Effects of diabetes and recurrent hypoglycemia on the regulation of the sympathoadrenal system and hypothalamo-pituitary-adrenal axis

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Although the effects of recurrent hypoglycemia on counter-regulation have been well characterized, the molecular mechanisms underlying these defects are not well understood. To investigate these mechanisms, we examined the differential effects of recurrent hypoglycemia and recurrent hyperinsulinemia per se on counterregulation in streptozotocin (STZ)-diabetic rats (29). It was important to differentiate the effects of insulin from those of hypoglycemia, since insulin can influence counterregulatory responses to hypoglycemia (14, 15, 24, 25, 34). Not only can insulin have an acute effect to enhance counterregulatory responses during hypoglycemia (14, 24, 34), but antecedent exposure to insulin can enhance responses to subsequent hypoglycemia (15, 25). In diabetic rats, we observed blunted glucagon, epinephrine, norepinephrine, and corticosterone responses to hypoglycemia compared with normal rats (29). Recurrent hypoglycemia further impaired epinephrine responses. This defect occurred despite a protective effect of the insulin doses used to induce recurrent hypoglycemia, since diabetic rats exposed to recurrent hyperinsulinemic hyperglycemia exhibited fully normalized epinephrine responses.

The present study aimed to identify the molecular adaptations underlying 1) the defective epinephrine, norepinephrine, and corticosterone responses to hypoglycemia in diabetic rats and 2) the further impairment of epinephrine counterregulation after exposure of diabetic rats to recurrent hypoglycemia. We examined the effects of diabetes and recurrent hypoglycemia on the regulation of sympathoadrenal and hypothalamo-pituitary-adrenal (HPA) function. Animals were treated identically to those in our previous study (29), but, instead of undergoing hypoglycemic clamps for determination of counterregulatory responses, they were euthanized with no further manipulation. We hypothesized that the impaired epinephrine responses in diabetes and after recurrent hypoglycemia were related to altered regulation of adrenal catecholamine synthesis. Thus we measured adrenal expression of mRNA for the catecholamine-synthesizing enzymes tyrosine hydroxylase (TH), which catalyzes the first and rate-limiting step of catecholamine synthesis; dopamine β-hydroxylase (DBH), which converts dopamine to norepinephrine; and phenylethanolamine N-methyltransferase (PNMT), which converts norepinephrine to epinephrine. In addition, adrenal glucocorticoid receptor (GR) mRNA levels were examined. Glucocorticoids are known to increase TH (55), DBH (32, 39), and PNMT (22, 30) mRNA expression.

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Hypoglycemia is the most common adverse effect of intensively treated type 1 diabetic (16, 27, 41). A major contributor to hypoglycemia in type 1 diabetes is defective glucose counterregulation (10). In established type 1 diabetes, glucagon responses to hypoglycemia are absent (3, 26), and epinephrine responses are often impaired (3, 11, 23, 33, 42). Defects in cortisol and norepinephrine responses may also exist (23, 33, 42). Recent exposure to hypoglycemia is a primary cause of defective counterregulation (11, 23, 35, 42, 45). In diabetic subjects, antecedent hypoglycemia not only reduces neuroendocrine and autonomic responses to subsequent hypoglycemia (11, 13, 35, 45) but also increases glycemic thresholds for counterregulatory responses (11) and causes hypoglycemia unawareness (35, 42). Together, these defects increase the patient’s susceptibility to further bouts of hypoglycemia (10).
Thus alterations in GR levels would influence these interactions. At the level of the HPA axis, we hypothesized that the defective corticosterone responses to hypoglycemia in diabetic rats were related to altered regulation of HPA function. In the HPA axis, stressors such as hypoglycemia induce the release of corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus (PVN). CRH then acts on the pituitary gland to stimulate the synthesis of ACTH from proopiomelanocortin (POMC). ACTH, in turn, stimulates the secretion of corticosterone (cortisol in humans) from the adrenal cortex. HPA activity is inhibited by negative feedback of glucocorticoids at mineralocorticoid receptors (MR) in the hippocampus and GR in the hippocampus, hypothalamus, and pituitary (18).

