

Basal plasma ACTH (13.5 ± 1.7, 20.4 ± 4.2, and 16.6 ± 2.1 pmol/l for normal, STZ, and STZ + insulin, respectively) and corticosterone (320.7 ± 69.6, 618.3 ± 71.1, and 418.8 ± 95.1 nmol/l, respectively) concentrations were significantly ($P < 0.05$) elevated in diabetic compared with control and insulin-treated diabetic animals. During the fall in plasma glucose levels, the magnitude of the ACTH and corticosterone responses to hypoglycemia were similar in control and insulin-treated diabetic animals. However, the magnitude of the responses in diabetic animals was significantly ($P < 0.01$) blunted compared with control and insulin-treated diabetic animals (Fig. 2*A* and *B*). It was 67% lower for peak ACTH and 50% lower for peak corticosterone levels. When absolute values were compared, the responses of diabetic rats were still significantly ($P < 0.02$) lower than controls. In addition, significant differences were also observed during termination of the stress response. During this period, ACTH responses of control and insulin-treated diabetic animals were terminated significantly ($P < 0.05$) quicker than in diabetic animals.

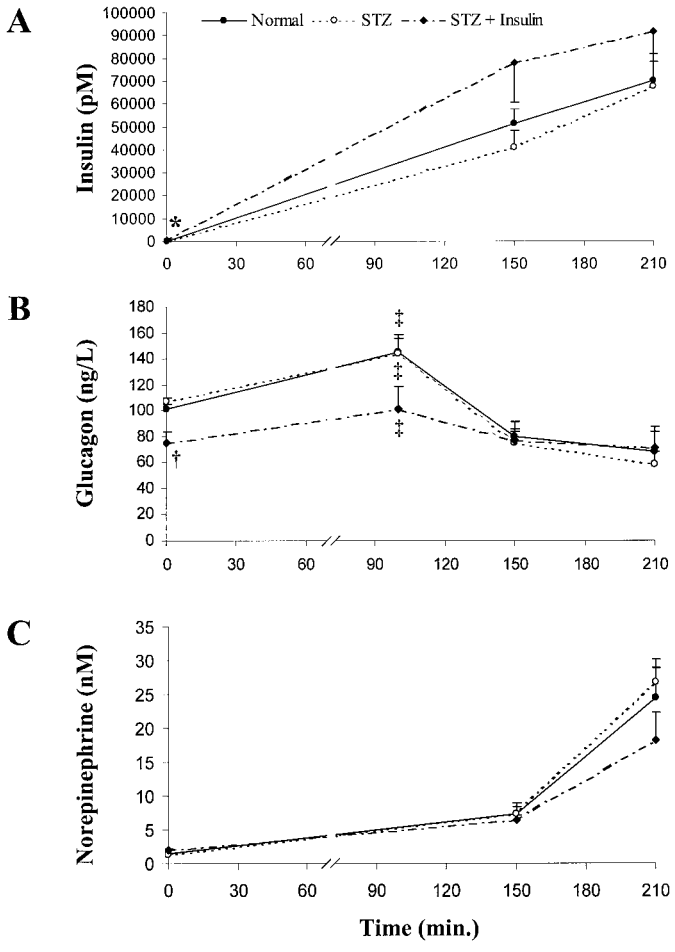

FIG. 3. Plasma insulin (*A*), glucagon (*B*), and norepinephrine concentrations (*C*) in normal (solid line), STZ-induced diabetic (dotted line), and insulin-treated diabetic rats (dashed line) during the hyperinsulinemic-hypoglycemic glucose clamp. Results are expressed as means ± SE. **P* <0.05 vs. normal and diabetic rats; †*P* < 0.05 vs. normal controls; ‡*P* < 0.05 vs. 0 min.

Fasting plasma insulin levels were similar between normal and diabetic rats. In insulin-treated diabetic rats, basal plasma insulin concentrations were eightfold higher ($P < 0.05$) than in normal and uncontrolled diabetic rats (Fig. 3*A*). These levels are comparable to postprandial insulin concentrations (25). The insulin levels achieved during the glucose clamp were similar between the three groups. Although plasma insulin levels appeared to be almost twice as high in the insulin-treated group at the 150-min time point, the difference was not statistically significant. The higher insulin levels may be attributed to the insulin implant. Although supraphysiological insulin doses can mildly stimulate the HPA axis (26), the response to hypoglycemia is much more pronounced and will likely override the response to hyperinsulinemia per se.

