Prolonged Prenatal Hypernatremia Alters Neuroendocrine and Electrolyte Homeostasis in Neonatal Sheep[1]

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Arginine vasopressin (AVP) is a neuroendocrine hormone synthesized in the hypothalamus, and is stored and secreted by the posterior pituitary gland in response to stimuli such as plasma hypertonicity and hypotension. The primary physiologic roles of AVP include plasma osmolality and blood pressure regulation. We have previously demonstrated that chronic prenatal plasma hypertonicity alters the AVP regulatory pathway in newborn lambs. The objectives of the present study were to evaluate prolonged effects of antenatal plasma hypertonicity on neonatal plasma osmoregulation. Pregnant ewes at 119 ± 3 days of gestation were water restricted to achieve and maintain hypertonicity until normal-term delivery. After delivery, ewes were provided food and water *ad libitum* and lambs were allowed maternal nursing. At the age of 28 days, blood samples were obtained for the analysis of plasma osmolality, electrolytes, and AVP levels from study ($n = 5$) and age-matched control ($n = 6$) lambs. Subsequently, lambs were euthanized, and the pituitary and hypothalamus were processed for the determination of pituitary AVP content by radioimmunoassay, and AVP gene expression by Northern analysis. In response to water restriction, maternal plasma osmolality significantly increased (306 ± 1.1 to 326 ± 1.2 mOsm/kg, $P < 0.001$). At the age of 28 days, plasma sodium level was higher in study (prenatally dehydrated) than control lambs (144.6 ± 0.4 vs 142.6 ± 0.3, $P < 0.05$). Study lambs had higher plasma AVP concentrations than the control lambs (4.1 ± 0.4 vs 1.7 ± 0.4 pg/ml, $P < 0.05$). Similarly, total pituitary AVP content was higher in the *in utero* hypertonic lambs than in the control lambs (6.5 ± 1.0 vs 2.8 ±1.2 μg, $P < 0.05$). However, there was no difference in hypothalamic AVP mRNA levels between the two groups. The present study demonstrates that chronic maternal and fetal plasma hypertonicity has prolonged effects on pituitary and plasma AVP, as well as plasma sodium in neonatal lambs, providing further evidence suggesting prenatal imprinting of osmoregulation through at least 1 month of age. Exp Biol Med 228:41–45, 2003

Key words: vasopressin; hypothalamus; osmoregulation; prenatal imprinting; neuroendocrine

Arginine vasopressin (AVP) is a neuroendocrine hormone synthesized in the hypothalamus, and is stored and secreted by the posterior pituitary in response to stimuli such as plasma hypertonicity and hypotension (1). The primary physiologic roles of AVP include plasma osmolality and blood pressure regulation. In addition to water and electrolyte dysregulation, altered AVP regulation may predispose to cardiovascular diseases (2, 3). AVP secretion is stimulated in response to increased plasma osmolality or decreased systemic blood pressure (4). Hypothalamic AVP synthesis is regulated at the level of gene expression. In adult mammals, plasma hyperosmolality significantly increases hypothalamic AVP mRNA (5). Conversely, plasma hypoosmolality decreases hypothalamic AVP mRNA levels (6, 7).

Several studies suggest that prenatal exposure to plasma osmolality alterations may permanently alter (imprint) osmoregulatory pathways. Dehydration of pregnant rats increased the salt appetite (8, 9) and blood pressure (10, 11) of adult offspring. In humans, prenatal and/or neonatal exposure to conditions resulting in plasma hyperosmolality enhances the salt preference of offspring at later life (12–14). We recently reported that after exposure to prolonged *in utero* plasma hypertonicity, neonatal lambs had higher pituitary AVP content and lower hypothalamic AVP gene expression compared with control newborns (15).These data are contrary to the results from the studies of neonatal and adult rats. In response to chronic plasma hypertonicity,

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adult rat pituitary AVP decreased and hypothalamic AVP mRNA increased (16). Similarly, studies of neonatal rats suggest a reduction in pituitary AVP content in response to prolonged postnatal dehydration (17). The different effects on AVP synthesis and secretion of prenatal versus postnatal hypertonicity exposure suggest prenatal imprinting of the AVP osmoregulatory pathway.

