Hyperglycemia does not increase basal hypothalamic-pituitary-adrenal activity in diabetes but it does impair the HPA response to insulin-induced hypoglycemia

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Submitted 1 October 2004; accepted in final form 1 March 2005

Chan, Owen, Karen Inouye, Eitan M. Akirav, Edward Park, Michael C. Riddell, Stephen G. Matthews, and Mladen Vranic. Hyperglycemia does not increase basal hypothalamic-pituitary-adrenal activity in diabetes but it does impair the HPA response to insulin-induced hypoglycemia. Am J Physiol Regul Integr Comp Physiol 289: R235–R246, 2005; doi:10.1152/ajpregu.00674.2004.—Recently, we established that hypothalamic-pituitary-adrenal (HPA) and counter-regulatory responses to insulin-induced hypoglycemia were impaired in uncontrolled streptozotocin (STZ)-diabetic (65 mg/kg) rats and insulin treatment restored most of these responses. In the current study, we used phloridzin to determine whether the restoration of blood glucose alone was sufficient to normalize HPA function in diabetes. Normal, diabetic, insulin-treated, and phloridzin-treated diabetic rats were either killed after 8 days or subjected to a hypoglycemic (40 mg/dl) glucose clamp. Basal: Elevated basal ACTH and corticosterone in STZ rats were normalized with insulin but not phloridzin. Increases in hypothalamic corticotrophin-releasing hormone (CRH) and inhibitory hippocampal mineralocorticoid receptor (MR) mRNA with STZ diabetes were not restored with either insulin or phloridzin treatments. Hypoglycemia: In response to hypoglycemia, rises in plasma ACTH and corticosterone were significantly lower in diabetic rats compared with controls. Insulin and phloridzin restored both ACTH and corticosterone responses in diabetic animals. Hypothalamic CRH mRNA and pituitary pro-opiomelanocortin (POMC) mRNA expression increased following 2 h of hypoglycemia in normal, insulin-treated, and phloridzin-treated diabetic but not in untreated diabetic rats. Arginine vasopressin mRNA was unaltered by hypoglycemia in all groups. Interestingly, hypoglycemia decreased hippocampal MR mRNA in control, insulin-, and phloridzin-treated diabetic rats but not untreated diabetic rats. Glucocorticoid receptor mRNA was not altered by hypoglycemia. In conclusion, despite elevated basal HPA activity, HPA responses to hypoglycemia were markedly reduced in uncontrolled diabetes. We speculate that defects in the CRH response may be related to a defective MR response. It is intriguing that phloridzin did not restore basal HPA activity but it restored the HPA response to hypoglycemia, suggesting that defects in basal HPA function in diabetes are due to insulin deficiency, but impaired responsiveness to hypoglycemia appears to stem from chronic hyperglycemia.

phloridzin; hypothalamic-pituitary-adrenal axis; streptozotocin
affected by diabetes using phloridzin treatment alone, suggesting that dysregulation of basal HPA function in diabetes may stem from the complex metabolic changes that occur in diabetes (11, 12) and not to hyperglycemia. On the other hand, even a partial improvement in plasma glucose can restore the HPA response to hypoglycemia in diabetic rats. This indicates that proper glucose control is crucial for the maintenance of proper HPA responses to insulin-induced hypoglycemia.

**RESEARCH DESIGN AND METHODS**

**Experimental Animals and Design**

Male Sprague-Dawley rats (Charles River, Québec, Canada) initially weighing between 325 and 375 g were individually housed in opaque cages in temperature (22–23°C)- and humidity-controlled rooms. 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 0700 and 1900) for a period of 1 wk before experimental manipulation. All experiments were approved by the Animal Care Committee at the University of Toronto and were conducted in accordance with guidelines set forth by the Canadian Council for Animal Care.

**Basal Studies**

Four groups of rats were used: 1) normal controls (n = 6), 2) untreated diabetic (n = 6), 3) insulin-treated diabetic (n = 6), and 4) phloridzin-treated diabetic rats (n = 6). Diabetes was induced with 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 to drink for the first 24 h after STZ injection to prevent hypoglycemia. This is a model of moderate diabetes characterized by fasting hyperglycemia and near normal fasting plasma insulin but reduced fed-state plasma insulin levels (16).

Four days following the induction of diabetes, two subgroups of diabetic animals received either a slow-release insulin implant or were started on a phloridzin treatment regimen. Insulin-treated diabetic rats received Linplants (~2.5 U of insulin/day); Linshin Canada, Scarborough, Ont., Canada), a sustained release bovine insulin preparation, under light ketamine/acepromazine/xylazine anesthesia. The rationale and technical aspects of the insulin implant have been detailed previously (59). The phloridzin treatment regimen consisted of two daily subcutaneous injections of phloridzin (0.4 g/kg per injection; Sigma) dissolved in warm 40% propylene glycol (52). These injections were administered at 0900 and 1300 and blood glucose was monitored using a glucometer (Glucometer Elite 3903, Bayer, Etobicoke, ON, Canada) every hour to ensure that normoglycemia was maintained and that hypoglycemia did not occur during the treatment period. Blood glucose levels in the other groups were monitored twice daily (at 0800 and 1300) to ensure that fasting normoglycemia was maintained in the control and insulin-treated diabetic groups and that appropriate hyperglycemia (>15 mM) was achieved in the uncontrolled diabetic group. Although every effort was made to ensure that hypoglycemia did not arise in diabetic animals treated with either insulin or phloridzin by frequent monitoring of blood glucose levels, this does not exclude the possibility that hypoglycemia may have occurred. As rats primarily feed in the dark phase, the chances of hypoglycemia occurring at night were far more remote than during the day, the time during which they were monitored. Those animals that experienced hypoglycemic episodes were excluded from the study. To maintain parity with the glucose clamp studies performed in fasted animals in the second part of this study, the rats were fasted for 24 h before being euthanized on day 8. During the fasting period, insulin and phloridzin-treated animals received a 5% sucrose solution in place of their regular drinking water to prevent hypoglycemia and maintain normal blood glucose levels (25).

