Dissociation of hypothalamic noradrenergic activity and sympathoadrenal responses to recurrent hypoglycemia

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INTENSIVE INSULIN THERAPY is used in the treatment of type 1 diabetes to improve metabolic control and to reduce long-term diabetic complications (8a). Unfortunately, frequent iatrogenic antecedent hypoglycemia diminishes warning symptoms and endogenous compensatory responses to subsequent hypoglycemia (7, 14). This syndrome of hypoglycemia-associated autonomic failure (HAAF), which involves suppressed secretion of epinephrine (Epi), growth hormone (GH), and glucocorticoids, further impairs the individual’s ability to regulate blood glucose concentration safely (7, 12).

Under experimental conditions, one or more episodes of hypoglycemia inhibited symptomatic and glucoregulatory responses in nondiabetic humans (13), as well as neuroendocrine responses in rats (20, 27). Impaired glucoregulation after repeated hypoglycemia does not simply result from secretory fatigue, because neuroendocrine responses to hypoglycemia are not affected by prior sympathetic activation with exercise (21). The inhibitory effects are also stimulus specific. Antecedent hypoglycemia does not inhibit responses of Epi secretion to a subsequent bout of physical exercise or blood hypoglycemia, the release of cortisol to systemic injection of ACTH, or the increase in plasma glucagon to arginine (20, 21, 30).

Still very little is known about the neurophysiology that underlies this syndrome. The wide range of compensatory processes affected suggests that the site of critical modulation is situated at a high level in the glucoregulatory circuitry of the central nervous system. The hypothalamus is known to be an important mediator in autonomic control of glucose balance (reviewed in Refs. 4, 16), and noradrenergic systems in the ventromedial hypothalamus (VMH), in particular, may be important. Blood glucose levels increased after bilateral injection of norepinephrine (NE) into the VMH (28), and Epi release from the adrenal glands in response to exercise (25) was inhibited by injecting noradrenergic antagonists directly into the VMH.

During recurrent insulin-induced hypoglycemia, the neural activity in the medial hypothalamus, as revealed by c-fos expression, was suppressed (9). In addition, lower activity levels of NE-synthetic enzyme tyrosine hydroxylase under these conditions may point toward impaired noradrenergic neurotransmission (11). Attenuated neural activity in the hypothalamus could be responsible for blunting of at least some of the compensatory hormonal responses observed after recurrent hypoglycemia. The responses of plasma Epi and NE to hypoglycemia, but not corticosterone or glucagon, were blunted when the neurons in the PVN were inactivated with lidocaine (10).

Earlier work from our laboratory has revealed, with the use of microdialysis, that extracellular concentrations of NE and GABA in the hypothalamus increase in a characteristic bi-modal response to glucoprivation with 2-deoxyglucose (2-DG) (1), as well as during the first 60 min of a single episode of insulin-induced hypoglycemia (2). The present study was designed to test whether the manifestation of HAAF is mediated, at least in part, by diminished noradrenergic activity in the hypothalamus. The first objective was to evaluate whether sympathoadrenal responses to insulin-induced hypoglycemia in nondiabetic rats would be suppressed by recurrent daily hypoglycemic episodes on four consecutive days. The second objective was to investigate the effect of recurrent episodes of hypoglycemia on the increase in extracellular NE in PVN and VMH.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the University of Illinois. Male Sprague-Dawley rats
(Charles River Laboratories), 250–300 g, were housed singly in Plexiglas cages (30 × 30 × 38 cm) in a light (12:12-h light/dark cycle; lights on at 0700)- and temperature (26 ± 2°C)-controlled room. The animals had free access to fresh water and rodent diet (Harlan Teklabs, Madison, WI) at all times, except where stated otherwise.

