The Effects of Dehydroepiandrosterone Sulfate on Counterregulatory Responses During Repeated Hypoglycemia in Conscious Normal Rats

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We previously determined that both antecedent hypoglycemia and elevated cortisol levels blunt neuroendocrine and metabolic responses to subsequent hypoglycemia in conscious, unrestrained rats. The adrenal steroid dehydroepiandrosterone sulfate (DHEA-S) has been shown in several studies to oppose corticosteroid action. The purpose of this study was to determine if DHEA-S could preserve counterregulatory responses during repeated hypoglycemia. We studied 40 male Sprague-Dawley rats during a series of 2-day protocols. Day 1 consisted of two 2-h episodes of 1) hyperinsulinemic (30 pmol · kg⁻¹ · min⁻¹) euglycemia (6.2 ± 0.2 mmol/l; n = 12; ANTE EUG), 2) hyperinsulinemic euglycemia (6.0 ± 0.1 mmol/l; n = 8) plus simultaneous intravenous infusion of DHEA-S (30 mg/kg; ANTE EUG + DHEA-S), or 3) hyperinsulinemic hypoglycemia (2.8 ± 0.1 mmol/l; n = 12; ANTE HYPO), or 4) hyperinsulinemic hypoglycemia (2.8 ± 0.1 mmol/l; n = 8) with simultaneous intravenous infusion of DHEA-S (30 mg/kg; ANTE HYPO + DHEA-S). Day 2 consisted of a single 2-h hyperinsulinemic hypoglycemia (2.8 ± 0.1 mmol/l) clamp. During the final 30 min of day 2, hypoglycemia norepinephrine levels were significantly lower in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S group (2.0 ± 0.2 vs. 3.3 ± 0.6 mmol/l; P < 0.05). In addition, epinephrine (8 ± 1 vs. 17 ± 2, 14 ± 3, and 15 ± 3 mmol/l), glucagon (91 ± 8 vs. 273 ± 36, 231 ± 42, and 297 ± 48 ng/l), and corticosterone (1,255 ± 193 vs. 1,915 ± 212, 1,557 ± 112, and 1,668 ± 119 pmol/l) were significantly lower in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (P < 0.05). Endogenous glucoses production was also significantly less in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S, ANTE HYPO + DHEA-S groups (13 ± 5 vs. 32 ± 3, 38 ± 7, and 29 ± 8 mmol/l · kg⁻¹ · min⁻¹; P < 0.05). Consequently, the amount of exogenous glucose needed to maintain the glycemic level during the clamp studies was significantly higher in the ANTE HYPO versus the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (57 ± 8 vs. 22 ± 5, 18 ± 6, and 18 ± 3 mmol/l · kg⁻¹ · min⁻¹; P < 0.05). In summary, day-1 antecedent hypoglycemia blunted neuroendocrine and metabolic responses to next-day hypoglycemia. However, simultaneous DHEA-S infusion during antecedent hypoglycemia preserved neuroendocrine and metabolic counterregulatory responses during subsequent hypoglycemia in conscious rats. Diabetes 53:679–686, 2004

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The Diabetes Control and Complications Trial established that intensive glucose control in type 1 diabetic patients can slow the progression or significantly reduce the onset of diabetic microvascular complications (e.g., retinopathy, nephropathy, neuropathy) (1). Unfortunately, the study also established that intensive glucose treatment causes an approximate threefold increase in the frequency of severe hypoglycemia (2). This increased frequency of hypoglycemia is at least partially caused by deficient autonomic nervous system (ANS) counterregulatory responses to hypoglycemia (3–5). Repeated exposure to hypoglycemia reduces neuroendocrine, ANS, and metabolic (endogenous glucose production [EGP], lactate, and glyceral) counterregulatory responses to subsequent hypoglycemia by as much as 50% in nondiabetic and type 1 diabetic subjects (6–9). These blunted counterregulatory responses are significant contributing factors to a vicious cycle of hypoglycemia for type 1 diabetic patients (9).

