Intact osmoregulatory centers in the preterm ovine fetus: Fos induction after an osmotic challenge

A. CASTON-BALDERRAMA,1 M. J. M. NJILAND,2 T. J. MCDONALD,2 AND M. G. ROSS1
1Perinatal Research Laboratory, Department of Obstetrics and Gynecology, Harbor-University of California Los Angeles Medical Center, Torrance, California 90502; and 2Laboratory for Pregnancy and Newborn Research, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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CASTON-BALDERRAMA, A., M. J. M. NJILAND, T. J. MCDONALD, AND M. G. ROSS. Intact osmoregulatory centers in the preterm ovine fetus: Fos induction after an osmotic challenge. Am J Physiol Heart Circ Physiol 281: H2626–H2635, 2001.—We previously demonstrated a functional systemic dipsogenic response in the near-term fetal sheep (128–130 days; 145 days = full-term) with swallowing activity stimulated in response to central and systemic hypertonic saline. Preterm fetal sheep (110–115 days) do not consistently demonstrate swallowing in response to hypertonic stimuli, and it is unclear whether this is due to immaturity of osmoreceptor mechanisms or neuronal pathways activating swallowing motor neurons. To determine whether osmoreceptive regions in the preterm fetus are activated by changes in plasma tonicity, we examined Fos expression with immunostaining in these neurons in response to an osmotic challenge. Nine preterm fetal sheep [five hypertonic saline-treated fetuses (Hyp) and four isotonic saline-treated fetuses (Iso)] were prepared with vascular and intraperitoneal catheters. Seventy-five minutes before tissue collection, hypertonic (1.5 M) or isotonic saline was infused (12 ml/kg) via an intraperitoneal catheter to fetuses. Brains were examined for patterns of neuronal activation (demonstrated by Fos protein expression). Hyp demonstrated increases in plasma osmolality (~10 mosmol/kg H2O) and Na concentrations (5 meq/l). Increased Fos expression was detected in Hyp in the organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), median preoptic nucleus (MnPO), supraoptic (SON), and paraventricular nuclei (PVN) compared with Iso animals. Neuronal activation within the OVLT, SFO, and MnPO indicates intact osmoregulatory mechanisms, whereas activation of the SON and PVN suggests intact fetal neural pathways to arginine vasopressin neurons. These results suggest that preterm fetal swallowing insensitivity to osmotic stimuli may be due to immaturity of integrated motor neuron pathways.

immunocytochemistry; circumventricular organs; thirst; swallowing

FUNDAMENTAL BIOLOGICAL SYSTEMS regulating body water volume and tonicity homeostasis include thirst-mediated drinking behavior in conjunction with arginine vasopressin (AVP)-mediated antidiuresis. Systemically, there are two principal regulatory mechanisms for the maintenance of body water homeostasis in the adult mammal. Small increases in extracellular fluid osmolality (~2%) stimulate AVP secretion (and urinary antidiuresis) and thirst. Somewhat larger decreases (~10%) in circulating volume, sensed by arterial and low-pressure baroreceptors, initiate AVP secretion and thirst via stimulation of renin release and direct activation of central neural thirst systems (9, 49). Physiological and neuroanatomic studies in several adult species indicate that both osmotic and ANG II dipsogenic effects are mediated via sites within the brain circumventricular organs (CVOs); i.e., organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO); small midline organs positioned on the surface of the cerebral ventricles; and interfacing subarachnoid, ventricular, and vascular spaces. CVOs, which have fenestrated capillaries, are in an unique position in the central nervous system to function as receptor sites for central neural signals, cerebrospinal fluid signals, and transduction of blood-borne signals to neural signals. Putative systemic dipsogens (hypertonicity, ANG II) initiate responses via CVO osmoreceptors or ANG II receptors (9). In sheep and dogs, hypertonicity-induced drinking behavior is mediated via the OVLT, the adjacent midline regions of anteroventral third ventricle (26, 44, 50), and perhaps the SFO (27). Although the SFO is not essential for angiotensin-mediated ovine dipsogenesis (27), direct injections of ANG II to the rat SFO elicits drinking (42). Neural efferent projections from both the OVLT and SFO evoke thirst and AVP release, and it is thought that many of these efferent projections synapse in the medullary organ of Cantor (MnPO) lying inside the blood-brain barrier (21). Extensive neural connectivity of the CVOs and MnPO suggests that this is a highly active local circuit involved in processing information from multiple neural inputs. Furthermore, anatomic evidence indicates that the supraoptic (SON) and both the parvicellular and magnocellular components of the paraventricular nucleus (PVN) receive afferents from each of the sensory CVOs as well as from the neuroendocrine modulators within the blood-
brain barrier (i.e., MnPO, parabrachial nucleus, and the nucleus of the solitary tract) with extensive projections and interconnections.

