Antecedent short-term central nervous system administration of estrogen and progesterone alters counterregulatory responses to hypoglycemia in conscious male rats

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Sandoval DA, Gong B, Davis SN. Antecedent short-term central nervous system administration of estrogen and progesterone alters counterregulatory responses to hypoglycemia in conscious male rats. Am J Physiol Endocrinol Metab 293: E1511–E1516, 2007. First published October 16, 2007; doi:10.1152/ajpendo.00340.2007.—The aim of this study was to test the hypothesis that antecedent short-term administration of estradiol or progesterone into the central nervous system (CNS) reduces levels of neuroendocrine counterregulatory hormones during subsequent hypoglycemia. Conscious unrestrained male Sprague-Dawley rats were studied during randomized 2-day experiments. Day 1 consisted of an 8-h lateral ventricle infusion of estradiol (1 μg/μl; n = 9), progesterone (1 μg/μl; n = 9), or saline (0.2 μl/min; n = 10). On day 2, a 2-h hyperinsulinemic (30 pmol·kg⁻¹·min⁻¹) hypoglycemic (2.9 ± 0.2 mM) clamp was performed on all rats. Central administration of estradiol on day 1 resulted in significantly lower plasma epinephrine levels during hypoglycemia compared with saline, whereas central administration of progesterone resulted in increased levels of plasma norepinephrine and decreased levels of corticosterone both at baseline and during hypoglycemia. Glucagon responses during hypoglycemia were unaffected by prior administration of estradiol or progesterone. Endogenous glucose production following day 1 estradiol was significantly lower during day 2 hypoglycemia, and consequently, the glucose infusion rate to maintain the hypoglycemia was significantly greater after estradiol administration compared with saline. These data suggest that 1) CNS administration of both female reproductive hormones can have rapid effects in modulating levels of counterregulatory hormones during subsequent hypoglycemia in conscious male rats, 2) forebrain administration of reproductive hormones can significantly reduce pituitary adrenal and sympathetic nervous system drive during hypoglycemia, 3) reproductive steroid hormones produce differential effects on sympathetic nervous system activity during hypoglycemia, and 4) reduction of epinephrine resulted in significantly blunted metabolic counterregulatory responses during hypoglycemia.

WOMEN, COMPARED WITH MEN, have reduced autonomic responses to psychological stress (22), exercise (8, 19, 40), and hypoglycemia (14). We have previously shown that women taking estrogen replacement therapy have reduced autonomic counterregulatory activity in response to insulin-induced hypoglycemia compared with age- and body mass index-matched men and women not taking estrogen replacement therapy (35), thus suggesting that chronic elevations in estradiol may be an important mechanism for sexually dimorphic responses to stress.

RESEARCH DESIGN AND METHODS

Animals. Twenty-nine male Sprague-Dawley rats (300–350 g) bred and purchased from Harlan (Indianapolis, IN) were studied. The rats were housed and individually caged in the Vanderbilt University Animal Care Facility under controlled conditions (12:12-h light-dark cycle, 50–60% humidity, 25°C) with free access to food and water. All procedures for animal use were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

Animal preparation. At least 1 wk before each study, each rat had catheters placed in the carotid artery (for blood sampling) and the external jugular vein (for infusions) under a general anesthesia mixture (5 mg/kg acepromazine, 10 mg/kg xylazine, and 50 mg/kg ketamine). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ketamine). Catheter lines were kept patent by flushing with 150 U/ml heparin. Immediately after the catheter placements, rats were placed on a stereotaxic frame (KOPF Instruments, Tujunga, CA) for placement of a 6-mm stainless steel guide cannula at stereotaxic coordinates corresponding to the lateral cerebral ventricle (−0.9 mm anteroposterior, +1.4 mm mediolateral, and −4.5 mm dorsoventral from bregma according to the atlas of Paxinos and Watson; Ref. 27). The intracranial cannulas were held in place with cranioplastics cement and three skull screws. Rats had free access to rat chow on the days before surgery and experiments. Seven days postsurgery, only rats with >90% of their presurgery body weight were used for the 2-day experiments.

Experimental design. Three groups of male rats were studied during a 2-day experimental protocol. Day 1 consisted of lateral cerebral ventricular infusion of estrogen (1 mg/ml; estradiol; E2; n = 9), progesterone (1 mg/ml; P4; n = 9), or saline (n = 10). Water-soluble estrogen and P4 (0.01 g; Sigma, St. Louis, MO) was diluted with 10 ml of 0.9% saline and infused at a rate of 0.06 mg/h during the morning and afternoon glycemic clamps. Using saline avoids any potential confounding side effects of the dissolving medium. On day 2, all rats were exposed to a 2-h hyperinsulinemic hypoglycemic clamp. Rats were fasted overnight before each day of the 2-day study and remained conscious and unrestrained throughout the experimental protocols. To prevent a fall in hematocrit, after each blood draw, washed red blood cells plus normal saline were reinfused through the carotid cannula of the rat. The morning of the study, extensions were placed on the exteriorized catheters for ease of access and were removed between day 1 and day 2 studies.

