Glucocorticoid-deficient corticotropin-releasing hormone knockout mice maintain glucose requirements but not autonomic responses during repeated hypoglycemia

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Glucocorticoid-deficient corticotropin-releasing hormone knockout (CRH KO) mice. Conscious, chronically cannulated, unrestrained WT and CRH KO mice underwent a euglycemic (Prior Eu) or hypoglycemic clamp (Prior Hypo) on day 1 followed by a hypoglycemic clamp on day 2 (blood glucose both days, 65 ± 1 mg/dl). Baseline epinephrine and glucagon were similar, and norepinephrine was elevated, in CRH KO vs. WT mice. CRH KO corticosterone was almost undetectable (<1.5 µg/dl) and unresponsive to hypoglycemia. CRH KO glucose requirements were significantly higher during day 1 hypoglycemia despite epinephrine and glucagon responses that were comparable to or greater than those in WT. Hyperinsulinemic euglycemia did not increase hormones or glucose requirements above baseline. On day 2, Prior Hypo WT had significantly higher glucose requirements and significantly lower corticosterone and glucagon responses. Prior Hypo and Prior Eu CRH KO mice had similar day 2 glucose requirements. However, Prior Hypo CRH KO mice had significantly lower day 2 epinephrine and norepinephrine vs. Prior Eu CRH KO and tended to have lower glucagon than on day 1. We conclude that glucocorticoid insufficiency in CRH KO mice correlates with 1) impaired counterregulation during acute hypoglycemia and 2) complex effects after repeated hypoglycemia, neither preventing decreased hormone responses nor worsening glucose requirements.

hypoglycemia unawareness; hypoglycemia-associated autonomic failure; catecholamines; adrenal insufficiency

HYPOGLYCEMIA-INDUCED AUTONOMIC FAILURE is an emerging challenge in diabetes management. Although intensive insulin therapy has been identified to reduce long-term complications from diabetes, more aggressive insulin use has also increased the risk of debilitating or even fatal hypoglycemia (9). The ability to decrease insulin, the first physiological defense against falling glucose levels, is absent in type 1 diabetes and in the insulin-dependent stages of advanced type 2 diabetes. Glucagon responses to hypoglycemia are also impaired with increasing duration of insulin-dependent diabetes (8). After glucagon, sympathetic nervous system activation remains the most rapid, and therefore the most effective, defense against decreases in blood glucose (8). However, both the threshold and the absolute magnitude of sympathetic nervous system responses decrease after as few as one or two prior episodes of hypoglycemia (12, 15). Other defenses against hypoglycemia, such as glucocorticoid and growth hormone secretion, exhibit similar impairment after repeated hypoglycemia, although these responses typically have less impact on immediate counterregulation (9). Decrement in sympathetically mediated norepinephrine and epinephrine release, along with the diminution of associated physical symptoms of sympathetic activation, are major contributors to hypoglycemia unawareness, in which diabetic patients lose both the physiological defenses and the subjective warning signs that blood sugar is dangerously low (9).

Glucocorticoids have been shown to inhibit sympathetic activity (4, 31), and Davis et al. (14) have proposed that hypoglycemia-induced increases in glucocorticoids contribute to the inhibition of sympathetic responses to recurrent hypoglycemia. Davis et al. have shown that cortisol infusion mimicked the suppressive effects of prior hypoglycemia on counterregulatory hormone responses (14) and have reported that counterregulatory responses were preserved in primary adrenocortical failure patients (13). These findings seem to contradict the established role of glucocorticoids not only as counterregulatory hormones but also as essential positive regulators of adrenomedullary epinephrine (18, 47), a key counterregulatory defense when glucagon secretion is defective (9).