We (36, 37) previously demonstrated a functional systemic dipsogenic response in the near-term ovine fetus (129–133 day gestation; full-term = 145–150 days), with swallowing activity stimulated in response to intravenous or intracarotid hypertonic saline infusion. Despite a functional response, the near-term fetus exhibits distinctly different dipsogenic responses from that of the adult. Whereas a 2 to 3% increase in plasma osmolality stimulates adult water intake (6), only a brief (5 min) stimulation of fetal swallowing activity occurs in response to acute and chronic increases in plasma osmolality above this threshold level. Similarly, in response to maternal water deprivation, chronic increases in fetal plasma osmolality (3–4% above basal) do not stimulate fetal swallowing activity (34). Furthermore, the threshold for fetal osmotic dipsogenic stimulation was recently calculated to be markedly greater than the 2 to 3% increase (30). These studies indicate that the ovine fetus, in contrast to the adult, exhibits a relatively high threshold for swallowing stimulation requiring greater increases in osmolality. We (17) recently explored the thresholds for AVP secretion and fetal swallowing stimulation in the preterm ovine fetus. In 60% of the preterm fetuses, no swallowing threshold was observed, i.e., no intracarotid dose through 2.55 M hypertonic saline elicited swallows. Spontaneous swallowing activity was noted during the control period in all preterm animals confirming that the swallowing mechanism was functional. These studies suggest that the development of osmotic dipsogenic mechanisms occurs between 100 and 130 days gestation and may develop acutely rather than by a gradual increase in sensitivity.

Immunocytochemical localization of immediate early gene products, such as Fos, provides a powerful tool with which to demonstrate activation of neuronal populations in response to a wide variety of stimuli. Numerous studies in adult animals, e.g., rodent, sheep, vole, chicken, and nonhuman primate, have established the utility of using immunocytochemical staining to anatomically identify populations of activated neurons at the cellular level (12, 15, 16, 41) with fewer studies having been conducted in fetal or neonatal brain tissue (2, 24, 50). We, and others, have demonstrated that for adults and near-term fetuses, Fos expression increases within distinct subsets of hypothalamic neurons along the lamina terminalis in response to systemic hypertonicity (2, 32) or chronic hypertonic saline loading (40). In the adult rat, systemic hypertonicity results in a concentration-related increase in Fos expression in the OVLT, SFO, MnPO, and the SON and PVN nuclei (32). Moreover, systemic infusion of dipsogenic ANG II has been shown to increase Fos expression in neurons throughout the lamina terminalis as well as neurosecretory sites in the hypothalamus (31, 48). These findings indicate that the CVOs, MnPO, and AVP-containing hypothalamic neurosecretory neurons are activated directly or indirectly by systemic hypertonicity, and are involved in osmoregulation.

Despite the importance of in utero fetal dipsogenic and AVP secretory maturation, there is little information about the sequence of events during neurogenic development that leads to a functional fetal osmotic response. Many mechanisms are involved in the formation and maintenance of synaptic connectivity of the central nervous system, yet it remains unclear how this intricate pattern of neuronal connections is established during development. The last third of ovine gestation is likely a critical period in the maturation of dipsogenic mechanisms. Specifically, during the ovine gestational period of 110–120 days, a marked maturation of several fetal endocrine systems occurs. In the present study, we examined the involvement of central osmoregulatory mechanisms in response to an osmotic stimulus in the preterm ovine fetus (110 days gestation) to determine whether preterm osmotic insensitivity is due to immaturity of osmoreceptors. We examined neuronal activation, as demonstrated by Fos protein expression of the immediate early gene c-fos within select hypothalamic CVOs and neurosecretory regions in premature fetal sheep after an intraperitoneal hypertonic saline infusion.

