Antecedent Hypercortisolemia Is Not Primarily Responsible for Generating Hypoglycemia-Associated Autonomic Failure

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Hypoglycemia-associated autonomic failure (HAAF) occurs commonly in patients with longstanding diabetes, placing affected patients at increased risk for severe hypoglycemia. Previous studies have suggested that hypoglycemia-induced hypercortisolemia may be responsible for blunting subsequent sympathoadrenal responses to hypoglycemia; however, this view remains highly controversial. In this work, we sought to better define the role of antecedent hypercortisolemia in generating HAAF, using two complimentary experimental models in nondiabetic human subjects: 1) antecedent hydrocortisone infusions (simulating physiologic cortisol responses to hypoglycemia) and 2) antecedent hydrocortisone, with and without concurrent blockade of endogenous cortisol production using oral metyrapone. Our results showed no effect of antecedent hypercortisolemia on epinephrine responses to subsequent hypoglycemia (area under the curve/time 280 ± 53 vs. 337 ± 57 pg/ml, P = 0.16). Of particular importance, selective blockade of endogenous cortisol production during antecedent hypoglycemia had no effect on subsequent counterregulatory responses to hypoglycemia. Compared with epinephrine responses following antecedent euglycemia (area under the curve/time 312 ± 38 pg/ml), epinephrine responses were comparably blunted following antecedent hypoglycemia, regardless of whether concurrent metyrapone blockade was employed (198 ± 28 vs. 192 ± 28 pg/ml, P = NS). Similar results were obtained for glucagon and ACTH levels. Considered together, these observations provide strong evidence that hypoglycemia-induced hypercortisolemia is not primarily responsible for the generation of HAAF. Diabetes 55:1121–1126, 2006
In this work, we sought to better define the role of antecedent hypercortisolemia in generating HAAF, using two complimentary experimental models in nondiabetic human subjects: 1) antecedent hydrocortisone infusions (targeting cortisol levels typically seen during hypoglycemia) and 2) antecedent hypercortisolemia, with and without concurrent blockade of endogenous cortisol production using oral metyrapone.

RESEARCH DESIGN AND METHODS
Healthy nondiabetic subjects aged 18–49 years were screened with a history and physical examination, fasting blood samples, a urine pregnancy test (if female), and a 12-lead electrocardiogram. Exclusion criteria included pregnancy, BMI $>28\ \text{kg/m}^2$, a history of systemic illness (including impaired fasting glycose, diabetes, and HPA axis disease), and medications that could affect either the HPA axis or counterregulatory responses to hypoglycemia. All subjects provided verbal and written informed consent before study participation. Both study protocols took place in the Yale University General Clinical Research Center (GCRC) and were approved by the Yale University School of Medicine Human Investigation Committee.

Hydrocortisone infusion study. A total of eight subjects (four men and four women) completed this study protocol. They had a mean ($\pm$SD) age of 27 $\pm$ 9 years, BMI of 24.2 $\pm$ 3.2 kg/m$^2$, fasting plasma glucose level of 88 $\pm$ 3 mg/dl, and morning cortisol levels of 18 $\pm$ 10 $\mu$g/dl. Subjects presented to the GCRC on two occasions. After an overnight fast, subjects first presented for their “control” clamp study at 7:00 A.M. Two intravenous catheters were inserted into opposite arms. The first catheter was used to deliver both a continuous insulin infusion (2 mU $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) and a variable infusion of 20% dextrose by infusion pump (Alaris Medical Systems, San Diego, CA). The second catheter was used for blood sampling; the involved hand was heated continuously to permit sampling of arterialized venous blood. After a 1-h rest period, at study time zero (8:30 A.M.), the insulin and dextrose infusions were started, with an initial goal of maintaining plasma glucose levels of 100 $\pm$ 3 mg/dl. At study time 30 min, the dextrose infusion rate was reduced, allowing plasma glucose levels to fall to 50 $\pm$ 3 mg/dl, where they were kept until the end of the clamp (study time 150 min). Throughout the study, blood samples were obtained for measurement of insulin, glucagon, catecholamine, growth hormone, cortisol, and ACTH levels.

After a minimum of 7 days, subjects returned for a 2-day inpatient protocol. On day 1, subjects received two identical 4-h infusions of hydrocortisone sodium phosphate (Merck, Whitehouse Station, NJ), beginning at 8:00 A.M. and 3:00 P.M. For each infusion, 20 mg of hydrocortisone was diluted in 250 ml of 0.9% sodium chloride then administered as a variable rate: 10 mg/h during the 1st h, then 3.33 mg/h for the last 3 h. On day 1, blood samples were obtained for measurement of cortisol levels only. On day 2, all subjects then completed a second, identical hydrocortisone clamp study, or “posthydrocortisone” study.

