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

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Abstract

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 $mU \cdot kg^{-1} \cdot min^{-1}$) 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 glycemic 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.

Keywords

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

HYPOGLYCEMIA-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

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(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 hind-brain 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 multi-factorial.

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

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postabsorptive 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 $mU \cdot kg^{-1} \cdot min^{-1}$ 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 10% for the low control (~30 pg/ml) and 3.4% for the high control (~500 pg/ml). The interassay coefficient of variation for norepinephrine 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

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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).

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 ± 163 vs. 1,385 ± 116; *n* = 6/group). 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-Hypo and Eu-Hypo mice, this decrease was not significant (*day 1* vs. *day 2*, 203 ± 45 vs. 149 ± 41 pg/ml, *n* = 6/group; *P* = 0.048 before and 0.096 after correction). Comparison of other endpoints between *day 1* and *day 2* in Hypo-Hypo mice did not reveal any further effects of repeated hypoglycemia on counterregulatory hormones beyond those discussed above. More severe hypoglycemia (final hour average blood glucose, 42 ± 2 mg/dl; *n* = 4/group) increased *day 1* peak catecholamine, corticosterone, and glucagon responses by ~1,400, 55, and 450%, respectively, but did not result in greater inhibition of plasma catecholamines or other counterregulatory hormones on *day 2* (data not shown).

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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 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 comparable to those measured at midday in similarly fasted, unoperated mice (L. Jacobson, unpublished observations). Baseline epinephrine and corticosterone levels were ~30- and 3-fold lower, respectively, than those reported in mice that were restrained and sampled from the tail tip (16). Neither plasma catecholamines nor glucocorticoids increased significantly during a euglycemic clamp combined with repeated blood sampling. 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 glucagon in male C57BL/6 mice. Counterregulatory hormone responses were also related to stimulus intensity, with deeper hypoglycemia eliciting higher hormone levels. Although the frequency and total number of hormone samples in our experiments were insufficient to derive a precise glycemic threshold, correlation of hormone levels against blood glucose indicated that epinephrine, corticosterone, and glucagon increased in mice at glucose levels that were close to 80 mg/dl. This level is notably higher than the 60–70 mg/dl threshold reported for responses of these counterregulatory hormones to insulin- induced hypoglycemia in humans and dogs (25,39) and suggests that the same blood glucose level may not represent the same stimulus severity in all species. We did not observe hypoglycemia-induced increases in plasma norepinephrine, but elevated norepinephrine is not a consistent feature of counterregulation in other species (14,29,37–39). Thus counterregulatory hormone responses to controlled, acute hypoglycemia in the mouse are largely comparable to those in other species.

Mice also resemble other species in exhibiting evidence of impaired glucose regulation after limited antecedent exposure to hypoglycemia. Studies in nondiabetic humans indicate that as little as a single or two brief (10–30 min) prior episodes of hypoglycemia are sufficient to diminish counterregulatory hormone and endogenous glucose production responses to subsequent hypoglycemia (5,6). We observed counterregulatory deficits in mice after a single 2-h episode of hypoglycemia, the most dramatic being the marked increase in glucose infusion requirements, which are likely to reflect decreased endogenous glucose production (4–6,23, 46). Comparable to the blunting of counterregulatory hormones described in humans (7,23) and rats (14,35,38,40), mice also had significantly lower glucocorticoid and glucagon responses after exposure to repeated 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 between previously euglycemic and previously hypoglycemic mice. However, we did detect decreases in epinephrine responses between the first and second exposures to hypoglycemia within the Hypo-Hypo group, suggesting that repeated