Eight days following the initial injection of saline or STZ, the rats were euthanized between 1000 and 1100 by decapitation. The brain and pituitary gland were quickly removed under sterile conditions. The hippocampus was dissected out from the left half of the brain to allow for comparison of corticosterone receptor protein levels with receptor mRNA expression in the right half of the brain. Tissues were frozen on dry ice and stored at −80°C until processing for in situ hybridization or Western blot analysis.

**Hyperinsulinemic-Hypoglycemic Glucose Clamp Studies**

In this series of studies, we used some data for normal, uncontrolled diabetic, and insulin-treated diabetic rats that were obtained from a previous study (7) and now include two additional animals in each group to form an expanded study group (n = 8, 7, and 12, respectively) to allow proper comparisons to be made.

Briefly, on day 0, catheters were placed into the left carotid artery and right jugular vein, as described previously (24). Diabetes was induced at the end of surgery with a single injection of STZ (65 mg/kg) dissolved in sterile saline, through the penile vein. Four days after surgery, two subgroups of diabetic animals started either the insulin or phloridzin (n = 6) treatment regimen, as described above.

The contents of the catheters were aspirated and reprimed with 60% polyvinyl-pyroli dine solution (wt/vol of 1,000 U/ml heparin) on days 2, 4, and 6 to maintain catheter patency and to acclimatize the rats to being handled.

**Hypoglycemia.** On day 7, the rats were fasted for 24 h before the start of the experiment as mentioned above. Subsequently, on day 8, we carried out the glucose clamp experiments in conscious, unrestrained rats. Catheters were extended outside of the cage to minimize investigator interaction and were connected to infusion pumps. The animals were then left undisturbed for 2.5 h before taking basal hormone samples at 1030 and 1100 and just before the start of the insulin infusion (1100). The rats then underwent a hyperinsulinemic-hypoglycemic glucose clamp. A constant insulin (50 mU·kg−1·min−1) and variable dextrose (25%) infusion through the jugular vein catheter were used to maintain plasma glucose levels at 40 ± 5 mg/dl 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. Arterial blood samples were obtained from the carotid catheter at regular time intervals throughout the glucose clamp. Plasma samples were collected for determination of ACTH, corticosterone, glucagon, insulin, and catecholamines at regular time intervals throughout the course of the experiment. Plasma was stored at −20°C (or −80°C for catecholamine determination). Red blood cells, after removal of plasma, were resuspended in heparinized saline (10 U/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 above 35%. At the end of the experiment, the rats were euthanized 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 previously in detail (37). Briefly, coronal cryosections (12 μm) were obtained through selected hypothalamic (bregma ~2.00 mm) and hippocampal (bregma ~3.80 mm) regions according to the stereotaxic coordinates of Paxinos and Watson (41). The sections were then thaw-mounted onto (poly)-l-lysine (Sigma)-coated slides, fixed for 5 min in 4% phosphate-buffered paraformaldehyde, rinsed in phosphate-buffered saline (2 min), dehydrated in an ethanol series (70 and 95%), and stored in 95% ethanol at 4°C until use.

The 45 α-mers antisense corticotrophin-releasing hormones (CRH; bases 536–580) (32), arginine vasopressin (AVP; bases 588–632) (32), proopimelanocortin (POMC; bases 572–616) (32), mineralocorti-
corticoid receptor (MR; bases 2942–2986) (36), and glucocorticoid receptor (GR; bases 1321–1365) (38) 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 [35S]deoxyadenosine 5’-(α-thio)triphosphate (1,300 Ci/mmol, New England Nuclear, DuPont Canada, Mississauga, Ontario, Canada) to a specific activity of 1.0 × 10^6 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 being washed in 1× SSC (20 min at room temperature), 1× SSC (35 min at 55°C), the slides were rinsed twice with 1× SSC and once with 0.1× SSC at room temperature, then 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, 35 days; AVP, 2 days; POMC, 2 h; MR, 14 days; and GR, 28 days).

To determine the molecular HPA responses to insulin-induced hypoglycemia using in situ hybridization, we simultaneously ran the "basal" tissue sections collected from the first part of this study with tissue sections collected from animals that underwent the hypoglycemic clamp. These in situ hybridization experiments were run using new slides containing hippocampal, hypothalamic, and pituitary sections from normal, uncontrolled diabetic, and insulin-treated diabetic animals collected from a previous study (7) in which the animals underwent the same hypoglycemic clamp as the phloridzin-treated animals collected from a previous study (7) in which the animals were developed using standard procedures (exposure: CRH, 35 days; AVP, 2 days; POMC, 2 h; MR, 14 days; and GR, 28 days).