To further investigate potential prenatal imprinting of osmoregulation, and to explore the mechanism of imprinting, the present study was designed to examine the effect of prolonged maternal plasma hypertonicity on plasma composition and AVP gene expression in 1-month-old lambs.

Material and Methods

Animals. Western mixed-breed sheep were obtained from a local source (Nebeker Ranch, Palmdale, CA). Animals were housed indoors in individual steel study cages and were acclimated to a 12:12-hr light:dark cycle. Food (alfalfa pellets) was provided *ad libitum* and water was provided as described below. Surgical procedures and experimental studies were approved by the Harbor-UCLA Animal Care and Use Committee. All animal studies used time-dated pregnant ewes with singleton fetuses. Following spontaneous vaginal delivery, neonatal lambs were subsequently studied.

Prenatal Dehydration. The animal model for the preparation of chronic prenatal dehydration has been previously described (15). Briefly, at 119 ± 1 days of gestation (term = 150 days), ewes (n = 5) with singleton pregnancy were surgically prepared with femoral vein catheters. Maternal blood samples were drawn daily to monitor plasma tonicity and electrolytes. After establishing the baseline plasma osmolality, water was removed from the ewes for 2 days, followed by water restriction of approximately 1 liter daily throughout the remainder of pregnancy. The water intake was titrated to achieve and maintain the plasma osmolality at 6%–10% above baseline level. Control ewes (n = 6) were not prepared with vascular catheters and were allowed *ad libitum* food and water intake throughout the pregnancy. In both study and control groups, ewes were allowed to deliver naturally. After giving birth, ewes were provided *ad libitum* food and water intake.

Newborn lambs were allowed to nurse naturally after birth. At the age of 28 days, under sedation by intramuscular ketamine injection to avoid stress, blood samples were drawn from the study and age-matched control lambs to measure plasma AVP, osmolality electrolytes, and hematocrit, after which lambs were euthanized by an intravenous injection of pentobarbital. The brain was quickly removed and the pituitary and hypothalamus were immediately dissected, frozen in liquid nitrogen, and stored at –70°C.

Northern Analysis of AVP Gene Expression. Total cellular RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. AVP and β-actin cRNA probes labeled with P^{32} were prepared by *in vitro* transcription using a T7 Maxiscript in Vitro Transcription kit (Ambion, Austin, TX). Samples of total RNA (15 μg) were denatured and size fractionated on a 1.2% agarose gel containing 18% formaldehyde in 1× MOPS-EDTA-sodium acetate buffer. After electrophoresis, RNA was capillary transferred to a 0.45-μm nylon membrane in 10× sodium citrate and sodium chloride buffer (SSC) overnight. The membrane was dried in a vacuum oven at 80°C for 1 hr. Hybridization of membrane with AVP cRNA probe was carried out in UltraHyb hybridization solution (Ambion) at 42°C overnight. Membrane was washed in solution containing 1× SSC and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 min twice, followed by washing in 0.1× SSC and 0.1% SDS at 65°C for 15 min twice. Hybridization signals were captured and analyzed with Multi-Imager and QuantityOne Software (Bio-Rad, Hercules, CA). The membrane was stripped of AVP probe by washing in 0.5% SDS at 95°C for 15 min and rehybridizing with ^{32}P-labeled β-actin cRNA probe. Hypothalamic AVP mRNA levels were expressed as AVP:β-actin ratios. All samples were analyzed on one gel and thus one membrane.

Plasma and Pituitary AVP Content. Plasma and total pituitary AVP content was determined by radioimmunoassay (18). The entire pituitary gland was homogenized in 4% acetic acid at 4°C, and a series of dilution of the homogenate was used for the AVP radioimmunoassay.

Plasma osmolality was measured by freezing point depression on an Advanced Digimatic osmometer (model 3 MO; Advanced Instruments, Needham Heights, MA). Plasma sodium, potassium, and chloride concentrations were determined by a NOVA 5 electrolyte analyzer (Nova Biomedical, Waltham, MA).

Statistical Analysis. Data are presented as the mean ± SEM. Comparison of hypothalamic AVP gene expression, pituitary AVP content, plasma osmolality, and electrolytes between the study and control groups was performed by unpaired t test.