We have studied counterregulation in the corticotropin-releasing hormone knockout (CRH KO) mouse to develop an animal model to resolve the paradoxical effects of glucocorticoids on adrenomedullary function. The primary metabolic phenotype of the CRH KO mouse is consistent with glucocorticoid deficiency (25, 26, 35). Male CRH KO mice have negligible plasma levels of glucocorticoids under most circumstances, including after hypoglycemia (28, 29, 36). However, CRH KO mice have relatively normal adrenomedullary morphology (34) and marked epinephrine responses to hypoglycemia (28, 29), making them an ideal model to investigate the effects of selective glucocorticoid deficiency on adrenomedullary function. We (28, 29) have previously used bolus insulin injection to test counterregulatory hormone responses to acute
and repeated hypoglycemia in CRH KO mice. However, because hypoglycemia induced in this manner is relatively uncontrolled, and because the handling, injection, and blood sampling required by this approach might have been stresses (10, 40) that affected counterregulatory responses (20, 23, 45), we have now assessed counterregulation during glucose clamps in conscious, unrestrained, chronically cannulated CRH KO and wild-type (WT) mice. We hypothesized that, in contrast to WT mice, CRH KO mice would exhibit impaired counterregulation during acute hypoglycemia but would not exhibit counterregulatory deficits after repeated hypoglycemic clamps.

METHODS

Animals. All procedures were approved by the Institutional Animal Care and Use Committees of Albany Medical College and Vanderbilt University. Male CRH WT and KO mice were bred as littermates from heterozygous parents (34). The breeding colony had been maintained on a C57BL/6 background for at least 10 generations. Mice were bred and genotyped at Albany Medical College and were subsequently studied at Vanderbilt University. Mice were 5–8 mo of age at the time of study, representing the time required for weaning, genotyping, quarantine after receipt at Vanderbilt, and surgical preparation. Mice were housed on a 12:12-h light-dark cycle throughout.

Mice were implanted with chronic indwelling carotid artery and jugular vein catheters and recovered for 5–7 days until they had regained their presurgery body weight, as previously described (24). The breeding colony had been maintained on a C57BL/6 background for at least 10 generations. Mice were bred and genotyped at Albany Medical College and were subsequently studied at Vanderbilt University. Mice were 5–8 mo of age at the time of study, representing the time required for weaning, genotyping, quarantine after receipt at Vanderbilt, and surgical preparation. Mice were housed on a 12:12-h light-dark cycle throughout.

Mice were implanted with chronic indwelling carotid artery and jugular vein catheters and recovered for 5–7 days until they had regained their presurgery body weight, as previously described (24). Catheters were filled with heparinized saline (200 U/ml), tunneled under the skin, and exteriorized suprascapularly. Mice were injected subcutaneously with 25 μg/day enoxaparin sodium (Sanofi-Aventis, Bridgewater, NJ) to prevent blood clots.

Glucose clamps. CRH WT and KO mice underwent two hyperinsulinemic glucose clamps separated by 24 h. For each clamp, regular insulin (Humulin-R; Lilly, Indianapolis, IN) was infused intravenously at a rate of 20 mU/kg/min for at least 10 generations. Mice were subcutaneously with 25 μg/day enoxaparin sodium (Sanofi-Aventis, Bridgewater, NJ) to prevent blood clots.

Glucose clamp. CRH WT and KO mice underwent two hyperinsulinemic glucose clamps separated by 24 h. For each clamp, regular insulin (Humulin-R; Lilly, Indianapolis, IN) was infused intravenously at a rate of 20 mU/kg/min for at least 10 generations. Mice were fasted for 6 h before the clamp by being transferred within 1-h flights-on to 1-liter chambers with bedding but without food or water. Extension lines were attached to the arterial and venous catheters at the time of transfer and exteriorized through the chamber lid. Mice were studied in the postabsorptive state 6 h later (7 h after lights-on). Whenever possible, CRH KO and WT littermates were studied together on the same days.