Although diabetic rats exhibited glucagon concentrations similar to those of control rats under basal and hypoglycemic conditions, the insulin-treated diabetic rats had significantly lower ($P < 0.05$) basal glucagon levels (Fig. 3*B*). Glucagon responses to hypoglycemia were not significantly different between the three groups; all groups exhibited significant ($P < 0.05$) rises from basal levels at the 100-min time point.

Plasma norepinephrine concentrations were similar in all groups under basal conditions and in response to hypoglycemia (20-fold increase) (Fig. 3*C*). Basal plasma epinephrine concentrations, however, were significantly ($P < 0.05$) lower in diabetic animals and elevated in insulin-treated diabetic animals, as compared with controls. In response to hypoglycemia, diabetic rats exhibited significantly ($P < 0.05$) diminished epinephrine responses at 210 min. Insulin treatment of diabetic animals did not restore the epinephrine response to hypoglycemia, which, in normal rats, increased by >40-fold (Fig. 2*C*).

Hypothalamic and pituitary neuropeptide expression. CRH mRNA was expressed throughout the hypothalamic paraventricular nucleus (PVN), with the highest abundance in the medial parvocellular region. After 8 days of moderate diabetes, CRH mRNA levels were significantly higher ($P < 0.01$) in diabetic animals compared with controls. Insulin treatment of diabetic rats did not restore basal CRH mRNA levels to normal. Hypoglycemia for 2 h resulted in significant ($P < 0.05$) elevations in CRH mRNA expression in control and insulin-treated diabetic animals, but not in diabetic animals (Fig. 4*A*).

AVP mRNA expression was observed in both the PVN and supraoptic nucleus (SON) of all groups. Insulin-treated diabetic rats had significantly ($P < 0.05$) elevated basal AVP mRNA levels in both the PVN and the SON compared with control and untreated diabetic rats (PVN: 5.6 ± 1.1, 6.5 ± 1.3, and 11.6 ± 1.3 ROD for normal, STZ, and STZ + insulin, respectively; SON: 13.6 ± 1.2, 14.7 ± 1.4, and 18.7 ± 0.7 ROD, respectively). After 2 h of hypoglycemia, there were no significant changes in AVP mRNA expression in the three groups when compared with their respective baseline levels (PVN: 7.7 ± 1.2, 7.0 ± 0.9, and 13.4 ± 1.3 ROD for normal, STZ, and STZ + insulin, respectively; SON: 17.4 ± 0.6, 19.5 ± 0.9, and 19.4 ± 0.6 ROD, respectively).

Expression of POMC mRNA was measured in the pars distalis (Fig. 4*B*). No significant differences in POMC mRNA expression were observed between the three groups under basal conditions. Control and insulin-treated diabetic animals exhibited significant ($P < 0.05$) increases in POMC mRNA content in response to hypoglycemia that were not seen in untreated diabetic animals.

Corticosteroid receptor expression. GR1 mRNA expression was localized to limbic structures in the rat brain—particularly, the CA1/2, CA3, and CA4 fields of the hippocampus and the dentate gyrus (Fig. 5). Basal GR1 mRNA levels were significantly ($P < 0.05$) elevated in diabetic and insulin-treated diabetic animals. After 2 h of hypoglycemia, GR1 mRNA expression decreased markedly ($P < 0.05$) in control and insulin-treated diabetic animals compared with their respective basal values. Conversely, in untreated diabetic animals, no changes in GR1 mRNA levels were observed in response to hypoglycemia.

