Effects of antecedent hypoglycemia, hyperinsulinemia, and excess corticosterone on hypoglycemic counterregulation

KATHY SHUM,*1 KAREN INOuye,*1 OWEN CHAN,1 JULIAN MATTHEWS,2 AND MLADEN Vranic3

Departments of 1Physiology, 2Obstetrics and Gynecology, and 3Medicine, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Received 29 November 2000; accepted in final form 19 April 2001

Shum, Kathy, Karen Inouye, Owen Chan, Julian Mathoo, Debra Bilinski, Stephen G. Matthews, and Mladen Vranić. Effects of antecedent hypoglycemia, hyperinsulinemia, and excess corticosterone on hypoglycemic counterregulation. Am J Physiol Endocrinol Metab 281: E455–E465, 2001.—This study aimed to differentiate the effects of repeated antecedent hypoglycemia, antecedent marked hyperinsulinemia, and antecedent increases in corticosterone on counterregulation to subsequent hypoglycemia in normal rats. Specifically, we examined whether exposure to hyperinsulinemia or elevated corticosterone per se could impair subsequent counterregulation. Four groups of male Sprague-Dawley rats were used: 1) normal controls (N) had 4 days of sham antecedent treatment; 2) an antecedent hyperinsulinemia group (AH) had 7 episodes of hyperinsulinemic hypoglycemia over 4 days; 3) an antecedent hyperinsulinemia group (AE) had 7 episodes of hyperinsulinemic euglycemia; and 4) an antecedent corticosterone group (AC) had 7 episodes of intravenous corticosterone to simulate the hypoglycemic corticosterone levels in AH rats. On day 5, hyperinsulinemic euglycemic-hypoglycemic clamps were performed. Epinephrine responses to hypoglycemia were impaired (P < 0.05 vs. N) after antecedent hypoglycemia and hyperinsulinemia. This correlated with diminished (P < 0.05 vs. N) absolute glucose production responses in AH rats and diminished incremental glucose production responses in AE rats. Paradoxically, norepinephrine responses were increased (P < 0.05 vs. N) after antecedent hypoglycemia. Glucagon and corticosterone responses were unaffected by antecedent hypoglycemia and hyperinsulinemia. In AC rats, incremental but not absolute glucose production responses were decreased (P < 0.05 vs. N). However, neuroendocrine counterregulation was unaltered. We conclude that both antecedent hypoglycemia and hyperinsulinemia impair epinephrine and glucagon production responses to subsequent hypoglycemia, suggesting that severe recurrent hyperinsulinemia may contribute to the development of hypoglycemia-associated autonomic failure.

glucose clamp; insulin; catecholamines; glucocorticoids; glucose production

HYPOGLYCEMIA IS A MAJOR ACUTE COMPLICATION of type 1 diabetes and is the predominant limiting factor in the improvement of glycemic control (7, 18, 19). In type 1 diabetes, hypoglycemia arises from the interplay of excess insulin and deficient counterregulation (8). There is considerable evidence indicating that antecedent hypoglycemia further impairs counterregulation in diabetic subjects (9, 12, 25, 34) and increases the risk of subsequent hypoglycemia (18). Recent antecedent hypoglycemia is in fact the major underlying factor in the increased incidence of hypoglycemia in intensively treated type 1 diabetes (18). The effects of recurrent hypoglycemia may also occur independently of diabetes, as demonstrated by clinical studies in nondiabetic subjects (11, 17, 24, 27, 30, 47). Despite the well-documented effects of antecedent hypoglycemia to impair counterregulation, the mechanisms underlying the development of impaired counterregulation are not fully understood.

It is not known whether exposure to hypoglycemia per se or whether exposure to other factors associated with insulin-induced hypoglycemia underlies the development of defective counterregulation. Although hypoglycemia typically occurs under hyperinsulinemic conditions, information on the effects of insulin per se on counterregulatory responses to subsequent hypoglycemia is scarce. To our knowledge, only Fruehwald-Schultes et al. (23) have investigated the extent to which insulin levels during antecedent hypoglycemia influence subsequent counterregulation. They demonstrated in normal subjects that counterregulatory increases in ACTH, cortisol, and norepinephrine were blunted after a single episode of low-dose insulin antecedent hypoglycemia, but not after high-dose insulin antecedent hypoglycemia. The authors concluded that insulin exerts a protective effect on subsequent counterregulation, and they postulated that hyperinsulinemia may prevent the development of hypoglycemia-associated counterregulatory failure. Others have demonstrated a similar protective effect of antecedent exposure to a single episode of hyperinsulinemic euglycemia (13). It is not known whether repeated exposure to hyperinsulinemia has similar effects on subsequent counterregulation.

*K. Shum and K. Inouye are to be considered first authors of this manuscript.