Arterial blood glucose was measured every 10 min (Hemocue, Lake Forest, CA) during each clamp. The glucose meter coefficient of variation was 1.6–3.5% for glucose values up to 400 mg/dl. Larger arterial blood samples (160 μl) were drawn for hormone analysis at 0 (before insulin infusion), 30, and 120 min on day 1 and at 0, 15, 30, and 120 min on day 2. Blood volume was replaced by donor red blood cells that were washed, resuspended in heparinized saline, warmed, and infused intravenously continuously with the insulin and glucose at 6 μl/min. Between the day 1 and day 2 clamp, euglycemia was restored in hypoglycemic mice by continuing glucose without insulin infusion, and all mice were returned to their home cages overnight with unrestricted access to food and water. After collection of the 120-min blood sample on day 2, mice were immediately killed by pentobarbital sodium overdose (100 mg/kg iv) and decapitation.

Plasma assays. Plasma epinephrine and norepinephrine (detection limit 20 pg/ml) were measured by HPLC according to previously described methods (22). Plasma corticosterone (MP Biomedical, formerly ICN, Irvine, CA), insulin, and glucagon (Linco, St. Louis, MO) were measured with previously described radioimmunoassays (5, 25, 29). All assays had been validated for small volumes of mouse plasma and had inter-assay coefficients of variation no higher than 10%.

Statistics. Data were analyzed by two-way ANOVA with repeated measures across time, with single post hoc comparisons performed by t-test (Statview 5.0; SAS Institute, Cary, NC). Significance was defined as P < 0.05. Data are presented throughout as means ± SE. Where no error bars are visible, the scale of the figure exceeded that of the error.

RESULTS

Day 1: effect of combined CRH and glucocorticoid deficiency on responses to controlled, acute hypoglycemia or euglycemia. WT and CRH KO mice had similar body weights at the time of study (WT vs. CRH KO, 28.1 ± 0.4 vs. 28.9 ± 0.6 g; n = 15 and 9, respectively). Blood glucose levels were similar 7 h after lights-on in 6-h-fasted WT and CRH KO mice (Fig. 1, bottom, and Table 1). During a 2-h hyperinsulinemic hypoglycemic clamp on day 1, blood glucose fell to the same absolute levels and at the same rate in both genotypes (Fig. 1, bottom). Blood glucose averaged 62 ± 1 and 63 ± 1 mg/dl in WT and CRH KO mice, respectively, during the last 60 min of the clamp. The glucose infusion rate required to maintain blood glucose increased gradually over time in both genotypes but was consistently and significantly higher in CRH KO mice during the first 70 min of hypoglycemia (Fig. 1, top). The glucose infusion rates required to maintain euglycemia did not differ between genotypes (Table 1). Plasma insulin was significantly lower in CRH KO than in WT mice at baseline but was similar in all mice after glucose clamps regardless of genotype or target glucose level (Table 2).

On day 1, there was a significant main effect of hypoglycemia on all counterregulatory factors measured except for norepinephrine (Fig. 2; symbols omitted for clarity). Basal plasma epinephrine was similar between WT and CRH KO mice and near the lower limit of detection. Plasma epinephrine increased...
to similar levels in both genotypes during the hypoglycemic clamp but did not increase during the euglycemic glucose clamp on day 1 (Fig. 2, top). Plasma norepinephrine did not change over time in either genotype, although norepinephrine was significantly higher in CRH KO than in WT mice both basally and after 120 min of hyperinsulinemic euglycemia (Fig. 2, 2nd from top). WT mice exhibited sustained elevations in plasma corticosterone during hypoglycemia, whereas plasma corticosterone was near the lower detection limit and did not change in CRH KO mice (Fig. 2, 3rd on left). In contrast to the response to hypoglycemia, WT mice did not maintain elevated corticosterone levels during euglycemia; corticosterone levels were significantly higher in hypoglycemic WT mice at 120 min (Fig. 2, 3rd from top; significance symbols omitted for clarity). Basal plasma glucagon was also near the lower detection limit and increased approximately threefold in WT mice during hypoglycemia. However, CRH KO mice exhibited transiently but significantly higher glucagon levels than did WT mice by 30 min of the hypoglycemic clamp (Fig. 2, bottom left). Plasma glucagon was low and did not change at any time during day 1 euglycemia (Fig. 2, bottom right).