GR2 mRNA expression was detected in the limbic system (CA1, CA2, CA3, and CA4 fields of the hippocampus and the dentate gyrus), the PVN, the ventromedial hypothalamus, the arcuate nucleus, and the pars distalis. Basal GR mRNA levels were significantly ($P < 0.05$) higher in the hippocampal CA3 region, PVN, and pars distalis of insulin-treated diabetic rats compared with normal and untreated diabetic rats. However, no significant changes in

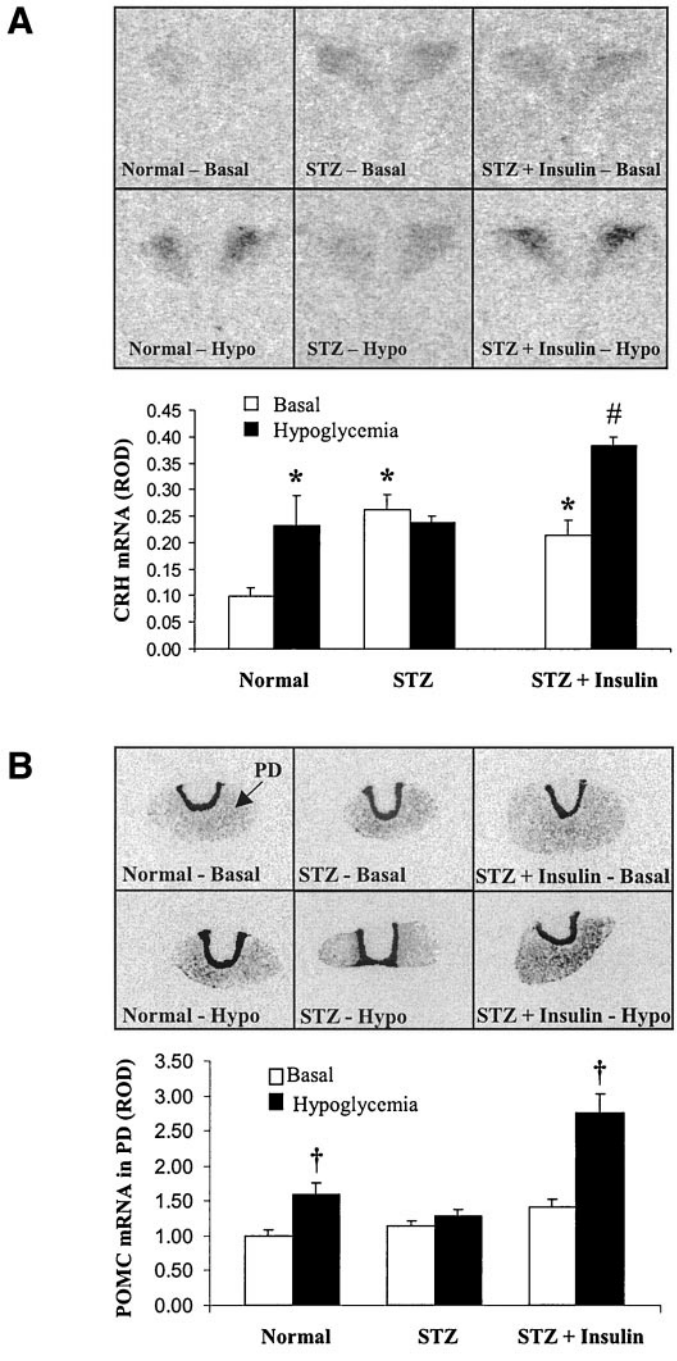

FIG. 4. Computerized images and densitometric analysis following in situ hybridization of CRH (*A*) and POMC mRNA expression (*B*) in the PVN and pars distalis (PD), respectively, of normal, STZ-induced diabetic, and insulin-treated diabetic rats under basal conditions and after insulin-induced hypoglycemia (Hypo). Results are expressed as means ± SE. **P* < 0.01 vs. normal − basal; #*P* < 0.05 vs. STZ + insulin − basal; †*P* < 0.05 vs. basal.