Address for reprint requests and other correspondence: M. Vranic, Professor of Physiology and Medicine, Univ. of Toronto, Rm. 3358, Medical Sciences Bldg., 1 King’s College Circle, Toronto, Ontario, Canada M5S 1A8 (E-mail: mladen.vranic@utoronto.ca).

http://www.ajpendo.org 0193-1849/01 $5.00 Copyright © 2001 the American Physiological Society
Exposure to glucocorticoids during antecedent hypoglycemia also affects counterregulation to subsequent hypoglycemia (14, 16, 38, 39). A series of studies by Davis et al. in nondiabetic humans (14, 16) and rats (38, 39) suggests that exposure to elevated plasma glucocorticoids during antecedent hypoglycemia contributes to the development of hypoglycemia-associated autonomic failure. In nondiabetic subjects exposed to high plasma cortisol levels similar to those observed during hypoglycemia, epinephrine, norepinephrine, glucagon, and muscle sympathetic nerve activity responses to subsequent hypoglycemia were blunted (14). Conversely, prevention of increases in cortisol during antecedent hypoglycemia (16), or treatment with the anti-glucocorticoid dehydroepiandrosterone (DHEA) during antecedent hypoglycemia (38), preserved counterregulatory responses. In these studies, however, glucocorticoids were infused on a background of hyperinsulinemia. It is not known whether administration of glucocorticoids alone has similar effects on subsequent counterregulation.

The objectives of the current study were to differentiate the effects of repeated episodes of antecedent hypoglycemia, antecedent hyperinsulinemia, and antecedent corticosterone on counterregulatory responses to subsequent hypoglycemia. We hypothesized that hyperinsulinemic episodes per se, without hypoglycemia, could affect subsequent counterregulation. Moreover, we postulated that antecedent increases in corticosterone alone contribute to the development of aspects of deficient counterregulation in normal rats.

RESEARCH DESIGN AND METHODS

Animal Care and Maintenance

Male Sprague-Dawley rats (Charles River Laboratories, Quebec, QC, Canada) weighing 350–450 g were used for experiments. Rats were individually housed in a temperature- and light-controlled environment (12:12-h light-dark schedule) and maintained on a standard rat diet (Rodent Laboratory Chow 5001, LabChows, Agribrands Canada, Strathroy, ON, Canada), with free access to drinking water. The rats were allowed 1–2 wk to acclimatize to the environment and husbandry regimen in the animal facility before surgery. During this time, observations were made to ensure that food and water intake was normal and that the rats were free of signs of clinical disease. All procedures were in accordance with the Canadian Council on Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.

Surgical Procedures

Intravascular cannulation was performed under general anesthesia (100 mg/kg ketamine hydrochloride, 1 mg/kg xylazine, and 1 mg/kg acepromazine maleate ip) 3 days before antecedent treatment. Catheters made from Silastic tubing (length 3 cm, ID 0.51 mm; Dow Corning, Midland, MI) connected to polyethylene tubing (PE-50, length 25 cm, ID 0.58 mm; Becton-Dickinson, Sparks, MD) were inserted into the left carotid artery and right jugular vein for blood sampling and infusion of test substances, respectively. The catheters were tunneled subcutaneously and exteriorized at the dorsal surface of the neck. To prevent blood clot formation, the catheters were primed with a 60% polyvinylpyrrolidone (Sigma Chemical, St. Louis, MO) and heparin (1,000 USP U/ml) solution.

Antecedent Treatment

Three days after surgery, the rats were divided into four groups.

Control group (N, n = 10). These rats received 4 days of sham antecedent treatment, in which subcutaneous insulin injections were replaced with saline injections. To control for the potential effects of mild stress inflicted by repeated measurement of blood glucose during antecedent treatment in the other groups, control rats’ tails were handled every 30 min throughout each sham antecedent episode to simulate the blood sampling procedure in the other groups.

Antecedent hypoglycemia group (AH, n = 8). Starting on the 3rd day after surgery (day 1), antecedent hypoglycemic episodes were induced by subcutaneous injection of regular insulin (Iletin II, 100 U/ml; Eli Lilly, Indianapolis, IN) at a dose of 0.12 U per 100 g body weight. This dose of insulin was necessary to decrease blood glucose to ~2.7 mM and to maintain this glycemic level for 2 h. Two hypoglycemic episodes (2 h each) were induced daily at 10 AM and 1 PM for 3 consecutive days (days 1–3). Only one episode was induced on the morning of day 4 to allow insulin and counterregulatory hormones to return to basal levels before the start of the experiment on day 5. Blood glucose was monitored from tail-nick samples collected every 30 min throughout each 2-h antecedent episode (Glucometer Elite 3903; Miles Canada, Etobicoke, ON; range 2.1–25 mM). Tail-nick samples were collected by initially making a small nick in the tip of the rat’s tail. Blood was then collected every 30 min by gently applying pressure to the rat’s tail. This procedure did not cause any distress in the rats and avoided potential complications associated with carotid artery sampling. During day 1 of treatment, blood samples were collected at 15- to 30-min intervals for measurement of plasma insulin and corticosterone. The blood was centrifuged immediately, and packed blood cells were resuspended in heparinized saline and rein fused into the rats after each sampling to prevent volume depletion and anemia.

Antecedent hyperinsulinemia group (AE, n = 7). These rats underwent treatment similar to the AH group. Antecedent hyperinsulinemic euglycemia was induced by subcutaneous injection of regular insulin at 0.2 U per 100 g of body weight combined with a variable intravenous infusion of 50% dextrose to maintain a euglycemic level of ~5 mM during the 2-h antecedent episodes. This group delineated the effects of antecedent hyperinsulinemia from those of antecedent hypoglycemia per se. Blood glucose was monitored from tail-nick samples collected every 30 min throughout each 2-h antecedent episode and was continued for 1 h after each episode to ensure that hypoglycemia would not occur upon withdrawal of exogenous glucose. During day 1 of treatment, blood samples were collected as described above for measurement of plasma insulin and corticosterone.