Day 2: effect of combined CRH and glucocorticoid deficiency on responses to repeated hypoglycemia. Blood glucose was similar among all mice subjected to a hypoglycemic glucose clamp on day 2 regardless of genotype or prior exposure to hypoglycemia (Fig. 3). Blood glucose levels during day 2 hypoglycemia (65 ± 1 mg/dL during the last 60 min) were also comparable to those during the day 1 hypoglycemic clamp. WT mice exposed to hypoglycemia on day 1 (Prior Hypo) required significantly higher glucose infusion rates to maintain blood glucose during the first 40 min of hypoglycemia on day 2 than did WT mice that had been euglycemic on day 1 (Prior Eu; Fig. 4, left). Prior Hypo CRH KO mice did not

Table 1. Day 1 blood glucose and GIR in WT and CRH KO mice subjected to a hyperinsulinemic euglycemic glucose clamp on day 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Time, min</th>
<th>0</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
<th>110</th>
<th>120</th>
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<tbody>
<tr>
<td>Glucose, mg/dL</td>
<td>WT</td>
<td>172±5</td>
<td>202±31</td>
<td>158±33</td>
<td>157±18</td>
<td>173±16</td>
<td>166±9</td>
<td>173±7</td>
<td>168±11</td>
<td>169±8</td>
<td>151±8</td>
<td>168±12</td>
<td>178±17</td>
<td>180±12</td>
<td>172±14</td>
</tr>
<tr>
<td>GIR, mg/kg/min</td>
<td>WT</td>
<td>55</td>
<td>61±2</td>
<td>67±5</td>
<td>68±4</td>
<td>68±0</td>
<td>72±5</td>
<td>71±6</td>
<td>74±7</td>
<td>75±8</td>
<td>79±9</td>
<td>79±8</td>
<td>77±6</td>
<td>76±5</td>
<td>76±5</td>
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</table>

Values are means ± SE; n = 5 wild-type (WT) and 4 glucocorticoid-deficient, corticotropin-releasing hormone knockout (CRH KO) mice per group. GIR, glucose infusion rate. There were no significant genotype effects on either blood glucose levels or glucose infusion rates.

Table 2. Plasma insulin in WT and CRH KO mice subjected to hyperinsulinemic euglycemic or hypoglycemic glucose clamps

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Group</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Hypoglycemia</td>
<td>0.9±0.2</td>
<td>10.7±0.5</td>
</tr>
<tr>
<td></td>
<td>Euglycemia</td>
<td>2.2±0.5</td>
<td>9.7±1.5</td>
</tr>
<tr>
<td>CRH KO</td>
<td>Hypoglycemia</td>
<td>0.2±0.02*</td>
<td>14.8±2.5</td>
</tr>
<tr>
<td></td>
<td>Euglycemia</td>
<td>0.1±0.04*</td>
<td>11.7±0.6</td>
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</tbody>
</table>

Values are means ± SE (pg/ml); n = 10 WT, hypoglycemia, 5 WT, euglycemia, 5 CRH KO, hypoglycemia, and 4 CRH KO, euglycemia. Insulin was measured in plasma drawn either before (0 min) or after (120 min) the 2-h glucose clamp on day 1. *P < 0.05 vs. WT.
exhibit further increases in glucose infusion requirements relative to their Prior Eu controls (Fig. 4, right). Nevertheless, glucose infusion requirements in all CRH KO mice closely resembled those in Prior Hypo WT mice.