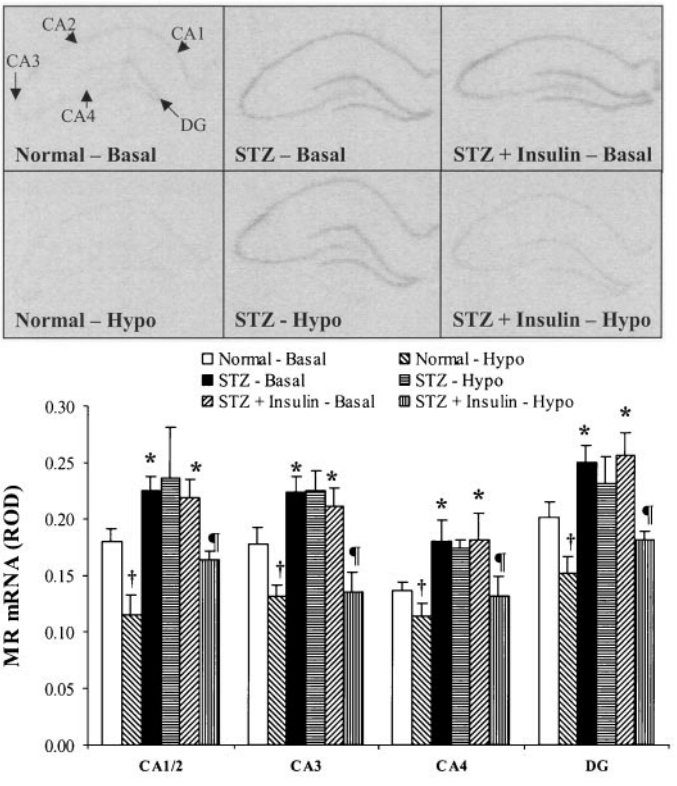

FIG. 5. Computerized images and densitometric analysis following in situ hybridization of mineralocorticoid receptor (MR) mRNA expression in the hippocampus (CA1–CA4) and dentate gyrus (DG) of normal, STZ-induced diabetic, and insulin-treated diabetic rats under basal conditions and after insulin-induced hypoglycemia (Hypo). Results are expressed as means ± SE. **P* < 0.05 vs. basal normal controls; †*P* < 0.05 vs. normal − basal; ¶*P* < 0.05 vs. STZ + insulin − basal.

GR2 mRNA expression were observed in response to hypoglycemia in the three treatment groups (Table 1).

DISCUSSION

The Diabetes Control and Complications Trial (DCCT) demonstrated that >60% of diabetic patients receiving intensive insulin therapy are at a threefold greater risk of severe hypoglycemia compared with those receiving conventional insulin treatment. With increased duration of diabetes and with poor glycemic control, most counterregulatory mechanisms become defective (27). As such, the reliance of these patients on the secretion of gluconeogenic hormones, such as epinephrine and glucocorticoids, is increased. Defects in these mechanisms can further compromise responses to hypoglycemia.

Hypoglycemia is a potent activator of the HPA axis (6). Studies in rats revealed both facilitated and inhibited HPA responses to hypoglycemia when a prior stressor was administered (28). Whether the response is facilitated or inhibited appears to depend on the intensity and the magnitude of the corticosterone response to the first stimulus (10). It has been suggested that exposure to high (stress) glucocorticoid levels suppresses the HPA response to hypoglycemia (10). Diabetes is characterized by high glucocorticoid concentrations and a lack of circadian periodicity in HPA activity, which may underlie unresponsiveness of the axis to stress (22,29–34). Davis et al. (35–37) suggested that partial impairment of the HPA counterregulatory response is due to recurrent exposure to high cortisol levels during hyperinsulinemic-euglycemic clamps. However, our work indicated that repeated exposure to elevated corticosterone in the absence of hyperinsulinemia does not diminish the HPA counterregulatory response (38). We therefore postulate that elevations in corticosterone affect the axis only in the presence of hyperinsulinemia. Therefore, the mechanism whereby elevated corticosterone concentrations in diabetes affect

TABLE 1
Densitometric analysis after in situ hybridization of glucocorticoid receptor mRNA expression in the limbic system (hippocampal CA1/2, CA3, and CA4 fields and dentate gyrus), the hypothalamus (arcuate nucleus, PVN, and ventromedial hypothalamus) and the pars distalis of normal, STZ-induced diabetic, and insulin-treated diabetic rats under basal conditions and after insulin-induced hypoglycemia