MATERIALS AND METHODS

Animals. Nine Western mixed-breed pregnant ewes and their preterm fetuses [hypertonic saline-treated fetuses (Hyp), n = 5; isotonic saline-treated fetuses (Iso), n = 4; 110 days of gestational age = 0.76 to 0.79 of gestation] were used in this study. Animals were housed indoors in individual steel study cages and were acclimated to a 12:12-h light-dark cycle. Water was available ad libitum, and food (alfalfa pellets) was provided on a twice-daily schedule, except during the 24-h period immediately preceding surgery. These experiments were conducted within National Institutes of Health guidelines for animal research and were approved by the Harbor-UCLA Animal Care Committee.

Catheterization. For the surgical procedures, atropine sulfate (0.05 mg/kg im) was administered before induction of anesthesia with ketamine hydrochloride (20 mg/kg im). After endotracheal intubation, anesthesia was maintained with isoflurane (1–2%) and oxygen (1–2 l/min) through a mask. Polyethylene catheters (inner diameter 1.0 mm, outer diameter 1.8 mm) were placed in the fetal dorsal hindlimb vein and artery and threaded to the inferior vena cava and abdominal aorta, respectively. The maternal femoral vein and artery were similarly catheterized (inner diameter 1.3 mm, outer diameter 2.3 mm) at the time the fetal catheters were implanted. Fetal sheep were fitted with indwelling intraperitoneal catheters (7-Fr). All fetal and maternal incisions were closed in layers and catheters were externally accessed via the ewes’ flank incisions.

Immediately preoperatively and twice daily during the initial 2 days of recovery, gentamicin (8 mg/kg) and oxacillin (33 mg/kg) were administered to the fetus, and gentamicin (72 mg/kg), oxacillin (1 g/kg), and chloramphenicol (1 g/kg) were administered to the ewe. Methods for maintenance of catheters and protocols employed for the sampling of blood have been detailed previously (37). All animals were allowed a minimum of 5 days for postoperative recovery before initiation of experiments.
Fetal sheep were studied with their unanesthetized maternal subsets of hypothalamic neurons involved in osmoregulation. Employed to determine the induction of Fos expression in neural hypertonic infusion delivered to the preterm fetus was persists for 2–5 h (40). Thus animals were killed term fetal sheep, in general, the production of Fos protein determine the expression of Fos protein after a robust intra-
influence on fetal neuronal activation.

Anesthesia of the dam to minimize any potential confounding until effluent ran clear. Care was taken to begin fixation of perfused (700 ml) (13). The perfusate bottle was placed 1.0 in potassium phosphate-buffered saline (KPBS), pH 6.8 was vasodilator. After this, acrolein (2.5% vol/vol; 00016; Poly-
as an anticoagulant and sodium nitrite (2.0% wt/vol) as a

15-gauge indwelling catheter pointed toward the brain. The fetal head and neck were oxygen (1–2 l/min). A midline abdominal incision was made of the infusion, the dams were rapidly anesthetized with ketamine hydrochloride (20 mg/kg iv) and then ventilated

In potassium, sodium (Na), chloride (Cl), and plasma osmolality, sodium (Na), potassium (K), chloride (Cl), AVP, and ANG II concentrations.

Perfusion procedures. Although a time-course study to determine the expression of Fos protein after a robust intra-
erotonic osmotic stimulus has not been conducted in pre-
term fetal sheep, in general, the production of Fos protein reaches a maximum within 60–90 min after stimulation and persists for 2–5 h (40). Thus animals were killed 60 min after the osmotic stimulus to provide sufficient time for Fos protein expression. Seventy-five minutes after the initiation of the infusion, the dams were rapidly anesthetized with ketamine hydrochloride (20 mg/kg iv) and then ventilated through a mask with a mixture of isofluorane (2–5%) and oxygen (1–2 l/min). A midline abdominal incision was made to gain access to the uterus. The fetal head and neck were exposed, and one fetal carotid artery was catheterized with a 15-gauge indwelling catheter pointed toward the brain. The nonsurgically catheterized carotid artery was occluded, and both jugular veins were cut (14). The fetal head was perfused with 700 ml of normal saline containing heparin (10 IU/ml) as an anticoagulant and sodium nitrite (2.0% wt/vol) as a vasodilator. After this, acrolein (2.5% vol/vol; 00016; Polysciences; Warrington, PA) and paraformaldehyde (4% wt/vol) in potassium phosphate-buffered saline (KPBS), pH 6.8 was perfused (700 ml) (13). The perfusate bottle was placed 1.0 meter above the brain with the perfusion catheter wide open until effluent ran clear. Care was taken to begin fixation of brain tissue within 30 min of the initial administration of anesthesia of the dam to minimize any potential confounding influence on fetal neuronal activation.