Metyrapone study. A total of 13 subjects (7 men and 6 women) completed this study protocol. They had a mean ($\pm$SD) age of 30 $\pm$ 8 years, BMI of 23.6 $\pm$ 1.9 kg/m$^2$, fasting plasma glucose levels of 85 $\pm$ 10 mg/dl, and morning cortisol levels of 12 $\pm$ 5 $\mu$g/dl. All 13 subjects completed three 2-day inpatient protocols at the Yale GCRC, which took place in random order and were separated by a minimum of 4 weeks. Each of the 2-day protocols consisted of three 3-h hyperinsulinemic (2 mU $\cdot$ kg$^{-1}$ $\cdot$ min$^{-1}$) clamp studies, with two clamps on day 1 and a third on day 2. The two day 1 clamps were designed to maintain either euglycemia (target plasma glucose 100 $\pm$ 3 mg/dl) or hypoglycemia (target plasma glucose 50 $\pm$ 3 mg/dl). The third clamp, on day 2, always targeted hypoglycemia. Figure 1 summarizes the three 2-day study protocols, using a simplified study timeline. On the night before each admission, at 11:00 p.m., subjects ingested either 30 mg/kg (maximum dose 3 g) of metyrapone (Alliance Pharmaceuticals, Webster, TX), or matching placebo pills. During the day 1 clamp studies, subjects received two additional doses of oral study drug (750 mg metyrapone or placebo) at 11:00 A.M. and 3:00 P.M. To maintain a double-blind study design, all study pills were prepared by the Yale New Haven Hospital Investigational Drug Pharmacy. All study investigators, GCRC research nurses, and study subjects were blinded to all oral medications throughout the study.

Following an overnight fast, subjects were admitted to the GCRC at 7:30 A.M. On day 1, subjects underwent two 3-h hyperinsulinemic clamp studies (9:00 A.M. to 12:00 P.M., then 3:00–6:00 P.M.), as described in detail above, with target plasma glucose levels of either 50 $\pm$ 3 mg/dl (hypoglycemia) or 100 $\pm$ 3 mg/dl (euglycemia). Throughout each day 1 clamp study, at 60-min intervals, blood samples were obtained for measurement of epinephrine, norepinephrine, ACTH, cortisol, and 11-deoxycorticisol (11-DOC) levels. Symptoms of hypoglycemia were also assessed every 60 mins, using a modified Edinburgh Hypoglycemia Scale (22). Upon completion of the day 1 clamps, meals were served at 6:00 and 9:00 p.m., then subjects were again fasted overnight. At 9:00 a.m. on day 2, all subjects underwent a third 3-h hypoglycemic clamp study. Throughout the day 2 clamps, blood samples were obtained at 30-min intervals for measurement of insulin, counterregulatory hormone (epinephrine, norepinephrine, glucagon, growth hormone, ACTH, and cortisol), and 11-DOC levels. Hypoglycemia symptom scores were also recorded at 30-min intervals.

Laboratory methods. Plasma glucose levels were measured using a glucose oxidase method (Beckman Instruments, Fullerton, CA). Insulin and glucagon (Linco Research, Fullerton, CA), ACTH (Diagnostic Systems Laboratories, Webster, TX), cortisol (Diagnostic Products, Los Angeles, CA), and 11-DOC levels.
and growth hormone (ICN Pharmaceuticals, Costa Mesa, CA) levels were measured using double-antibody radioimmunoassays. Plasma epinephrine and norepinephrine levels were assayed using high-performance liquid chromatography (ESA, Acton, MA).

Statistical methods. For the first study, baseline and posthydrocortisone hormone data were compared using paired Student's t tests. For the metyrapone study, hormone data and symptom scores obtained from the three different study conditions were analyzed using standard, parametric, three-way ANOVA utilizing a repeated-measures design. Paired Student's t tests were then employed to compare the individual study conditions. For both studies, statistical significance was defined by P < 0.05. Except where noted, all data are reported as means ± SE.