The mechanisms responsible for the blunted counterregulatory responses seen after antecedent hypoglycemia are not fully understood. Controversy exists regarding the role played by corticosteroids in the pathophysiology of blunted ANS responses during hypoglycemia (10–14). Reports in rodents (12) and humans (15) have demonstrated that, similar to antecedent hypoglycemia, antecedent increases of cortisol can blunt neuroendocrine and metabolic counterregulatory responses to next day hypoglycemia. In addition, patients with adrenocortical failure have preserved catecholamine, pancreatic polypeptide, glucagon, growth hormone, and muscle sympathetic nerve activity responses to hypoglycemia after antecedent hypoglycemia (16). Other animal studies have found that corticosteroids can blunt catecholamine responses to a variety of stressors, including insulin-induced hypoglycemia in sheep (17) and immobilization stress in rats (18,19). However, in contrast to the above studies, three other reports have indicated that prior corticosterone administration has little or no effect on ANS responses to subsequent hypoglycemia in the conscious rat (10,13,14).
Dehydroepiandrosterone (DHEA) and its corresponding sulfate ester (dehydroepiandrosterone sulfate; DHEA-S) are steroid hormones secreted from the adrenal gland. After being orally or intravenously administered, the two hormones are interconverted at a high rate and have similar physiological effects (20,21). Little is known about their biological significance, but many studies have shown that they work contrary to corticosteroid actions. For example, in rats, a simultaneous infusion of DHEA-S counteracts the detrimental effects of corticosterone on neuronal function (22) and neurogenesis (23) in the dentate gyrus. In Zucker rats, simultaneous infusion of DHEA and dexamethasone prevents the dexamethasone-induced increase in hepatic tyrosine aminotransferase activity (24). In addition, intracerebroventricular infusion of DHEA has been shown to increase norepinephrine and epinephrine levels in the paraventricular nucleus and ventromedial hypothalamus in obese Zucker rats (25). Although corticosteroids have been found to increase gluconeogenesis (26), proteolysis (27), and net fat deposition in humans (28), DHEA has been found to elicit the opposite effect on these metabolic processes (29–32). Based on the wealth of existing evidence, we therefore hypothesized that if DHEA-S can antagonize corticosteroid actions, it may also prevent any corticosteroid-induced inhibition of neuroendocrine and metabolic responses to repeated hypoglycemia. Thus, the aim of this study was to determine if peripheral infusion of DHEA-S during antecedent hypoglycemia could preserve counterregulatory responses during a subsequent bout of hypoglycemia in conscious, unrestrained rats.

**RESEARCH DESIGN AND METHODS**

We studied 40 male Sprague-Dawley rats (300–350 g) bred and purchased from Harlan (Indianapolis, IN). The rats were housed and individually caged under a 12:12-h light-dark cycle, with room humidity and temperature at 50–60% and 25°C, respectively. All animals had access to water in the Vanderbilt University Animal Care Facility. All procedures for animal use were approved by the Institutional Animal Care and Use Committee at Vanderbilt University.

At 1 week before experiments, catheters were placed in the rats’ 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). Catheter lines were kept patent by flushing them with 150 unit/ml of heparin every 3 days. Rats had free access to rat diet on the days preceding surgery and the experiments. All rats used for the 2-day experiments maintained >90% of their presurgery body weight.

Four groups of rats were studied after an overnight fast during a 2-day experimental protocol, as outlined in Fig. 1. Rats were fasted overnight before each day of the 2-day study and remained conscious and unrestrained throughout the 2-day protocols. The morning of each study day, extensions were placed on the exteriorized catheters for ease of access and were removed between day-1 and -2 studies. At t = 0 min, rats were moved to an experimental cage and allowed to become acclimated to the surroundings.