Tissue processing. After perfusion, the brains were removed, blocked rostral to the optic chiasm and caudal to the mammillary bodies, and dehydrated in 25% sucrose solution for 4–6 days. With the use of a cryostat microtome, coronal sections (12 μm) were thaw-mounted on slides and stored at −20°C until staining. Serial sections were analyzed for (1) mean number of Fos-positive cells within specific hypotha-
lamic nuclei, and (2) percentage of activated Fos-containing cells double-stained for Fos protein in the selected nuclei. Mounted tissue was stained for Fos and AVP using modified methods previously reported (3, 4, 12, 15, 25, 33). Briefly, mounted tissue sections were treated with sodium borohy-
dride (1% in KPBS) to remove any traces of the aldehydes from the sections. After rinsing, the tissue was incubated in primary anti-Fos antisera (Antiserum Ab-5, concentration 1:40,000; Oncogene Research Products; Cambridge, MA) di-
luted in 0.4% Triton X-100 in KPBS for 24 h at 4°C. After rinsing, slides were incubated for 1 h at room temperature in biotinylated IgG (anti-rabbit IgG, Vector Laboratories; Burlingame, CA) at a concentration of 1:600 in KPBS with 0.4% Triton X-100, rinsed and incubated for 1 h in avidin-biotin complex solution (Elite ABC Kit model PK6100, Vector Lab-

Experimental protocol: Osmotic challenge. The intraperito-
nidal hypertonic infusion delivered to the preterm fetus was employed to determine the induction of Fos expression in subsets of hypothalamic neurons involved in osmoregulation. Fetal sheep were studied with their unanesthetized maternal ewes. Experiments were undertaken only if the fetal arterial blood pH value was ~7.3. On the day of study, either hypertonic (1.5 M) or isotonic (0.15 M) saline solution, 12 ml/kg of predicted fetal body wt, was infused (1.8 ml/min-10 min) through the fetal intraperitoneal catheter. We projected that this dosage of hypertonic saline would induce a rise in plasma osmolality of ~10 mosmol/kg H2O, correlating to a 2–3% increase in extracellular fluid osmolality. At minute 15, the increase in fetal plasma osmolality was similar to that observed in the near-term fetus at 6 ml/kg or adult at 18 ml/kg dose of hypertonic saline and had been shown to stimulate near-term fetal swallowing (2, 37).

Serial blood samples were withdrawn throughout the study from the fetuses for measurement of blood gases, elec-
trolytes, and hormones with an equivalent volume of hepa-
rinized maternal blood (withdrawn before the study) re-
placed after each sample. Maternal ewe arterial blood was

Immunohistochemical analyses. To evaluate the patterns of neuronal activation in response to the intraperitoneal hypertonic saline in the preterm fetal sheep, we examined the expression of the immediate early gene product, c-fos, in areas of the hypothalamus that are known to respond to changes in body fluid homeostasis, in particular, the OVLT, SFO, MnPO, SON, and PVN. A minimum of 2 to 3 sections per nucleus were analyzed in the fetal sheep. To analyze Fos induction, the number of Fos positive cells was counted within a grid (400 × 400 μm) placed over each section in a standardize
donated from helpdesk@ajpheart.org by /2020.25.0 April on 6, 2017
Table 1. Preterm fetal sheep arterial values before and after intraperitoneal hypertonic saline infusion