Antecedent corticosterone group (AC, n = 7). To simulate the increase in plasma corticosterone seen in AH rats during antecedent hypoglycemia, corticosterone was infused intravenously at a rate of 4.05 μg/min for 2 h per antecedent episode. The glucocorticoid infusate was prepared according to the method of Plotsky et al. (41) by dissolving 7.5 mg of corticosterone (4-pregnene-11β,21-diol-3,20-dione; Sigma Chemical) in 0.5 ml of absolute ethanol, followed by dilution to 25 ml with normal saline, giving rise to a 300 μg/ml solution. Blood glucose was monitored from tail-nick samples...
collected every 30 min throughout each 2-h antecedent episode. During day 1 of treatment, blood samples were collected as described above for measurement of plasma insulin and corticosterone.

Experimental Protocol

On day 5, after 4 days of treatment, hyperinsulinemic euglycemic-hypoglycemic clamp experiments were performed in all groups. At 8 AM, overnight-fasted rats were weighed and connected to the infusion and sampling apparatus. All procedures were carried out with minimal disturbance to the rats to avoid stress. After setup, the animals were allowed to rest for 2 h before experimentation. Rats were conscious and allowed to roam freely in their cages during the experiment.

At 10:30 AM, basal arterial samples for plasma glucose and hormones were collected over a 20-min period. Hyperinsulinemic euglycemia was then induced by a constant intravenous infusion of insulin at a rate of 50 mU·kg⁻¹·min⁻¹. This dose has previously been used in normal rats to maintain constant hypoglycemia at ~2.5 mM (29). Meanwhile, a primed (4 μCi) infusion of HPLC-purified [3-3H]glucose (New England Nuclear, Boston, MA) was started at 0.07 μCi/min. Blood was sampled from the carotid artery every 5 min to measure glucose levels, and a variable infusion of 50% dextrose was adjusted accordingly to maintain euglycemia at 5.7 ± 0.5 mM. After a period of >1 h of tracer equilibration, and once target glucose levels were maintained for ≥20 min, samples for tracer and hormones were collected over the next 20 min of euglycemia. Plasma glucose was then allowed to drop to a hypoglycemic target level of 2.5 ± 0.2 mM. To ensure accurate calculation of glucose turnover rates, changes in specific activity (SA) during the transition from euglycemia to hypoglycemia were minimized by reducing the rate of [3-3H]glucose infusion from 0.07 μCi/min to 0.035 μCi/min. Once target glycemia was attained, blood samples for tracer, glucagon, catecholamines, and corticosterone were collected at regular intervals over a period of 105 min. ACTH and insulin samples were collected at the end of the hypoglycemic period. All blood samples were centrifuged immediately after collection. The plasma was transferred to tubes for storage at −20°C (or −70°C for catecholamines) until assayed, and packed blood cells were resuspended in heparinized saline and reinfused into the rats after each sampling to prevent volume depletion and anemia.

Analytical Methods

Plasma glucose concentrations were measured in triplicate by the glucose oxidase method (31) (Glucose Analyzer II; Beckman Instruments, Fullerton, CA). [3-3H]glucose specific activity was determined in assays as previously described (1). Plasma glucagon (Diagnostic Products, Los Angeles, CA), ACTH (DiaSorin, Stillwater, MN), and corticosterone (ICN Biomedicals, Costa Mesa, CA) were measured by radioimmunoassay with commercially available radioimmunoassay kits, with interassay coefficients of variation (CV) of 16, 6, and 7%, respectively. Insulin radioimmunoassay was performed according to the method described by Herbert et al. (28), with an interassay CV of 11%. Plasma catecholamines were determined using a radioenzymatic assay for simultaneous measurement of epinephrine and norepinephrine (37), and the CV was calculated to be 17%.

Calculations

Data for SA and plasma glucose concentration were smoothed using the optimized Optimal Segments program (5). Data smoothing was required for the accurate calculation of glucose appearance and disappearance. Smoothing eliminated abrupt data fluctuations that might otherwise lead to inaccurate estimation of the rates of change of SA and blood glucose (22). All calculations were carried out according to Steele’s non-steady-state equations (20, 44). Both smoothing and Steele’s non-steady-state calculations were required because, although mean plasma glucose levels and SA were relatively constant, some variation occurred in the individual data. Because SA was kept relatively constant during the clamps, the total rate of glucose appearance (Ra) was similar to the rate of glucose disappearance (Rd). Therefore, turnover rate (= Ra = Rd) was mainly equivalent to the tracer infusion rate divided by SA, and the pool fraction correction was minimal (20). Because the input of glucose comprised both endogenous glucose production and exogenous glucose infusion, the rate of hepatic glucose production was calculated by subtracting the glucose infusion rate from Ra (20). The metabolic clearance rate (MCR) of glucose, a measure of glucose utilization (GU = Rd) that partially corrected for the mass effect of plasma glucose, was calculated by dividing glucose utilization by plasma glucose concentration (21).

Statistical Analysis

All data were expressed as means ± SE. Statistical analysis was performed with SAS software (Statistical Analysis System; SAS Institute, Cary, NC). Significance was assumed at P < 0.05. Comparisons of basal and euglycemic parameters were made using analysis of variance (ANOVA) in conjunction with Tukey’s Studentized Range (HSD) test. Counterregulatory responses to hypoglycemia and glucose turnover parameters during hypoglycemia were compared using repeated-measures analysis.