**Day-1 procedures.** Day 1 consisted of two 2-h (120–240 and 360–480 min) hyperinsulinemic-euglycemic clamps (ANTE EUG; n = 12), hyperinsulinemic-hypoglycemic clamps (ANTE HYPO; n = 12), hyperinsulinemic-euglycemic clamps plus infusion of DHEA-S (30 mg/kg) (ANTE EUG + DHEA-S; n = 8) or hyperinsulinemic-hypoglycemic clamps plus DHEA-S infusion (ANTE HYPO + DHEA-S; n = 8). The DHEA-S was continuously infused into the jugular vein starting at t = 0 min throughout day-1 procedures. DHEA-S rather than DHEA was infused because of the former’s water solubility. DHEA-S (30 mg/kg) was dissolved in 1.5 ml of normal saline and infused at a rate of 3 μg/min. This dosage of DHEA-S was chosen because it has been previously shown to have anticytotoxic effects on the brain (22). At the conclusion of day-1 procedures, rats were fed 5–8 g of rat diet. To maintain hematocrit, after each blood draw the rats’ own erythrocytes plus normal saline were reinfused through the jugular cannula. If a rat’s hematocrit was <20% at the beginning of the day-2 studies, that rat was removed from the study. Plasma measurements of glucose were taken every 5 min during the clamp periods and at t = 240 and 480 min for insulin levels. Between morning and afternoon clamps, plasma glucose was measured every 15–30 min and glucose infusion was adjusted to maintain euglycemia at 6.1 mmol/l.

**Day-2 procedures.** At t = 0 min, rats were moved to an experimental cage and allowed to become acclimated to the surroundings. The study consisted of a basal period (t = 90–120 min) and an experimental period (t = 120–240 min), during which time a hyperinsulinemic-hypoglycemic clamp (described below) was performed. To measure glucose kinetics during the clamp, a primed (10 μCi), constant (0.2 μCi/min) infusion of [3-3H] glucose (PerkinElmer, Boston, MA) purified by high-performance liquid chromatogra-
Phy (HPLC) was administered via a precalibrated infusion pump (Harvard Apparatus, South Natick, MA) at \( t = 0 \) min and continued through \( t = 240 \) min. During the experimental period, blood was drawn every 5 min for measurement of plasma glucose, every 10 min during the basal period and every 15 min during the experimental period for \([3-3^\text{H}]\text{glucose}\), and at \( t = 90, 120, 180, 210, \) and \( 240 \) min for counterregulatory hormones. For clarity of presentation and because counterregulatory hormones were not significantly different between \( t = 210 \) and \( 240 \) min (indicating steady state), these time points were averaged in RESULTS. Rats were killed after the day-2 procedures and the placement of carotid and jugular cannulae was verified.

Glycemic clamping procedures. At \( t = 120–240 \) min (days 1 and 2) and \( 360–480 \) min (day 1 only), a primed (60 pmol \( \cdot \) kg \(^{-1} \) \cdot min \(^{-1} \)) continuous (30 pmol \( \cdot \) kg \(^{-1} \) \cdot 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). Plasma glucose was measured every 5 min. For the euglycemic clamp, a 50% dextrose infusion was adjusted to maintain glucose at \( 6.1 \) mmol/l. For the hypoglycemic clamp, glucose levels were allowed to fall and a 20% dextrose infusion was adjusted to maintain glucose at \( 2.9 \) mmol/l for 90 min.

Tracer calculations. The rate of glucose appearance (\( R_a \)), EGP, and glucose utilization was calculated according to the methods of Wall et al. (33). EGP was calculated by determining the total \( R_a \), which comprises both EGP and any exogenous glucose infused to maintain the desired hypoglycemia, and subtracting from it the amount of exogenous glucose infused. It is now recognized that this approach is not fully quantitative, as underestimates of total \( R_a \) and the rate of glucose disposal (\( R_d \)) can be obtained. Using a highly purified tracer and taking measurements under steady-state conditions (i.e., constant specific activity) in the presence of low glucose flux eliminates most, if not all, of the problems. In addition, to maintain a constant specific activity, isotope delivery was increased proportionally with increases in exogenous glucose infusion.