**Surgical procedures.** After a 1-wk acclimation period, rats were fitted with a jugular vein catheter and a microdialysis guide cannula as previously described (2). Briefly, rats were anesthetized with a mixture of ketamine HCl, xylazine HCl, and acepromazine (30:6:1 mg/kg im) and a 4-cm segment of Silastic tubing (0.64 mm ID; 0.94 mm OD) was inserted into the isolated right jugular vein. The catheter was exteriorized through an incision on top of the head, with a piece of 21-gauge stainless steel tubing inserted onto the end of the catheter. Patency was maintained by filling the catheter with a 40% polyvinylpyrrolidone (PVP) solution containing 500 units/ml heparin and capping it with a sealed piece of Tygon tubing. The rat was placed into a stereotaxic instrument (ASI Instruments, Warren, MD), and a guide cannula was positioned 2 mm dorsal to either the left VMH or left PVN using the stereotaxic atlas of Paxinos and Watson (18). Coordinates for the tip of the guide cannula were VMH [anterioposterior (AP) = −2.4, lateral (L) = 0.8, dorsal (D) = 6.4 mm relative to bregma], or PVN (AP = −1.8, L = 0.5, D = 5.2 mm). The tips of the microdialysis probes were designed to extend 2 mm beyond the end of the guide. This directs the surface of the membrane either into the lateral edge of the anterior portion of the VMH (immediately anterior to the perifornical region), or into both magnocellular and parvocellular regions of the PVN. Location of cannula placement is depicted in Fig. 1. The guide cannula and the end of the venous catheter were fixed in position with dental acrylic cement and anchored to the skull with four stainless steel screws (Small parts, Miami Beach, FL). After surgery, rats were monitored until they had completely recovered from the anesthesia. Postsurgical analgesia was provided by Bannamine (1.5 mg/kg sc). At the end of the study, cannula placement was verified histologically. Cannula placements were verified histologically at the end of the study. Typical cannula placements are shown in Fig. 1. Only data from animals with a cannula in the appropriate target area were included for statistical analysis.

**Sample collection.** Rats were allowed 5–7 days to recover from surgery and only animals with body weights greater than on the day of surgery were used. During this time, the animals were handled daily and adapted to the experimental procedures. Blood and dialysate samples were collected from unrestrained animals in their home cages using sampling lines through a liquid swivel (Instech, Plymouth Meeting, PA) connected to a weighted counterbalance lever. Experiments were conducted during the mid-light phase to minimize possible confounding diurnal-associated changes. Microdialysis probes of 0.20 × 1 mm were constructed with cuprophan membrane (AKZO-Nobel, Wuppertal, Germany). Probe efficiencies were determined, in vitro, after their use. To minimize the effect of tissue disruption at the sampling site, probes were in place at least 3 h before samples were collected. The probes were connected through a liquid swivel to a 1-ml gas-tight syringe on a microinfusion pump (Bioanalytical Systems, West Lafayette, IN) and continuously perfused with Krebs Ringer buffer (in mmol/l: 147 NaCl, 4 KCl, 2.4 CaCl₂; pH 6.4) at 1.5 μl/min. Dialysate was collected at 10-min intervals into chilled microtubes containing 3 μl 0.01 N perchloric acid and kept at −84°C until assayed for NE. Food was removed from cages when probes were placed into the rat brains and returned after the last sample had been collected.

Thirty minutes before the induction of hypoglycemia the PVP-heparin solution was flushed from the catheter and replaced by sterile 0.9% saline. Insulin (1.0 units/kg in sterile saline; Humulin, Eli Lilly, Indianapolis, IN), or an equal volume of saline, was injected via the catheter at t = 0 min. Blood samples (250 μl) for the monitoring of glucose, Epi, and NE were taken at t = −15, 0, 5, 15, 30, 45, 60, and 90 min. Blood glucose (50 μl) was immediately analyzed using a hand-held glucose analyzer (Roche Diagnostics, Indianapolis, IN). The remaining 200 μl was transferred to chilled centrifuge tubes containing heparin, EDTA, and aprotinin. From these samples, plasma was stored at −84°C until assayed for catecholamines. Donor blood from littermates was infused after each sample to avoid diminution of blood volume.

**Experimental design.** Rats with a guide cannula in either the VMH or PVN were divided into two groups. The first group received a once daily administration of 1.0 units/kg insulin on each of 4 days. The second group received equal administrations of saline on the first 3 days and insulin (1.0 U/kg) on day 4. All infusions were given in the middle of the light phase through the jugular vein catheter. Blood glucose, plasma catecholamines, and extracellular NE in VMH and PVN were measured on day 4.