Prior exposure to hypoglycemia on day 1 did not significantly affect basal hormone levels in either genotype the following day (Fig. 5). At the times monitored, we did not detect any differences in epinephrine responses to hypoglycemia between Prior Eu and Prior Hypo WT mice (Fig. 5, top left). However, hypoglycemia-induced epinephrine levels were significantly higher at 15 min in Prior Eu vs. Prior Hypo CRH KO mice (Fig. 5, top right). Plasma norepinephrine did not increase during hypoglycemia in either genotype and did not differ between Prior Eu and Prior Hypo WT mice (Fig. 5, left, 2nd from top). Day 2 norepinephrine levels were initially elevated and declined over time in Prior Eu CRH KO mice (Fig. 5, right, 2nd from top), similar to changes in norepinephrine in hypoglycemic CRH KO mice on day 1. In contrast, Prior Hypo CRH KO mice tended to have lower day 2 norepinephrine levels, which differed significantly from those in Prior Eu CRH KO mice at 15 min (Fig. 5, right, 2nd from top). Prior Hypo WT mice had delayed corticosterone responses to day 2 hypoglycemia, with 15-min corticosterone levels being significantly lower than those in corresponding Prior Eu controls (Fig. 5, left, 3rd from top). Plasma corticosterone remained near the lower limit of detection and was unaffected by acute or repeated hypoglycemia in CRH KO mice (Fig. 5, right, 3rd from top). Glucagon responses to day 2 hypoglycemia were also transiently but significantly reduced in Prior Hypo vs. Prior Eu WT mice (Fig. 5, bottom left). Prior hypoglycemia did not cause similar impairment of the glucagon response in Prior Hypo vs. Prior Eu CRH KO mice (Fig. 5, bottom right). However, peak glucagon levels in CRH KO mice subjected to two hypoglycemic clamps still tended to be lower on day 2 than on day 1, although these differences were not statistically significant.

We also measured food intake overnight between the day 1 and day 2 glucose clamps to determine whether differences in feeding among groups could have influenced our counterregulatory measures. There was a significant main effect of genotype on overnight food consumption (Table 3). However, CRH KO mice actually ate less than WT mice, and differences between WT and CRH KO mice were not significant by post hoc testing in either Prior Eu or Prior Hypo groups.
action, on overnight food consumption.

acute hypoglycemia (responses to subsequent hypoglycemia. 

activation is not essential to explain blunted autonomic re-

axis is required for protection against hypoglycemia, its prior 

ecedent hypoglycemia are not associated with further increases 

counterregulation during acute hypoglycemia and does not 

prevent decreases in counterregulatory hormones after repeated 

hypoglycemia. These findings are consistent with our prior 

studies of this model using bolus insulin injection to induce 

hypoglycemia (29). However, we have found that, unlike in 

WT mice, decreased counterregulatory hormones after ante-

cedent hypoglycemia are not associated with further increases 

in glucose requirements in CRH KO mice. Our current and 

previous data suggest that the role that glucocorticoids play in 

the impaired counterregulatory response to repeated hypogly-

cemia is complex. Although activation of the adrenocortical 

axis is required for protection against hypoglycemia, its prior 

activation is not essential to explain blunted autonomic re-

sponses to subsequent hypoglycemia.

CRH KO mice exhibited impaired counterregulation during 

acute hypoglycemia (day 1). This impairment was evident 

from the significantly elevated glucose infusion rates required 

during the first hour of hypoglycemia. Additional counterregu-

latory abnormalities were also suggested by the fact that 

glucose requirements were increased despite relatively normal 

epinephrine and significantly greater glucagon responses to 

hypoglycemia in CRH KO vs. WT mice.

Counterregulatory deficits in CRH KO mice during acute 

hypoglycemia were consistent with their profound glucocorti-

coid insufficiency. Due to their slower actions, glucocorticoids 

are not typically considered critical to acute counterregulation 

(9). However, there is evidence that glucocorticoids contribute 

acutely to increasing net glucose production during hypogly-

cemia in normal subjects independently of changes in epineph-

rine (16). Chronic glucocorticoid insufficiency also increases 

risks of hypoglycemia; although hypoglycemia risk is most 

common in children, it is not unheard of in adult patients (21, 

43). Both global and hepatic loss of glucocorticoid action in 

mice causes fasting hypoglycemia (34, 39).

