Counterregulatory deficits occur within 24 h of a single hypoglycemic episode in conscious, unrestrained, chronically cannulated mice

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Counterregulatory deficits occur within 24 h of a single hyperglycemic episode in conscious, unrestrained, chronically cannulated mice. Am J Physiol Endocrinol Metab 290: E678–E684, 2006; doi: 10.1152/ajpendo.00383.2005.—Hypoglycemia-induced counterregulatory failure is a dangerous complication of insulin use in diabetes mellitus. Controlled hypoglycemia studies in gene knockout models, which require the use of mice, would aid in identifying causes of defective counterregulation. Because stress can influence counterregulatory hormones and glucose homeostasis, we developed glucose clamps with remote blood sampling in conscious, unrestrained mice. Male C57BL/6 mice implanted with indwelling carotid artery and jugular vein catheters were subjected to 2 h of hyperinsulinemic glucose clamps 24 h apart, with a 6-h fast before each clamp. On day 1, blood glucose was maintained (euglycemia, 178 ± 4 mg/dl) or decreased to 62 ± 1 mg/dl (hypoglycemia) by insulin (20 mU·kg⁻¹·min⁻¹) and variable glucose infusion. Donor blood was continuously infused to replace blood sample volume. Baseline plasma epinephrine (32 ± 8 pg/ml), corticosterone (16.1 ± 1.8 µg/dl), and glucagon (35 ± 3 pg/ml) were unchanged during euglycemia but increased significantly during hypoglycemia, with a glycermic threshold of ~80 mg/dl. On day 2, all mice underwent a hypoglycemic clamp (blood glucose, 64 ± 1 mg/dl). Compared with mice that were euglycemic on day 1, previously hypoglycemic mice had significantly higher glucose requirements and significantly lower plasma glucagon and corticosterone (n = 6/group) on day 2. Epinephrine tended to decrease, although not significantly, in repeatedly hypoglycemic mice. Pre- and post-clamp insulin levels were similar between groups. We conclude that counterregulatory responses to acute and repeated hypoglycemia in unrestrained, chronically cannulated mice reproduce aspects of counterregulation in humans, and that repeated hypoglycemia in mice is a useful model of counterregulatory failure.

hypoglycemia-associated autonomic failure; hypoglycemia unawareness; catecholamines; norepinephrine; glucocorticoids

HYP戈LICYCMA-ASSOCIATED COUNTERREGULATORY FAILURE is an increasingly common complication of intensive insulin therapy and poses new obstacles to glucose control in insulin-dependent diabetes. Counterregulatory failure is evident as decreases in either the absolute level or the threshold for activation of sympathoadrenal, glucocorticoid, and glucagon responses to hypoglycemia (3). Of these responses, the glucagon and sympathoadrenal responses are the most critical because of the rapidity with which they are activated and serve to increase blood glucose (4). Counterregulatory failure is not unique to individuals with type 1 diabetes and can be demonstrated experimentally in nondiabetic subjects after only one to two episodes of hypoglycemia (5, 6). However, because type 1 diabetes patients lack the ability to decrease insulin and often lose hypoglycemia-induced glucagon secretion with increased duration of their disease (3), they are in danger of the central nervous system effects of severe hypoglycemia, including loss of consciousness, coma, or even death, when sympathoadrenal responses are impaired by recurrent hypoglycemia. Similar risk factors have also been demonstrated for late-stage type 2 diabetics who require insulin therapy (3).

Identifying the factors that contribute to counterregulatory failure will aid in reducing the potentially fatal risks of insulin therapy. Several mechanisms have been proposed, including increased brain glucose uptake (1), elevations in nonglucose cerebral energy substrates (13, 44), hypoglycemia-induced release of glucocorticoids (7), corticotropin-releasing hormone (CRH), or CRH-related peptides (17) and inhibition of hindbrain noradrenergic neurons (36). However, support for these mechanisms has not been documented consistently (3, 11), and the causes of counterregulatory failure are likely to be multifactorial.

