Effects of prenatal ethanol exposure and sex on the arginine vasopressin response to hemorrhage in the rat

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Submitted 19 October 2005; accepted in final form 4 February 2006

Bird, Danielle N., Aileen K. Sato, Daniel S. Knee, Catherine F. T. Uyehara, Donald A. Person, and John R. Claybaugh. Effects of prenatal ethanol exposure and sex on the arginine vasopressin response to hemorrhage in the rat. Am J Physiol Regul Integr Comp Physiol 291: R77–R82, 2006. First published February 9, 2006; doi:10.1152/ajpregu.00740.2005.—AVP synthesis, storage, and osmotically stimulated release are reduced in young adult rats exposed prenatally to ethanol (PE). Whether the reduced release of AVP to the osmotic stimulus is due to impairment of the vasopressin system or specifically to an osmoreceptor-mediated release is not known. The present experiments were done, therefore, to determine whether a hemorrhage-induced AVP response would also be diminished in PE-exposed rats. Pregnant rats were fed either a control liquid diet [no prenatal ethanol (NPE)] or a liquid diet with 35% of the calories from ethanol from days 7–21 of pregnancy. Offspring were weaned at 3 wk of life. At 11 wk of age, femoral arterial catheters were surgically placed, and blood volumes were determined at 12 wk. Three days later, two hemorrhages of 10% of the blood volume were performed with samples taken before and 10 min after the hemorrhages. After a 20% blood loss, plasma AVP was 19% higher in NPE rats than in the PE rats despite no differences in mean arterial blood pressure (MABP). Also, hypothalamic AVP mRNA and pituitary AVP content were reduced in PE rats. Furthermore, confirming an earlier report of sex differences in AVP release, the hemorrhage-induced hormone response was twofold greater in female rats than male rats, regardless of previous ethanol exposure. These studies demonstrate that the AVP response to hemorrhage is reduced in PE rats independently of differences in MABP. The data are compatible with a theory of a reduced number of hemorrhage-responsive vasopressinergic neurons capable of stimulated AVP release in PE rats.

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fetal alcohol syndrome; vasopressin mRNA; neurohypophysis; hypothalamus; blood pressure

FETAL ALCOHOL SYNDROME (FAS) is a condition characterized by facial abnormalities, growth restriction, and evidence of neurodevelopment disorders. According to the Centers for Disease Control and Prevention, this condition affects 0.2 to 1.5 infants per 1,000 live births. Less severe consequences of prenatal ethanol, such as alcohol-related neurodevelopmental disorder and alcohol-related birth defects are believed to occur approximately three times as often as FAS (15).

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Animals. The animals were housed at 22°C with lights on from 0600 to 1800 in a separate facility from the laboratory. For surgery or when used in experiments, they were transported in the morning approximately 1/2 mile to the laboratory before 0800.

Breeding and Diet Procedures

Sprague-Dawley rats were acquired from a single vendor (Taconic, Germantown, NY). Vaginal smears were performed on 11 female rats to precisely time their pregnancies. Females were paired with males until the presence of sperm was detected in the daily vaginal smear. This day was counted as day 0 of pregnancy. On day 7 of pregnancy the females were changed to a liquid diet. The experimental females (6 total) were fed a liquid diet in which 35% of the calories were derived from ethanol (Bio-Serv, Frenchtown, NJ). The control females (5 total) were fed a similar liquid diet in which the calories were provided by carbohydrates instead of ethanol. The liquid diet was continued until day 21 of pregnancy, at which time the females were returned to regular rat chow. The pups remained with their biological mothers until day 21 of life, at which time the offspring were weaned and fed regular rat chow throughout the remainder of the experiment. The offspring of the ethanol-fed experimental dams were referred to as PE rats and those of the dams fed the control diet were designated as nonprenatal ethanol (NPE) rats.

Surgical Procedure

A catheter was surgically placed in the left femoral artery of all offspring during days 74–86 of life (28). Five percent isoflurane anesthesia was administered through an animal anesthetic gas delivery system (Surgivet/Anesco, Waukesha, WI). Briefly, the femoral artery was exposed through an incision on the medial thigh and catheterized with flexible Tygon tubing (OD 0.03 in.). The catheter was tied in place, and the distal end was tunneled under the skin along the animal’s left side and exited through an incision just anterior to the scapulae on the dorsal surface. The catheter was filled with a solution of 500 U/ml heparin in 25% dextrose, to prevent clotting and maintain patency, and sealed with a metal pin until the animal was used in experiments. The rats received ibuprofen dissolved in a cube of Jell-O (7.2 mg in 2 ml), and in their drinking water (120 mg in 200 ml) for 48 h postoperatively for analgesia.