Chronic glucocorticoid insufficiency in CRH KO mice could 

also increase glucose requirements during insulin-induced hy-

poglycemia by increasing insulin sensitivity, decreasing sub-

strates for glucose production, or impairing the action of other 

counterregulatory hormones. The significantly lower baseline 

insulin levels in CRH KO mice are consistent with increased 

insulin sensitivity (1, 11, 29). However, glucose requirements 

during euglycemic hyperinsulinemic clamps on day 1 were 

similar, suggesting that peripheral insulin responsiveness was 

normal in CRH KO. Hepatic insulin action could differ be-

tween CRH KO and WT mice, but such differences would not 

have been detected at the insulin infusion rates used in this 

study. Glucocorticoids also promote the deposition of hepatic 

glycogen (2, 20), which is the largest and most rapidly acces-

sible source of glucose during acute hypoglycemia (21). Al-

though liver glycogen content is similar in fed WT and CRH 

KO mice (29), it is possible that CRH KO mice had lower 

hepatic glycogen levels after the limited fasting in the present 

studies. In addition, glucocorticoids help to maintain respon-

siveness of glycogenolytic signaling pathways to glucagon and 

epinephrine (2, 38); these actions, in combination with possible 

glycogen depletion, could account for the higher glucose re-

quirements despite normal or enhanced autonomic responses to 

hypoglycemia in CRH KO mice.

Although glucocorticoids are also required to maintain epi-

nephrine synthesis (18, 46), CRH KO mice did not show any 

deficits in acute hypoglycemia-induced epinephrine levels. 

These findings are unexpected in light of clinical and animal 

evidence that glucocorticoid deficiency impairs adrenomedul-

lary development and function (3, 13, 18, 46), including other 

studies in CRH KO mice (30). We suspect that the hypogly-

cemic stimulus in the current experiments was sufficiently 

limited and controlled as to be within the adrenomedullary 

capacity of CRH KO mice. In experiments combining the 

stimuli of retroorbital blood sampling and more severe hypo-

glycemia induced by bolus insulin injection, we did observe 
significant deficits in CRH KO epinephrine secretion (29).

WT mice exhibited counterregulatory failure after two epi-

sodes of hypoglycemia, evident as elevated glucose infusion 

requirements and transient but significant decreases in hypo-
glycemia-evoked glucocorticoid and glucagon levels. Al-

though not the focus of our analyses, counterregulatory hor-

mone responses were similar between day 1 hypoglycemia in 

Prior Hypo WT mice and day 2 hypoglycemia in Prior Eu WT 

mice (compare Figs. 2 and 5). Baseline corticosterone levels in 

WT mice tended to be elevated compared with levels typically 

reported in rodents (37), but these elevations probably reflect 

the combined effects of fasting and sampling at a later time in 

the light cycle (27). WT corticosterone levels were similar to 

those measured at midday in similarly fasted but otherwise 

unmanipulated C57BL/6 mice (L. Jacobson, unpublished ob-

servations). Both the overall pattern of results and the absolute 

levels of counterregulatory hormones were entirely consistent 

with our findings from repeated hypoglycemic glucose clamps 

in C57BL/6 mice (27). Taken together, our data indicate that 

controlled, mild hypoglycemia elicits defined, predictable 

counterregulatory responses in the mouse and that these re-

sponses are comparable across a range of ages up to 8 mo.