<table>
<thead>
<tr>
<th>Time, min</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmolality, mosmol/kg H2O</td>
<td>294.8 ± 2.0</td>
<td>304.4 ± 1.4</td>
<td>304.9 ± 1.1</td>
<td>305.5 ± 1.7</td>
<td>301.6 ± 1.8</td>
</tr>
<tr>
<td>AVP, pg/ml</td>
<td>2.8 ± 1.8</td>
<td>6.1 ± 1.6</td>
<td>5.1 ± 1.0</td>
<td>3.5 ± 0.6</td>
<td>3.5 ± 0.8</td>
</tr>
<tr>
<td>ANG II, pg/ml</td>
<td>19.9 ± 2.9</td>
<td>104.9 ± 52.4</td>
<td>43.7 ± 14.8</td>
<td>28.4 ± 5.0</td>
<td>25.2 ± 5.0</td>
</tr>
<tr>
<td>Hct, %</td>
<td>33.6 ± 2.1</td>
<td>31.5 ± 2.0</td>
<td>31.2 ± 1.6</td>
<td>30.7 ± 1.6</td>
<td>30.7 ± 1.3</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>7.8 ± 0.6</td>
<td>8.5 ± 0.5</td>
<td>8.7 ± 0.5</td>
<td>8.6 ± 0.4</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>pH</td>
<td>7.36 ± 0.01</td>
<td>7.34 ± 0.01</td>
<td>7.33 ± 0.01</td>
<td>7.33 ± 0.01</td>
<td>7.32 ± 0.01</td>
</tr>
<tr>
<td>Po2, mmHg</td>
<td>22.3 ± 1.2</td>
<td>23.6 ± 1.1</td>
<td>24.1 ± 1.4</td>
<td>24.2 ± 1.3</td>
<td>23.9 ± 1.1</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>48.1 ± 1.6</td>
<td>47.6 ± 0.8</td>
<td>47.6 ± 0.9</td>
<td>47.7 ± 0.7</td>
<td>46.7 ± 0.8</td>
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<td>Na, meq/l</td>
<td>137.7 ± 0.4</td>
<td>142.6 ± 0.6</td>
<td>143.8 ± 0.8</td>
<td>142.9 ± 0.7</td>
<td>141.8 ± 0.7</td>
</tr>
<tr>
<td>Cl, meq/l</td>
<td>105.2 ± 1.0</td>
<td>111.7 ± 0.8</td>
<td>113.5 ± 0.7</td>
<td>113.0 ± 0.9</td>
<td>112.4 ± 1.1</td>
</tr>
<tr>
<td>K, meq/l</td>
<td>3.8 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>3.5 ± 0.3</td>
<td>3.5 ± 0.2</td>
<td>3.5 ± 0.3</td>
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Values are means ± SE. AVP, arginine vasopressin; Hct, hematocrit; Hb, hemoglobin. *Significant difference from baseline; †significant difference from baseline rank.

Plasma samples for ANG II extraction were acidified with 1 N of HCl and extracted on C18 Sep-Pak columns (Waters; Milford, MA) as described by Cain et al. (1). Before use, columns were washed with methanol (10 ml), followed by distilled water (10 ml). Acidified plasma samples were added slowly to the columns, and the columns were washed with 10 ml of 0.1% trifluoroacetic acid (TFA). The absorbed peptide was eluted with 3 ml of 80% methanol and 0.1% TFA, and the eluates were dried in a Speed-Vac concentrator. Dried fractions were stored (−20°C) until they were dissolved in radioimmunoassay buffer immediately before assay.

The ANG II radioimmunoassay procedure has been previously described (35). The standard curve ranged from 1 to 200 pg/tube; sensitivity of the assay was 3 pg/tube. The intra- and interassay coefficients of variation were 7 and 9%, respectively.

Statistical analyses. Physiological measures were analyzed for differences over time using repeated-measures ANOVA (P < 0.05 considered significant). The number of Fos-positive cells counted per field were averaged to obtain a single independent number for each structure from each fetal sheep. Data are presented as means ± SE. Averages for each nucleus were compared among treatment groups using ANOVA.

RESULTS

Physiological parameters. Arterial plasma osmolality, electrolytes, AVP, ANG II, and blood Hct, Hb, pH, Po2, and PCO2, were within normal limits for euhydrated preterm fetal sheep during the baseline periods. In response to the intraperitoneal hypertonic saline infusions, arterial plasma osmolality and Na and Cl concentrations significantly increased; arterial Hct, pH, and plasma K concentrations decreased in Hyp animals. At time minute 15, fetal plasma osmolality and Na concentrations increased by ~10 mosmol/kg H2O and 5 meq/l, respectively (Fig. 1). Fetal plasma osmolality and Na concentration demonstrated only slight increases after the 15-min sample. By time minute 15, fetal plasma AVP concentrations rose above baseline and remained elevated throughout minute 30 (ANOVA on log-transformed data, P < 0.01). At time minute 15, ANG II concentrations rose above baseline values (ANOVA on ranks, P < 0.05).

With the exception of a small physiological rise in arterial Cl concentration, there were no other significant fetal arterial responses to the intraperitoneal isotonic saline infusions. Results indicate there were no significant changes in blood Hb during the study.