**Analytic methods.** Plasma catecholamines were obtained from samples of 100 μl plasma each, by solid phase extraction with aluminum oxide (Bioanalytical Systems) and eluted with 0.2 N perchloric acid. DHBA was used as internal standard to determine extraction efficiency (~50%). These extracts were analyzed for NE, Epi, and DHBA. Plasma catecholamines and dialysate NE were analyzed on a Dynamax SD-200 system (Varian Instruments, Woburn, MA) by reverse-phase HPLC and electrochemical detection. Samples (10 μl dialysate or 20 μl plasma extract) were injected onto a 150 × 2 mm C₁₈ (3 μm) Hypersil column (Keystone Scientific, Bellefonte, PA) fitted with a 2-mm C₁₈ (3 μm) Hypersil javalin guard column (Keystone Scientific). Mobile phase (pH = 3.0) was 75 mM NaH₂PO₄, 1.7 mM 1-octanesulfonic acid, 25 μM Na₂EDTA, 7% (vol/vol) acetonitrile, and 0.1% (vol/vol) tetrahydrofuran. A DECADE electrochemical detector fitted with a VT-03 glassy carbon electrode (Antec Leyden, Leiden, The Netherlands) set at +0.75 V was used with Dynamax MacIntegrator II and “C” module programs (Rainin Instruments, Woburn, MA) for peak integration and quantitation. The interassay coefficient of variation was ≤3%.

**Data analysis.** Results are presented as means ± SE. Changes in blood glucose and plasma catecholamines in response to the first and
fourth hypoglycemic episodes were analyzed by repeated-measures ANOVA, followed by Scheffé’s post hoc test. The overall magnitude of the responses to each episode was determined by calculating the area under the curve (AUC) after insulin administration, as is appropriate for biomedical parameters (15), and analyzed by Student’s t-test. Baseline concentrations of extracellular NE were calculated by taking the average of the first three samples obtained before insulin administration. Under these steady-state conditions the obtained dialysate concentrations were corrected for the individual probe’s efficiency, and differences within a brain area were analyzed by Student’s t-test. Concentrations of dialysate NE in response to hypoglycemia are expressed as percentage of (uncorrected) baseline within each animal. The effect of recurrent hypoglycemia on changes of NE within a brain area was analyzed by repeated-measures ANOVA, followed by Scheffé’s post hoc test. P<0.05 was considered to be significant.

**Supplies.** Ketamine, acepromazine, and butorphenol were obtained from Aveco (Fort Dodge, IA). Xylazine was obtained from Vedco (St. Joseph, MO). All other reagents were purchased from Sigma Chemical (St. Louis, MO).

**RESULTS**

**Blood glucose.** Baseline blood glucose concentrations were similar among treatment groups and decreased (F$_{7,112}$ = 214.26, P < 0.001) rapidly after the administration of insulin (Fig. 2, top). During the first hypoglycemic episode the measured glycemic nadir of 3.2 ± 0.3 mmol/l was reached 15 min after injection of 1.0 units/kg insulin. This low glucose level was maintained until 60 min postinjection, followed by a small increase measured at 90 min postinjection. Blood glucose was reduced to a similar nadir during the fourth hypoglycemic episode, also followed by a small, transient increase toward 90 min postinjection. The total decrease in blood glucose, as determined by AUC, was not different between first and fourth episodes (t$_{15}$ = 0.61, P = 0.447).