To assess the central regulation of HPA function, we measured levels of GR and MR mRNA in the hippocampus, CRH and GR in the hippocampus, hypothalamus, and pituitary (18). To assess the central regulation of HPA function, we measured levels of GR and MR mRNA in the hippocampus, CRH and GR mRNA in the PVN, and POMC and GR mRNA in the pituitary.

RESEARCH DESIGN AND METHODS

Animals

Male Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada) initially 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 food (Rodent Laboratory Chow 5001, LabChows; Agribrands Purina, Woodstock, ON, Canada) and water. Groups were 1) normal (n = 9), 2) diabetic control (n = 6), 3) diabetic exposed to recurrent hyperinsulinemic hypoglycemia (D-hypo; n = 7), and 4) diabetic exposed to recurrent hyperinsulinemic hypoglycemia (D-hyper; n = 7). Diabetes was induced by intraperitoneal injection of STZ (65 mg/kg; Sigma Chemical, St. Louis, MO) dissolved in saline. This dose produced diabetes with fed-state glucose levels ranging from 18 to 25 mM. All procedures were in accordance with Canadian Council on Animal Care standards and were approved by the Animal Care Committee of the University of Toronto.

Surgical Procedure for Implantation of Jugular Catheters

Fourteen days after induction of diabetes, or 3 days before the first day of antecedent treatment, all rats were implanted with catheters into the right jugular vein. In D-hyper rats, the catheters allowed for infusions during treatment. Surgery was performed under general anesthesia [100 mg/kg ketamine chloride (MPAC Pharmaceuticals, Cambridge, ON, Canada), 1 mg/kg acepromazine maleate (Wyeth-Ayerst, Montreal, QC, Canada), and 1 mg/kg xylazine (Bayer, Etobicoke, ON, Canada)], as described previously (52).

Treatment Protocol

Three days after surgery, on day 17 after induction of diabetes, diabetic rats were assigned to one of three treatment groups. Normal rats underwent sham treatment. The treatments are described below. D-hypo rats. D-hypo (n = 7) rats underwent 4 days of 2 episodes/day of hyperinsulinemic hypoglycemia. Insulin (~2 U/100 g body wt, Iletin II Regular Insulin injection 100 U/mg; Eli Lilly, Indianapolis, IN) was injected subcutaneously at 0900 and 1300 to yield two ~90-min episodes at blood glucose levels of ~2.5 mM. For each episode, blood glucose was measured (Glucometer Elite Blood Glucose Meter, Bayer; range 2.1–29 mM) every 30 min over a 3-h period from a single tail nick made at the time of the first sample. To ensure that hypoglycemia did not last longer than 90 min, morning and afternoon episodes were terminated by providing the rats with food and 10% sucrose water. Sucrose water was replaced with plain water overnight.

D-hyper rats. D-hyper (n = 7) rats controlled for the insulin doses administered to D-hypo rats and thus differentiated the effects of hyperinsulinemia per se from those of hypoglycemia. D-hyper rats underwent treatment identical to that of D-hypo rats but received intravenous infusions of 40% d-glucose (Abbott Laboratories, Montreal, QC, Canada) after insulin injections, so that basal hyperglycemic levels could be maintained. Hyperglycemia, rather than euglycemia, was maintained so that the effects of insulin, independently of its effects to normalize glucose, could be determined. Glucose levels were kept at 21.5 ± 1.2 mM during treatment.

Diabetic control rats. Diabetic control (n = 6) rats underwent 4 days of sham treatment, in which insulin injections were replaced with saline injections. To control for the effect of repeated measurement of blood glucose in D-hyper and D-hypo rats, diabetic controls underwent identical handling every 30 min throughout each treatment episode to simulate the blood-sampling procedure in D-hypo and D-hyper animals.