Day 1 procedures. At time 0 min, rats were moved to the experimental cage, and the intracerebroventricular (ICV) infusion of saline, estrogen, or P4 was started. Plasma measurements of glucose were taken at 0, 240, 360, and 480 min of ICV infusion. At the conclusion of day 1 procedures, rats were fed 5–8 g of rat chow.

Day 2 procedures. At time 0 min, rats were moved to an experimental cage and allowed to acclimate to the surroundings. The experiment consisted of a basal period (time 90–120 min) and an experimental period (time 120–240 min) during which a hyperinsulinemic hypoglycemic clamp (described below) was performed. To measure glucose kinetics during the clamp, a primed (10 μCi) constant (0.1 μCi/min) infusion of HPLC-purified [3-3H]glucose (Perkin Elmer Life Sciences, Boston, MA) was administered via a precalibrated infusion pump (Harvard Apparatus, South Natick, MA) at time 0 min and continued through 240 min. Blood was drawn every 5–15 min during the experimental period for measurements of plasma glucose, every 10 min during the baseline period and every 15 min during the experimental period for [3-3H]glucose, and at 110, 120, 180, 210, and 240 min for counterregulatory hormones. Rats were euthanized after day 2 procedures, and placement of ICV (by injection of cresyl violet), carotid, and jugular cannulas was verified.

Glycemic clamping procedures. On day 2, from 120 to 240 min, a primed (60 pmol·kg⁻¹·min⁻¹) continuous (30 pmol·kg⁻¹·min⁻¹) infusion of insulin (Eli Lilly, Indianapolis, IN) containing 9.7% (vol/vol) rat plasma was administered via a precalibrated infusion pump (Harvard Apparatus). After the start of insulin, glucose levels were allowed to fall (reaching nadir in ~30 min), and a 20% dextrose infusion was adjusted to maintain glucose at ~2.9 mM for 90 min.

Tracer calculations. Rates of glucose appearance, endogenous glucose production (EGP), and glucose utilization were calculated according to the methods of Wall et al. (43) and as described previously (37).

Analytic methods. Plasma glucose was measured in duplicate by the glucose oxidase technique on a Beckman glucose analyzer. Catecholamines were determined by HPLC with an interassay coefficient of variation (CV) of 12% for both epinephrine and norepinephrine as described previously (36). Corticosterone (ICN Biomedicals, Irvine, CA) (interassay CV = 7%), insulin (interassay CV = 11%), and glucagon (Linco Research, St. Louis, MO) (interassay CV = 15%) were all measured using radioimmunoassay techniques described previously (36).

Statistical analysis. Data are expressed as means ± SE and were analyzed using standard parametric two-way analysis of variance (ANOVA) with repeated measures where appropriate. A Tukey’s post hoc analysis was used to delineate statistical significance. A P value ≤ 0.05 was accepted as statistical significance.

RESULTS

Glucose and insulin. All rats remained euglycemic during day 1 lateral ventricle infusions (6.5 ± 0.1 mmol/l) with no change from baseline glucose levels. Glucose (2.9 ± 0.1 mmol/l) and insulin (50 ± 8 at baseline and 945 ± 61 pmol/l) levels were also similar among all groups during day 2 hypoglycemic clamps (Fig. 1).

Counterregulatory hormones. Plasma norepinephrine levels were significantly greater at baseline and during the final 30 min of hypoglycemia in the P4 group compared with both saline and E2 (0.5 ± 0.05 vs. 0.3 ± 0.06 and 0.2 ± 0.02 at baseline and 0.7 ± 0.05 vs. 0.5 ± 0.07 and 0.4 ± 0.08 mmol/l for P4 vs. saline and E2, respectively; P < 0.05; Fig. 2). Basal levels of day 2 plasma epinephrine were similar among the groups (0.2 ± 0.03, 0.2 ± 0.03, and 0.2 ± 0.05 nmol/l for saline, E2, and P4 groups, respectively). However, plasma epinephrine levels were significantly lower in E2 vs. both saline and P4 at 210 and 240 min of hypoglycemia (P < 0.05; Fig. 2). Plasma glucagon levels at baseline and during hypoglycemia were similar among the three groups across all time points (Fig. 3). Plasma corticosterone levels were reduced at baseline (2 ± 1 vs. 5 ± 2 and 8 ± 2 nmol/l, respectively; P < 0.05; Fig. 3) and during the final 30 min of day 2 hypoglycemia (15 ± 1 vs. 21 ± 2 and 22 ± 1 nmol/l, respectively; P < 0.05; Fig. 3) in P4 vs. both saline and E2 (P < 0.05; Fig. 3).