**Plasma catecholamines.** There was a significant increase in plasma Epi in response to insulin (F$_{1,175}$ = 35.50, P < 0.001) (Fig. 2, middle). During the first hypoglycemic episode, plasma Epi increased rapidly to 2,561 ± 410 pg/ml at 45 min after administration of insulin, followed by a steady decline. This increase was much reduced during the fourth hypoglycemic episode (774 ± 134 pg/ml at 45 min) compared with the first episode (F$_{1,175}$ = 26.58, P < 0.001). The total increase in plasma Epi, as determined by AUC, was also smaller during the fourth compared with the first episode (t$_{15}$ = 10.61, P =

![Fig. 2. Effect of recurrent hypoglycemia on changes in blood glucose (top), plasma epinephrine (Epi; middle), and plasma norepinephrine (NE; bottom) during the first (n = 11) or fourth (n = 16) hypoglycemic episodes. Insulin (1.0 units/kg regular insulin) was administered into the jugular vein at time 0. Total change in catecholamine concentration from baseline [area under the curve (AUC)] is shown at right of each panel. Data are means ± SE, and were analyzed by repeated-measures ANOVA and Scheffé’s post hoc test. *Different between episodes at P < 0.01; †different between episodes at P < 0.05.](image-url)
There was a modest increase in plasma NE in response to insulin injection (F_{7,175} = 16.81, P < 0.001) (Fig. 2, bottom). The increase of plasma NE to insulin was not significantly reduced by multiple episodes of hypoglycemia, although only at 90 min postinjection were the plasma NE levels significantly reduced by multiple episodes of hypoglycemia (F_{1,112} = 4.28, P < 0.001). The release of NE in the PVN followed an episodic pattern throughout the 90-min collection period. The increase of extracellular NE to insulin was not significantly different after multiple hypoglycemic episodes (F_{1,110} = 1.46, P = 0.15). Although the response pattern seemed to have a somewhat broader shape, there was no difference in the total change in NE, represented by similar AUCs (Fig. 3).

### DISCUSSION

The main finding we report here is that the characteristic increases in extracellular NE in the PVN and VMH to insulin-induced hypoglycemia were not diminished after 4 days of repeated hypoglycemic episodes. We previously demonstrated that extracellular concentrations of NE and GABA in the hypothalamus were increased in a bimodal pattern within 60 min of the onset of a single episode of hypoglycemia (2). The present study confirms our earlier findings on NE. During the first hypoglycemic episode, the timing and pattern of NE release in the PVN and VMH were the same as reported before. Between 60 and 90 min after insulin administration, levels of extracellular NE in the VMH tend to gradually return to baseline, whereas in the PVN two more peaks were observed during the 50- to 60- and 80- to 90-min intervals. The responses of NE in the VMH were not affected by recurrent hypoglycemia, but the pattern of NE release in the PVN seemed to be smoothed out. This effect is not due to diminished NE release, but can be entirely ascribed to small temporal

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Table 1. Norepinephrine concentrations in extracellular space of VMH and PVN under baseline conditions, before first and fourth hypoglycemic episodes

<table>
<thead>
<tr>
<th></th>
<th>1st Episode</th>
<th>4th Episode</th>
<th>t</th>
<th>P</th>
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<tbody>
<tr>
<td>VMH</td>
<td>7.27±0.72 (7)</td>
<td>9.07±0.56 (7)</td>
<td>1.96</td>
<td>0.07</td>
</tr>
<tr>
<td>PVN</td>
<td>10.75±0.78 (6)</td>
<td>15.68±1.63 (8)</td>
<td>6.00</td>
<td>0.03</td>
</tr>
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Data are means ± SE (n values in parentheses). Extracellular norepinephrine in ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN) was determined by dialysate concentrations corrected for in vivo probe recoveries.

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The NE increase in the VMH in response to insulin was not different after multiple hypoglycemic episodes (F_{1,123} = 1.27, P = 0.25). Extracellular levels of NE in the PVN were also increased in response to insulin-induced hypoglycemia (F_{1,121} = 4.28, P < 0.001). The release of NE in the PVN followed an episodic pattern throughout the 90-min collection period. The increase of extracellular NE to insulin was not significantly different after multiple hypoglycemic episodes (F_{1,110} = 1.46, P = 0.15). Although the response pattern seemed to have a somewhat broader shape, there was no difference in the total change in NE, represented by similar AUCs (Fig. 3).
shifts of peak concentrations in individual animals, leading to a smooth curve that depicts the group means.