	Normal		STZ		STZ + insulin	
	Basal	Hypoglycemia	Basal	Hypoglycemia	Basal	Hypoglycemia
n	6	6	6	5	6	10
Limbic system						
CA1/2	15.0 ± 2.5	10.0 ± 1.3	13.5 ± 2.8	11.3 ± 0.5	20.0 ± 1.8	12.0 ± 0.5
CA3	7.8 ± 0.8	6.5 ± 0.5	6.8 ± 0.5	8.3 ± 0.5	8.8 ± 0.3*	6.6 ± 0.3
CA4	8.0 ± 1.5	6.5 ± 0.3	6.8 ± 0.8	7.8 ± 0.8	8.8 ± 0.5	6.8 ± 0.3
DG	15.5 ± 2.3	11.3 ± 1.3	13.8 ± 2.3	14.8 ± 0.5	19.8 ± 1.8	11.3 ± 0.8
Hypothalamus						
ARC	9.0 ± 1.5	9.5 ± 7.8	10.0 ± 0.8	15.8 ± 3.8	10.3 ± 1.5	14.0 ± 1.3
PVN	11.5 ± 0.75	9.8 ± 0.5	10.5 ± 1.3	12.0 ± 1.0	17.8 ± 2.5†	13.8 ± 0.8
VMH	8.0 ± 0.5	14.0 ± 2.0	6.5 ± 0.5	15.0 ± 1.5	8.3 ± 0.8	15.3 ± 1.0
Pars distalis	7.3 ± 1.5	10.3 ± 1.0	6.8 ± 0.5	11.0 ± 1.0	25.5 ± 1.5†	25.0 ± 1.0

Data are means ± SE of ROD. ARC, arcuate nucleus, DG, dentate gyrus, VMH, ventromedial hypothalamus. *$P < 0.05$ vs. STZ − basal; †$P < 0.05$ vs. normal − basal and STZ − basal.

HPA counterregulatory responses needs to be further explored.

In the present study, we identified a number of central molecular mechanisms that may contribute to the defective HPA response to hypoglycemia in diabetes. We report for the first time that basal hyperactivation of the HPA axis in STZ-induced diabetes results in profound changes in synthetic and secretory processes in response to hypoglycemia. These changes are characterized by reductions in ACTH and corticosterone secretion and a marked inability to increase hypothalamic CRH mRNA synthesis and decrease hippocampal GR1 mRNA expression. Impaired responsiveness of the diabetic HPA axis was normalized with insulin treatment.

ACTH and corticosterone responses to hypoglycemia were significantly reduced in diabetic animals compared with controls. Insulin treatment of diabetic animals normalized both responses. It has been suggested that the magnitude of the HPA response to hypoglycemia may be related to the rate of fall in blood glucose levels. In diabetic animals, blood glucose levels fell faster than in normal and insulin-treated diabetic animals. However, even with this greater rate of fall in blood glucose levels, the response in the diabetic animals was still smaller than in the other two groups. More importantly, peak corticosterone responses occurred at a time when plasma glucose levels were similar in the three groups. Peak corticosterone levels were still lower in the untreated diabetic group. Thus, we do not believe that an initial reduction in duration of hypoglycemia during the fall in plasma glucose levels influenced our results.

Our results are similar to observations made by Akana et al. (39), who demonstrated that intact rats treated chronically with stress levels of corticosterone were unable to generate an adequate HPA response to restraint stress. Moreover, Keller-Wood et al. (10) established in dogs that prior stimulation of the HPA axis with nitroprusside or insulin-induced hypoglycemia decreased the pituitary-adrenal response to a subsequent episode of hypoglycemia. These studies indicate that prior exposure to stress levels of glucocorticoids may impair the system's ability to respond to stress. In our study, not only was there a marked difference in the magnitude of pituitary-adrenal responses between control and diabetic animals, but these responses also terminated significantly more rapidly in control and insulin-treated diabetic rats than in diabetic animals. This is likely the result of decreased glucocorticoid negative-feedback sensitivity in diabetic rats (30,40,41). Thus, both activation and termination of the pituitary-adrenal response to hypoglycemia are impaired in the early stages of STZ-induced diabetes. The fact that insulin restored the pituitary-adrenal response to hypoglycemia in diabetic rats suggests that hypoinsulinemia and/or hyperglycemia may also play a role in attenuating the diabetic HPA response to hypoglycemia. In other studies, we exposed diabetic rats to repeated episodes of hyperinsulinemic hyperglycemia for 4 days and then measured the counterregulatory response to hypoglycemia (42). This treatment restored the corticosterone response to hypoglycemia, suggesting that hypoinsulinemia, rather than hyperglycemia, may impair the HPA response.