Western Blot Analysis

Western Blot analysis was undertaken as described previously (40). Frozen hippocampal tissues were homogenized in radioimmunoprecipitation (RIPA) lysis buffer. Protein content was assessed with the Bradford protein assay. Equal amounts of protein were resolved under reducing conditions in an 8% SDS-polyacrylamide gel. Protein samples were then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Success of the protein transfer was assessed by exposure of the nitrocellulose membranes to Ponceau S solution (Sigma). This was followed by a thorough washing before soaking in 5% blocking solution overnight.

The membranes were washed using 0.01 M PBS-Tween (PBS-T) and incubated with primary antibody for 1 h. Primary antibodies used were as follows: rabbit anti-MR (1:200 dilution, MCR H-300, sc-11412, Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-GR (1:500 dilution, GR H-300, sc-8992, Santa Cruz Biotechnology), and rabbit anti-tubulin (1:5,000 dilution, T-3526, Sigma, Oakville, ON, Canada). Each primary antibody was run on a separate day. Membranes were then washed with PBS-T and incubated for an additional hour with a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (1:5,000 dilution, NEF812, New England Nuclear Life Science Products, Boston, MA). After incubation with the secondary antibody, the membranes were washed and immersed into chemiluminescence (PerkinElmer, Life Sciences, Boston, MA). The membranes were then exposed to X-ray film (X-Omat LS, Kodak, Eastman Kodak). Film analysis was undertaken using a computerized image analysis system (Imaging Research) by individuals who were blinded to the experimental treatment groups. Protein levels were expressed as a ratio between the protein of interest and tubulin to correct for loading and transfer.

**Results**

**Basal**

Plasma hormone concentrations. STZ-diabetic rats exhibited significantly (P < 0.001) elevated basal blood glucose concentrations throughout the course of the day compared with control animals (Fig. 1A). “Fed” blood glucose values (taken at 0800 or 1 h after lights on) were moderately elevated in insulin- and phloridzin-treated animals compared with normal controls (P < 0.05). Both treatments normalized “fasting” blood glucose levels (taken at 1300 or 6 h after lights on) in diabetic animals. As rats are primarily nocturnal feeders, we took the “fed” blood glucose levels as close to the time of lights on as possible. Because the animals were fasted for 24 h just before being studied on day 8, this protocol allowed us to obtain fed and fasted blood glucose measurements without subjecting the animals to a second fast during the 1-wk duration of this study.

Basal plasma ACTH and corticosterone (Fig. 2) concentrations were significantly (P < 0.05) higher in uncontrolled diabetic rats compared with normal controls. Although insulin treatment restored both basal ACTH and corticosterone levels to normal, phloridzin treatment failed to do so.

Fasting basal plasma insulin concentrations were similar between normal, uncontrolled diabetic, and phloridzin-treated diabetic rats (Table 1). Insulin treatment resulted in significantly (P < 0.01) elevated insulin levels compared with all other treatment groups. These latter insulin levels are similar to fed-state insulin concentrations.

No significant treatment effect (P = 0.16) was observed for plasma free fatty acid levels and plasma ketone levels came up negative for all treatment groups (Table 2).

Basal plasma glucagon concentrations in uncontrolled diabetic rats were not significantly different from normal controls.
(Table 3). However, glucagon levels were significantly \( P < 0.05 \) lower in insulin- and phloridzin-treated diabetic rats compared with the other two treatment groups.

Basal norepinephrine levels were not altered by diabetes and neither insulin nor phloridzin treatments modified these levels significantly (Table 1). Basal plasma epinephrine concentrations were, however, significantly \( P < 0.05 \) lower in diabetic rats compared with normal controls (Fig. 3). Insulin, but not phloridzin treatment, raised basal plasma epinephrine levels markedly above normal \( P < 0.01 \).

No significant treatment effects were noted for plasma free fatty acid levels \( (P = 0.16) \) or plasma osmolality \( (P = 0.44) \); Table 2). Results for the presence of ketone bodies came up negative for all treatment groups (Table 2).

**Hypothalamic and Pituitary Neuropeptide Expression**

After 8 days of moderate diabetes, basal CRH mRNA levels were significantly \( P < 0.05 \) higher in diabetic animals compared with controls (Fig. 4). Fisher LSD post hoc analysis revealed that insulin treatment further increased \( P < 0.05 \) and that phloridzin treatment failed to decrease CRH mRNA levels in diabetic animals.

AVP mRNA was highly expressed in both the magnocellular and parvocellular neurons of the PVN and the magnocellular neurons of the SON. No differences in AVP mRNA levels were observed between normal, uncontrolled diabetic, and phloridzin-treated diabetic rats in the PVN (Table 4). Insulin treatment did, however, increase AVP mRNA levels in both the PVN \( (P < 0.02) \) and SON \( (P < 0.01) \), whereas phloridzin treatment decreased AVP mRNA levels significantly \( P < 0.001 \) in the SON compared with the other treatment groups.