Elucidation of counterregulatory mechanisms would be aided by well-controlled studies of hypoglycemia in gene knockout models, which require the use of mice. Glucose clamps in mice commonly involve either acute vascular catheterization surgery and anesthesia (34) or tail nick sampling and restraint (12, 16, 30). However, surgery, pain, handling, and restraint are all stresses that can activate sympathoadrenal and adrenocortical activity (24, 33). Stress and autonomic activation can also stimulate glucagon secretion (42, 45). The potential impact of elevations in these counterregulatory hormones is supported by experimental evidence in humans and animals that stress can induce hyperglycemia (41). The balance between catecholamines, glucocorticoids, and glucagon can have distinct effects on glycogenolysis, gluconeogenesis, and glucose flux across specific tissues (18, 21), such that, depending on its nature and duration, stress could confound the characterization of metabolic as well as hormonal aspects of counterregulation. Therefore, glucose clamp techniques that minimize potential confounding effects of stress on counterregulatory responses are needed in mice. We have developed techniques for glucose clamps and remote blood sampling in chronically cannulated, conscious, unrestrained C57BL/6 mice. With these techniques, we have tested the effects of acute and repeated hypoglycemia on counterregulation in the mouse.
METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committees of Albany Medical College and Vanderbilt University. Male C57BL/6 mice (JAX Research Systems, Bar Harbor, ME) were housed on a 12:12-h light-dark cycle (lights on at 6 AM) and used at 2 mo of age (24 ± 0.4 g body wt). Mice were implanted with chronic indwelling carotid artery and jugular vein catheters under pentobarbital sodium anesthesia (50 mg/kg, ip), as previously described (22). The catheters were tunneled under the skin to exit in the suprascapular area. Catheters were flushed daily with heparin (200 U/ml) after surgery. Mice were used for glucose clamp studies 5–7 days after surgery, when they had regained their preoperative body weight.

Glucose clamps. On the day of the study, mice were transferred within 1 h of lights-on to 1-liter chambers with bedding but no food or water. At this time, extension lines were attached to the arterial and venous catheters and exteriorized through a lid on the chamber. Mice were studied in the postsorbptive state starting 7 h after lights-on. After a baseline arterial blood sample (160 μl), regular insulin (Humulin-R; Lilly, Indianapolis, IN) was infused intravenously at 20 μU·kg⁻¹·min⁻¹ for 120 min, combined with a variable infusion of glucose to match the baseline incoming blood glucose for each mouse (euglycemia) or to maintain target blood glucose levels of 70 mg/dl (hypoglycemia). In preliminary experiments, we determined that the insulin infusion rate was necessary to decrease glucose consistently and maintain target levels for ≥60 min, the length of hypoglycemia indicated by our previous work to encompass peak and plateau counterregulatory hormone responses (28). Glucose was measured every 10 min in whole arterial blood (5 μl) with a glucose meter (Hemocue, Lake Forest, CA). Larger arterial samples (160 μl) were drawn for analysis of plasma hormones at 30 and 120 min. To replace volume and red cells lost in sampling, heparinized blood cells from donor mice were resuspended in saline and infused intravenously, along with the insulin and glucose, at 6 μl/min.

Once the 120-min blood sample had been collected on day 1, insulin infusion was stopped and glucose infusion was continued to restore euglycemia in hypoglycemic mice. Mice were then returned to their home cages with food and water overnight. To assess effects of repeated hypoglycemia on counterregulatory responses, all mice were subjected on the next day (day 2) to a hyperinsulinemic hypoglycemic glucose clamp. All day 2 procedures were the same as they were for the day 1 hypoglycemic clamp, except that an additional 160-μl blood sample was collected at 15 min for blood glucose and plasma hormones. Mice were killed immediately after the 120-min blood sample on day 2 by pentobarbital sodium overdose (100 mg/kg iv) and decapitation.

Plasma assays. Plasma epinephrine and norepinephrine (lower detection limit, 20 pg/ml) were measured in 50-μl plasma samples by HPLC according to previously described methods (20). The interassay coefficient of variation for epinephrine was 8.5% for the low control (~300 pg/ml) and 4.0% for the high control (~800 pg/ml). Plasma corticosterone [MP Biomedical (formerly ICN), Irvine, CA] and insulin (Linco, St. Louis, MO) were measured in 2.5- and 25-μl samples, respectively, using radioimmunoassays previously validated in mice for these volumes (2, 26, 28). Glucagon was assayed in 20-μl plasma samples, using a radioimmunoassay from Linco; the assay had a sensitivity of 10 pg/ml and interassay coefficients of variation of 8.6% for samples in the 55–70 pg/ml range and 8.2% for samples in the 215–250 pg/ml range (2, 26, 28).