RESULTS

Animal Weights During Antecedent Treatment

There were no differences in the effects of the various antecedent treatments on animal weights. Although initial weights on surgery day were higher (P < 0.05) in AH and AE rats compared with controls, and in AE rats compared with AC rats (P < 0.05), all groups displayed similar weight changes throughout treatment (Table 1). Weights dropped in all groups from surgery day to day 1 of treatment. However, there were no weight changes in any of the groups from day 1 to day 5.

<table>
<thead>
<tr>
<th>Antecedent Treatment</th>
<th>Presurgery</th>
<th>Day 1 Antecedent Treatment</th>
<th>Day 4 Antecedent Treatment</th>
<th>Day 5 Glucose Clamp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (N)</td>
<td>332 ± 18</td>
<td>325 ± 16</td>
<td>324 ± 17</td>
<td>317 ± 20</td>
</tr>
<tr>
<td>Antecedent hypoglycemia (AH)</td>
<td>394 ± 15*</td>
<td>369 ± 13</td>
<td>375 ± 15</td>
<td>358 ± 12</td>
</tr>
<tr>
<td>Antecedent hyperinsulinemia (AE)</td>
<td>412 ± 11†</td>
<td>402 ± 13†</td>
<td>394 ± 11†</td>
<td>379 ± 10†</td>
</tr>
<tr>
<td>Antecedent corticosterone (AC)</td>
<td>355 ± 14</td>
<td>346 ± 16</td>
<td>355 ± 17</td>
<td>331 ± 13</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed in g. *P < 0.05 vs. N; †P < 0.05 vs. AC.
day 4. Weights dropped in all groups from day 4 to the day 5 hypoglycemic clamp due to overnight fasting.

Blood Glucose and Plasma Insulin and Corticosterone Levels During Antecedent Treatment

Figure 1A represents the average of morning and afternoon blood glucose levels over 4 days of treatment. Figure 1, B and C, represents the average of morning and afternoon insulin and corticosterone levels on day 1 of treatment. Morning and afternoon data were combined because there were no differences between morning and afternoon glucose and hormone levels.

Blood glucose was maintained at \(~4.5\) mM during antecedent hyperinsulinemia and at \(~5.5\) mM during antecedent corticosterone. At 30 min into the monitoring period in AE rats, mean glucose was at \(6.7 \pm 0.2\) mM. By 60 min, mean glucose was at \(7.3 \pm 0.2\) mM. Antecedent hyperinsulinemic glucose levels were lower \((P < 0.05)\) than during antecedent corticosterone but were higher \((P < 0.05)\) than during antecedent hypoglycemia. During antecedent hypoglycemia, glucose was maintained at \(~2.7\) mM.

Subcutaneous insulin injections during antecedent treatment resulted in significant elevations in plasma insulin concentrations in both the AH and AE groups (Fig. 1B). Plasma insulin in the AH and AE groups increased from basal levels of \(439 \pm 111\) and \(184 \pm 27\) pM, respectively, and reached peak values at 30 min after injection before gradually declining toward basal levels over the 2nd h of treatment. In AC rats, which did not receive insulin injections, plasma insulin increased slightly but significantly \((P < 0.05)\) from basal levels (Fig. 1B, inset). Nonetheless, the increase in insulin was negligible compared with the increase in AH and AC rats.

Starting from similar baseline values, corticosterone levels in the AC group initially rose significantly \((P < 0.05)\) above the other groups during the first 30 min of the 2-h episode, reaching a plateau of \(~750\) nM. Corticosterone levels rose more gradually in the AH group but also reached a relatively stable level of \(~700\) nM over the final 90 min of the treatment period. Corticosterone levels in the AE group rose moderately but significantly \((P < 0.05)\) to a peak of \(386 \pm 61\) nM and remained elevated compared with baseline throughout antecedent treatment. The increase in corticosterone in the AE group was lower \((P < 0.05)\) than in the AH and AC groups.

Basal Plasma Glucose and Hormone Levels After Antecedent Treatment

Basal plasma glucose and hormone levels after 4 days of antecedent treatment are summarized in Table 2. Samples were collected on the morning of the glucose clamp experiment 2 h after connection of the animals to the infusion apparatus. Plasma glucose levels were similar in controls, AE rats, and AC rats, but they were elevated \((P < 0.05)\) in AH rats compared with controls. There were no significant differences in insulin levels among the groups. Basal glucagon levels were decreased \((P < 0.05)\) in AH rats compared with AE rats. ACTH levels were increased \((P < 0.05)\) in all antecedent groups compared with controls and were further increased \((P < 0.05)\) in AC rats compared with AH and AE rats. Corticosterone levels were increased \((P <
0.05) in AC rats compared with AE rats. Epinephrine levels were increased ($P < 0.05$) in AC rats compared with controls and AE rats, whereas norepinephrine levels did not differ among the groups.

**Plasma Glucose and Hormone Levels During Day 5 Hyperinsulinemic Euglycemic-Hypoglycemic Clamp Experiments**

Plasma glucose for each experimental group was maintained within the target range throughout the euglycemic ($5.7 \pm 0.5$ mM) and hypoglycemic ($2.5 \pm 0.2$ mM) periods of the glucose clamp experiment (Fig. 2A). Insulin concentrations increased from baseline values of $70–130$ pM (Table 2) to over $20,000$ pM during the clamps (Fig. 2B). This supraphysiological insulin level was required to maintain constant hypoglycemia at $2.5$ mM (29). For control rats, AH rats, and AE rats, there were no significant differences between euglycemic and hypoglycemic insulin levels. However, in AC rats, hypoglycemic insulin levels were increased ($P < 0.05$) approximately twofold compared with euglycemic levels.