Analytical methods. Plasma glucose was measured in duplicate by the glucose oxidase technique on a Beckman glucose analyzer. Catecholamines were determined by HPLC (34) with an interassay coefficient of variation (CV) of 12% for both epinephrine and norepinephrine. We made two modifications to the procedure for catecholamine determination: 1) we used a five-point rather than a one-point standard calibration curve, and 2) we spiked the initial and final samples of plasma with known amounts of epinephrine and norepinephrine so that accurate identification of the relevant catecholamine peaks could be made. Corticosterone (ICN Biomedicals, Irvine, CA; interassay CV 7%), insulin interassay (CV 11%) (35), glucagon (Linco Research, St. Louis, MO; interassay CV 15%), and DHEA-S (Diagnostic Systems, Webster, TX; interassay CV 5%) were all measured using radioimmunnoassay techniques.

Statistical analysis. Data are expressed as means \( \pm \) SE and were analyzed using standard, parametric, one-way ANOVA with repeated measures. A Tukey’s post hoc analysis was used to delineate statistical significance. \( P \leq 0.05 \) was accepted as statistically significant.

RESULTS

Glucose and insulin. Plasma glucose levels were similar during morning and afternoon day-1 euglycemic clamps in
the ANTE EUG and ANTE EUG + DHEA-S groups (6.2 ± 0.2 and 6.1 ± 0.2 mmol/l) and during hypoglycemic clamps in the ANTE HYPO and ANTE HYPO + DHEA-S groups (2.8 ± 0.1 and 2.8 ± 0.1 mmol/l). Glucose levels were also similar during day-2 hypoglycemic clamps in all groups (2.8 ± 0.1 mmol/l) (Fig. 2). In addition, day-1 and -2 insulin levels were similar in all four groups of rats (day 1: 726 ± 38 pmol/l; day 2: 750 ± 28 pmol/l for final 30 min).

**Counterregulatory hormones.** The increase in plasma norepinephrine during the final 30 min of day-2 hypoglycemia was significantly lower in the ANTE HYPO versus the ANTE HYPO + DHEA-S group (2.0 ± 0.2 vs. 3.3 ± 0.6 nmol/l; $F = 7.31; P < 0.05$) (Fig. 3). Also, the increase in epinephrine levels during the final 30 min of day-2 hypoglycemia was significantly lower in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (8 ± 1 vs. 17 ± 2, 14 ± 3, and 15 ± 3 nmol/l; $F = 8.0; P < 0.0001$) (Fig. 3). Similarly, glucagon and corticosterone responses during the final 30 min of day-2 hypoglycemia were significantly lower in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (91 ± 8 vs. 273 ± 36, 231 ± 42, and 297 ± 78 ng/l; and 1,255 ± 93 vs. 1,915 ± 212, 1,557 ± 112, and 1,668 ± 119 nmol/l, respectively; $F = 4.37; P < 0.01$) (Fig. 4).

**Glucose kinetics.** Specific activity was stable during both the basal and the final 30-min periods for all groups, with a CV of <5 ± 1% (Table 1). EGP was significantly lower in the ANTE HYPO group versus the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (13 ± 5 vs. 32 ± 3, 38 ± 7, and 29 ± 8 μmol/l · kg$^{-1}$ · min$^{-1}$; $F = 7.3; P < 0.001$) (Fig. 5). The $R_g$ was similar among groups and did not change during hypoglycemia (Fig. 5). As a consequence of the lower EGP, the exogenous glucose infusion rate needed to maintain the glycemic level during the clamp was significantly greater in the ANTE HYPO group compared with the ANTE EUG, ANTE EUG + DHEA-S, and ANTE HYPO + DHEA-S groups (57 ± 8 vs. 18 ± 3, 18 ± 6, and 22 ± 5 μmol/l · kg$^{-1}$ · min$^{-1}$; $F = 13.43; P < 0.0001$) (Fig. 5).