The absence of an effect of antecedent hypoglycemia on the 

epinephrine response is probably not attributable to group size 

or variability. We did not observe significant differences in day 

2 plasma epinephrine between Prior Eu and Prior Hypo mice, 

even after pooling the current data with those from the previous 

studies in C57BL/6 mice (27), for respective total group sizes 

of 11 and 16. This pooling was justified by the statistical 

similarity of hormone levels, as discussed above, and by the 

generic similarity from breeding WT mice on a C57BL/6 

background (see METHODS). The similarity of epinephrine re-

sponses to acute and repeated hypoglycemia most likely re-

flects either the greater importance of glucagon in the mouse or

<table>
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<th>Genotype</th>
<th>Group</th>
<th>Food Intake, g/g body wt</th>
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</thead>
<tbody>
<tr>
<td>WT</td>
<td>Prior Hypo</td>
<td>0.19±0.02</td>
</tr>
<tr>
<td></td>
<td>Prior Eu</td>
<td>0.20±0.03</td>
</tr>
<tr>
<td>CRH KO</td>
<td>Prior Hypo</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td></td>
<td>Prior Eu</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 WT Prior Hypo, 5 WT Prior Eu, 5 CRH KO Prior Hypo, and 4 CRH KO Prior Eu. There was a significant main effect of genotype, but not of prior hypoglycemia or genotype × hypoglycemia interaction, on overnight food consumption.
the inability of our limited sampling to detect transient differences in epinephrine. Our model is not unique in lacking recurrent hypoglycemia-induced suppression of epinephrine. Epinephrine has been found to be unchanged or even increased in humans and rats exhibiting other evidence of counterregulatory failure after repeated hypoglycemia (12, 44).

Although it may also be questioned whether the early and transient decreases in counterregulatory hormones reflect impaired counterregulation in our model, there is also considerable variation in the literature as to whether, or when, counterregulatory responses to hypoglycemia differ after prior eu- and hypoglycemia. The composite picture of counterregulatory failure, in which all counterregulatory hormone responses to hypoglycemia exhibit sustained inhibition, has emerged through intensive investigation and is not illustrated by every study. Studies in humans as well as in rats have found epinephrine (12, 44), norepinephrine (13, 17, 32, 42, 44), glucocorticoids (15, 42, 44), or glucagon (12, 19, 44) to be unaffected even when other counterregulatory hormones were reduced. There are very few studies of counterregulatory hormones during hypoglycemic clamps in mice. As this number expands, we expect the findings will reinforce the validity of our model of counterregulatory failure.

Combined CRH and glucocorticoid deficiency had complex effects on counterregulation after repeated hypoglycemia. Unlike WT mice, Prior Hypo CRH KO mice did not require additional glucose infusion compared with their Prior Eu counterparts. Because of the additional blood volume required, we could not evaluate glucose flux to determine how glucose requirements might have been maintained in Prior Hypo CRH KO mice. However, the lack of change in glucose requirements, despite decreases in counterregulatory hormone levels, suggested that aspects of counterregulation were preserved in CRH KO mice after repeated hypoglycemia.

Contrary to our hypothesis, CRH KO mice had significant decreases in their epinephrine response to a second hypoglycemic exposure. The decline in epinephrine could have been an artifact of slightly higher day 2 epinephrine levels in Prior Eu CRH KO mice. However, epinephrine levels in Prior Hypo CRH KO mice on day 1 were similar to those in Prior Eu CRH KO mice on day 2 and (although also not the focus of our analyses) were still higher than day 2 levels in Prior Hypo CRH KO mice. Elevated levels of norepinephrine in CRH KO mice were also suppressed after antecedent hypoglycemia. These trends are consistent with inhibition of sympathoadrenal activity. Glucagon levels in CRH KO mice did not differ between Prior Eu and Prior Hypo groups during day 2 hypoglycemia, but there was still a tendency for plasma glucagon to decrease between the first and second episodes of hypoglycemia in Prior Hypo CRH KO mice. We cannot exclude the possibility that the apparent decreases in counterregulatory hormones are due to chance variation among the relatively few CRH KO mice studied or that our sampling missed increases in counterregulatory hormones that would have maintained glucose requirements. Nevertheless, our data also do not strongly support the preservation of counterregulatory hormone responses to recurrent hypoglycemia in CRH KO mice. If combined CRH and glucocorticoid deficiency can preserve counterregulation, our current data indicate that this deficiency has more pronounced effects on glucose requirements than on counterregulatory hormone levels. Any decreases in counterregulatory hormones in CRH KO mice might account for why glucose requirements were not lower in CRH KO than in WT mice after repeated hypoglycemia.