Experimental Procedure

During the week before surgery and after surgery, the rats were placed in plastic experimental chambers (Braintree Scientific, Braintree, MA) for increasing times up to 90 min to allow them to become familiar with the confinement. On the third and final day of this conditioning of the rats, blood volume was measured using the Evans Blue dye dilution technique, as previously described (20), but modified to require removal of only 1 ml of blood (18).

Hemorrhage experiments were then performed 3 days later as previously described (4). The rats were placed in the plastic chambers, and the arterial catheter was accessed for determination of mean arterial blood pressure (MABP) and heart rate (HR) and for blood removal for the hemorrhage and samples. The catheter was connected to a MacLab/8 recording instrument (ADInstruments, Castle Hill, Australia) for MABP and HR recordings. After 60 min of acclimation, baseline MABP and HR were obtained, and blood sample 1 was drawn, representing a 10% hemorrhage. Ten minutes later, MABP and HR were again recorded followed by sample 2, achieving a total 20% hemorrhage. A third sample was drawn 10 min later for purposes of measuring the effects of the total 20% hemorrhage, and the rats were then euthanized within 3 min of the last sample with an overdose of pentobarbital. All blood volume determinations and hemorrhage experiments were performed between the hours of 0830 and 1130.

Hematocrit was determined from each blood sample by centrifugation of microcapillary tubes. The remainder of the specimen was placed in a 3-ml Vacutainer containing sodium heparin (BD Vacutainer, Franklin Lakes, NJ), and the plasma was separated after centrifugation. To 400 μl of plasma, 40 μl of 1 N HCl were added, and the aliquot was frozen for later determination of plasma AVP concentration. Approximately 100 μl of plasma was apportioned for determination of plasma osmolality by freezing point depression (Advanced Micro-Osmometer, 3M01, Norwood, MA). The remainder (minimum 250 μl) was placed in a third microcentrifuge tube and frozen for later determination of plasma corticosterone concentration.

Tissue Harvesting and Assays

After euthanasia, the brain was removed, and the posterior lobe of the pituitary was dissected away from the rest of the gland and frozen at −70°C for later analysis of pituitary AVP content. The brain was then dissected into a block containing the supraoptic, paraventricular, and suprachiasmatic nuclei, and the block was placed in a microcentrifuge tube containing RNAlater (Ambion, Austin, TX). This tube was also stored at −70°C for later analysis of AVP mRNA. Plasma AVP concentration and pituitary AVP content were determined by radioimmunoassay procedures and hypothalamic AVP mRNA quantification by real time PCR (iCycler, Bio-Rad Laboratories, Hercules, CA), as previously described (12). The data are expressed as a ratio of relative copies of AVP mRNA to β-actin mRNA.

Blood-Alcohol Determinations

In three control female rats used to produce NPE offspring, after delivery of their litters and weaning of the pups, a femoral arterial catheter was surgically placed as described in Surgical Procedure. The animals were placed on the liquid diet containing ethanol 3 days after surgery. The diet was the same as that given to the pregnant rats receiving ethanol in their diets, as described Breeding and Diet Procedures. The diets were refreshed daily at 0900. The ethanol diet began at 0900 on day 1, and blood samples were taken on the day of surgery and at 0900 on day 2, and at 0900, 1700, and 2100 on day 3 and 0900 on day 4. Blood samples were analyzed for ethanol using an alcohol analyzer (Analog Instruments AM1, London).

Statistical Methods

The data were analyzed by a two-way ANOVA with sex and type of prenatal exposure, that is, PE exposed or NPE exposed, as the two factors. In addition, MABP, HR, and plasma concentrations of AVP were analyzed by a three-way ANOVA with repeated measures over the added factor of time to assess the response to hemorrhage. ANOVA procedures were followed by post hoc analysis with a least significant difference Student’s t-test to determine differences between individual means. The level of significance was set at P < 0.05. All tests were performed using the JMP statistical software (SAS Institute, Cary, NC). Values are given as means ± SE.