**DISCUSSION**

In the current study, we compared the counterregulatory responses of four groups of rats during 2 h of hyperinsulminic hypoglycemia on day 2 after different interventions on day 1. The day-1 interventions included antecedent euglycemia, antecedent euglycemia plus DHEA-S infusion, antecedent hypoglycemia, and antecedent hypoglycemia plus DHEA-S infusion. Similar to previous findings from human studies, we found in these conscious, unrestrained rats that two 2-h episodes of hypoglycemia blunted neuroendocrine and metabolic responses to a subsequent episode of hypoglycemia occurring 1 day later. Peripheral infusion of DHEA-S during hypoglycemia on day 1, however, prevented counterregulatory failure and preserved...
catecholamine, glucagon, corticosterone, and EGP responses to next day hypoglycemia.

In this study, DHEA-S rather than DHEA was infused because the former is water soluble. Using DHEA-S avoids the potentially confounding variable of using a substance (e.g., alcohol or DMSO) that has independent effects on the central nervous system as a vehicle for administering the DHEA-S. Although the structures of DHEA-S and DHEA differ slightly, studies have shown similar physiological effects of DHEA-S and DHEA (20,21) and in humans the hormones are interconverted at a high rate.

The two euglycemic control groups, ANTE EUG and ANTE HYPO, produced similar neuroendocrine and metabolic counterregulatory responses during day-2 hypoglycemia. This indicated that DHEA-S did not have any direct action on modulating counterregulatory responses during day-2 hypoglycemia. However, DHEA-S infused during day-1 hypoglycemia preserved counterregulatory function during day-2 hypoglycemia. The mechanism for this effect is unknown, as a specific DHEA-S receptor has not been identified. It is interesting that many studies have shown that DHEA and its sulfated ester can antagonize corticosteroid actions (22,24,25,36). The role of glucocorticoids in blunting physiological responses to stress are somewhat controversial, with a number of studies both supporting (11,17,19,37) and contradicting (14) this finding (10,13). Several studies, in addition to our own, have shown that corticosteroids can reduce ANS

data-2 hypoglycemia. This indicated that DHEA-S did not

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<th>Group</th>
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<tr>
<td></td>
<td>100</td>
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<tr>
<td>ANTE EUG</td>
<td>334 ± 61</td>
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<tr>
<td>ANTE HYPO + DHEA-S</td>
<td>397 ± 47</td>
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<tr>
<td>ANTE HYPO</td>
<td>503 ± 40</td>
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<tr>
<td>ANTE EUG + DHEA-S</td>
<td>403 ± 53</td>
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Data are means ± SD. Glucose specific activity given as dpm/mmol. Hyperinsulinemic hypoglycemia is defined as 2.8 ± 0.1 mmol/l.
EFFECTS OF DEHYDROEPIANDROSTERONE DURING HYPOGLYCEMIA

![Graphs showing glucose production and disappearance rates during hypoglycemia with and without DHEA-S infusion.](image)

**FIG. 5.** EGP, $R_d$, and $R_a$ during the final 30 min of day-2 hyperinsulinemic hypoglycemia after ANTE EUG, ANTE HYPO, ANTE HYPO + DHEA-S, or ANTE EUG + DHEA-S in conscious, unrestrained rats. $*P < 0.05$ vs. ANTE HYPO + DHEA-S, ANTE EUG + DHEA-S, and ANTE EUG.