RESULTS

Hydrocortisone infusion study. Plasma cortisol levels during the day 1 hydrocortisone infusions rose from 18 ± 3 to 36 ± 3 μg/dl (peak levels) during the morning, and from 11 ± 2 to 29 ± 3 μg/dl during the afternoon. While steady-state (60–150 mins) plasma glucose levels were similar during the two hypoglycemic clamps (50 ± 1 vs. 49 ± 1 μg/dl, P = NS), insulin levels were slightly higher posthydrocortisone than at baseline (166 ± 13 vs. 142 ± 12 μU/ml, P = 0.04). As expected, ACTH responses to day 2 hypoglycemia were suppressed following the antecedent hydrocortisone infusions (area under the curve [AUC]/time = 78 ± 8 vs. 102 ± 12 pg/ml, P = 0.04). Additionally, though absolute cortisol responses were somewhat lower during the posthydrocortisone study, incremental AUCs/time for day 2 cortisol responses were not significantly different (4 ± 1 μg/dl posthydrocortisone vs. 3 ± 2 μg/dl control, P = 0.59).

As shown in Fig. 2, epinephrine responses to hypoglycemia were not significantly lower following antecedent hydrocortisone infusion, whether analyzed by peak level (631 ± 129 vs. 686 ± 119 pg/ml, P = 0.45), mean level between 90 and 150 min (529 ± 110 vs. 535 ± 84 pg/ml, P = 0.93), or AUC/time (280 ± 53 vs. 337 ± 57 pg/ml, P = 0.16). Norepinephrine responses were similarly unaffected. Glucagon (AUC/time 91 ± 13 vs. 94 ± 13 pg/ml, P = 0.36) and growth hormone (AUC/time 14.6 ± 3.2 vs. 16.4 ± 3.1 ng/ml, P = 0.46) responses to hypoglycemia were also unaltered by antecedent glucocorticoid administration.

Metyrapone study

Clamp studies. On day 1 (Fig. 3A), steady-state (45–180 min [A.M.], 405–540 min [P.M.]) plasma glucose levels were 98 ± 1 and 100 ± 1 mg/dl during the two euglycemic clamps,
On day 2 (Table 1; Fig. 4, top left panel), plasma glucose and insulin levels were similar for all three experimental conditions.

Day 1 hormone levels and symptom scores.

Mean 11-DOC levels rose to 105 ± 8 and 155 ± 9 ng/ml during the two day 1 clamps employing metyrapone blockade but remained flat (<2 ng/ml) during the other two experimental conditions. As expected, peak ACTH levels were significantly higher during metyrapone blockade (568 ± 60 and 805 ± 114 pg/ml) than during either euglycemia (90 pg/ml) or hypoglycemia alone (181 ± 31 and 131 ± 27 pg/ml).

Day 1 cortisol levels are shown in Fig. 3B. During euglycemia, mean cortisol levels were 13 ± 2 μg/dl in the

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**TABLE 1**

Metyrapone study, day 2 results

<table>
<thead>
<tr>
<th></th>
<th>Antecedent euglycemia</th>
<th>Antecedent hypoglycemia</th>
<th>Antecedent hypoglycemia with metyrapone</th>
<th>Antecedent euglycemia versus antecedent hypoglycemia</th>
<th>Antecedent hypoglycemia versus antecedent hypoglycemia with metyrapone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma glucose (mg/dl)</td>
<td>51 ± 1</td>
<td>49 ± 1</td>
<td>49 ± 1</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Insulin (μU/ml)</td>
<td>162 ± 7</td>
<td>153 ± 7</td>
<td>153 ± 8</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>ACTH (pg/ml)</td>
<td>115 ± 18</td>
<td>85 ± 16</td>
<td>90 ± 15</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Cortisol (μg/dl)</td>
<td>23.0 ± 2.6</td>
<td>20.0 ± 2.6</td>
<td>23.6 ± 1.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Epinephrine (pg/ml)</td>
<td>312 ± 38</td>
<td>192 ± 28</td>
<td>198 ± 28</td>
<td>0.006</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Norepinephrine (pg/ml)</td>
<td>233 ± 18</td>
<td>237 ± 28</td>
<td>236 ± 16</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>95 ± 11</td>
<td>67 ± 7</td>
<td>69 ± 9</td>
<td>0.003</td>
<td>0.004</td>
</tr>
<tr>
<td>Growth hormone (ng/ml)</td>
<td>18.9 ± 3.5</td>
<td>13.7 ± 1.5</td>
<td>14.2 ± 2.0</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Rise in symptom score (units)</td>
<td>12 ± 2</td>
<td>11 ± 3</td>
<td>8 ± 2</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are means ± SE. Plasma glucose and insulin levels, hormone levels, and rises in symptom scores measured during the day 2 hypoglycemic clamp studies. Plasma glucose and insulin are reported as steady-state values (45–180 mins), while hormone levels and rises in symptom scores are expressed as AUC/time. When significant, P values comparing each pair of experimental conditions are shown in the three rightmost columns. NS, nonsignificant.