Three episodes of antecedent recurrent hypoglycemia reduced the increase of plasma Epi concentrations in response to subsequent insulin-induced hypoglycemia. The sympathoadrenal response was not completely absent, but merely suppressed throughout the hypoglycemic period. The modest increase in plasma NE concentration to hypoglycemia was not affected by multiple hypoglycemic episodes. These results are consistent with earlier reports from human and animal studies. For example, Shum et al. (26) reported the sympathoadrenal responses to be significantly reduced following seven recurrent episodes of hypoglycemia. Only 2 days of repeated hypoglycemia was sufficient to inhibit elevation of plasma Epi levels, but adrenal sympathetic nerve activity was not reduced (27). In patients with diabetes mellitus (14) and in nondiabetic individuals (13) peak plasma Epi levels were reduced during hypoglycemia.

Several possible explanations can be offered for the preservation of noradrenergic activity to recurrent hypoglycemia in the face of attenuated sympathoadrenal responses. The first possibility is that increased noradrenergic activity is mediated by direct insulin action in the brain. With computerized densitometry $^{125}$I-insulin binding sites have been identified in tissue section of rat hypothalamus (6). Highest densities were obtained in PVN, arcuate nucleus, and suprachiasmatic nucleus. Intermediate densities were obtained in VMH and median eminence. However, with the use of euglycemic hyperinsulinemic clamps we have shown in the past that, whereas noradrenergic activity in the PVN was somewhat elevated to insulin-induced glucoprivation (2), alternatively, modulation of autonomic activation to recurrent hypoglycemia may be taking place downstream of hypothalamic NE release. It could, for example, involve postsynaptic mechanisms including noradrenergic receptors or intracellular signal transduction. This view is supported by blockade of sympathoadrenal outflow to hypoglycemia during application of noradrenergic antagonists into the PVN (5). A third possibility is to assume the key modulation to take place in a glucose responsive pathway collateral to hypothalamic NE outflow. Some glucose responsive neurons in hindbrain cell groups A1/C1, C2, and C3 extend efferent projections to the hypothalamus (23); others project to sympathetic motoneurons in the intermediolateral column of the spinal cord (22, 29), Sanders et al. (24) noted attenuated c-Fos expression in these hindbrain centers to recurrent glucoprivation with 2-DG.

In earlier studies it was reported that neural activity in the medial hypothalamus, normally elevated to insulin-induced hypoglycemia, is not increased after repeated bouts of hypoglycemia (9). It has been postulated that the activity of hypothalamic noradrenergic neurons is suppressed after recurrent hypoglycemia, because activity levels of tyrosine hydroxylase, rate-limiting enzyme for NE synthesis, are indistinguishable from saline controls (11). However, in these studies tissue homogenates were used that were taken from relatively large brain regions. Here we demonstrate that the increase in neurotransmission in the noradrenergic fiber shell immediately surrounding the VMH is preserved after recurrent hypoglycemia. This NE is released from hindbrain projections onto the medial hypothalamus (17, 19), and is not dependent on local NE synthesis. We previously showed that NE released in this area stimulates local GABAergic neurotransmission (1), which in turn may indeed silence interneurons or hypothalamic output. It is interesting to note that baseline concentrations of extracellular NE are increased after multiple hypoglycemic episodes, most clearly in the PVN. Inasmuch as responses to hypoglycemia are preserved when expressed as percent of baseline, total noradrenergic activity actually increases to recurrent hypoglycemia. This is consistent with our recent finding that extracellular glucose is further decreased to multiple episodes of hypoglycemia compared with a single episode (8). We believe increased neural activity may be responsible for increased glucose utilization, thereby causing the exacerbated glucose dip.

In summary, 4 days of recurrent daily hypoglycemic episodes reduced sympathoadrenal responses to systemic hypoglycemia. However, the concurrent increase in noradrenergic activity in the PVN and VMH was not attenuated. We therefore suggest that inhibition of sympathoadrenal responses in HAAF is caused by a postsynaptic mechanism downstream of hypothalamic NE release, or is located in a collateral pathway in the neural circuitry that regulates glucose balance.

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GRANTS

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REFERENCES

HYPOTHALAMIC NOREPINEPHRINE DURING HAAF