Normal rats. Normal (n = 9) rats underwent the same sham treatment as diabetic control rats.

After the fourth day’s afternoon treatment, all rats were fasted from 1800 before being euthanized between 1500 and 1600 the next day. Fasting enabled direct comparison of the current experiment with our previous antecedent hypoglycemia study (29), in which animals were fasted before undergoing hypoglycemic glucose clamps on the fifth day. The euthanasia time matched the time at which animals underwent hypoglycemia during euglycemic-hypoglycemic clamps in our previous study (29). This was close to the circadian peak of HPA activity (12). After decapitation, trunk blood was collected for measurement of basal plasma insulin, glucagon, ACTH, and corticosterone concentrations. Plasma epinephrine and norepinephrine levels were not measured in trunk blood, as levels increase too rapidly on handling (44). However, we have reported catecholamine levels in identical groups of animals previously (29). Plasma was stored at −20°C until assayed. The brain, pituitary, and adrenal glands were removed under aseptic conditions and stored at −80°C until sectioning.

Tissue Sectioning and In Situ Hybridization

For brain sectioning, coronal brain cryosections (12 μm) were obtained through selected hypothalamic (bregma −1.90 to −2.10 mm) and hippocampal (bregma −3.70 to −4.00 mm) regions according to the stereotaxic coordinates of Paxinos and Watson (43). Pituitary sections (12 μm) containing intermediate and anterior lobe and adrenal sections (12 μm) containing medulla and cortex were obtained. The method of in situ hybridization has been described in detail previously (36). Briefly, 45-mer antisense oligonucleotide CRH (bases 536–580), POMC (bases 572–616), MR (bases 2942–2986), GR (bases 1321–1365), TH (bases 905–949), DJH (bases 1514–1558), and PNMT (bases 265–309) (Sigma Genosys; Sigma Aldrich, Oakville, ON, Canada) probes were labeled with 35S-labeled deoxyadenosine 5’-α-thio) triphosphate (1,300 Ci/mmol; NEN DuPont, Mississauga, ON, Canada) to a specific activity of 1.0 × 109 counts/min/mg (cpm) using terminal deoxynucleotidyl transferase (Pharmacia Biotech, Baie d’Urfe, QC, Canada). Labeled probe was applied to each slide. Sections containing hypothalamic PVN were hybridized with CRH and GR probes. Hippocampal brain sections were hybridized with GR and MR probes. Pituitary sections were hybridized with POMC and GR probes. Adrenal sections were hybridized with TH, DJH, PNMT, and GR probes. Slides were incubated overnight at 42.5°C. They were then washed in SSC and dehydrated in 70% and 95% ethanol. Air-dried slides were exposed (exposure time: CRH 5 wk; POMC 2 h; MR 2 wk; GR 4 wk; and TH, DJH, and PNMT 24 h) to autoradiographic film (Biomax MR;
Eastman Kodak, Rochester, NY). Films were developed using standard procedures.

**Plasma Hormone Analysis**

Plasma insulin was measured by radioimmunoassay, according to the method of Herbert et al. (28). Plasma glucagon (Diagnostics Products, Los Angeles, CA), ACTH (Diasorin, Stillwater, MN), and corticosterone (Diagnostics Products) were measured by commercially available radioimmunoassay kits.

**Data Analysis**

For in situ hybridization, tissue sections were processed simultaneously for each probe to allow for direct comparison between groups. For each probe, six to eight sections were selected from each animal, based on visual inspection of the desired region. Sections were exposed together with 14C 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 the film was quantified, after subtraction of background values, using a computerized image analysis system (Imaging Research, St. Catharines, ON, Canada).

Data are presented as means ± SE. For each parameter, measurements from all four rat groups were analyzed by one-way ANOVA. Duncan’s post hoc test for multiple comparisons was performed if the ANOVA revealed a P value < 0.05. Significance was set at P < 0.05. Statistical analysis was performed with Statistica software (Statsoft, Tulsa, OK).