POMC mRNA was expressed at high levels in the anterior pituitary. There were no differences in basal POMC mRNA levels between the four treatment groups (Fig. 5).

**Corticosteroid Receptor Expression**

MR mRNA expression was almost exclusively localized to limbic structures in the rat brain (Table 5 and Fig. 6). Compared with control animals, diabetic rats had significantly increased MR mRNA content throughout all fields of the hippocampus (CA1/2, CA3, and CA4) and in the dentate gyrus \( (P < 0.05) \). In the insulin- and phloridzin-treated diabetic animals, limbic MR mRNA levels remained elevated above control values \( P < 0.05 \) and were similar to those observed in untreated diabetic animals.

Expression of GR mRNA was observed in several regions within the rat brain. High levels of GR mRNA were detected in the hippocampus, PVN, and anterior pituitary (Table 6). No significant differences in GR mRNA expression were detected between control and diabetic animals in any of the limbic regions. Insulin treatment, however, resulted in significantly \( (P < 0.05) \) increased GR mRNA expression in the PVN and the anterior pituitary in diabetic animals compared with normal controls.

The relationship between hippocampal corticosteroid receptor mRNA and protein levels was established in this study by comparing mRNA expression in the right half of the brain (as assessed by in situ hybridization) with receptor protein levels in the left half of the brain (as assessed by Western blot analysis) in each animal. Mineralocorticoid and glucocorticoid receptor protein levels mirrored the results found in the mRNA data (Fig. 7). Hippocampal MR protein levels were significantly \( (P < 0.05) \) elevated in all diabetic groups compared with controls, whereas no difference in hippocampal GR proteins were seen between the four treatment groups. Correlation analysis revealed that both hippocampal MR mRNA \( (r = 0.89; P < 0.0001) \) and GR mRNA \( (r = 0.67; P < 0.001) \) expression was strongly associated with receptor protein expression.

**Hypoglycemia Studies**

**Plasma hormone and catecholamine concentrations.** Plasma glucose levels were maintained at similar levels between normal and diabetic animals during the hypoglycemic clamping period from 80 to 210 min (Fig. 1B).

During the fall in plasma glucose levels, the magnitude of the ACTH and corticosterone responses to hypoglycemia were greatly diminished in uncontrolled diabetic animals \( P < 0.01 \). However, insulin and phloridzin treatments normalized the pituitary-adrenal response to hypoglycemia in diabetic rats (Fig. 2).

Glucagon responses to hypoglycemia, as determined by both ANOVA and AUC, were not significantly different between the treatment groups (Table 3). Insulin levels achieved during the glucose clamp were similar between normal and all diabetic groups (Table 1).

Plasma norepinephrine responses to hypoglycemia, as determined by both ANOVA and AUC, were similar between all
treatment groups (Table 1). In response to hypoglycemia, diabetic rats exhibited significantly ($P < 0.05$) diminished epinephrine responses at 210 min (Fig. 3). Insulin treatment did not, whereas phloridzin treatment did, reverse the epinephrine response to hypoglycemia in diabetic rats ($P < 0.05$).

Hypothalamic and pituitary neuropeptide expression. Following hypoglycemia, we observed a significantly ($P < 0.01$) attenuated CRH response to hypoglycemia in diabetic animals. Both insulin and phloridzin treatments restored this defect in diabetic animals (Fig. 4).

AVP mRNA expression was observed in both the PVN and SON of all groups. No significant changes in AVP mRNA expression in the four treatment groups were observed following hypoglycemia (Table 4).

Table 1. Plasma insulin and norepinephrine concentrations in normal (n = 8), STZ (n = 7), STZ + insulin (n = 12), and STZ + phloridzin (n = 6) rats during the hyperinsulinemic-hypoglycemic glucose clamp

<table>
<thead>
<tr>
<th>Time, min</th>
<th>0</th>
<th>150</th>
<th>210</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin, µU/ml</td>
<td>16.9±4.6</td>
<td>8,025.1±850.0</td>
<td>10,540.3±1,262.6</td>
</tr>
<tr>
<td>STZ</td>
<td>14.2±2.6</td>
<td>6,623.3±1,000.4</td>
<td>10,229.0±2,208.4</td>
</tr>
<tr>
<td>STZ + insulin</td>
<td>111.5±18.5*</td>
<td>13,012.3±2,931.6</td>
<td>13,972.6±1,972.3</td>
</tr>
<tr>
<td>STZ + phloridzin</td>
<td>8.0±1.1</td>
<td>10,633.3±3,536.8</td>
<td>13,395.8±4,097.3</td>
</tr>
<tr>
<td>Norepinephrine, pg/ml</td>
<td>222.3±19.3</td>
<td>1,147.3±137.6</td>
<td>3,308.9±768.8</td>
</tr>
<tr>
<td>STZ</td>
<td>185.4±26.3</td>
<td>1,093.1±213.8</td>
<td>3,499.9±213.8</td>
</tr>
<tr>
<td>STZ + insulin</td>
<td>303.9±49.5</td>
<td>1,069.9±94.5</td>
<td>2,516.4±94.5</td>
</tr>
<tr>
<td>STZ + phloridzin</td>
<td>217.9±44.4</td>
<td>4,210.4±1,076.0</td>
<td>7,747.3±2,165.9</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. STZ, streptozotocin-diabetic; STZ + insulin, insulin-treated diabetic; STZ + phloridzin, phloridzin-treated diabetic. *$P < 0.01$ vs. normal, STZ and STZ + phloridzin.