Results

Maternal Dehydration. Similar to our previous study (15), water deprivation for 2 days resulted in significant increase of maternal plasma osmolality (306 ± 1.1 to 326 ± 1.2 mOsm/kg; $P < 0.001$) and plasma sodium (146 ± 1 to 154 ± 1 mEq/L; $P < 0.001$) and chloride concentrations (108 ± 1 to 117 ± 1 mEq/L; $P < 0.001$). With water intake restriction adjusted to approximately 1 liter per day, plasma osmolality and sodium levels of the ewes were maintained at significantly elevated levels throughout the remaining gestation (Fig. 1). There were no significant changes in maternal plasma K^+ (4.3 ± 0.1 mEq/L) or hematocrit (28.8% ± 1.2%) in response to water restriction.

Newborn Studies. Lambs were born naturally. There was no difference in pregnancy length between control and study animals (147 ± 1 vs 146 ± 1 days). After 1 month of *ad libitum* maternal nursing, lambs of prenatally dehydrated ewes had significant higher plasma sodium con-

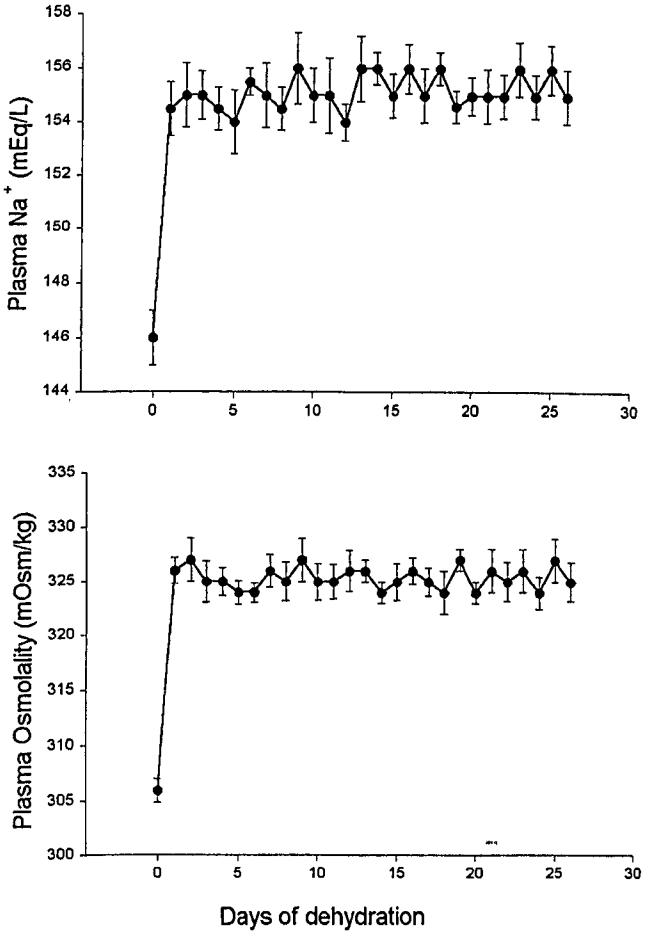

Figure 1. Effect of water restriction on maternal plasma Na^+ concentration (top) and osmolality (bottom). Maternal blood was withdrawn for monitoring plasma electrolytes and osmolality daily during the period of water restriction of approximately 1 liter per day. 0 day on the *x* axis represents baseline. Following water restriction, both maternal plasma sodium and osmolality increased significantly ($P < 0.001$). The maternal and fetal hypernatremia were maintained throughout the last 20% of pregnancy.

centrations, but lower chloride levels than the age matched controls (Table I). There were no significant difference in plasma osmolality, potassium concentration, or hematocrit between study and control groups.

At the time of sacrifice (1 month of age), lambs of prenatally dehydrated ewes had higher plasma AVP levels than the control lambs (4.1 ± 0.4 vs 1.7 ± 0.4 pg/ml, $P < 0.05$; Table I). Similarly, total pituitary AVP content was higher in the *in utero* dehydrated lambs than in the control lambs (6.5 ± 1.0 vs 2.8 ± 1.2 μg, $P < 0.05$; Table I).

Northern blot analysis of hypothalamic AVP gene expression is shown in Figure 2. AVP gene expression level in prenatal dehydrated lambs (i.e., AVP:β-actin ratio) was similar to the control lambs (0.34 ± 0.03 vs 0.33 ± 0.03).