**RESULTS**

**Glucose Levels and Insulin Doses During Antecedent Treatment**

Morning and afternoon blood glucose levels averaged over days 1–4 of recurrent hyperinsulinemic hypoglycemia (D-hypo) and recurrent hyperinsulinemic hyperglycemia (D-hyper) are shown in Fig. 1. In D-hypo rats, during morning hypoglycemia, glucose levels fell to 3.3 ± 0.3 mM by 120 min after insulin injection and remained at ~2.7 mM for 60 min thereafter. In the afternoon, glucose levels fell to 3.7 ± 0.3 mM by 60 min after injection and remained at ~2.6 mM for 120 min thereafter. Glucose levels were at least ~3.5 mM for a total of 3 h/day. During recurrent hyperglycemia, the mean blood glucose level was 21.5 ± 1.2 mM. Insulin doses over the 4 days of treatment did not differ (D-hypo 2.1 ± 0.2, D-hyper 2.1 ± 0.2 U/100 g body wt; P = 0.9).

**Table 1. Plasma glucose and hormone levels**

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 6)</th>
<th>Diabetic (n = 9)</th>
<th>D-Hypo (n = 7)</th>
<th>D-Hyper (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mM</td>
<td>4.4 ± 0.2</td>
<td>16.6 ± 3.2</td>
<td>20.3 ± 2.8</td>
<td>19.3 ± 3.5</td>
</tr>
<tr>
<td>Insulin, pM</td>
<td>74 ± 16</td>
<td>44 ± 5†‡</td>
<td>139 ± 14‡</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>Glucagon, ng/l</td>
<td>69 ± 11</td>
<td>100 ± 14</td>
<td>86 ± 14</td>
<td>117 ± 11</td>
</tr>
<tr>
<td>ACTH, pM</td>
<td>11.4 ± 3.5</td>
<td>11.3 ± 2.5</td>
<td>9.5 ± 2.1</td>
<td>4.3 ± 1.5</td>
</tr>
<tr>
<td>Corticosterone, nM</td>
<td>490 ± 168</td>
<td>902 ± 107‡</td>
<td>552 ± 182</td>
<td>444 ± 95</td>
</tr>
</tbody>
</table>

Values are means ± SE. Afternoon plasma glucose and hormone levels in overnight-fasted normal rats, diabetic control rats, and diabetic rats exposed to recurrent hyperinsulinemic hypoglycemia (D-hypo) or recurrent hyperinsulinemic hyperglycemia (D-hyper). *P < 0.05 vs. normal, †P < 0.05 vs. D-hypo, and ‡P < 0.05 vs. D-hyper.

**Fasting Blood Glucose and Hormone Levels After Antecedent Treatment**

Afternoon fasting blood glucose and hormone levels after antecedent treatment are summarized in Table 1. Glucose levels were elevated (P < 0.05) in all diabetic groups compared with normal rats. Plasma insulin levels were decreased (P < 0.05) in diabetic controls vs. normal rats. D-hypo and D-hyper rats displayed increased (D-hypo, P < 0.0001; D-hyper, P < 0.05) insulin levels compared with diabetic controls. Despite identical insulin doses during treatment, insulin levels in D-hypo rats were nearly twofold greater (P < 0.005) than in D-hyper rats, the insulin levels of which did not differ from normal rats. These data suggest that insulin clearance was decreased in D-hypo rats. Plasma glucagon concentrations did not differ among the groups. There were also no significant differences in afternoon plasma ACTH levels. However, corticosterone levels were increased (P < 0.05) in diabetic controls compared with all groups. Epinephrine and norepinephrine levels were not measured, since animals did not have blood-sampling catheters and were euthanized by decapitation (44).