RESULTS

NPE dams consumed an average 305 ± 7 kCal·kg−1·day−1 and PE dams consumed 292 ± 13 kCal·kg−1·day−1 (P = 0.44). All animals displayed the same lengths of gestation with parturition occurring on day 22. Despite the similarity in caloric intake, NPE dams gained an average of 112 ± 7 g from day 7 of pregnancy to day 21, whereas PE dams gained 62 ± 8 g (P < 0.002). The average litter sizes of the PE litters were slightly smaller (P = 0.018) with average NPE litter size 13.6 ± 0.8 and PE litters of 10.5 ± 0.7. The average weight per pup on day 7 of life among the five litters comprising the NPE group was 12.9 ± 0.4 g per pup, whereas the average weight per pup among the six litters of the PE group was 13.8 ± 0.6 g per pup (P = 0.24). Using these average values, ~31 g of the discrepant weight gain between the two groups during preg-
nancy may be explained mainly by the difference in litter size. The alcohol diet did not affect the sex distribution among the litters; mean of 56.8 ± 0.01 and 49.2 ± 0.9% females in the NPE and PE litters respectively (P = 0.476) The rats were weighed again at the time of the experiment, and there were no weight differences between the NPE and PE groups, but weights were different when comparing between males and females. (Table 1).

In both groups, MABP was maintained after the 10% hemorrhage but was significantly decreased 10 min after the 20% hemorrhage (Table 2). Relative to baseline values, HR was elevated after the 10% hemorrhage (P < 0.05, all groups combined) and decreased after the 20% hemorrhage (P < 0.05). After the 20% hemorrhage, the PE rats had higher heart rates than the NPE animals (Table 2). Although the hematocrit (Hct) at each sample time showed no significant difference between the two groups, the Hct decreased significantly for both groups after both the 10% and 20% hemorrhage sample. Across the three samples in each group, plasma osmolality did not change significantly, nor was there a significant difference between the NPE and PE groups, but the combined responses of both groups demonstrated that female rats had a lower plasma osmolality than male rats over all periods (P < 0.001) and specifically at the 20% hemorrhage sample period as shown in Table 2.

Basal levels of plasma AVP were similar in both groups and between sexes (Fig. 1). After the first 10% hemorrhage, AVP was significantly elevated in the female NPE rats, but the other groups did not change significantly. After the 20% hemorrhage, both groups and both sexes had elevated plasma AVP levels. The AVP response to hemorrhage was significantly greater in female rats than males regardless of group. The AVP response to hemorrhage as a function of group is less clear than that of sex. When the overall response is considered, including both sexes, the NPE animals revealed a significantly greater AVP response to hemorrhage than the PE animals. However, the female rats had a similar AVP response regardless of prenatal exposure to ethanol, and only the male rats had a significantly impaired AVP response to the 20% hemorrhage in the PE animals. To more thoroughly analyze the potential sex difference in the response to prenatal ethanol exposure, the 0% and 10% hemorrhage data were analyzed without the 20% data to remove the overall increase in experimental variability brought about by the larger responses and greater variability about the means following the second hemorrhage. When this was performed, both male and female NPE rats were found to respond with significant increases in AVP, and both were significantly greater than the corresponding PE groups. There was no detectable effect of sex at the 10% hemorrhage with this analysis.

Although male rats in the NPE group tended to have MABP over the course of the entire experiment, both groups of animals, and both sexes maintained MABP 10 min after the 10% hemorrhage, and all decreased a similar degree after the 20% hemorrhage (Table 2).

Pituitary AVP content was reduced in the PE group compared with the NPE group, and the male glands had higher contents of the hormone with the magnitude of these group and sex differences remaining constant, and therefore no interaction was noted (Table 3). Finally, the hypothalamic AVP mRNA levels were assayed as an index of AVP synthesis. The AVP mRNA in the PE group was ~30% lower than the NPE group (Table 3).

The blood alcohol levels measured in the three nonpregnant animals, consuming similar volumes of liquid diet, were variable, ranging from nondetectable to 112 mg%. All values obtained during the daytime in the present experiments were undetectable.

**DISCUSSION**

The aims of this study were twofold. First, we wished to determine whether prenatal exposure to ethanol reduced the AVP response to an acute hemorrhage stimulus in the adult rat. Second, we intended to determine whether there was a sex-based difference in the AVP response to hemorrhage in the PE rats. The latter may be expected because others have shown that the AVP response to hemorrhage, in the experimental model we have used, is greater in the female than in male Sprague-Dawley rats (4).