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hypoglycemia had the expected inhibitory effects on sympathoadrenal activity, even if those effects did not reach statistical significance in multiple comparisons. It is unlikely that hypoglycemia was insufficiently prolonged or severe enough to impair sympathoadrenal responsiveness. Mice were hypoglycemic for as long as the time shown to reduce hypoglycemia-induced plasma catecholamines in humans (6,8), and antecedent 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 glucagon responses did not exceed 300 pg/ml, responses to even milder hypoglycemia might have been too low to detect decrements 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 hypoglycemia 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. Comparisons 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 counterregulatory mechanisms in mice differ from those in humans and other 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 after recurrent hypoglycemia (44). However, because counterregulatory impairment can occur independently of changes in lactate or ketones (6,13), these factors do not appear to be major mediators of hypoglycemia-induced decreases in counterregulatory hormones. These considerations notwithstanding, it is equally plausible that the decrements in epinephrine, corticosterone, and glucagon, regardless of whether they were statistically significant, had a cumulative impact to increase glucose requirements. Other mechanisms, including alterations in insulin secretion, endogenous glucose production, or tissue glucose uptake, can be readily investigated in our model.

In summary, we have developed a model for remote infusion and blood sampling to evaluate counterregulation with minimal stress in the conscious, unrestrained mouse. We have demonstrated that counterregulatory hormone responses and glucose requirements during

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acute and repeated hypoglycemia in mice resemble those in humans and other species. This model will be valuable for testing the role of individual genes in normal and defective counterregulation through the use of knockout and transgenic models.

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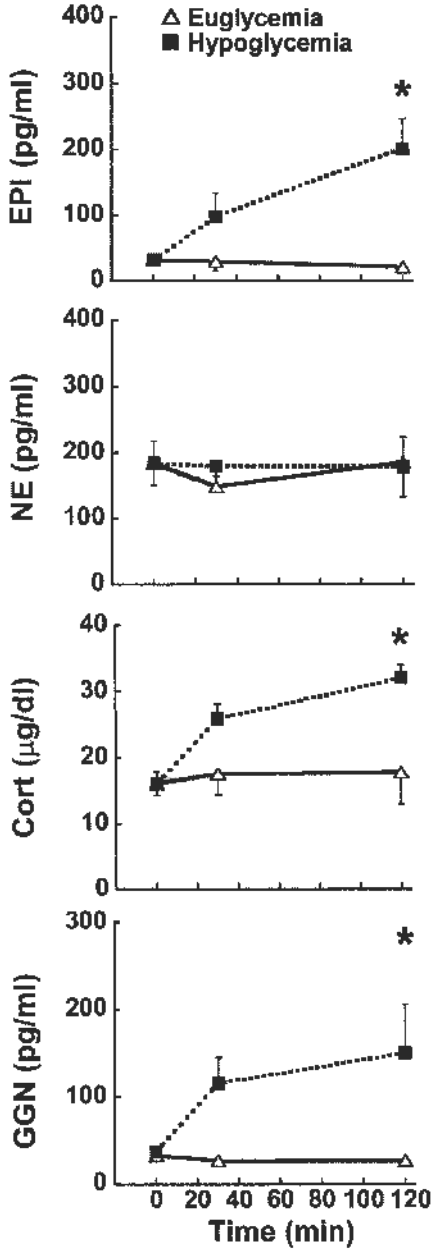

Fig. 1.

Plasma levels of epinephrine (EPI; *top*), norepinephrine (NE; *upper middle*), corticosterone (Cort; *lower middle*), and glucagon (GGN; *bottom*) in mice exposed to a hypoglycemic (▪) or a euglycemic (▵) clamp on day 1. ANOVA main effects are summarized in Table 3; $n = 6$/group. *$P < 0.05$, Eu-Hypo vs. Hypo-Hypo at the same time point.