**Adrenal Weights and Levels of TH, DBH, PNMT, and GR mRNA**

Adrenal weights did not differ among the groups, indicating that neither diabetes nor the recurrent hypoglycemia or hyperglycemia regimens altered adrenal size (normal 0.144 ± 0.006, diabetic 0.136 ± 0.010, D-hypo 0.137 ± 0.013, D-hyper 0.133 ± 0.015 mg adrenal/g body wt). In contrast, adrenal medulla TH mRNA levels were reduced by ~25% in all diabetic groups vs. normal rats (normal vs. diabetic, D-hypo, P < 0.05; normal vs. D-hyper, P < 0.01; Fig. 2A). PNMT mRNA levels were similar among normal, diabetic control, and D-hyper rats but were decreased (P < 0.05) in D-hypo rats by ~40% compared with the other groups (Fig. 2B). Paradoxically, DBH mRNA levels in D-hypo rats were increased compared with those in diabetic control and D-hyper rats (D-hypo vs. diabetic control, P < 0.05; D-hypo vs. D-hyper, P < 0.005; Fig. 2C). The apparent increase in DBH mRNA in D-hypo rats vs. normal rats was not significant. GR mRNA levels in the cortex and medulla did not differ among the groups (Table 2).
Fig. 2. Representative images and densitometric analysis of tyrosine hydroxylase (TH; A), phenylethanolamine N-methyltransferase (PNMT; B), and dopamine β-hydroxylase (DβH; C) mRNA levels in the adrenal medulla of normal rats, diabetic control rats, and D-hypo or D-hyper rats. Values are means ± SE, expressed as relative optical density (ROD). *P < 0.05 vs. normal, †P < 0.05 vs. diabetic, ‡P < 0.05 vs. D-hypo, and §P < 0.05 vs. D-hyper.
Brain and Pituitary Levels of MR, GR, CRH, and POMC mRNA

MR mRNA levels were increased ($P < 0.001$ to $P < 0.05$) in all limbic regions examined (CA1/2, CA3, and CA4 fields of hippocampus, and dentate gyrus (DG)) in all three diabetic groups compared with normal rats (Fig. 3). Conversely, hippocampal GR mRNA levels did not differ (Table 3). Levels of GR mRNA in the hypothalamic PVN and anterior pituitary, CRH mRNA in the PVN, and POMC mRNA in the anterior and intermediate lobes of the pituitary also did not differ between groups.

DISCUSSION

The present study aimed to identify a number of the molecular adaptations in the sympathoadrenal system and HPA axis underlying 1) the defective epinephrine, norepinephrine, and corticosterone responses to hypoglycemia in untreated diabetic rats and 2) the further impairment of epinephrine counterregulation in diabetic rats exposed to recurrent hypoglycemia. We have demonstrated for the first time that, in diabetic rats, defects in epinephrine counterregulation are associated with reduced adrenal expression of TH mRNA. Further blunting of epinephrine counterregulation after recurrent hypoglycemia is not only associated with decreases in TH mRNA but also reduced PNMT mRNA. In the brain, hippocampal MR mRNA levels were elevated in all diabetic groups compared with normal rats. Despite the differences in hippocampal MR mRNA, hippocampal GR mRNA, PVN GR and CRH mRNA, and pituitary GR and POMC mRNA levels did not differ. Because mRNA and protein levels for MR (2), GR (2, 38), CRH (50), and POMC (37) in these areas are closely correlated, we suggest that the diminished corticosterone responses

### Table 2. Densitometric analysis of GR mRNA levels in rat adrenal glands

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 4)</th>
<th>Diabetic (n = 9)</th>
<th>D-Hypo (n = 7)</th>
<th>D-Hyper (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenal cortex</td>
<td>0.63 ± 0.04</td>
<td>0.61 ± 0.04</td>
<td>0.61 ± 0.06</td>
<td>0.62 ± 0.05</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>1.07 ± 0.14</td>
<td>0.83 ± 0.07</td>
<td>0.76 ± 0.09</td>
<td>0.92 ± 0.04</td>
</tr>
</tbody>
</table>

Values are means ± SE (relative optical density). GR, glucocorticoid receptor.