Our results reveal a blunted AVP release in response to hemorrhage in prenatal ethanol-exposed animals. Although the impairment appeared to occur only in the male rats after the 20% hemorrhage, we feel it is premature to suggest that the impairment does not occur in female animals. In support of this possibility, a separate statistical analysis in which the 20% values were eliminated, revealed that the nonhypotensive hemorrhage of 10% caused increased AVP levels in both male and female NPE rats, but not in the PE rats. This resulted in significantly greater responses in either sex in the NPE group compared with the PE group. Taken together, the data suggest an impaired AVP response to hemorrhage of both sexes of rats when prenatally exposed to ethanol.

The release of AVP when stimulated by hemorrhage has been classically viewed as a response to reduced stretch of the aortic arch and carotid baroreceptors and the cardiac atrial receptors (24). Although the present study did not include an assessment of atrial stretch, the high pressure receptors would appear to be similarly affected by the hemorrhage since MABP was reduced similarly in both groups. We observed a 5% greater blood volume in the PE rats compared with NPE animals. This slightly expanded blood volume could blunt the effect of hemorrhage via atrial receptors, but since the hemorrhage was a similar percentage of the blood volume in both

### Table 1. Effects of prenatal ethanol exposure and sex on body weight in the rat

<table>
<thead>
<tr>
<th>NPE(M)</th>
<th>PE(M)</th>
<th>NPE(F)</th>
<th>PE(F)</th>
<th>Group</th>
<th>Sex</th>
<th>Group × Sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td>n = 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight, g</td>
<td>234 ± 3</td>
<td>378 ± 7</td>
<td>223 ± 3</td>
<td>374 ± 11</td>
<td>0.364</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. NPE, nonprenatally exposed to ethanol; PE, prenatally exposed to ethanol; F, female; M, male; Group, main effects of NPE vs. PE on two-way ANOVA; Sex, main effects of sex; and Group × Sex, statistical interaction.
groups, the effect would seem to be negligible. Noteworthy is the better maintained heart rate in the PE group after the 20% hemorrhage. This may reflect a lesser degree of sympathetic withdrawal that is known to accompany this moderate, hypertensive, level of hemorrhage (23). The relatively greater heart rate would be expected to produce a better maintained firing rate of afferent nerves from atrial receptors, and this mechanism, therefore, may have contributed to inhibition of the release of vasopressin. Because the osmolality was not different between the two groups, it is unlikely to be a factor in different AVP responses to hemorrhage seen in the present experiments.

Despite some possibilities of baroreceptor-mediated contribution to the impaired AVP response to hemorrhage in the PE rats of the present experiments, consequences of central neurological damage are likely. The present experiments confirm earlier observations of a reduced hypothalamic synthesis of AVP (12, 13). In the experiments by Lee et al. (13), the determination of reduced AVP synthesis was made by in situ hybridization and quantification of optical density of the paraventricular nucleus. In the present experiments and those of Knee et al. (12), the entire hypothalamic content of AVP mRNA was assessed and was ~30% lower in PE animals compared with NPE animals regardless of sex. It is probable, therefore, that the number of cells producing AVP is reduced. This could lead to fewer axonal projections to the posterior pituitary, accounting for the reduced pituitary stores of the hormones, as observed in the present and previous study (12). The reduced osmoreceptor (12) and baroreceptor-mediated AVP release observed in PE-exposed rats is consistent with a reduced number of AVP-producing cells that can be depolarized by appropriate stimuli.

A prominent sex difference was observed in the AVP response to hemorrhage in both NPE and PE rats. This observation has been previously reported by Crofton and Share (4) who originally described the hemorrhage sequence, used in the present studies, based on individual animal blood volume assessments. They found a greater AVP response to hemorrhage in female rats during the proestrus, diestrus, and metestrus, but not during estrus compared with males. These investigators were unable to repeat their observations in subsequent experiments (25), but, as the authors indicate, the sampling times were different between the two studies. Also, in their second study, a constant 8.2 ml/kg of blood was removed for each estimated 10% hemorrhage regardless of sex. We subsequently observed, in a larger study, that the blood volume of female Sprague-Dawley rats is ~14% larger than males (18). Removal of a constant blood volume per kg body wt, therefore, would tend to produce a larger relative hemorrhage in male rats, and thus cancel out the sex-based differences that we observed in these studies, as well as those that they had observed in their initial study (4). Unfortunately, the phase of the estrus cycle was not determined in the current experiments.
but despite the inclusion of all estrus cycle phases, the current results support earlier observations.