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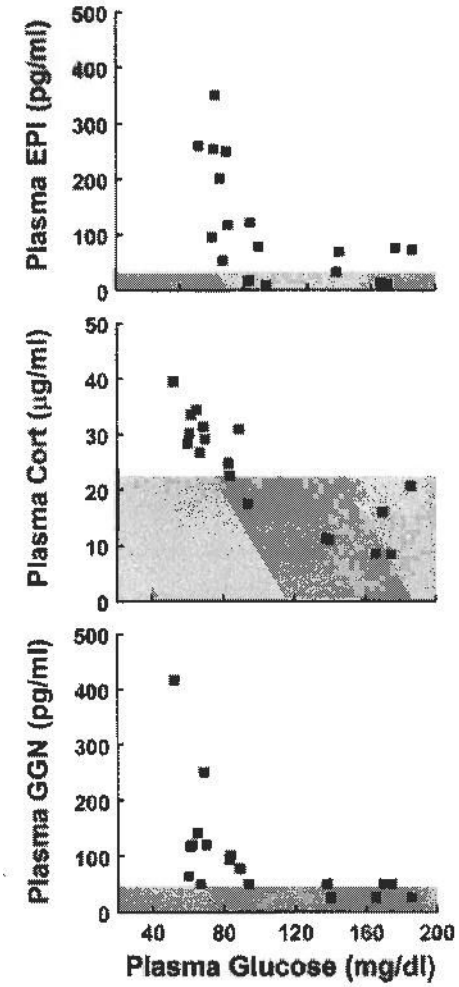

Fig. 2.

Plasma EPI (*top*), Cort (*middle*), and GGN (*bottom*) vs. blood glucose levels in individual Hypo-Hypo mice on *day 1*. Shaded area indicates upper 95% confidence limit for levels of each hormone measured in Eu-Hypo mice on *day 1*. Plasma NE is not shown because it did not change significantly during hypoglycemia; $n = 6$/group

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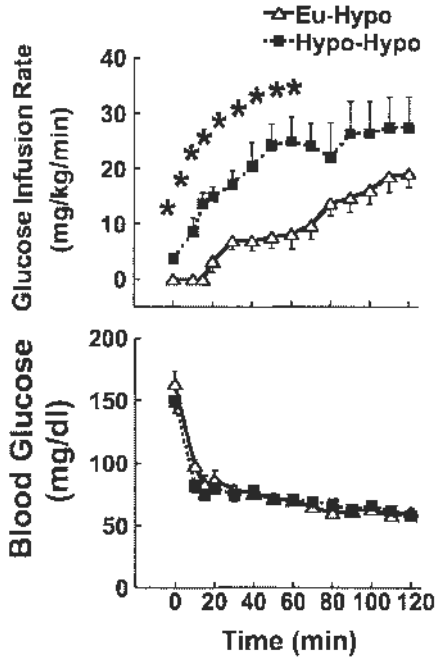

Fig. 3.

Glucose infusion rates (*top*) and blood glucose levels (*bottom*) during hypoglycemia in mice previously exposed to either hypoglycemia (Hypo-Hypo; ▪) or euglycemia (Eu-Hypo; ▵) 1 day earlier. Insulin was infused for 120 min at 20 mU·kg^{-1}·min^{-1} starting at *time 0*. ANOVA main effects are summarized in Table 3. Blood glucose did not differ between Hypo-Hypo and Eu-Hypo mice at any time by post hoc testing despite significant main effects by ANOVA; $n = 6$/group. *$P < 0.05$, Eu-Hypo vs. Hypo-Hypo at the same time point.