Table 2. Basal plasma osmolarity, FFA, and ketone body concentrations in normal (n = 6), STZ (n = 6), STZ + insulin (n = 6), and STZ + phloridzin (n = 6)

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>STZ</th>
<th>STZ + Insulin</th>
<th>STZ + Phloridzin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmolarity, mOsm</td>
<td>310±2</td>
<td>318±6</td>
<td>309±6</td>
<td>324±12</td>
</tr>
<tr>
<td>FFA, mM</td>
<td>0.9±0.1</td>
<td>1.1±0.2</td>
<td>0.6±0.1</td>
<td>1.3±0.4</td>
</tr>
<tr>
<td>Ketones</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

All values are expressed as means ± SE. FFA, free fatty acid.
Anterior pituitary POMC mRNA levels did not increase in untreated diabetic rats following 2 h of hypoglycemia (Fig. 5). In contrast, control, insulin-treated, and phloridzin-treated diabetic animals exhibited significant ($P < 0.04$) increases in pituitary POMC mRNA levels in response to hypoglycemia.

Corticosteroid Receptor Expression

Following 2 h of hypoglycemia, hippocampal MR mRNA expression decreased markedly ($P < 0.05$) in all regions of control, insulin-treated, and phloridzin-treated diabetic animals compared with their respective basal values (Table 5 and Fig. 6). However, no changes in MR mRNA levels were observed in untreated diabetic animals in response to hypoglycemia.

No significant changes in GR mRNA expression were observed in response to hypoglycemia in the four treatment groups (Table 6).

**DISCUSSION**

We previously showed that dysregulation of basal HPA function in diabetes is associated with increased plasma ACTH and corticosterone and elevated hypothalamic CRH and hippocampal MR mRNA (8). Although we were able to normalize pituitary-adrenal function using insulin, central alterations in HPA function remained upregulated. Moreover, the HPA response to insulin-induced hypoglycemia was diminished in uncontrolled STZ-diabetic rats and insulin restored the response to normal (7). However, with insulin treatment, we restored both insulin and fasting glucose levels. Therefore, the question remained as to whether hypoinsulinemia and/or hyperglycemia are responsible for HPA dysfunction in diabetes. In the current study, we used phloridzin to normalize fasting blood glucose levels without insulin. We report for the first time that dysregulation of basal HPA function in diabetes may not be mediated by hyperglycemia per se, as partial correction of plasma glucose with phloridzin failed to reestablish basal HPA parameters to normal. On the other hand, hyperglycemia appeared to play a crucial role in impairing the HPA response to insulin-induced hypoglycemia in diabetes.
intermediate between normal and uncontrolled STZ animals, supporting the notion that basal plasma epinephrine levels may be affected by a balance between plasma insulin and glucocorticoid concentrations.

The lower basal plasma glucagon levels in insulin-treated diabetic animals may stem from suppressed glucagon release by the high plasma insulin levels resulting from the insulin implant. As seen previously, plasma glucagon levels were normal in STZ-diabetic rats treated with phloridzin (17, 52). The decrease in plasma glucagon in the current study was surprising but it may be the result of postprandial hyperglycemia.

Activation of the HPA axis is due to the release of CRH and AVP. Basal hypothalamic CRH mRNA levels were elevated in all diabetic animals. Because insulin did not lower CRH mRNA in STZ rats, the results in the phloridzin group were not surprising, as changes in other metabolic parameters with diabetes may contribute centrally to dysregulation of the HPA axis. In the rat, AVP is thought to play a synergistic role with CRH to enhance the release of ACTH from pituitary corticotrophs. By itself, AVP does not have a significant stimulatory effect on ACTH secretion. In some chronic stress paradigms, the HPA axis may actually switch from being primarily driven by CRH to being driven by AVP (26, 34, 35). However, in our short-term model of diabetes, we did not observe changes in AVP mRNA in the mixed parvocellular and magnocellular neuronal populations of the PVN or the magnocellular neurons of the SON. Insulin itself may have contributed to the increase in AVP mRNA in the PVN and SON as we did not see these changes in phloridzin-treated animals nor did we see any changes in plasma osmolarity between treatment groups.

Despite differences in plasma ACTH concentrations, no corresponding changes in POMC mRNA levels were detected in the anterior pituitary. The abundance of POMC and ACTH that are stored in pituitary corticotrophs may have masked subtle changes that occurred over the short duration of our study (1, 54).