The sex difference was not evident in plasma AVP responses after the nonhypotensive 10% hemorrhage despite a fourfold increase in AVP levels. Somewhat comparable experiments in human subjects have shown that a nonhypotensive head-up tilt in human subjects, producing a small increase in AVP levels of approximately twofold, produced a greater AVP response in men than in women of similar ages between 23 and 50 yr (7). The nonhypotensive models of head-up tilt and 10% hemorrhage are different, however, because men, being taller than women, may have a more pronounced fluid shift due to a larger hydrostatic gradient, and consequently, greater stretch receptor unloading. Thus a proportionately larger fluid shift in men could have overshadowed any sex-related difference that may have existed with equal stretch receptor activation of the AVP response.

It is well established that prenatal ethanol exposure enhances the activity of the hypothalamic-pituitary-adrenal in response to stress (6, 8, 10, 11, 13). Furthermore, physiological levels of glucocorticoids can suppress the AVP response to a hypotensive stimulus in the dog (19). Because hypotensive stress stimulates glucocorticoid production (e.g., 19), it is reasonable to postulate that the hemorrhage-induced glucocorticoid production would be more elevated in rats prenatally exposed to ethanol and may have contributed to the reduction in AVP release to hemorrhage in the PE rats of the present experiments. Attempts were made to measure corticosterone in the present experiments, but the results were confounded by an inability to attain true basal levels of the hormone before hemorrhage. Although similar levels of corticosterone were observed in NPE and PE rats following hemorrhage (data not shown), we could not rule out possible involvement of enhanced hypothalamic-pituitary axis activity on the reduced AVP response to hemorrhage in the PE rats.

Blood alcohol levels were determined in three nonpregnant animals during alcohol administration for 4 days, indicating elevated blood alcohol levels only at night when food intake was greatest. The absolute values obtained in nonpregnant rats may, however, be higher than those of pregnant animals. More comprehensive studies by others (3, 9) have shown that pregnant rats have an increased clearance of ethanol compared with nonpregnant rats. Badger et al. (3) demonstrated that the increased clearance was particularly evident when the alcohol is administered intragastrically as opposed to intravenously. Their experimental objectives required bolus administrations of ethanol that may be different than the response to ad libitum consumption of the alcohol. Nevertheless, the blood-alcohol levels achieved by the feeding regimen used in the present experiments, were similar to reports of others using similar feeding regimens in pregnant Sprague-Dawley rats (5, 26), although peak levels may not have been obtained in our small number of observations. All values obtained during the daytime in the present experiments were undetectable, which is similar to previous reports by others (26). Thus the alcohol exposure would appear to be uneven, and, characteristically, relatively low during most of the day with a peak occurring after feeding.

In summary, this study confirms earlier studies suggesting that PE results in a defect in the vasopressin system evident in the young adult animal. PE impairs not only the osmotically stimulated release, as previously reported, but also the hypovolemic stimulated release of the hormone. The blunted AVP release seems to be independent of differences in high-pressure baroreceptor or osmoreceptor activity. The reduced AVP release is likely due to central neuronal damage and probably is not confined to the vasopressinergic neurons involved in release of the hormone from the posterior pituitary, but also due to AVP production by cells in the suprachiasmatic nucleus (22) and possibly throughout the central nervous system. Also this study confirms an earlier study by others demonstrating a sex-based difference in the vasopressin response to hemorrhage (4).

Perspectives

The vasopressin system, specifically that part of the system with neuronal axons terminating in the posterior pituitary, is impaired in rats exposed prenatally to ethanol. The current work and previous work indicate that a condition of central diabetes insipidus (DI) is created by prenatal ethanol exposure in the rat. It is important to determine whether a similar condition exists in humans and to determine whether the process can be reversed. Nephrogenic DI has been reported in humans with FAS (2), but concurrent central DI was not ruled out. Markers of DI may be helpful in the identification of more subtle manifestations of prenatal ethanol exposure in humans.

ACKNOWLEDGMENTS

The authors are grateful for the expert veterinary support of Major R. J. Probst and the animal care and technical support provided by Staff Sergeant David Walters and Robin Grain.

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The views expressed in this manuscript are those of the authors and do not reflect the official policy or position of the Department of the Army, Department of Defense, the U.S. Government, the National Center for Research Resources, or the National Institutes of Health.

GRANTS

This work was supported in part by grants from the Hawaii Community Foundation Grant 20030617 and from a Research Centers in Minority Institutions award P20 RR11091 from the National Center for Research Resources, National Institutes of Health.
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