There is considerable evidence that glucose requirements during hypoglycemia can be met in part independently of counterregulatory hormones. Hepatic autoregulation, or hormone-independent changes in hepatic glucose production evoked by changing glucose levels (7, 33), is one of the best characterized examples of this phenomenon. Hepatic autoregulation can be due to autonomic nerve activity or to non-neurally-mediated changes in hepatic responses to hormones and gluconeogenic substrates (33). Although there is disagreement on the importance of hepatic autoregulation to defenses against hypoglycemia, hepatic autoregulation may play a significant role during severe hypoglycemia (33). Hepatic autoregulation could occur in CRH KO mice at less severe hypoglycemia as an adaptation to chronic glucocorticoid insufficiency. If involved, the effects of hepatic autoregulation on glucose requirements in CRH KO mice could be identified by analyzing metabolic responses to repeated hypoglycemia and defined counterregulatory hormone levels. However, initial evaluation of glucose and metabolite kinetics could not be done in the current experiments because of the additional blood volume required.

It may seem puzzling that the effects of repeated hypoglycemia on counterregulatory hormones in CRH KO mice did not resemble those reported by Davis et al. (13) in subjects with primary adrenocortical insufficiency. These subjects were studied after 5 days of basal cortisol infusion; it is possible that CRH KO mice have adapted to chronically low glucocorticoid levels differently than have patients whose replacement was modified 5 days earlier. Our hormone data are consistent with the majority of studies (17, 19, 41, 44) that have not found the inhibitory effects of glucocorticoids on counterregulation reported by Davis et al. (14) and Sandoval et al. (42).

Nevertheless, the defense of glucose requirements in repeatedly hypoglycemic CRH KO mice, despite the decreased hormonal responses, suggests that glucocorticoid deficiency might prevent further deficits without actually normalizing counterregulation.

It is unlikely that CRH deficiency rather than glucocorticoid deficiency accounts for the response of CRH KO mice to repeated hypoglycemia. Although findings on the role of CRH in autonomic responses hypoglycemia are contradictory (6, 19), recent studies indicate that CRH may suppress counterregulatory responses to recurrent hypoglycemia (19). These findings make it doubtful that CRH deficiency counterbalanced or obscured the hypothesized effects of glucocorticoid deficiency in the CRH KO mouse. It is unlikely that glucocorticoid replacement would shed light on this issue, since other counterregulatory responses after repeat hypoglycemia in CRH KO mice were so low and so similar to WT that further changes would have been difficult to detect. We did not find evidence of other processes, such as compensatory food intake, that might have accounted for the suppression of counterregulatory hormones in repeatedly hypoglycemic CRH KO mice. In sum, our data corroborate a significant impact of glucocorticoid deficiency on counterregulatory responses to acute hypoglycemia but do not demonstrate protection against defective counterregulatory hormone secretion after recurrent hypoglycemia.
It remains possible that the low but detectable glucocorticoid levels in CRH KO mice mediated the observed inhibition of counterregulatory hormones after repeated hypoglycemia. This interpretation seems unlikely in view of the number of studies that did not find counterregulatory inhibition even in subjects with normal adrenocortical function given additional glucocorticoids (17, 19, 41, 44). Even if low glucocorticoid levels contributed to counterregulatory hormone suppression after repeated hypoglycemia in CRH KO mice, these low levels also correlated with impaired counterregulation. The contribution of glucocorticoids to counterregulatory failure may be subtle if such a narrow margin exists between glucocorticoid levels that do not permit normal counterregulation during either acute or repeated hypoglycemia.

ACKNOWLEDGMENTS

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