49 ± 1 and 49 ± 1 mg/dl during hypoglycemia, and 50 ± 1 and 48 ± 1 mg/dl during hypoglycemia with metyrapone blockade.

**FIG. 4.** Metyrapone study, day 2 counterregulatory hormone levels. Mean (±SE) plasma glucose levels (top left panel), epinephrine levels (top right panel), ACTH levels (bottom left panel), and glucagon levels (bottom right panel) measured on day 2 for the three study conditions: 1) antecedent euglycemia, 2) antecedent hypoglycemia, and 3) antecedent hypoglycemia with metyrapone blockade.

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The image contains graphs and data tables related to the study on hypercortisolemia and its role in HAAF.
morning and 8 ± 1 μg/dl in the afternoon, while during hypoglycemia, cortisol levels peaked at 31 ± 2 and 26 ± 2 μg/dl. During hypoglycemia with metyrapone blockade, mean cortisol levels were indistinguishable from those observed during euglycemia (12 ± 1 and 7 ± 1 μg/dl, \( P = \text{NS} \)). Considered as a combined AUC/time for both clamps, total cortisol exposure was identical during euglycemia (10 ± 1 μg/dl) and during hypoglycemia with metyrapone blockade (10 ± 1 μg/dl, \( P = 0.73 \)). Both conditions resulted in ~50% less cortisol exposure than during hypoglycemia alone (20 ± 1 μg/dl, \( P < 0.0001 \) for both).

Epinephrine levels, norepinephrine levels, and symptom scores remained predictably flat during euglycemia. During hypoglycemia with metyrapone blockade, peak epinephrine levels were somewhat higher (831 ± 114 and 755 ± 96 pg/ml) than those observed during hypoglycemia alone (557 ± 63 and 478 ± 74 pg/ml, \( P = 0.02 \) for both). However, norepinephrine responses and symptom scores during the two hypoglycemic conditions were not significantly different.

**Day 2 hormone levels and symptom scores.** On day 2, high 11-DOC levels (58 ± 9 ng/ml) persisted following metyrapone blockade, whereas they remained low (<2 ng/ml) during the other two study arms. Day 2 ACTH responses following both antecedent hypoglycemia and antecedent hypoglycemia with metyrapone blockade were blunted by ~30% versus those following antecedent euglycemia. On the other hand, cortisol responses following day 2 hypoglycemia were not significantly different among the three study conditions (Table 1; Fig. 4).

Epinephrine responses following antecedent hypoglycemia were blunted by 38% compared with those following antecedent euglycemia. Endogenous cortisol blockade had no effect, since an identical degree of suppression (36%) was observed following antecedent hypoglycemia with metyrapone blockade. In parallel fashion, metyrapone blockade also failed to reverse blunted day 2 glucagon responses induced by antecedent hypoglycemia. As shown in Table 1, a similar trend was observed for growth hormone responses, though these results did not reach statistical significance. Lastly, day 2 norepinephrine levels and symptom scores did not differ significantly among any of the three experimental conditions.

**DISCUSSION**

Physiologic mechanisms leading to hypoglycemia unawareness and HAAF are incompletely understood. However, antecedent hypoglycemia has been clearly identified as a causative factor. In 1991, Heller and Cryer (9) demonstrated in nondiabetic human subjects that two 2-h episodes of hypoglycemia were sufficient to blunt subsequent neuroendocrine and symptomatic responses to hypoglycemia. Subsequent studies confirmed that antecedent hypoglycemia reduces neuroendocrine responses to hypoglycemia, both in nondiabetic subjects (9–11) and in patients with diabetes (12–14). Lending further credence to antecedent hypoglycemia as a primary instigator of HAAF, intensive insulin therapy has been clearly associated with suppressed counterregulatory responses (7,8,23), while meticulous avoidance of hypoglycemia can restore symptom and hormonal responses to hypoglycemia within a matter of weeks (24–26).

It has recently been suggested that hypoglycemia-induced hypercortisolism is at least partially responsible for generating HAAF. In 1996, Davis et al. (16) reported that nondiabetic human subjects receiving two 2-h cortisol infusions (2 μg · kg⁻¹ · min⁻¹ or ~9 mg/h) exhibited blunted day 2 neuroendocrine responses to hypoglycemia. Lending further support to their cortisol hypothesis, these authors later reported that following day 1 hypoglycemia, patients with Addison’s disease (who could not mount endogenous cortisol responses) had preserved day 2 counterregulatory responses to hypoglycemia (27). However, the latter study’s findings were complicated by profoundly suppressed epinephrine responses (<200 pg/ml or >60% lower than the epinephrine responses from our control studies) in the Addison’s patients, present even during the control studies; this may have limited the ability of antecedent hypoglycemia to exert an additional effect. In a separate study, McGregor et al. (17) showed that day 1 infusions of α-(1–24)-ACTH (producing high endogenous cortisol levels of 45 ± 3 μg/dl) also blunted day 2 catecholamine responses to hypoglycemia, lending additional support to antecedent hypercortisolism as a possible mediator of HAAF.