In response to hypoglycemia, plasma concentrations of all counterregulatory hormones (glucagon, epinephrine, norepinephrine, ACTH, and corticosterone) increased ($P < 0.05$) from euglycemic levels. All groups displayed a two- to fourfold increment in glucagon from similar euglycemic values, with peak incremental responses of $\sim 100$ ng/l (Fig. 3). Glucagon responses were similar among the groups. Epinephrine levels increased ($P < 0.05$) in all groups during hypoglycemia (Fig. 4A). There was an 85-fold increase in epinephrine in the controls, reaching a peak concentration of $>36,000$ pM. Epinephrine responses in AH and AE rats were impaired ($P < 0.05$) by nearly 50% compared with controls. Epinephrine responses in AC rats did not differ from control responses. Plasma norepinephrine concentrations during the first 45 min of hypoglycemia did not differ among the groups (Fig. 4B). However, AH rats displayed increased norepinephrine levels during the final 60 min of the clamp, such that the overall norepinephrine responses in AH rats were greater ($P < 0.05$) than in control and AE rats.

During hyperinsulinemic euglycemia, only control rats displayed increased ($P < 0.05$) plasma ACTH levels compared with baseline (Fig. 5A). In response to hypoglycemia, ACTH levels increased in all groups. Although hypoglycemic ACTH levels were decreased ($P < 0.05$) in AC rats compared with AE rats, increases from euglycemic levels did not differ among the groups. In all groups, euglycemic corticosterone levels were increased ($P < 0.05$) from basal levels (Fig. 5B). However, euglycemic corticosterone levels were greater ($P < 0.05$) in control rats than in AH rats and tended to be greater than in AE and AC rats. In response to

![Fig. 2. Plasma glucose (A) and insulin (B) during day 5 hyperinsulinemic euglycemic-hypoglycemic glucose clamp experiments. Plasma glucose was maintained within target euglycemic (Eugly; $5.7 \pm 0.5$ mM) and hypoglycemic (Hypo; $2.5 \pm 0.2$ mM) levels for each group. AC rats displayed increased ($P < 0.05$) insulin levels during the hypoglycemic vs. the euglycemic period of the clamp. Values are expressed as means ± SE.](http://ajpendo.physiology.org/)

**Table 2. Effects of days 1–4 antecedent hypoglycemia, antecedent hyperinsulinemia, and antecedent corticosterone on basal plasma glucose and hormone levels on day 5**

<table>
<thead>
<tr>
<th></th>
<th>Controls (N, n = 10)</th>
<th>Antecedent Hypoglycemia (AH, n = 8)</th>
<th>Antecedent Hyperinsulinemia (AE, n = 7)</th>
<th>Antecedent Corticosterone (AC, n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma glucose, mM</strong></td>
<td>6.1 ± 0.2</td>
<td>7.0 ± 0.2*</td>
<td>6.4 ± 0.2</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td><strong>Insulin, pM</strong></td>
<td>85 ± 18</td>
<td>129 ± 20</td>
<td>115 ± 11</td>
<td>68 ± 13</td>
</tr>
<tr>
<td><strong>Glucagon, ng/l</strong></td>
<td>69 ± 7</td>
<td>54 ± 5.5</td>
<td>74 ± 5</td>
<td>73 ± 3</td>
</tr>
<tr>
<td><strong>ACTH, pM</strong></td>
<td>7.9 ± 2.1</td>
<td>15.6 ± 1.3*</td>
<td>14.1 ± 1.3*</td>
<td>21.2 ± 1.8**†‡</td>
</tr>
<tr>
<td><strong>Corticosterone, nM</strong></td>
<td>50 ± 12</td>
<td>101 ± 29</td>
<td>40 ± 15</td>
<td>158 ± 28‡‡</td>
</tr>
<tr>
<td><strong>Epinephrine, pM</strong></td>
<td>428 ± 43</td>
<td>564 ± 99</td>
<td>520 ± 92</td>
<td>1,189 ± 287‡‡</td>
</tr>
<tr>
<td><strong>Norepinephrine, nM</strong></td>
<td>1.48 ± 0.13</td>
<td>2.55 ± 0.69</td>
<td>1.59 ± 0.30</td>
<td>2.86 ± 0.93</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats/group. *$P < 0.05$ vs. N; †$P < 0.05$ vs. AH; ‡$P < 0.05$ vs. ARE.
hypoglycemia, plasma corticosterone further increased in all groups. Incremental corticosterone responses from euglycemia to hypoglycemia did not differ among the groups.

Glucose Kinetics During Day 5 Hyperinsulinemic Euglycemic-Hypoglycemic Clamp Experiments

Glucose SA was relatively stable throughout the experiment (peak rate of increase <0.8% per min) and was not statistically different among the groups (Fig. 6A). During hyperinsulinemic euglycemia, hepatic glucose production in the controls was maximally suppressed (Table 3). Hepatic glucose production under basal conditions in normal rats has been reported to be $50 \pm 1.7 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ (46). In AE and AC rats, glucose production during hyperinsulinemic euglycemia was increased ($P < 0.05$) compared with controls. Glucose production in AH rats tended to be increased compared with controls. In response to hypoglycemia, hepatic glucose production increased in all groups (Fig. 6B). During the first 45 min of hypoglycemia, absolute rates of glucose production in AH rats were decreased ($P < 0.05$) compared with control and AE rats. Incremental glucose production responses from euglycemia during the first 45 min of hypoglycemia were diminished ($P < 0.05$) in all antecedent groups compared with controls (Fig. 6C).