Statistics. Data were analyzed by two-way ANOVA with repeated measures across day and time. Post hoc tests comparing two groups of mice at individual time points were performed by t-test with Bonferroni correction (Statview 5.0; SAS Institute, Cary, NC). Significance was defined as P < 0.05. Data are presented throughout as means ± SE.

RESULTS

Two groups of mice were studied. One group was exposed to hyperinsulinemic hypoglycemia on both day 1 and day 2 (Hypo-Hypo). The second group was exposed to a hyperinsulinemic euglycemic clamp on day 1 and a hypoglycemic hyperinsulinemic clamp on day 2 (Eu-Hypo). Blood glucose levels for day 1 of the sequential clamps are shown in Table 1. Baseline blood glucose was similar between groups and averaged 173 ± 6 mg/dl for all mice (n = 12). Average glucose levels on day 1 during the last hour of the clamp in Hypo-Hypo mice were 62 ± 1 (n = 6). Average glucose levels in Eu-Hypo mice during the last hour (178 ± 4 mg/dl; n = 6/group) were comparable to initial blood glucose levels. Glucose requirements in Hypo-Hypo mice on day 1 increased to a plateau of 15 ± 1 mg·kg⁻¹·min⁻¹ during the last hour of study. The glucose infusion required to maintain euglycemia was higher at all times (Table 1) and was 79 ± 2 mg·kg⁻¹·min⁻¹ during the last hour of the clamp on day 1 (n = 6/group).

Baseline levels of epinephrine were low and near the limit of detection (pooled 0-min levels for all mice on day 1, 32 ± 8 pg/ml; n = 12). Plasma norepinephrine was <300 pg/ml in all but two mice (pooled 0-min levels for all mice on day 1, 185 ± 33 pg/ml; n = 12). Plasma glucagon was also near the lower detection limit in most samples (pooled 0-min levels for all mice on day 1, 35 ± 3 pg/ml; n = 12). Initial plasma corticosterone levels on day 1 were 16.1 ± 1.8 μg/dl (n = 12).

Only mice subjected to hypoglycemia exhibited increases in counterregulatory hormones on day 1 (Fig. 1). Plasma epinephrine, corticosterone and glucagon increased in Hypo-Hypo but not in Eu-Hypo mice on day 1. Plasma norepinephrine did not change in either group (Fig. 1). We estimated the threshold for epinephrine, corticosterone, and glucagon responses to hypoglycemia by determining the glucose level at which each hormone exceeded the 95% confidence limit (39) of levels measured in euglycemic mice. Plasma epinephrine, corticosterone, and glucagon were consistently increased at glucose levels in the 70–80 mg/dl range (Fig. 2).

| Glucose, mg/dl | Eu-Hypo | 183±2 | 232±16 | 173±11 | 154±9 | 157±7 | 156±8 | 167±8 | 184±6 | 191±11 | 184±9 | 184±7 | 182±9 | 169±12 | 149±13 |
| Glucose, mg/dl | Hypo-Hypo | 162±8 | 94±6 | 83±6 | 85±4 | 82±4 | 79±3 | 74±4 | 68±2 | 64±2 | 60±3 | 61±2 | 61±4 | 62±2 | 62±2 |

| GFR, mg·kg⁻¹·min⁻¹ | Eu-Hypo | 56±1 | 54±2 | 60±3 | 70±3 | 75±4 | 82±5 | 84±4 | 83±5 | 79±4 | 79±4 | 78±5 | 76±5 | 78±3 | 78±3 |
| GFR, mg·kg⁻¹·min⁻¹ | Hypo-Hypo | 0 | 0 | 0 | 0.7±7 | 4±2 | 4±1 | 6±2 | 6±2 | 9±2 | 12±1 | 15±2 | 15±2 | 17±2 | 19±3 |