### Table 3. Densitometric analysis of mRNA levels in rat hippocampus, PVN, and pituitary gland

<table>
<thead>
<tr>
<th></th>
<th>Normal (n = 6)</th>
<th>Diabetic (n = 9)</th>
<th>D-Hypo (n = 7)</th>
<th>D-Hyper (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR in hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA1/2</td>
<td>2.80 ± 0.11</td>
<td>2.97 ± 0.18</td>
<td>2.99 ± 0.18</td>
<td>3.52 ± 0.29</td>
</tr>
<tr>
<td>CA3</td>
<td>1.45 ± 0.14</td>
<td>1.68 ± 0.13</td>
<td>1.79 ± 0.07</td>
<td>1.81 ± 0.10</td>
</tr>
<tr>
<td>CA4</td>
<td>1.51 ± 0.14</td>
<td>1.68 ± 0.12</td>
<td>1.77 ± 0.07</td>
<td>1.81 ± 0.13</td>
</tr>
<tr>
<td>DG</td>
<td>3.22 ± 0.18</td>
<td>3.75 ± 0.20</td>
<td>3.69 ± 0.16</td>
<td>3.87 ± 0.25</td>
</tr>
<tr>
<td>PVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>4.7 ± 0.2</td>
<td>4.2 ± 0.5</td>
<td>5.1 ± 0.5</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>GR</td>
<td>2.7 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior lobe</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>POMC</td>
<td>2.60 ± 0.42</td>
<td>2.45 ± 0.36</td>
<td>2.56 ± 0.43</td>
<td>2.59 ± 0.35</td>
</tr>
<tr>
<td>GR</td>
<td>3.1 ± 0.2</td>
<td>3.0 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>Intermediate lobe</td>
<td>0.233 ± 0.044</td>
<td>0.206 ± 0.035</td>
<td>0.219 ± 0.051</td>
<td>0.235 ± 0.034</td>
</tr>
<tr>
<td>POMC</td>
<td>0.233 ± 0.044</td>
<td>0.206 ± 0.035</td>
<td>0.219 ± 0.051</td>
<td>0.235 ± 0.034</td>
</tr>
</tbody>
</table>

Values are means ± SE (relative optical density). PVN, paraventricular nucleus; CRH, corticotropin-releasing hormone; DG, dentate gyrus; POMC, proopiomelanocortin.

Fig. 3. Representative images and densitometric analysis of mineralocorticoid receptor (MR) mRNA levels in the hippocampus (CA1/2, CA3, and CA4 fields) and dentate gyrus (DG) of normal rats, diabetic control rats, and D-hypo or D-hyper rats. Values are means ± SE, expressed as ROD. *$P < 0.05$ vs. normal, and **$P < 0.001$ vs. normal.
to hypoglycemia in untreated diabetic rats (7, 29) are not due to altered basal expression of these receptors and neuropeptides in the hippocampus, PVN, and pituitary.

Previously, we demonstrated that epinephrine responses to hypoglycemia are markedly impaired in rats that had been diabetic for 3 wk (29). Diabetic rats in the current study were treated identically to those in our earlier study. However, instead of undergoing hypoglycemic clamps for determination of counterregulatory responses, they were euthanized for analysis of adaptations in the sympathoadrenal system and HPA axis. The present data suggest that the blunted epinephrine responses in diabetic rats may be due to defects in adrenal catecholamine synthesis, particularly at the rate-limiting step catalyzed by TH. Although we did not measure TH protein levels or enzyme activities, others have shown in rats that exposure to chronic treatment regimens, such as cold (1), repeated immobilization (48), and nicotine treatment (56), lasting from 5 to 14 days induces similar changes in TH mRNA, protein, and enzyme activity. Thus it is likely that, in 3-wk-diabetic rats, the decreases in TH mRNA were accompanied by reductions in TH protein and enzyme activities. It is important to note, however, that TH mRNA was also reduced in diabetic rats exposed to recurrent hyperinsulinemia with hyperglycemia (hereafter referred to as recurrent hyperinsulinemia). In our earlier study, these animals exhibited fully normalized epinephrine responses to hypoglycemia (29). We cannot rule out that the improved epinephrine responses after the comparatively shorter 4 days of recurrent hyperinsulinemia were due to increases in catecholamine-synthesizing enzyme activities or adaptations in other aspects of catecholamine synthesis, secretion, or degradation.