Glucose utilization ($R_d$) and MCR were similar in all groups during the euglycemic period (Table 3). Glucose utilization decreased ($P < 0.05$) to similar levels in all groups during hypoglycemia. In control rats, AH rats, and AE rats, MCR did not change from euglycemia to hypoglycemia, because the decline in $R_d$ was proportional to the decrease in glucose concentration. Although AC rats displayed decreased ($P < 0.05$) hypoglycemic MCR compared with the euglycemic period, hypoglycemic MCR did not differ among the groups.

The lower hypoglycemic MCR in AC rats was due to a tendency toward increased euglycemic MCR.

DISCUSSION

The aim of this study was to differentiate the effects of seven episodes of antecedent hypoglycemia, antecedent hyperinsulinemia, and antecedent increases in plasma corticosterone on counterregulation to subsequent hypoglycemia in normal rats. Specifically, we examined whether antecedent hyperinsulinemia or corticosterone per se could impair subsequent counterregulation. Previous studies have mainly examined the effects of one or two antecedent hypoglycemia (17, 27, 30, 45) or antecedent glucocorticoid (14, 39) episodes induced on the day before the subsequent hypoglycemic clamp. In the present study, administration of seven antecedent episodes over 4 days allowed us to investigate the effects of chronic recurrent antecedent treatment. Such adaptations may differ from those occurring after one or two episodes. We demonstrated...
that both antecedent hypoglycemia and antecedent hyperinsulinemia could impair epinephrine and incremental glucose production responses to subsequent hypoglycemia, suggesting that antecedent hyperinsulinemia per se contributes to the development of hypoglycemia-associated autonomic failure. Selective increases in corticosterone reduced incremental, but not absolute, glucose production responses to subsequent hypoglycemia. Glucose production was impaired despite unaltered neuroendocrine responses.

Antecedent hypoglycemia produced similar hypoglycemic glucose levels each day, resulting in invariable mean antecedent glucose levels. The similar daily glucose levels were unexpected, given that counterregulation likely became impaired as antecedent treatment progressed. The lack of variability was partly due to the large number of glucose measurements that comprised each data point (n animals per group × 7 glucose measurements). In addition, the fact that we were unable to measure glucose levels below 2.1 mM during antecedent treatment, because of the limitations of the glucose meter used, may also have contributed to the invariability. Surprisingly, in AC rats, there was a small, but significant, increase in plasma insulin during corticosterone infusion. This increase may have been due to reduced insulin clearance. In Cushing’s patients with elevated cortisol, insulin clearance is decreased by as much as 33% (6). Corticosterone levels during day 1 antecedent hypoglycemia increased markedly and were matched in AC rats. AE rats also displayed a small, but significant, rise in corticosterone after insulin injection. Although rats normally exhibit a rise in afternoon corticosterone levels (10), in both the morning and afternoon episodes in AE rats, corticosterone levels increased after insulin injection and then decreased. Negative feedback of elevated corticosterone on the hypothalamic-pituitary-adrenal (HPA) axis (43) after insulin injection may have inhibited afternoon corticosterone release.

Exposure of rats to severe electric shock stress results in elevated plasma corticosterone even 24 h post-stress (36). To reduce the possibility of elevated insulin and stress hormone levels at the beginning of the day 5 glucose clamp, we omitted the afternoon antecedent episode on day 4 to allow a 22-h period for hormone levels to decrease. Antecedent hypoglycemia, hyperinsulinemia, and corticosterone did not significantly affect day 5 basal insulin levels. Surprisingly, basal glucagon was decreased in AH rats compared with AE rats, and it tended to be decreased compared with the other groups. Although basal glucose was increased in AH rats, it is unlikely that this increase was sufficient to impair glucagon secretion. Despite the rest period, all antecedent groups displayed increased basal ACTH and corticosterone levels compared with controls. Furthermore, AC rats displayed elevated corticosterone and epinephrine. Although we cannot rule out that differences in handling during antecedent treatment contributed to the elevated stress hormone levels, the similar weight patterns among all groups during antecedent treatment suggests that blood sampling in AH, AE, and AC rats did not cause additional major stress. We hypothesize that the increased stress hormone levels in AH, AE, and AC rats were due to differences in the antecedent treatment paradigms themselves, rather than potential differences in handling stress. Hypoglycemia and hyperinsulinemia induce HPA activity, as demonstrated by the increased corticosterone levels in AH and AE rats during antecedent treatment. In addition, elevated corticosterone can have long-term effects on HPA (43) and sympathetic activity (32). Therefore, it is not surprising that recurrent hypoglycemia, hyperinsulinemia, and elevated corticosterone affected basal stress hormone levels.