Values are means ± SE; n = 6 per group. GFR, glucose infusion rate; Eu-Hypo, mice subjected to a euglycemic clamp on day 1 followed by a hypoglycemic clamp on day 2; Hypo-Hypo, mice exposed to a hypoglycemic clamp on days 1 and 2.
On day 2, baseline blood glucose levels were similar between Eu-Hypo and Hypo-Hypo groups (Fig. 3, bottom). Glucose levels were also similar between Eu-Hypo and Hypo-Hypo mice at all times during insulin-induced hypoglycemia on day 2, such that neither the absolute levels nor the rate of fall differed between groups (Fig. 3, bottom). The average glucose levels during the last hour of hypoglycemia on day 2 were 65 ± 1 and 63 ± 1 mg/dl in Hypo-Hypo and Eu-Hypo mice, respectively. A significantly higher rate of glucose infusion was required to maintain equivalent glucose levels in Hypo-Hypo vs. Eu-Hypo mice during the first 70 min of hypoglycemia on day 2 (Fig. 3, top). Total glucose requirements, calculated as the area under the glucose infusion rate curve, were also significantly higher in Hypo-Hypo mice (Hypo-Hypo vs. Eu-Hypo, 2,531 ± 374 vs. 1,161 ± 207 mg/kg, n = 6). On both study days, plasma insulin levels were similar at baseline and increased to comparable levels after each clamp (Table 2).

Baseline hormone levels on day 2 were not affected by prior exposure to hypoglycemia or euglycemia on day 1 (Fig. 4). Plasma epinephrine tended to be lower on day 2 in Hypo-Hypo vs. Eu-Hypo mice, but this effect was not significant (Fig. 4, top). Norepinephrine levels on day 2 also did not differ between Hypo-Hypo and Eu-Hypo mice. However, increases in plasma corticosterone on day 2 were significantly less by 120 min in Hypo-Hypo vs. Eu-Hypo mice (Fig. 4, lower middle). Plasma glucagon responses to hypoglycemia on day 2 were also blunted at 15 min in Hypo-Hypo mice (Fig. 4, bottom). Area under the plasma glucagon curve was also significantly reduced in Hypo-Hypo mice (Hypo-Hypo vs. Eu-Hypo, 426 ±
There were no significant differences in area under the curve for plasma epinephrine, corticosterone, or norepinephrine (not shown). Post hoc comparisons between day 1 and day 2 responses to hypoglycemia in Hypo-Hypo mice did show that 120-min epinephrine levels were lower after repeated hypoglycemia. However, after correcting post hoc test results for the comparison between Hypo-Hyp and Eu-Hyp mice at any time by post hoc testing despite significant main effects by ANOVA; \( n = 6 \) group. *\( P < 0.05 \), Eu-Hyp vs. Hypo-Hyp at the same time point.

**Table 2.** Baseline (0 min) and final (120 min) plasma insulin levels produced by 120-min hyperinsulinemic glucose clamps in Eu-Hyp and Hypo-Hyp mice

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>120 min</td>
</tr>
<tr>
<td>Eu-Hyp</td>
<td>1.9 ± 0.8</td>
<td>15.2 ± 2.7</td>
</tr>
<tr>
<td>Hypo-Hyp</td>
<td>1.0 ± 0.2</td>
<td>15.9 ± 1.6</td>
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Values are means ± SE in ng/ml and are as described in METHODS; \( n = 6 \) group.