In the pregnant ewes of the treated fetuses, there were no significant changes in measured parameters throughout either study protocol (Hyp: plasma osmolality 299.8 ± 2.3 mosmol/kg H2O; AVP 1.3 ± 0.3 pg/ml; ANG II 25.2 ± 14.3 pg/ml; Hct 28.9 ± 1.0%; Hb 8.4 ± 0.6 g/dL; pH 7.47 ± 0.01; Po2 114.8 ± 4.8 mmHg;...
Comparison with AVP-containing neurons in the PVN was activated significantly greater percentage of AVP-containing cells double-stained for Fos within the fetal OVLT, SFO, and MnPO was demonstrated that an intravenous hypertonic saline infusion resulted in intense Fos immunoreactivity in several areas of the hypothalamus involved in osmoregulation (Fig. 2). The mean number of cells stained for Fos within the fetal OVLT, SFO, and MnPO was significantly increased in the Hyp animals compared with the Iso group (P < 0.05, Fig. 3).

Percentage of AVP-containing cells double-stained for Fos. Within the SON, the Hyp group showed a significantly greater percentage of AVP-containing cells that were activated (59.5 ± 3.5%) compared with the Iso animals (6.0 ± 1.0%; P < 0.05) (Figs. 4 and 5). Moreover, in Hyp animals, we detected 64.5 ± 1.6% of AVP-containing neurons in the PVN were activated compared with ~20.0 ± 5.0% in the Iso animals.

**DISCUSSION**

Fetal swallowing activity is paramount to maintaining amniotic fluid balance and contributes to fetal gastrointestinal maturation and perhaps fetal somatic growth. The near-term ovine fetus demonstrates dipsogenic responses to systemic, intracarotid, or intrace-rebroventricular hypertonicity (36, 37). We previously demonstrated that an intravenous hypertonic saline injection stimulates near-term fetal swallowing activity with a concomitant increase in plasma osmolality of 14 mosmol/kg H2O (37). Interestingly, whereas fetal plasma osmolality remains 2 to 3% above basal osmolality (6–9 mosmol/kg H2O), a level that typically would induce prolonged ingestive behavior in adult animals (47), fetal swallowing response is abated within minutes. Despite the altered fetal swallowing response to osmotic challenges, studies indicate that the primary osmoregulatory mechanisms, involved in sensing hypertonicity, are functional in the near-term fetus (24, 30, 36, 37). Stimulation of fetal osmoreceptors, capable of responding appropriately, albeit briefly to hypertonicity, are therefore functional and Fos expression may be detected in fetal osmoreceptors in response to hypertonicity (2).

In contrast to the near-term fetus, the preterm fetus (110–115 days gestation) may not demonstrate dipsogenic responses to intracarotid hypertonicity. We previously demonstrated that intracarotid hypertonic saline injections did not consistently stimulate preterm swallowing activity (17). Specifically, only 30% of fetuses exhibited swallowing response to highly concentrated hypertonic saline. Nevertheless, all preterm fetuses exhibited spontaneous swallowing activity during the control period, confirming that brainstem integration of motor neuron activities is functional early in gestation as are other reflex fixed-action patterns of neural circuitry. This finding is also consistent with reported integration of the swallowing reflex in the human fetus as early as 11-wk gestation (28). These findings indicate that in the preterm fetus, the full complement of neural circuits has not reached a critical point of transition into a functional central dipsogenic system and, therefore, remains unresponsive to osmotic stimuli. However, these results alone cannot distinguish the nonfunctional sites within preterm osmoregulatory neural circuitry.