Using the glucose clamp technique, we examined the effects of various antecedent treatments on neuroendocrine responses to subsequent hypoglycemia. In the present study, glucagon responses were not affected by any of the antecedent treatments. We expected reduced
glucagon responses in AH rats, because antecedent hypoglycemia consistently impairs glucagon counterregulation in normal humans (11, 17, 27, 30). The effects of the antecedent treatments on glucagon secretion in the present study may have been masked by a suppressive effect of marked hyperinsulinemia during the glucose clamp. Using a lower insulin infusion rate of 20 mU·kg\(^{-1}\)·min\(^{-1}\), Powell et al. (42) demonstrated a nearly sixfold increase in glucagon levels in normal rats during hypoglycemia and an 84% reduction in glucagon responses after 3–4 wk of recurrent hypoglycemia. In our experiments, in which insulin was infused at 50 mU·kg\(^{-1}\)·min\(^{-1}\), glucagon levels in all groups increased by only two- to fourfold. We cannot exclude the possibility that a longer antecedent treatment period was required to affect glucagon counterregulation.

During the hyperinsulinemic euglycemic period of the glucose clamp, ACTH levels increased only in control rats. Likewise, hyperinsulinemic corticosterone levels in control rats were greater than in AH rats and tended to be greater than in AE and AC rats. These data indicate that pituitary-adrenal responses to hyperinsulinemia were reduced after all antecedent treatments. In contrast, pituitary-adrenal responses to hypoglycemia per se were unaffected by any of the antecedent treatments, because all antecedent groups displayed similar increments in ACTH and corticosterone from euglycemia to hypoglycemia.

In response to hypoglycemia, epinephrine levels in control rats increased by \(85\)-fold from baseline, whereas norepinephrine increased maximally by \(5\)-fold. After antecedent hypoglycemia, epinephrine responses were reduced by nearly 50%. Norepinephrine responses, in contrast, were enhanced in AH rats compared with controls and AE rats. This was unexpected, because most studies report unaltered (30, 47) or decreased (45) norepinephrine responses after antecedent hypoglycemia. It is conceivable that norepinephrine responses were increased in AH rats to compensate for the impaired epinephrine responses. Nonetheless, the improved norepinephrine responses did not appear to have any metabolic effects.

Surprisingly, epinephrine responses to hypoglycemia were also reduced by 50% in rats exposed to antecedent hyperinsulinemia. Although the decreased epinephrine responses after antecedent hypoglycemia are consistent with previous studies (11, 17, 27, 30), our finding that antecedent hyperinsulinemia per se can reduce epinephrine responses has not been reported previously. During antecedent hyperinsulinemia, considerable care was taken to avoid hypoglycemia. It is unlikely that hypoglycemia occurred after daily antecedent hyperinsulinemia, because blood glucose was at \(7\) mM by the end of the monitoring period after each antecedent episode, and most rats no longer required exogenous glucose to maintain normoglycemia at this point. Nonetheless, because mean glucose levels throughout antecedent hyperinsulinemia were below fed state levels, we cannot rule out that mild stimulation of counterregulation during antecedent

Fig. 6. Plasma [3-\(^3\)H]glucose specific activity (A), absolute hepatic glucose production responses to hypoglycemia (B), and incremental hepatic glucose production responses to hypoglycemia (C) during day 5 hyperinsulinemic euglycemic-hypoglycemic glucose clamp experiments. Specific activity was relatively stable and did not differ among groups. Absolute glucose production responses during the first 45 min of hypoglycemia were diminished in AH rats vs. controls \((P < 0.05)\) and AE rats \((P < 0.05)\). Incremental glucose production responses during the first 45 min of hypoglycemia were diminished \((P < 0.05)\) after all antecedent treatments. Values are means ± SE.

AJP-Endocrinol Metab • VOL 281 • SEPTEMBER 2001 • www.ajpendo.org
treatment may have influenced the epinephrine responses. On the other hand, we did not observe lower epinephrine responses in rats that demonstrated lower glucose levels during antecedent treatment. Therefore, we hypothesize that, in normal rats, marked antecedent hyperinsulinemia per se may contribute to the development of hypoglycemia-associated autonomic failure.

Acutely, higher degrees of hyperinsulinemia enhance neuroendocrine responses and symptom awareness to equivalent hypoglycemic stimuli (33). This effect may be related to an ability of insulin to suppress brain glucose utilization and hence cause neuroglycopenia (33). In rats exposed to hyperinsulinemic euglycemia, insulin suppresses glucose utilization in the medial basal hypothalamus (26) and tends to reduce glucose utilization in the lateral and ventromedial hypothalamus (VMH) (35). The effect of insulin on the VMH is of particular interest, because the VMH plays a critical role in the detection of and counterregulation against hypoglycemia (3, 4). In fact, diminished counterregulation after antecedent hypoglycemia may involve dysfunction of VMH glucose-sensing neurons or its efferent pathways as they become resistant to neuroglycopenia (2). It is conceivable that the reduced epinephrine responses in AE rats were due to desensitization of brain glucose-sensing neurons to neuroglycopenia after repeated hyperinsulinemia.

Recent studies by Davis and colleagues in nondiabetic humans (14, 16) and in rats (Ping and coworkers; 38, 39) suggest that antecedent increases in glucocorticoids contribute to the development of impaired counterregulation after antecedent hypoglycemia. They showed that antecedent increases in plasma cortisol in normal humans could blunt counterregulatory responses to subsequent hypoglycemia (14). In the present study, hormonal responses were unaffected by antecedent corticosterone. The discrepancies between our findings and those of Davis and colleagues may be due to differences in experimental protocols. During antecedent cortisol treatment, those investigators infused cortisol together with insulin (14, 16, 38, 39). In the present study, AC rats received corticosterone without insulin. In light of our observation of impaired epinephrine responses in AE rats, it is conceivable that the effects of cortisol in the studies of Davis and colleagues were enhanced by the coadministration of insulin. Nonetheless, we cannot rule out that, in the present study, the slight increases in corticosterone in AE rats during antecedent treatment contributed to the decreased epinephrine and incremental glucose production responses.