In the present study, basal epinephrine and its response to hypoglycemia were significantly lower in diabetic and insulin-treated diabetic rats than controls. This would suggest that the resulting reduction in sympathoadrenal system (SAS) activity may not be mediated solely by the actions of hypoinsulinemia and hyperglycemia, but it may involve glucocorticoid-mediated impairment of epinephrine release (4,35,43). The impaired epinephrine response is similar to the clinical situation in which epinephrine secretion in response to hypoglycemia is impaired with progression of type 1 diabetes (44–47). This is considered to be an acquired defect that is caused, at least in part, by alterations in glycemic thresholds (46). It is interesting that although insulin was effective at restoring the HPA response to hypoglycemia in diabetic animals, it failed to improve the epinephrine response. The fact that insulin concentrations were high during both the fasting and feeding state, and that blood glucose was higher than normal during feeding, could indicate the presence of residual insulin resistance. It is possible that deficiency of the epinephrine response to hypoglycemia could be related to incomplete correction of plasma glucose. It is of course possible that other hormonal and metabolic factors

were not normalized with insulin therapy. Further studies are required to determine the causal factor.

With respect to other counterregulatory hormones, no differences in glucagon or norepinephrine responses to hypoglycemia were observed between the three treatment groups. This suggests that in the presence of marked hyperinsulinemia in the early stages of diabetes, these counterregulatory systems are unaffected by the disorder (3). Insulin-treated diabetic rats did, however, have significantly lower basal plasma glucagon. This decrease is likely attributable to the suppressive actions of exogenous insulin on pancreatic α-cell function (48,49). In addition, high insulin concentrations during the hyperinsulinemic-hypoglycemic glucose clamp are presumably responsible for the small glucagon responses we observed. We previously reported much larger glucagon responses when less insulin was used (13). Despite the hyperinsulinemia, epinephrine, norepinephrine, and corticosterone responses were much greater than in the previous study (13), suggesting that this may be a more suitable model for the study of SAS and pituitary-adrenal responses to insulin-induced hypoglycemia.

Although basal hypothalamic CRH mRNA levels were higher in diabetic rats, there was no change in CRH mRNA levels in response to hypoglycemia. Conversely, CRH mRNA levels increased in response to hypoglycemia in both control and insulin-treated diabetic groups. The question remains of whether an increase in CRH mRNA is required to increase POMC synthesis, ACTH secretion, and, subsequently, corticosterone secretion during hypoglycemia. Reported CRH mRNA responses to hypoglycemia are varied and depend on experimental design and the duration of hypoglycemia. Our rats were fasted and conscious, and a great deal of care was taken to induce hypoglycemia without extraneous stress. We feel it is important that insulin be infused, rather than injected, to maintain consistent plasma insulin levels in all groups. Injection protocols result in the brain being exposed to inconsistent insulin levels because they tend to rise immediately after injection and fall thereafter. Our study indicates that an increase in CRH mRNA and, presumably, CRH is necessary to stimulate POMC synthesis, as was demonstrated by our diabetic rats. In these animals, CRH mRNA expression did not increase, despite exposure to the same level of hypoglycemia and hyperinsulinemia as normal and insulin-treated diabetic animals, resulting in unaltered POMC mRNA levels. Our work, however, also indicates that a small increase in ACTH can occur without an increase in CRH mRNA. Different stressors have been shown to engage different populations of hypophysiotropic neurons (50).

At this point, it is unclear whether other factors (such as leptin or urocortin) play a role in pituitary activation during hypoglycemia. Increases in CRH (51,52) and CRH mRNA (53) in response to hypoglycemia were not noticed in some studies. However, others have reported increased hypothalamic CRH mRNA levels in anesthetized rats (11) and after 3 h of hypoglycemia in conscious rats (12). These results indicate the importance of the duration of hypoglycemia and of avoiding anesthesia when analyzing HPA function. Presumably, changes in mRNA levels require ≥2 h to occur. Though studies have examined CRH mRNA responses to hypoglycemia, we are the first to compare the response in normal, diabetic, and insulin-treated diabetic rats.