The corticosteroid receptor system, which inhibits the HPA axis, was altered in STZ diabetes. Although diabetes itself did not affect GR mRNA expression in the brain, insulin increased expression in both the PVN and anterior pituitary. As suggested before, this regionally specific increase in GR mRNA

Table 4. Densitometric analysis following in situ hybridization of AVP mRNA expression in the PVN and SON of normal, STZ, STZ + insulin, and STZ + phloridzin rats under basal conditions and following Hypo

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>STZ</th>
<th>STZ + Insulin</th>
<th>STZ + Phloridzin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVN Basal</td>
<td>0.6±0.1</td>
<td>0.7±0.1</td>
<td>1.2±0.1‡</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>PVN Hypo</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
<td>1.4±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>SON Basal</td>
<td>1.4±0.1</td>
<td>1.5±0.1</td>
<td>1.9±0.1*</td>
<td>0.8±0.1†</td>
</tr>
<tr>
<td>SON Hypo</td>
<td>1.5±0.1</td>
<td>1.4±0.1</td>
<td>1.9±0.1</td>
<td>1.0±0.1</td>
</tr>
</tbody>
</table>

Results are presented as means ± SE relative optical density (ROD). *P < 0.01 vs. normal-basal and STZ-basal. ‡P < 0.001 vs. normal-basal and STZ-basal. †P < 0.02 vs. normal-basal. PVN, paraventricular nucleus; SON, supraoptic nucleus; Hypo, insulin-induced hypoglycemia.
may contribute to the normalization of pituitary-adrenal function in diabetes (8). Basal MR mRNA levels were elevated in the hippocampus of diabetic animals. This defect was not corrected with either insulin or phloridzin treatment. We postulate that the increase in hippocampal MR mRNA in STZ diabetes may be caused by factors other than insulin or hyperglycemia but is not related to changes in plasma osmolarity, ketones, or free fatty acids. More importantly, the current study also demonstrated that basal hippocampal corticosteroid receptor mRNA is highly correlated with receptor protein expression. This relationship is very important as it allows us to extrapolate our findings with mRNA to the protein level, at least when examining basal HPA regulation.

Hypoglycemia

Hyperglycemia alone appears sufficient to impair the HPA response to hypoglycemia. During insulin-induced hyperglycemia, rises in plasma ACTH and corticosterone levels were significantly impaired in diabetic animals. Insulin and phloridzin treatments were able to normalize the pituitary-adrenal response, suggesting that the HPA response to hyperglycemia is glucose sensitive. The mechanism by which this occurs remains unknown. It is possible that glucose toxicity may contribute to impairment of HPA counterregulation. As the sensing of falling blood glucose levels is crucial for glucose counterregulation, disruption of key glucose sensing mechanisms in hypothalamic neurons may be responsible for the attenuated response. The PVN contains neurons that are responsive to insulin and glucose administration (6). Increases in the number of Fos-like immunoreactive neurons in the PVN of rats with glucose infused into the brain have been reported (18, 31). In addition, the DMH has been shown to be important in regulating the HPA response to hypoglycemia, as inactivation of DMH neurons, which project to the PVN, impairs the HPA response to hypoglycemia (19). This suggests that these neurons may play a role in integrating and initiating the HPA response to hypoglycemia. Whether glucose itself can directly impair the ability of such neurons from responding to hypoglycemia remains to be elucidated. More importantly, impairment of the HPA response to hypoglycemia does not appear to stem from prior exposure to high glucocorticoid levels as phloridzin animals exhibited normal HPA responses to hypoglycemia, despite the fact that these animals maintained high circulating basal corticosterone levels.

After the induction of hypoglycemia, plasma insulin levels were not significantly different between normal and phloridzin-treated animals. Given the results we observed with ACTH and corticosterone, and the fact that insulin levels were not normalized with phloridzin, we hypothesize that hyperglycemia may represent the main factor impairing the HPA response to hypoglycemia in diabetes.

Uncontrolled diabetic animals exhibited impaired epinephrine responses to hypoglycemia. Although insulin failed to restore this response to normal, phloridzin treatment actually increased the sympathoadrenal response to hypoglycemia. A deficient epinephrine response to hypoglycemia occurs in diabetic patients and animals (33). In the present study, epinephrine responses in both diabetic and insulin-treated diabetic rats were attenuated. Antecedent hypoglycemia can result in reduced counterregulatory responses to subsequent hypoglycemia (21). It is possible that because of the presence of hyperinsulinemia, some of the insulin-treated rats may have developed hypoglycemia that was not detected by our periodic monitoring. Phloridzin treatment, however, produced epinephrine responses that were greater than in normal rats, suggesting that correction of hyperglycemia can improve epinephrine responses in diabetes. In contrast to our data, observations of an attenuated catecholamine response made in alloxan-diabetic dogs receiving phloridzin treatment for 1–2 days have been reported (23). Although the mechanism of these phloridzin-related changes in catecholamine levels is unknown, differences between this study and the current one may be attributed to the duration and severity of diabetes, as well as the shorter duration of treatment.