Fig. 1. Plasma glucose and insulin levels during day 2 exposure to hyperinsulinemic hypoglycemia after antecedent intracerebroventricular (ICV) infusion of either saline (SAL), estradiol (E2), or progesterone (P4).
Glucose kinetics. Specific activity, listed in Table 1, was stable and not statistically different between groups during baseline and the final 30 min of hyperinsulinemic hypoglycemia in all groups, with an average CV of 8 ± 1% for both periods. During the final 30 min of day 2 hypoglycemia, EGP in the E2 group was significantly less than saline (24 ± 5 vs. 36 ± 4 μmol·kg⁻¹·min⁻¹; P < 0.05; Fig. 4), but neither group differed from P4 (28 ± 4 μmol·kg⁻¹·min⁻¹). Glucose rate of disappearance during the final 30 min of hypoglycemia was similar among all groups (Fig. 4). The glucose infusion rate needed to maintain the glycemic level was significantly greater in E2 compared with saline (31 ± 4 vs. 19 ± 4 μmol·kg⁻¹·min⁻¹; P < 0.05; Fig. 4).

**DISCUSSION**

In the present study, we have used unrestrained, conscious male rats to determine the short-term effects of CNS delivery of E2 and P4 on hormonal and metabolic responses to next day hypoglycemia. Our results demonstrate that E2 in the CNS can rapidly induce blunting of epinephrine levels during next day hypoglycemia. These data are the first of which we are aware to demonstrate during clamped hypoglycemia in awake rats that E2 administered centrally can rapidly mediate differences in sympathoadrenal drive during hypoglycemia. We have additionally determined the short-term effects of lateral ventricle infusion of P4 in mediating differences in counterregulatory hormone levels during hypoglycemia. We found that, while E2 blunted epinephrine and as a result EGP, P4 enhanced both basal and end-of-clamp norepinephrine levels. On the other hand, P4 blunted basal and end-of-clamp plasma levels of corticosterone. Therefore, the present results provide novel data that E2 and P4 can have rapid differential effects on key mechanistic pathways regulating counterregulatory hormone levels during hypoglycemia. Additionally, these findings support the concept that estrogen may be considered as a factor responsible for causing a form of hypoglycemia associated autonomic failure.

Previous studies in humans have demonstrated that women have blunted catecholamine, growth hormone, cortisol, and glucagon responses to hypoglycemia compared with men (2, 12, 14). We have also shown that postmenopausal women taking E2 only replacement therapy have reduced epinephrine, glucagon, and muscle sympathetic nerve activity compared with age- and body mass index-matched women not taking E2 replacement therapy and with men (35). Adams et al. (1) have demonstrated that anesthetized 14-day-old ovariectomized female rats had elevated epinephrine responses to insulin bolus-induced hypoglycemia compared with nonovariectomized controls. Conversely, epinephrine responses to hypoglycemia were significantly reduced in ovariectomized rats with E2 replacement (1). In the present study, short-term forebrain E2 infusion in male rats reduced epinephrine but not glucagon levels, and forebrain P4 infusion blunted plasma corticosterone levels.
Thus our present data may explain why postmenopausal women taking only E2 replacement had preserved cortisol responses to hypoglycemia. Growth hormone was not measured because of a lack of increase during hypoglycemia in rats (unpublished observations). The lack of a signal to reduce glucagon in the present study vs. results in humans is interesting. One possibility is that the time course of the effects of E2 on plasma glucagon during hypoglycemia is different from that on sympathoadrenal responses. In other words, more prolonged exposure to E2 is required to downregulate glucagon responses to hypoglycemia. On the other hand, glucagon responses to hypoglycemia have been shown to be regulated by both changes in insulin levels within the pancreatic islets (28) and direct ANS regulation (18, 39) in rats and humans. Interestingly, E2 has also been shown to act directly on pancreatic alpha cells to reduce glucagon levels (31). Thus E2 may act centrally to blunt epinephrine but peripherally to blunt glucagon levels during hypoglycemia.