No change in AVP mRNA expression in response to hypoglycemia was observed in any of the treatment groups. This was consistent with previous studies (53). In contrast, Plotsky et al. (51) reported that hypoglycemia increased immunoreactive AVP concentration in the hypophyseal portal circulation of anesthetized rats. In the rat, it is thought that AVP plays more of a synergistic role, whereas CRH is essential for mediating ACTH secretion during hypoglycemia (54).

POMC mRNA expression was increased in control and insulin-treated diabetic rats, but not in uncontrolled diabetic rats during insulin-induced hypoglycemia. Although several studies have demonstrated increases in pars distalis POMC mRNA expression in response to insulin-induced hypoglycemia in both freely moving (53) and anesthetized normal rats (52,54), the factors that mediate the rise have yet to be determined. Some have reported increases in POMC mRNA levels in the absence of either increased hypothalamic CRH mRNA or plasma CRH (53), whereas others suggest it is the rise in CRH that is primarily responsible for increase in POMC mRNA levels (54). This latter observation is consistent with our data because POMC mRNA levels were only elevated in control and insulin-treated diabetic rats, which not only increased hypothalamic CRH mRNA but also had larger ACTH responses to hypoglycemia. Because both CRH and POMC mRNA levels in diabetic rats were unaltered in response to hypoglycemia, it is conceivable that the lack of CRH synthesis and, presumably, secretion may have impaired both ACTH synthesis and secretion.

Hippocampal GR1 mRNA expression in control and insulin-treated diabetic animals decreased in response to hypoglycemia. The effects of hypoglycemia on hippocampal GR1 mRNA expression have not been explored previously. Basal GR1 mRNA levels were elevated in all hippocampal regions of diabetic and insulin-treated diabetic animals compared with controls. In response to hypoglycemia, however, hippocampal GR1 mRNA levels decreased in control and insulin-treated diabetic animals. Interestingly, hypoglycemia did not modify GR1 mRNA levels in uncontrolled diabetic rats. Normally, GR1 is thought to provide tonic inhibitory input to the PVN. The fact that hippocampal GR1 expression decreased in those groups of animals that demonstrated full HPA activation, the control and insulin-treated diabetic groups, suggests that these receptors may be important in the response to hypoglycemia. We hypothesize that the ability to decrease GR1 expression, and thus relieve inhibitory influences, may be important in determining responsiveness of the HPA axis to insulin-induced hypoglycemia. Thus, we speculate that control and insulin-treated diabetic animals are capable of responding to episodes of glucopenia with greater HPA drive than diabetic animals because they can decrease synthesis of hippocampal GR1s. This observation may, in part, explain why diabetic rats are unable to fully activate the HPA axis in response to hypoglycemia. Further studies are required to define the role of GR1s in HPA counterregulation to hypoglycemia.

No significant alterations in GR2 mRNA expression

were seen in the hippocampus, hypothalamus, or pars distalis during hypoglycemia in any of the treatment groups. Little is known about the effects of hypoglycemia per se on GR2 mRNA expression. Although GR2 mRNA levels were not altered in response to insulin-induced hypoglycemia, this does not exclude the possibility that occupation of GR2s may alter sensitivity of the axis to feedback. Previously, we demonstrated that pituitary and adrenal sensitivity were indeed decreased in STZ-induced diabetes (40).

In conclusion, we have demonstrated that in short-term diabetes, despite chronic activation of the HPA axis, the response to hypoglycemia was significantly attenuated. This could be due to a number of factors, including the inability to increase central drive (CRH and POMC) in response to a fall in plasma glucose, which in turn may be related to their inability to relieve tonic inhibition on the axis by decreasing hippocampal GR1 expression. Interestingly, insulin treatment was effective at reversing the effects of diabetes on the HPA response to hypoglycemia but not on SAS output. This is important because during profound hypoglycemia, epinephrine is an important counterregulatory hormone. These observations have provided possible new mechanisms by which one component of counterregulation, activation of the HPA axis, is impaired in uncontrolled diabetes.

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