Discussion

To date, there have been limited studies investigating the hypothesis of prenatal imprinting of osmoregulation. The majority of published studies related to this hypothesis

Table I. Comparisons of Pituitary AVP, Plasma AVP, Plasma Electrolytes, Osmolality, Hematocrit, and Body Weight of Prenatal Dehydrated and Control Lambs

Parameter	Prenatal dehydrated ($n = 5$)	Control ($n = 6$)
Pituitary AVP (μg)	6.5 ± 1.0[a]	2.8 ± 1.2
Plasma AVP (pg/ml)	4.1 ± 0.4[a]	1.7 ± 0.4
Na^+ (mEq/l)	144.6 ± 0.4[a]	142.6 ± 0.3
K^+ (mEq/l)	5.2 ± 0.3	5.9 ± 0.4
Cl^- (mEq/l)	106.8 ± 0.6[a]	108.6 ± 0.3
Osmolality (mOsm/kg)	299.6 ± 1.0	301.1 ± 1.2
Hematocrit (%)	32.5 ± 0.8	32 ± 0.9
Body weight (kg)	10.3 ± 0.8	10.8 ± 0.9

Note. Values are means ± SEM. Data were analyzed by Student's unpaired *t* test.
[a] Indicates significant differences between prenatally dehydrated and control lambs ($P < 0.05$).

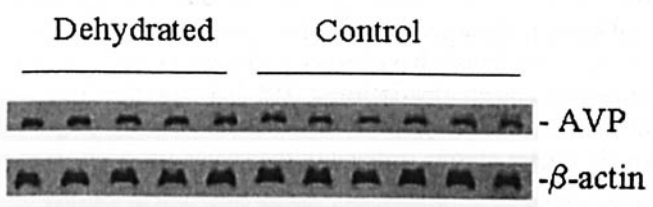

Figure 2. Comparison of hypothalamic AVP gene expression between the prenatally dehydrated and the control lambs. Figure shows the Northern blotting results of AVP and β-actin total RNA samples isolated from prenatally dehydrated ($n = 5$) and control ($n = 6$) lambs. There is no statistical difference in AVP gene expression level between the two groups (AVP/β-actin ratio of 0.34 ± 0.03 vs 0.33 ± 0.03).

have centered on the effect of maternal salt loading or salt depletion on offspring salt appetite. In humans, studies suggest that conditions that might result in maternal dehydration such as morning sickness may be associated with high salt preference of offspring (13, 19, 20). On the other hand, animal studies investigating effect of maternal osmotic alterations on salt appetite provided more contradictory results. Maternal salt loading either increased (9) or decreased (21, 22) salt preference of adult offspring. Similarly, maternal salt deprivation increased salt preference of adult offspring in one study (23), but not in another (24). Interestingly, Mouw *et al.* (25) found that prenatal and early postnatal sodium deprivation had no effect on salt preference or plasma osmolality and electrolytes, but resulted in higher fluid intake of adult offspring. This suggests that the elevated fluid intake was a result of higher urinary water excretion, although these results were not investigated by the authors. Despite the varying results, these studies suggest the potential for prenatal imprinting of salt preference and osmoregulation.

Our previous study demonstrated that prolonged prenatal dehydration results in the alteration of hypothalamic AVP gene expression regulation in the immediate newborn lamb (15). Despite the presence of plasma hypernatremia, prolonged fetal plasma hypertonicity increased newborn pi-

tuitary AVP content, yet decreased hypothalamic AVP gene expression. This response in newborn sheep to chronic plasma hypertonicity is different from adult animals. In adult rat, chronic hypertonicity decreases pituitary AVP content yet increases hypothalamic AVP mRNA level (26). The remarkable differences in response to chronic plasma hyperosmolality during the prenatal versus adult period is consistent to our hypothesis of imprinting of AVP osmoregulatory pathway in prenatal period. The current study was designed to directly evaluate whether prolonged prenatal plasma hyperosmolality has long-term effect on offspring osmoregulation at 1 month of age. The demonstration of such an effect would provide direct and objective evidence of prenatal imprinting of osmoregulation.