Despite the noted preterm fetal insensitivity to osmotic stimulation, in the present study, preterm fetal sheep responded to osmotic stimulation with a marked increase in Fos expression within the OVLT, SFO, and MnPO, which validates the use of Fos as a marker of neuronal activation after an intraperitoneal hyperosmotic challenge in the preterm fetal sheep. Activation of AVP-containing neurons with the SON and PVN, indicate intact fetal neural pathways to these secretory neurons. These results are consistent with studies in the adult rat (31, 32) and near-term ovine fetus (2, 24), employing a variety of methods to administer hypertonic stimuli and demonstrating significant increases in Fos activation within osmo-sensitive neurons. The detection of neural activation indicates that preterm fetal osmoreceptors are capable of appropriately responding to an osmotic stimulus. Thus preterm fetal swallowing insensitivity to osmotic-dipsogenic stimuli is not due to the primary osmoreceptor nor to select interhypothalamic connections, but rather may be due to a lack of functional neural projections to medullary swallowing centers. Further studies will be required to determine the final neural event in dipsogenic maturation.
Fig. 2. Fos immunoreactivity observed in the areas of organum vasculosum of the lamina terminalis (OVLT) (A), median preoptic nucleus (MnPO) (C), and subfornical organ (SFO) of preterm fetal sheep (110 days gestation) (E) after receiving the intraperitoneal hypertonic saline infusion (1.5 M NaCl, 12 ml/kg). In contrast, after an intraperitoneal isotonic saline infusion (0.15 M NaCl, 12 ml/kg) minimal Fos expression was observed in areas of organum vasculosum of the lamina terminalis (OVLT) (B), MnPO (D), and SFO (F) of the fetal sheep. AC, anterior commissure. Magnification ×10.
thirst challenges in the ovine fetus, we have shown that cellular activation and AVP secretion appear at preterm (110-days gestation), whereas stimulated swallowing develops near term (130 days). Importantly, anatomical evidence demonstrates a shift in neural innervation occurring late in gestation of developing sheep, with a larger proportion of fibers projecting to the internal lamina and destined for posterior pituitary innervation (8). Together, these studies suggest that a change in function may occur concomitant with changes in neurocytoarchitecture and the developmental status of the fetus.

In this study, intraperitoneal infusion of hypertonic saline induced increases in preterm fetal plasma osmolality and Na concentrations of ~10 mosmol/kg H2O and 5 meq/l, respectively, within 15 min after the infusion. These increases are consistent with a 2–3% change in systemic osmolality previously demonstrated to induce adult and near-term fetal osmoregulatory responses (activated neuronal centers, thirst, and AVP secretion). In the present study, we administered a reduced dosage of hypertonic saline to the preterm fetuses than had been previously administered to adults (18 ml/kg) due to fetal morbidity at the higher dose. Despite our previous report (3) of a 2–3% rise in plasma osmolality in near-term ovine fetuses (130 days gestation) induced by a 6 ml/kg dose of hypertonic saline, this concentration of hypertonic saline did not cause an adequate rise in preterm fetal plasma osmolality. During pilot studies, it was determined that a 12 ml/kg dose of hypertonic saline could be safely administered and would result in a rise of preterm fetal plasma osmolality (of ~10 mosmol/kg H2O). Nevertheless, in these studies, by minute 15, adults and fetuses demonstrate similar increases of plasma osmolality and Na concentrations (~10 mosmol/kg and 5 meq/l, respectively). In contrast with the extended rise in plasma osmolality observed in adult sheep after osmotic stimulation, fetal plasma osmolality reach a peak level by 30 min and then decreased toward baseline values. The fetal response is

Alternatively, during this stage of in utero development, an enhanced regulatory mechanism that suppresses swallowing activity may also be involved in fetal osmoregulation. Additionally, the possible modulatory role of ascending hindbrain and/or brainstem projections to the hypothalamus in response to the osmotic challenge, cannot be excluded. Neurosecretory neurons may be directly and intrinsically osmo-sensitive (22, 23). Recently, using a similar hypertonic infusion paradigm in near-term fetal sheep, we detected a 10- to 20-fold increase in neuronal activation in caudal catecholaminergic brainstem neurons, specifically in the commissural subnucleus of the solitary tract and ventrolateral medulla (3). Because these neurons have established projections to the hypothalamus (5, 39), it is likely that these ascending brainstem projections also play a modulatory role in hypothalamic osmotic sensing neuronal activation.

It has been hypothesized that fetal osmotic regulatory mechanisms mature acutely at a critical developmental stage during the last third of gestation. Our studies suggest that osmotic dipsogenic activity develops acutely in the ovine fetus between 110 and 130 days, rather than by gradually increasing sensitivity to osmotic stimuli. Were the critical maturational step the gradual appearance of osmoreceptors, one may have expected gradually increasing sensitivity to dipsogens. Although neurogenic development occurs as an extensive process, there may be a critical event after which the organism is capable of responding to stimuli. Little is known about the sequence of events leading up to the final structure of the central osmoregulatory mechanism, although in an altricial specie, the rat, acute development of dipsogenic responses during the neonatal period has been demonstrated (29). Specifically, responsiveness to thirst challenges appear in the suckling rat with a clear timetable: cellular dehydration at 2 days of age, hypovolemia at 4 days, intracerebroventricular angiotensin at 4–5 days, and β-adrenergic stimulation at 6 days (46). Similarly, Ellis et al. (7) demonstrated that shortly after pups become responsive to ANG II, they drink in response to doses that reliably elicit ingestion in the adult. In response to