In AE and AC rats, we observed elevated glucose production, and hence impaired hepatic insulin sensitivity, during the hyperinsulinemic-euglycemic period of the glucose clamp. Conversely, MCR, an indicator of peripheral insulin sensitivity, was not altered by any of the antecedent treatments. During the first 45 min of hypoglycemia, absolute and incremental glucose production responses were impaired in AH rats compared with controls. In AE and AC rats, incremental but not absolute glucose production responses were impaired.

These data indicate that antecedent hypoglycemia, hyperinsulinemia, and elevated glucocorticoids can all act to impair incremental glucose production responses to subsequent hypoglycemia. In AH and AE rats, the defects in glucose production may have been due to impaired epinephrine responses. The decreased incremental glucose production responses in AC rats were associated with twofold increased insulin levels during the hypoglycemic period compared with the euglycemic period. This increase in insulin may have been due to a defect in insulin clearance.

In summary, we demonstrate that, in normal rats, both antecedent hypoglycemia and marked antecedent hyperinsulinemia can impair epinephrine and incremental glucose production responses to subsequent hypoglycemia. In contrast, antecedent increases in corticosterone alone impair incremental, but not absolute, glucose production responses without altering neuroendocrine responses. Although we cannot rule out that differences in antecedent sampling and infusion procedures influenced our results, the similar weight patterns among the groups suggest that infusion and sampling did not cause additional major stress. It is conceivable that, in AH rats, the decreased epinephrine and glucose production responses were due to exhaustion of these responses caused by recurrent ex-
pose to hypoglycemia. However, our observation of impaired epinephrine and incremental glucose produc-
tion responses after antecedent hyperinsulinemia leads us to hypothesize that insulin may also be in-
volved. Our results suggest that marked antecedent hyperinsulinemia per se plays a role in the develop-
ment of hypoglycemia-associated autonomic failure.

This work was generously supported by research grants from the Canadian Institutes of Health Research, the Canadian Diabetes Association, and the Juvenile Diabetes Foundation International. Graduate students K. Shum, K. Inouye, and O. Chan were supported by scholarships from the University of Toronto's Department of Physiology, Canadian Institutes of Health Research, and by Novo-
Nordisk studentships from the Banting and Best Diabetes Centre in Toronto. J. Mathoo was supported by a summer scholarship from the University of Toronto's Faculty of Medicine.

REFERENCES

1. Bjorkman O, Miles PD, Wasserman D, Lickley HL, and Vranic M. Regulation of glucose turnover during exercise in pancreactomized, totally insulin deficient dogs: effects of beta-


2. Borg MA, Borg WP, Tamborlane WV, Brines ML, Shulman

GI, and Sherwin RS. Chronic hyperglycemia and diabetes im-
pair counterregulation induced by localized 2-deoxy-glucose per-
fusion of the ventromedial hypothalamus in rats. Diabetes 48:


3. Borg MA, Sherwin RS, Borg WP, Tamborlane WV, and Shulman


5. Bradley DC, Steil GM, and Bergman RN. Quantitation of measurement error with Optimal Segments: basis for adaptive time course smoothing. Am J Physiol Endocrinol Metab 264:


7. Cryer PE. Hypoglycemia: the limiting factor in the manage-

8. Cryer PE and Gerich JE. Hypoglycemia in insulin-dependent diabetes mellitus: interplay of insulin excess and compromised glucose counterregulation. In: Ellenberg & Rifkin’s Diabetes Meta-
bolism, edited by H Rifkin and D Porte Jr. East Norwalk, CT:


9. Dagogo-Jack SE, Craft S, and Cryer PE. Hypoglycemia-
associated autonomic failure in insulin-dependent diabetes mel-

10. Dallman MF, Akana SF, Cascio CS, Darlington DN, Jacob-


13. Davis SN, Dobbins R, Colburn C, Tarumi C, Jacobs J, Neal


15. Davis SN, Shavers C, Davis B, and Costa F. Prevention of an increase in plasma cortisol during hypoglycemia preserves sub-


18. DCCT Research Group. The effect of intensive treatment of diabetes on the development of long-term complications in insu-

19. Finegood DT, Bergman RN, and Vranic M. Estimation of endogenous glucose production during hyperinsulinemic-eugly-

20. Fisher SJ, Lekas M, Shi ZQ, Bilinski D, Carvalho G, Gi-


24. George E, Marques JL, Harris ND, Macdonald IA, Hard-

isty CA, and Heller SR. Preservation of physiological re-


26. Heller SR and Cryer PE. Reduced neuroendocrine and symp-

27. Herbert, Coated charcoal immunoadsorbent of insulin. J Clin En-


28. Hevener AL, Bergman RN, and Donovan CM. Portal vein splanchnic outflows are critical for the sympathoadrenal response to hypo-


29. Hvidberg A, Fanelli CG, Hershey T, Terkamp C, Craft S, and Cryer PE. Impact of recent antecedent hypoglycemia on hypoglycemic cognitive dysfunction in nondiabetic humans. Di-


32. Lingenfeller T, Overkamp D, Renn W, Buettner U, KIm-