When diabetic rats in our earlier study underwent repeated hypoglycemia, epinephrine responses became further impaired (29). Here, an identical regimen of recurrent hypoglycemia was not only associated with reduced TH mRNA but also with a 40% decrease in PNMT mRNA. These data suggest that the further epinephrine defect after repeated hypoglycemia was related to a specific defect in the norepinephrine-to-epinephrine conversion pathway. Paradoxically, recurrent hypoglycemia increased mRNA levels of the norepinephrine-synthesizing enzyme DBH. In response to immobilization stress, it has been shown that PNMT mRNA increases within 5 min, whereas DBH mRNA requires multiple exposures to be induced (47). These data raise the possibility that the opposite changes in PNMT and DBH mRNA were in part due to differences in the time courses of activation and inhibition of the biosynthesis of these enzymes. Previously, we observed fully normalized norepinephrine counterregulation in diabetic rats exposed to identical recurrent hypoglycemia (29). It is unlikely that the improved responses were solely related to the enhanced expression of DBH, since norepinephrine counterregulation was also restored in diabetic rats exposed to repeated hyperinsulinemia (29) that showed unaltered DBH mRNA levels. In humans, the adrenal medulla appears to be the primary source of norepinephrine released during hypoglycemia (19). However, in rats, the peripheral sympathetic nerve terminals can contribute 50% (31) to nearly all (40, 57) of the plasma norepinephrine. It may be that, at least in our diabetic rats, the norepinephrine defect in untreated animals and the improved responses after repeated insulin administration were partially due to peripheral sympathetic adaptations.

The mechanisms underlying the reduced TH mRNA levels in diabetes and the decreased PNMT mRNA levels after repeated hypoglycemia are not known. In bovine adrenomedullary cells, Stachowiak et al. (54) demonstrated an effect of moderate depletion of cellular catecholamine content to increase PNMT mRNA levels. However, when catecholamine concentrations were decreased by 50%, PNMT mRNA dropped to 50% of baseline levels. Although we did not measure adrenal catecholamine levels, others have shown in rats that hypoglycemia can reduce adrenal epinephrine content by 70% (57). These data suggest that the decrease in PNMT mRNA after recurrent hypoglycemia in diabetic rats may have been due at least in part to depletion of chromatinf cell catecholamine content. However, they also raise the possibility that the reduced PNMT, and perhaps TH, mRNA levels were the result, rather than the cause, of the blunted epinephrine responses. In type 1 diabetic humans with defective epinephrine counterregulation, adrenal epinephrine stores are reduced (17), suggesting that depletion of adrenal catecholamine content may indeed contribute to defective epinephrine counterregulation. Defects in epinephrine synthesis may therefore only be one aspect of impaired epinephrine counterregulation. Changes in other aspects of adrenal function, such as catecholamine content or even degradation, cannot be ruled out. Initially, we hypothesized that the decreases in PNMT and TH mRNA might be due to reduced glucocorticoid-mediated stimulation of PNMT (22, 30) and TH (55) synthesis. However, because adrenomedullary GR mRNA levels did not differ among the groups, and changes in GR mRNA are usually accompanied by similar changes in GR protein or binding capacity (20, 21, 53), this does not appear to have been the case.