**DISCUSSION**

We have established a hypoglycemic glucose clamp model that permits sequential glucose clamps and repeated hormone sampling with minimal extraneous stress in mice. Using this model, we have demonstrated hypoglycemia-specific stimulation of epinephrine, glucocorticoid, and glucagon secretion, as
deficits in mice after a single 2-h episode of hypoglycemia, the
quent hypoglycemia (5, 6). We observed counterregulatory
mone and endogenous glucose production responses to subse-
hypoglycemia are sufficient to diminish counterregulatory hor-
as little as a single or two brief (10–30 min) prior episodes of
regulation in other species (14, 29, 37–39). Thus counterregu-
elevated norepinephrine is not a consistent feature of counter-
duced hypoglycemia in humans and dogs (25, 39) and
is notably higher than the 60–70 mg/dl threshold reported for
mice at glucose levels that were close to 80 mg/dl. This level
epinephrine, corticosterone, and glucagon increased in
iments were insufficient to derive a precise glycemic threshold,
frequency and total number of hormone samples in our exper-
responses were also related to stimulus intensity, with deeper
glucagon in male C57BL/6 mice. Counterregulatory hormone
significantly increased plasma epinephrine, corticosterone, and
control hypoglycemia. The reduced glucagon response might
have accounted in part for the increased glucose requirements
during the first hour of day 2 hypoglycemia (4, 9), although
significant increases in glucose infusion rates before 15 min
suggest that glucagon or other counterregulatory factors could
have been reduced at even earlier times in Hypo-Hypo mice.
We did not observe significant hypoglycemia-associated
inhibition of epinephrine when responses were compared be-
tween previously euglycemic and previously hypoglycemic
mice. However, we did detect decreases in epinephrine re-
sponses between the first and second exposures to hypoglyce-
mia within the Hypo-Hypo group, suggesting that repeated
hypoglycemia had the expected inhibitory effects on sympa-
thoadrenal activity, even if those effects did not reach statisti-
cal significance in multiple comparisons. It is unlikely that
hypoglycemia was insuffi ciently prolonged or severe enough to
impair sympathoadrenal responsiveness. Mice were hypogly-
cemic for as long as the time shown to reduce hypoglycemia-
induced plasma catecholamines in humans (6, 8), and anteced-
tent hypoglycemia of ~40 mg/dl did not further inhibit day 2
epinephrine responses. Conversely, it also seems unlikely that
hypoglycemia was too severe. Because epinephrine and gluca-
gon responses did not exceed 300 pg/ml, responses to even
milder hypoglycemia might have been too low to detect dec-
rements due to repeated hypoglycemia. We doubt that limited
group size prevented us from detecting significant differences
between Hypo-Hypo and Eu-Hypo epinephrine responses. In
subsequent studies of gene knockout models, we have found
that wild-type controls on a C57BL/6 background exhibit
counterregulatory hormone responses to repeated hypoglyce-
mia that are statistically indistinguishable from those reported
here. Pooling data from these and the present experiments,
representing a total of 16 Hypo-Hypo mice and 11 Eu-Hypo
mice, did not alter results from those described above. Com-
parisons between Eu-Hypo and Hypo-Hypo mice still showed
significant reductions after repeated hypoglycemia only in
glucagon and corticosterone and not in epinephrine (28a).
The lack of more robust inhibition of epinephrine responses
by recurrent hypoglycemia could indicate that counterregula-
tory mechanisms in mice differ from those in humans and other

well as defective counterregulation after repeated exposure to
controlled hypoglycemia.
Baseline plasma epinephrine levels were typically low and
near the limit of detection on both days of sequential glucose
clamps. Although initial levels of plasma corticosterone in our
studies seem elevated compared with circadian trough levels
measured in fed mice (27, 32), this is probably an effect of
fasting. Glucocorticoid levels in the present study were com-
parable to those measured at midday in similarly fasted, unop-
erated mice (L. Jacobson, unpublished observations). Baseline
epinephrine and corticosterone levels were ~30- and 3-fold
lower, respectively, than those reported in mice that were re-
strained and sampled from the tail tip (16). Neither plasma
catecholamines nor glucocorticoids increased significantly dur-
ing a euglycemic clamp combined with repeated blood sam-
ping. Our model therefore minimizes stress-associated effects
on counterregulatory hormones and metabolic parameters.
As has been shown in other species (5, 15, 39, 40, 47),
clamping glucose at hypoglycemic but not euglycemic levels
significantly increased plasma epinephrine, corticosterone, and
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species. Nevertheless, many studies (5, 6, 8, 17, 38, 40, 46) that demonstrate evidence of counterregulatory deficits do not show decreases in all counterregulatory hormones at once. Even some human studies (5) do not find decreases in epinephrine when other counterregulatory hormones are decreased. Instead, we suspect that inconsistencies between the effects of repeated hypoglycemia on sympathoadrenal responses in mice and humans are due to blood sampling constraints in mice. Increased sampling frequency, made possible by larger blood volume in humans, increases the chances of detecting changes in absolute, incremental, or integrated hormonal responses. Because even significant hormonal differences on day 2 between Hypo-Hypo and Eu-Hypo mice were relatively transient, our limited hormone sampling schedule may have precluded the detection of more significant sympathoadrenal inhibition.

It is possible that factors we did not measure were more significantly affected by repeated hypoglycemia in mice. We did not analyze growth hormone, because it was reported to have a minor role in counterregulation in mice (43). However, this conclusion might not apply to recurrent hypoglycemia in the mouse. We also did not have any measurements of sympathetic activity other than plasma norepinephrine and epinephrine. Because plasma norepinephrine might not be a reliable indicator of sympathetic nerve activity, particularly during hypoglycemia (10, 19), we may have missed changes in sympathetic outflow induced by repeated hypoglycemia. Changes in lactate or ketone production, which we did not assess, have also been suggested to contribute to the suppression of counterregulatory responses to subsequent hypoglycemia in normal humans. Diabetologia 48: 1897–1903, 2000.