In response to hypoglycemia, all groups, except the uncontrolled diabetic rats, responded with an increase in CRH mRNA. These data correlate well with what we observed in the

### Table 5. Densitometric analysis following in situ hybridization of MR mRNA expression in the hippocampal CA1/2 and CA4 fields and DG of normal, STZ-diabetic, insulin-treated diabetic, and phloridzin-treated diabetic rats under basal conditions and following Hypo

<table>
<thead>
<tr>
<th></th>
<th>Normal Basal (n = 6)</th>
<th>Hypo (n = 8)</th>
<th>STZ Basal (n = 6)</th>
<th>Hypo (n = 7)</th>
<th>STZ + Insulin Basal (n = 6)</th>
<th>Hypo (n = 12)</th>
<th>STZ + Phloridzin Basal (n = 6)</th>
<th>Hypo (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1/2</td>
<td>6.0 ± 1.0</td>
<td>3.5 ± 0.4†</td>
<td>10.4 ± 1.5*</td>
<td>9.6 ± 1.0</td>
<td>12.6 ± 1.0*</td>
<td>3.9 ± 0.3†</td>
<td>10.2 ± 0.1†</td>
<td>5.0 ± 0.6†</td>
</tr>
<tr>
<td>CA4</td>
<td>4.5 ± 0.4</td>
<td>3.0 ± 0.4†</td>
<td>6.5 ± 0.6*</td>
<td>5.5 ± 0.5</td>
<td>7.5 ± 0.4*</td>
<td>3.1 ± 0.2†</td>
<td>7.4 ± 0.2*</td>
<td>4.0 ± 0.2†</td>
</tr>
<tr>
<td>DG</td>
<td>6.4 ± 0.9</td>
<td>3.5 ± 0.3†</td>
<td>11.3 ± 1.7*</td>
<td>10.3 ± 1.0</td>
<td>14.5 ± 1.3*</td>
<td>4.2 ± 0.4†</td>
<td>13.1 ± 0.2*</td>
<td>4.9 ± 0.4†</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SE ROD. *P < 0.05 vs. normal, MR, mineralocorticoid receptor; †P < 0.05 vs. basal, DG, dentate gyrus.
hormonal profiles. As CRH is thought to be the primary stimulator of pituitary ACTH synthesis and release, it is not surprising that uncontrolled diabetic animals that are unable to further increase CRH synthesis, and presumably release, during hypoglycemia also demonstrate impaired pituitary-adrenal responses. It is interesting that both insulin- and phloridzin-treated animals, despite displaying elevated baseline CRH mRNA levels, can further increase CRH synthesis. This supports the hypothesis that hyperglycemia is primarily responsible for impairing the central HPA responses to hypoglycemia.

No changes in AVP mRNA expression were observed during hypoglycemia. This is consistent with reports showing rats injected with insulin failed to exhibit any changes in AVP mRNA during hypoglycemia, despite increases in ACTH and corticosterone (45). In contrast, other laboratories reported increased ir-AVP in the hypophyseal portal circulation during hypoglycemia in anesthetized rats (43). Whether AVP plays a role in the response to acute hypoglycemia remains controversial. In the rat at least, parvocellular AVP seems to play a synergistic role, whereas CRH

Table 6. Densitometric analysis following in situ hybridization of GR mRNA expression in the limbic system (hippocampal CA1/2, CA3, and CA4 fields and DG, the hypothalamic PVN, and the AP of normal, STZ-diabetic, insulin-treated diabetic, and phloridzin-treated diabetic rats under basal conditions and after Hypo

<table>
<thead>
<tr>
<th>Limbic System</th>
<th>Normal Basal (n = 6)</th>
<th>Hypo (n = 8)</th>
<th>STZ Basal (n = 6)</th>
<th>Hypo (n = 7)</th>
<th>STZ + Insulin Basal (n = 6)</th>
<th>Hypo (n = 12)</th>
<th>STZ + Phloridzin Basal (n = 6)</th>
<th>Hypo (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1/2</td>
<td>15.0±2.5</td>
<td>10.0±1.3</td>
<td>13.5±2.8</td>
<td>11.3±0.5</td>
<td>20.0±1.8</td>
<td>12.0±0.5</td>
<td>20.0±1.8</td>
<td>12.0±0.5</td>
</tr>
<tr>
<td>CA3</td>
<td>7.8±0.8</td>
<td>6.5±0.5</td>
<td>6.8±0.5</td>
<td>8.3±0.5</td>
<td>8.8±0.3*</td>
<td>6.6±0.3</td>
<td>8.8±0.5*</td>
<td>6.6±0.3</td>
</tr>
<tr>
<td>CA4</td>
<td>8.0±1.5</td>
<td>6.5±0.3</td>
<td>6.8±0.8</td>
<td>7.8±0.8</td>
<td>8.8±0.5</td>
<td>6.8±0.3</td>
<td>8.8±0.5*</td>
<td>6.8±0.3</td>
</tr>
<tr>
<td>DG</td>
<td>15.5±2.3</td>
<td>11.3±1.3</td>
<td>13.8±2.3</td>
<td>14.8±0.5</td>
<td>19.8±1.8</td>
<td>11.3±0.8</td>
<td>19.8±1.8</td>
<td>11.3±0.8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVN</td>
<td>11.5±0.8</td>
<td>9.8±0.5</td>
<td>10.5±1.3</td>
<td>12.0±1.0</td>
<td>17.8±2.5†</td>
<td>13.8±0.8</td>
<td>17.8±2.5†</td>
<td>13.8±0.8</td>
</tr>
<tr>
<td>AP</td>
<td>7.3±1.5</td>
<td>10.3±1.0</td>
<td>6.8±0.5</td>
<td>11.0±1.0</td>
<td>25.5±1.5†</td>
<td>25.0±1.0</td>
<td>25.5±1.5†</td>
<td>25.0±1.0</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE ROD. *P < 0.05 vs. STZ-basal. †P < 0.05 vs. normal-basal and STZ-basal. GR, glucocorticoid receptor; AP, anterior pituitary.
is essential for mediating ACTH synthesis and secretion during stress.