Recently, several studies using rodent models have challenged the cortisol hypothesis for generating HAAF. In one study, Shum et al. (19) found that antecedent glucocorticoid administration had no discernable impact upon subsequent epinephrine responses to hypoglycemia. In another study, direct delivery of corticosterone into the hypothalamus also failed to blunt subsequent counterregulatory responses to hypoglycemia (20). From our lab, Flanagan et al. (21) reported that antecedent glucocorticoid exposure actually augmented subsequent epinephrine responses to hypoglycemia, whereas antecedent CRH exposure suppressed the sympathoadrenal response. Recent studies in human subjects have also challenged the cortisol hypothesis. In 2003, Raju et al. (18) found that low-dose cortisol infusions (1.0–1.4 μg · kg⁻¹ · min⁻¹, or ~4–6 mg/h) on day 1 had no effect upon day 2 sympathoadrenal responses to hypoglycemia. Our hydrocortisone infusion study results are consistent with those of Raju et al. In our hands, low-dose day 1 hydrocortisone infusions (averaging 5 mg/h) had no effect upon day 2 sympathoadrenal responses to hypoglycemia.

Considered together, these animal and human data suggest that antecedent hypercortisolism may not be the primary mediator of HAAF. We believe that the contrasting conclusions of earlier glucocorticoid infusion studies may be related to the dose of steroid administered. While supraphysiologic hypercortisolism (as achieved by McGregor et al. (17)) may play a role in modulating sympathoadrenal responses to hypoglycemia, more moderate cortisol elevations (as achieved by Raju et al. (18) and by our hydrocortisone infusion study, with cortisol responses closer to those typically observed during hypoglycemia) do not appear to exert a similar effect.

We acknowledge that our hydrocortisone infusion study is limited by its sequential design, in which control clamps always preceded the posthydrocortisone studies. In addition, a significant limitation of all glucocorticoid infusion studies, including our own, is that this experimental model raises cortisol levels while suppressing other components of the HPA axis. These conditions are in direct contrast with actual hypoglycemia, during which the entire HPA axis is concurrently activated. To address these methodologic shortcomings, we conducted serial hypoglycemic clamp studies (on nondiabetic human volunteers) with and without oral metyrapone, which specifically blocks hypoglycemia-induced hypercortisolism without suppressing central activation of the HPA axis. The primary goal of this study was to determine the specific impact of blocking endogenous cortisol...
production during antecedent hypoglycemia on subsequent neuroendocrine responses to hypoglycemia. We employed three doses of oral metyrapone to successfully block physiologic cortisol responses to day 1 hypoglycemia. In our study, metyrapone blockade produced euglycemic cortisol levels during day 1 hypoglycemia.

In our subjects, antecedent hypoglycemia blunted day 2 (AUC) epinephrine responses to hypoglycemia by 38% when compared with those observed following a control (antecedent euglycemia) study. This effect was completely unaltered by the addition of metyrapone, since hypoglycemia with metyrapone blockade blunted day 2 epinephrine responses by a nearly identical 36%. Similarly, no effects of metyrapone were observed on day 2 norepinephrine, glucagon, ACTH, or cortisol responses. (We were unable to demonstrate significant differences in symptom scores among the three study conditions.) When active metyrapone was given, high levels of 11-DOC were expectedly observed. However, while potentially a confounding factor, 11-DOC is not thought to have significant glucocorticoid activity (28).

In summary, we have shown that selective blockade of endogenous cortisol production during antecedent hypoglycemia does not alter the effect of antecedent hypoglycemia to blunt subsequent counterregulatory responses to hypoglycemia. In addition, we concur with prior authors that antecedent infusion of low-dose glucocorticoids does not dampen subsequent sympathoadrenal responses to hypoglycemia. Considered together, these observations provide strong evidence that hypoglycemia-induced hypercortisolemia is not primarily responsible for the development of HAAF.

Of course, our data do not exclude an etiologic role for the entire HPA axis. Mechanisms for generating defective counterregulation may reside further upstream, perhaps at the level of CRH, urocortin, or CRH receptors. Preliminary rodent data from our laboratory suggest that changes in the activation state of CRH receptors in the ventromedial hypothalamus modulate subsequent counterregulatory hormone responses to hypoglycemia (29).

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REFERENCES