Pituitary-adrenal function is dysregulated in diabetic rats (7, 8, 29). We have shown that after both 1 wk (7, 8) and 3 wk (29) of diabetes, morning plasma ACTH and corticosterone levels are elevated compared with normal rats. In contrast, corticosterone responses to hypoglycemia are nearly absent in diabetic animals (7, 29). The pituitary-adrenal defects appear to at least in part be due to a lack of insulin, since they are nearly normalized in diabetic rats exposed to repeated hyperinsulinemia, both with and without concurrent hypoglycemia (29). In the current study, hippocampal MR mRNA levels were elevated in all diabetic groups, indicating that diabetes, irrespective of glucose or insulin levels, increases hippocampal MR mRNA. The increases in mRNA were likely accompanied by increases in protein, since hippocampal MR mRNA and protein levels are closely correlated (2). These data confirmed our previous findings of elevated hippocampal MR mRNA in 1-wk-diabetic rats (7, 8). Despite the differences in MR mRNA between normal and diabetic rats, it does not appear that the increases in MR were responsible for the impaired corticosterone responses to hypoglycemia in untreated diabetic animals. This is because MR mRNA was also elevated in diabetic rats exposed to recurrent hyperinsulinemia, which showed nearly normalized corticosterone responses (29). Hippocampal GR mRNA and PVN and pituitary CRH, POMC, and GR mRNA levels did not differ among the groups. As with hippocampal MR, mRNA and protein levels of CRH (50), POMC (37), and GR (2, 38) in these areas are closely correlated. Thus the defective corticosterone responses to hypoglycemia in diabetic rats also do not appear to be due to altered basal expression of...
these neuropeptides and receptors. In 1-wk-diabetic rats that were euthanized in the morning, we previously observed increased PVN CRH mRNA levels (7, 8). The lack of differences in CRH mRNA in the present study was likely due to the fact that, unlike 1-wk-diabetic rats, the current animals were euthanized in the afternoon, near the circadian peak of HPA activity (12). In diabetes, the diurnal rhythm of HPA activity is disrupted (6, 9, 51, 58). As a result, differences in HPA function between normal and diabetic rats are known to be more pronounced in the morning, during the nadir (6, 9, 51, 58).

Our data suggest that the defective epinephrine responses to hypoglycemia in diabetes and after recurrent hypoglycemia and the concurrent decreases in TH and PNMT mRNA are not the result of changes in the brain and pituitary parameters we examined. Other central nervous system areas are known to play important roles in glucose sensing and counterregulatory responses (5, 46). The ventromedial hypothalamus (VMH) appears to be a key hypothalamic-sensing region that is important in eliciting counterregulatory hormone responses (5).

In addition, there is evidence to suggest that alterations in the VMH or its efferent pathways may contribute to impaired counterregulation after chronic hypoglycemia (4). In the hindbrain, specific catecholaminergic neurons respond to 2-deoxyglucose glucoprivation (46) and show decreased responsiveness after repeated glucoprivation (49). Adaptations in the VMH and hindbrain catecholaminergic sites may therefore contribute to the epinephrine, TH, and PNMT defects in diabetes and after recurrent hypoglycemia.

In summary, we report for the first time that the blunted epinephrine responses to hypoglycemia in untreated diabetic rats and diabetic rats exposed to recurrent hypoglycemia are associated with specific defects in adrenal catecholamine-synthesizing enzyme expression. Impaired epinephrine responses in diabetic rats are related to reduced expression of TH mRNA, whereas the further epinephrine defect after recurrent hypoglycemia is associated with decreases in both TH and PNMT mRNA. As for the blunted corticosterone response to hypoglycemia in untreated diabetes, the data suggest that the defect is not due to alterations in basal expression of MR or GR in the hippocampus or of CRH, POMC, or GR in the PVN and pituitary. We conclude that impaired epinephrine counterregulation in diabetes and after recurrent hypoglycemia may in part be due to defective adrenal catecholamine synthesis. Investigation of the mechanisms underlying the defects in TH and PNMT expression may aid in the development of treatments to improve epinephrine counterregulation in type 1 diabetic humans.

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