POMC mRNA expression increased in all groups but not in uncontrolled diabetic rats during hypoglycemia. While several studies have demonstrated increases in anterior pituitary POMC mRNA expression in response to hypoglycemia in normal rats (45, 56, 57), the correlation between CRH and POMC is somewhat contradictory. Some have reported increases in POMC mRNA levels in the absence of either increased hypothalamic CRH mRNA or plasma CRH (45), while others have suggested it is the rise in CRH that is primarily responsible for increasing POMC mRNA during hypoglycemia (56). This latter observation is consistent with our findings as POMC mRNA levels were only elevated in control, insulin-treated, and phloridzin-treated diabetic rats. These groups exhibited both increased hypothalamic CRH mRNA expression and larger pituitary-adrenal responses to hypoglycemia. As both CRH and POMC mRNA levels were unaltered in diabetic rats following 2 h of hypoglycemia, it is likely that the lack of a CRH response hindered both ACTH synthesis and secretion from corticotrophs.

Hippocampal MR mRNA expression in control, insulin-treated, and phloridzin-treated diabetic animals decreased in response to hypoglycemia. Interestingly, hypoglycemia did not modify MR mRNA in uncontrolled diabetic rats. The effects of hypoglycemia on hippocampal MR mRNA expression have not been previously explored. Normally, the MR is thought to provide tonic inhibitory input to the PVN (10, 14, 15). However, corticosteroid receptor mRNA levels in the dentate gyrus can be altered in as little as 30 min after the onset of stress (20). Because hippocampal MR expression decreased in those groups of animals that exhibited full HPA activation, the nondiabetic control, insulin-treated, and phloridzin-treated diabetic groups, suggests that these receptors may be important in modulating the response to hypoglycemia. Two possible explanations exist for this trend. First, the larger increase in plasma glucocorticoid levels in normal, insulin-, and phloridzin-treated diabetic rats may contribute to greater suppression of MR expression or second, the ability to decrease MR expression, and thus to relieve inhibitory influences, may be important in determining responsiveness of the HPA axis to hypoglycemia. Whether the latter is true, then this may, in part, explain why diabetic rats are unable to fully activate the HPA axis during hypoglycemia. We speculate that responsiveness of hippocampal MRs to hypoglycemia is glucose sensitive as treatment of diabetic animals with phloridzin was able to restore this response to normal. This is a novel observation. To our knowledge, no studies have examined the effects of glucose on hippocampal MR expression.

In conclusion, we established that in short-term diabetes, along with chronic activation of the HPA axis, the response to hypoglycemia was also 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

Table 7. Summary table of the main study results

<table>
<thead>
<tr>
<th>Condition</th>
<th>Normal</th>
<th>STZ</th>
<th>STZ + Insulin</th>
<th>STZ + Phloridzin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ACTH</td>
<td></td>
<td></td>
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<tr>
<td>Corticosterone</td>
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<td></td>
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<tr>
<td>Epinephrine</td>
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<td></td>
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</tr>
<tr>
<td>Glucagon</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POMC mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR mRNA</td>
<td></td>
<td></td>
<td></td>
<td>(PVN and AP)</td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticosterone</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Epinephrine</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucagon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POMC mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MR mRNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR mRNA</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

For the basal studies, the arrows indicate the direction of change relative to normal animals. In the hypoglycemia section, the arrows indicate the direction of change relative to their respective baseline levels and the number arrows indicate the magnitude of the response.
glucose. This, in turn, may be related to the inability to relieve tonic inhibition on the axis by decreasing hippocampal MR expression. Interestingly, insulin and phloridzin treatment were effective at reversing the effects of diabetes on the HPA response to hyperglycemia. This observation indicates that among the many disrupted metabolic parameters that are associated with the diabetic condition, it is probable that insulin deficiency per se may be primarily responsible for dysregulation of basal HPA function in diabetes. Chronic elevations in blood glucose with uncontrolled or poorly controlled diabetes, on the other hand, can critically impair the HPA response to blood glucose with uncontrolled or poorly controlled diabetes, its responsiveness to stress. Understanding the mechanisms underlying these defects will be important for developing future treatment strategies for patients with diabetes.

ACKNOWLEDGMENTS

We thank E. Burdett and D. Bilinski for excellent technical assistance.

GRANTS

This work was generously supported by research grants from the Juvenile Diabetes Foundation International (M. Vranic and S. G. Matthews) and the Canadian Institutes of Health Research’s Doctoral Research Award. O. Chan is also a recipient of the Ontario Graduate Scholarship for Science and Technology and the University of Toronto’s Department of Physiology Scholarship. E. Akirav is a recipient of the University of Toronto’s Department of Physiology Scholarship and E. Park is a recipient of the Ontario Graduate Scholarship.

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