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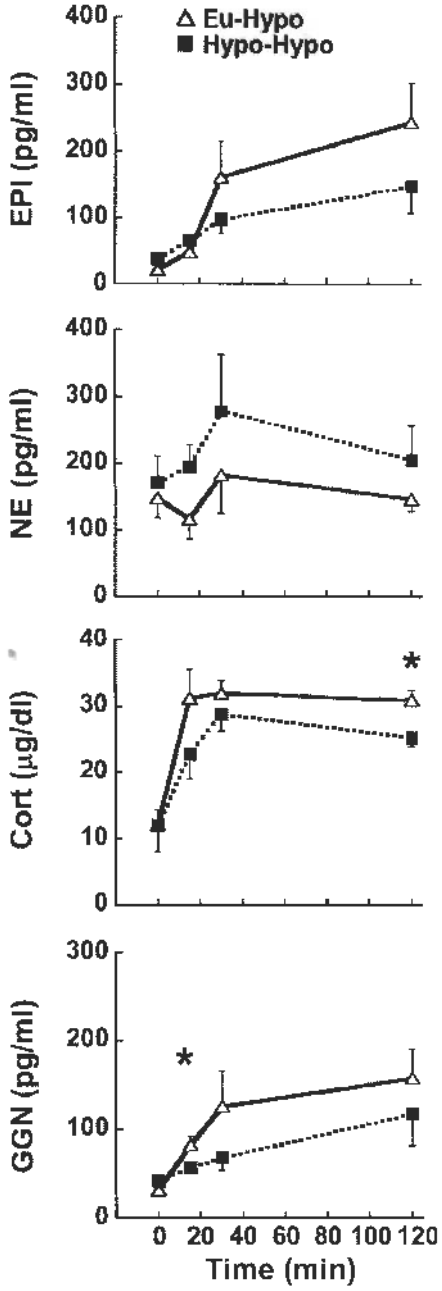

Fig. 4.

Plasma levels of EPI (*top*), NE (*upper middle*), Cort (*lower middle*), and GGN (*bottom*) during hypoglycemia in mice previously exposed to hypoglycemia (Hypo-Hypo; ▪) or euglycemia (Eu-Hypo; ▵) 1 day earlier. ANOVA main effects are summarized in Table 3; $n = 6$/group. *$P < 0.05$, Eu-Hypo vs. Hypo-Hypo at the same time point.

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Table 1

Day 1 blood glucose and GIR in mice, subjected to Eu-Hypo or Hypo-Hypo hyperinsulinemic glucose clamps

Time, min		**0**	**10**	**15**	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**	**110**	**120**
Glucose, mg/dl	Eu-Hypo	183±9	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
	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	61±2
GIR, $mg \cdot kg^{-1} \cdot min^{-1}$	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
	Hypo-Hypo	0	0	0	0.7±.7	4±2	4±1	6±2	9±2	12±1	15±2	15±2	17+2	19+3	19±3

Values are means ± SE; *n* = 6 per group. GIR, 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*.

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Table 2

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

	Day 1		*Day 2*	
	0 min	**120 min**	**0 min**	**120 min**
Eu-Hypo	1.9±0.8	15.2±2.7	1.4 ±0.2	16.4 ± 2.6
Hypo-Hypo	1.0±0.2	15.9±1.6	0.65 ±0.1	13.7 ± 1.8

Values are means ± SE in ng/ml and are as described in METHODS; *n* = 6/group.

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Table 3

P values of main effects of repeated-measures ANOVAs for end points reported in Figs. 1, 3, and 4

	P Values						
	Glucose	**GIR**	**EPI**	**NE**	**Cort**	**GGN**	**Insulin**
Group	**< 0.0001**	**< 0.0001**	0.2656	0.3721	0.7270	0.2161	0.3902
Day	**< 0.0001**	**< 0.0001**	0.0783	0.8777	0.1823	0.1828	0.8401
Group × day	**< 0.0001**	**< 0.0001**	**0.0427**	0.3355	**0.0290**	**0.0166**	0.7957
Time	**< 0.0001**	**< 0.0001**	**0.0015**	0.8083	**< 0.0001**	**0.0005**	**< 0.0001**
Group × time	**< 0.0001**	0.1997	0.877	0.2522	0.0994	0.5500	0.9381
Day × time	**< 0.0001**	0.7175	0.0927	0.6793	**0.0029**	0.4272	0.9446
Group × day × time	**<0.0001**	**< 0.0001**	**0.0151**	0.7859	**0.0019**	**0.0491**	0.6948

EPI, epinephrine; NE, norepinephrine; Cort, corticosterone; GGN, glucagon. Group refers to whether mice were hypoglycemic or euglycemic on *day 1*. Day indicates *day 1* vs. *day 2* hyperinsulinemic glucose clamps. Time refers to time during a given glucose clamp. Significant values ($P < 0.05$) are highlighted in bold.