![Graphical depiction of mean number of Fos-stained cells after saline infusion, for the preterm fetal sheep, in the areas of OVLT, SFO, and MnPO. Solid bars represent hypertonic saline-treated animals; open bars represent isotonic saline-treated animals. *Significant differences from isotonic saline-treated group.](http://ajpheart.physiology.org/)
likely a result of transplacental exchange of water and electrolytes with the isotonic pregnant ewe. Interestingly, due to the larger volume of distribution, maternal plasma values were not significantly impacted by fetal treatment. Moreover, after the hypertonic saline infusion, preterm fetuses demonstrated decreased Hct, consistent with plasma volume expansion and perhaps cellular dehydration; therefore, it is unlikely that hypovolemia contributed to central Fos stimulation. In response to intraperitoneal isotonic (0.15 M) saline infusion, there were no changes in preterm fetal plasma osmolality, whereas the infusate osmolality (284 mosmol/kg H\textsubscript{2}O) was slightly lower than basal plasma values. Although intraperitoneal hypertonic NaCl may be painful to young and adult animals, we have demonstrated that intravenous infusion of hypertonic NaCl without potential painful stimulation also induces \textit{c-fos} expression in the SFO, OVLT, MnPO, and SON in the near-term ovine fetus (47). These data support the interpretation that induction of \textit{c-fos} in the CVO in this study is primarily caused by osmotic stimulation. However, in the present study, plasma osmolality, Na, and Cl concentrations increased after hypertonic infusion. Thus part or all of the effects may be due to direct effects of Na and/or Cl concentration, rather than osmolality, per se.

As expected, in the Iso group, numerous activated neurons were detected within osmotic sensing neural

Fig. 5. Fos immunoreactivity observed within AVP-containing neurons in the SON (A) and the PVN (C) of preterm fetal sheep after the intraperitoneal hypertonic saline infusion. In contrast, after an intraperitoneal isotonic saline infusion, minimal Fos expression (arrow) was observed in areas of SON (B) and PVN (D). Magnification ×100.
areas. These results are consistent with the elevated Fos expression detected in the near-term fetal sheep after an isotonic saline infusion (3), but are in contrast to the minimal Fos expression in adults in osmo-sensitive neural regions after similar treatment. Moreover, the expression of Fos in neurons is generally low, or undetectable, at basal levels of neuronal activity (11, 38). In the absence of a rise in plasma AVP levels, the cause for elevated Fos expression detected in the Iso sheep remains unclear. Analysis of the SON and PVN indicated that in addition to activated AVP-containing neurons, numerous neurons of undetermined phenotype are activated after isotonic saline infusion. In adult rats, studies indicate that if the administration of hypertonic stimuli to adult rats results in Fos activation within both AVP- and oxytocin (OT)-containing neurons located in the SON and PVN parvocellular and magnocellular divisions (10, 12, 19, 20, 40), and that this is consistent with OT secretory responses to hypertonicity (45). Although in the present study we did not stain for OT, Fos expression within the SON and PVN was not exclusively detected within AVP-containing neurons for both treatment groups. These results may suggest a potential stress-induced AVP secretion in preterm fetal sheep and may explain the Fos activation in Iso animals. Further studies to delineate the phenotype of activated neurons without intraperitoneal infusions, may be required to explain elevated basal levels of activation in the preterm fetus.

Determining the time course for the development of an intact central and systemic dipsogenic mechanism in utero has important implications in the regulation of fetal fluid homeostasis. Understanding the consequences of altered osmotic environments (such as exposure to maternal thermal exposure, dehydration, or exercise-induced water loss) in modulating swallowing activity in utero and in developing adult sensitivities to thirst and AVP secretion, requires further study to elucidate the mechanisms modulating the development of central sensitivity and responsiveness to fetal body fluid composition.

In conclusion, we demonstrate select neuronal activation, after a hypertonic saline infusion, within the CVOs, the MnPO, as well as hypothalamic AVP secretory nuclei, in the preterm fetal sheep. Activation of AVP-containing neurons within the SON and PVN, indicate intact fetal neural pathways to these secretory neurons. These observations suggest that preterm fetal swallowing insensitivity to osmotic-dipsogenic stimuli may be due to a lack of functional neural projections to medullary swallowing centers.

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