The selection of gestational age of 120 days to initiate maternal dehydration is based upon our previous studies of the ovine fetal osmoregulatory pathway, which indicated that the last 20% of ovine gestation is a critical period of endocrine maturation. For example, near-term (142 day gestation) ovine fetuses have higher AVP secretion in response to plasma epinephrine infusion than 131 day (preterm) fetuses (27). Similarly, preterm ovine fetuses have significantly higher plasma osmolality thresholds for AVP secretion (28), fetal swallowing (29), and activation of central osmotic brain nuclei (30). Therefore, the preterm period (~130 days gestation age) represents a critical period of maturation of osmoregulation and therefore is potentially subject to imprinting.

To avoid excessive surgical intervention and potential fetal compromise, we did not catheterize the fetus and thus did not measure fetal blood values during the prenatal dehydration period. However, maternal plasma data were monitored daily and are known to closely reflect fetal plasma values (31, 32). Our previous studies have demonstrated that fetal plasma osmolality and electrolytes equilibrate rapidly to near maternal values (31, 32). Thus, the percentage of change in maternal plasma osmolality and electrolytes reflect those of the fetus. Our primary objective was to evaluate effect of chronic prenatal hypernatremia on baseline plasma osmolality, electrolytes, and neuroendocrine vasopressin after a 1-month period of normalization. We elected not to perform any physiological study of the neonatal lambs to eliminate potential stress-induced effects on AVP synthesis and secretion (33, 34).

Despite the 1-month period of normal nursing, the lambs of prenatally dehydrated ewes had higher plasma sodium levels, yet lower plasma chloride concentration than age-matched controls. More importantly, prenatally hypertonic lambs had higher pituitary and plasma AVP levels than the control lambs. These observations demonstrate that chronic prenatal dehydration has prolonged effect on osmoregulation long after birth. The elevated plasma sodium level in prenatally dehydrated lambs suggests a higher set point for sodium homeostasis. However, the reduction in plasma chloride and the lack of difference in plasma osmolality may indicate an alteration in plasma electrolyte regulation. Further studies of daily plasma composition, nursing, food and water intake, and urine output are necessary to confirm and explain the mechanism of the altered plasma electrolytes.

Physiologically, the elevated basal AVP levels would be expected to induce renal antidiuresis and reduce plasma sodium and osmolality. However, despite the higher baseline level of plasma AVP, the prenatally dehydrated lambs had higher plasma sodium levels and normal plasma osmolality. We hypothesize that the higher plasma sodium level in the presence of elevated plasma AVP is due to reduced renal AVP receptor quantity or receptor responsiveness, a result of the imprinting of prenatal hypertonicity. This hypothesis, based on the present results, is consistent with the previously published studies of adult rat. In adult rats, dehydration reduces renal AVP receptor concentration and binding efficiency (35–37), providing an mechanism of renal escape from vasopressin-induced antidiuresis.

Prenatally dehydrated lambs demonstrated increased pituitary AVP content, although there was no difference in hypothalamic AVP gene expression between the two groups. The higher pituitary content may be a result of alteration(s) of AVP posttranscription, such as stability of AVP mRNA or a higher level of protein translation. Alternatively, these results may indicate an increased plasma sodium set point for AVP secretion. Despite the increased pituitary AVP content, there was no difference in hypothalamic AVP gene expression, again suggesting an increased set point for pituitary AVP-mediated feedback regulation of hypothalamic gene expression.

Although these studies show a demonstrable difference in plasma composition under basal conditions, the mechanism for such alteration needs to be empirically determined. Further studies of prenatally dehydrated sheep responses to osmotic (e.g., osmotic threshold for pituitary AVP release, activation of dipsogenic centers, and renal antidiuretic responses) or plasma volume challenges will provide insight into mechanism of osmotic imprinting. Should any alteration in physiologic responses be demonstrable, the imprinting of sodium and osmolar homeostasis would be particular concern in human disease states.

In summary, the current study provides further evidence of prenatal imprinting of osmoregulation in sheep, as prenatal dehydration resulted in alterations of offspring plasma electrolytes and AVP, and pituitary AVP levels. Mechanisms of altered AVP/osmoregulation are potentially due to alteration of renal concentrating capability, resetting of the osmotic set point for hypothalamic AVP synthesis and pituitary AVP secretion, and/or resetting of the central dipsogenic threshold. Although longer term studies are needed to evaluate whether prenatal osmoregulatory imprinting is permanent, the present results indicate osmoregulatory imprinting through 1 month of age.

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