E2 and P4 had differential effects on sympathetic nervous system drive during hypoglycemia. Lateral ventricle E2 blunted plasma epinephrine levels (sympathoadrenal activation) but not plasma norepinephrine (sympathoadrenal and sympathetic neural activation), whereas lateral ventricle P4 increased norepinephrine levels but had no effect on epinephrine levels during hypoglycemia. The mechanism for these novel, divergent results with epinephrine and norepinephrine following the different steroid administration is unknown. It is well recognized that P4 and E2 can have opposite physiological effects. For instance, P4 administration has been reported to reverse E2-mediated increases in insulin sensitivity (29, 38). Chronic (3 wk) P4 plus E2 administration resulted in enhanced counterregulatory responses to hypoglycemia in dogs (3). While one study has shown that the source of norepinephrine in humans is primarily adrenomedullary (9), data from animal models suggest that central regulation of sympathetic output may be more complicated and organ specific (21). For example, increased levels of norepinephrine have been found within the brain during hypoglycemia, specifically in the paraventricular (11, 26) and ventromedial nuclei (10), and despite reduced systemic levels, these hypothalamic levels were not reduced after multiple daily episodes of hypoglycemia (11). These authors postulated that the mechanism for blunted sympathoadrenal responses with repeated hypoglycemia occurs downstream from the hypothalamus. With regard to our data, it is possible that E2 and P4 alter sympathetic outflow in different areas of the brain, leading to the divergent results. Thus the elevated basal levels of norepinephrine following P4 administration are likely to be caused by increased sympathetic neural outflow, whereas the elevated norepinephrine levels during hypoglycemia are likely to be caused by both increased sympathetic neural and sympathoadrenal activity.

The doses of E2 and P4 used in the present study are similar to those in previous reports (1, 3, 16). We do not know the volume of distribution of the E2 and P4 infused into the lateral ventricle of the brain. However, it is reasonable to assume that, because of cerebrospinal fluid circulation, areas of the forebrain, midbrain, and hindbrain could have been affected by the

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**Table 1. Glucose specific activity (dpm/mmol) at baseline and final 30 min during day 2 hyperinsulinemic hypoglycemia (2.8 ± 0.1 mmol) in conscious rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Time, min</th>
<th>100</th>
<th>110</th>
<th>120</th>
<th>210</th>
<th>225</th>
<th>240</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAL</td>
<td>650±183</td>
<td>649±178</td>
<td>631±165</td>
<td>1,047±126</td>
<td>1,057±72</td>
<td>1,026±89</td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>1,139±200</td>
<td>1,175±195</td>
<td>1,045±177</td>
<td>1,020±208</td>
<td>943±175</td>
<td>973±53</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>708±87</td>
<td>617±94</td>
<td>611±52</td>
<td>818±81</td>
<td>745±98</td>
<td>733±122</td>
<td></td>
</tr>
</tbody>
</table>

SAL, saline; E2, estradiol; P4, progesterone; dpm, disintegrations/min.
CNS ESTRADIOL AND HYPOGLYCEMIA

steroid action. These are all areas, specifically the paraventricular nucleus of the hypothalamus (13), the ventromedial hypothalamus (11, 17, 24), and the hindbrain (34), that have been implicated in regulating counterregulatory responses to hypoglycemia and contain E2 and P4 receptors. Central injections of E2 into hindbrain areas have been found to depress renal sympathetic drive and increase vagal nerve activity in male rats (30, 32). P4 receptors are also found in glucose-sensing areas within the hypothalamus and hindbrain regions and within the pituitary (18a). The decrease in corticosterone levels strongly suggests that P4 has effects on forebrain pathways to inhibit hypothalamic-pituitary-adrenal drive. Several studies have demonstrated the importance of hippocampal, thalamic, and hypothalamic regulation of hypothalamic-pituitary-adrenal axis responses to stress (26). However, the specific regions and receptors within the CNS that are responsible for the effects of E2 and P4 on counterregulatory responses to hypoglycemia remain unknown. Furthermore, we were unable to measure E2 and P4 levels in the plasma and cannot rule out potential peripheral actions of these sex steroids on counterregulatory hormones.

In summary, these data show that short-term (i.e., hours) central administration of female reproductive hormones in male rats can result in J) rapid, significant effects on levels of ANS and neuroendocrine counterregulatory hormones during subsequent hypoglycemia, 2) differential regulation of plasma catecholamine levels during hypoglycemia, and 3) significantly blunted metabolic (glucose production) counterregulatory responses during subsequent hypoglycemia. The effect of E2 is consistent with data in humans suggesting that E2 may be considered as a mechanism responsible for causing a partial subset of the condition known as hypoglycemia-associated autonomic failure (7).

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GRANTS

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