responses to differing forms of stress in a variety of species, such as rats (37), sheep (17), and dogs (38). However, DHEA-S does not antagonize corticosteroid actions by competitively binding to corticosteroid receptors, at least in hepatocytes (39). In addition, chronically administered DHEA has been shown to prevent stress-induced increases in glucocorticoid receptor number (40), which minimizes the biological effects of the elevated corticosteroids. Brain N-methyl-D-aspartate (NMDA) and $\gamma$-aminobutyric acid (GABA) receptors are known to have excitatory and inhibitory effects on neuronal norepinephrine release, and thus ANS function, respectively. Although DHEA-S has shown to have stimulatory effects on NMDA activity (21) and receptor number (41) and inhibitory effects on GABA receptor activity (42), corticosterone has been found to increase the affinity of GABA receptors (43). Therefore, DHEA-S could be stimulatory to the ANS (stimulatory to NMDA and inhibitory to GABA receptors), whereas cortisol may be inhibitory (i.e., may increase activity of GABA receptors). Future studies should be aimed at determining the mechanism for the protective effect of DHEA-S against repeated hypoglycemia in rats. A number of the effects observed in the present study with antecedent DHEA-S infusion appear to have been mediated via the ANS. Stimulation of the ANS increases plasma catecholamines, plasma glucagon, and, subsequently, EGP. ANS activation also results in reduced glucose uptake during hypoglycemia. Alterations in glucose flux are critical end points in the defense against hypoglycemia. During acute periods of hypoglycemia, increases in EGP provide the primary defense (44). However, during more prolonged hypoglycemia, both reduced glucose uptake and increased EGP become important (44). It is therefore relevant that during the present study DHEA-S infusion prevented the blunting of EGP during subsequent hypoglycemia. Regulation of glucagon responses during hypoglycemia is complex, with data demonstrating that direct $\alpha$-cell sensing (45), prevailing insulinemia (46), epinephrine, and the ANS (47) may all modulate levels of the hormone during stress. The finding that DHEA-S infusion preserved glucagon responses during our studies may also indicate an action of the compound on preventing ANS dysfunction during repeated episodes of hypoglycemia. The site of a DHEA-S action that protects the ANS cannot be determined from our study. However, DHEA-S readily crosses the blood-brain barrier and peripheral infusions have been found to affect various sites in the brain (20,23,41). We would therefore speculate that DHEA-S may influence ANS responses via direct brain sensing, but future studies will be needed to determine whether this is in fact the case.

DHEA-S also preserved the response of corticosterone during repeated hypoglycemia, thereby suggesting additional non–ANS-mediated effects. Although DHEA-S may antagonize corticosteroid actions, some (48) but not all (49,50) studies have found that DHEA-S can also decrease corticosteroid levels. If this is correct, it suggests that DHEA-S may have the ability to antagonize multiple levels of glucocorticoid negative feedback loops (i.e., on both the ANS and the hypothalamic-pituitary-adrenal axis).

It is interesting that rats do not secrete DHEA-S. This renders the rat a good animal model for testing the hypothesis that DHEA-S could preserve counterregulatory responses during repeated hypoglycemia because any effects of DHEA-S can be isolated. The fact that DHEA-S was infused before and during hypoglycemia on day 1 may be relevant. However, the goal of the present study was to determine if DHEA-S infused during hypoglycemia on day 1 could prevent hypoglycemia-associated autonomic failure 1 day later. The levels of DHEA-S that we infused peripherally reached 0.14 $\mu$mol/l, which is $\sim$5 times the basal levels found in humans. These levels are also similar with those seen in other studies that demonstrated an anticoncorticosteroid affect on the brain in rats (23) and with levels found with 100 mg of oral DHEA replacement in elderly (51) and young adult subjects (52). Thus, the finding that DHEA-S preserves counterregulatory responses during repeated hypoglycemia in conscious, unrestrained rats could have implications for future human studies.

In conclusion, the present study demonstrated that in the conscious rat, simultaneous DHEA-S administration...
during antecedent hypoglycemia preserves neuroendocrine (norepinephrine, epinephrine, glucagon, and corticosterone) and metabolic (EGP) responses to next day hypoglycemia. This preservation of counterregulatory function appears to involve the antagonism of mechanisms responsible for causing blunted counterregulatory responses during repeated hypoglycemia rather than the hormone’s directly stimulating neuroendocrine responses per se.

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