Osmotic threshold and sensitivity for vasopressin release and Fos expression by hypertonic NaCl in ovine fetus

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Received 5 April 2000; accepted in final form 7 August 2000

Xu, Zhice, Calvario Glenda, Linda Day, Jiaming Yao, and Michael G. Ross. Osmotic threshold and sensitivity for vasopressin release and Fos expression by hypertonic NaCl in ovine fetus. Am J Physiol Endocrinol Metab 279: E1207–E1215, 2000.—In adults, hyperosmolality stimulates central osmoreceptors, resulting in arginine vasopressin (AVP) secretion. Near-term fetal sheep have also developed mechanisms to respond to intravascular hypertonicity with stimulation of in utero AVP release. However, prior studies demonstrating fetal AVP secretion have utilized plasma tonicity changes greater than those required for adult osmotically induced AVP stimulation. We sought to examine near-term fetal plasma osmolality threshold and sensitivity for stimulation of AVP secretion and to correlate plasma hormone levels with central neuronal responsiveness. Chronically instrumented ovine fetuses (130 ± 2 days) and maternal ewes simultaneously received either isotonic or hypertonic intravascular NaCl infusions. Maternal and fetal plasma AVP and angiotensin II (ANG II) levels were examined at progressively increasing levels of plasma hypertonicity. Intravenous hypertonic NaCl gradually elevated plasma osmolality and sodium levels. Both maternal and fetal plasma AVP increased during hypertonicity, whereas ANG II levels were not changed. Maternal AVP levels significantly increased with a 3% increase in plasma osmolality, whereas fetal plasma AVP significantly increased only at higher plasma osmolality levels (over 6%). Thus the slope of the regression of AVP vs. osmolality was greater for ewes than for fetuses (0.232 vs. 0.064), despite similar maternal and fetal plasma osmolality thresholds for AVP secretion (302 vs. 304 mosmol/kg). Hyperosmolality induced Fos immunoreactivity (FOS-ir) in the circumventricular organs of the fetal brain. FOS-ir was also demonstrated in the fetal supraoptic and paraventricular nuclei (SON and PVN), and double labeling demonstrated that AVP-containing neurons in the SON and PVN expressed Fos in response to intravenous NaCl. These results demonstrate that, in the ovine fetus at 130 days of gestation, neuroendocrine responses to cellular dehydration are functional, although they evidence a relatively reduced sensitivity for AVP secretion compared with the adult.

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the plasma osmolality threshold that stimulates AVP secretion) appear to be different (18, 22).

There are likely additional factors that mediate hypertonicity-induced AVP release. In addition to direct effects of hypertonicity, one possible mediator of the rise in plasma AVP is angiotensin. Dehydration may increase circulating angiotensin (5), and hypertonic saline infusions may increase angiotensin II (ANG II) levels (3, 12). In addition, intravenous ANG II evoked AVP release in sheep (14), likely by stimulation of the hypothalamic-neurohypophysial system via circumventricular organs (SFO, OVLT) (34) and the area postrema (AP), central nuclei that lack blood-brain barriers. Furthermore, blockade of angiotensin receptors with angiotensin antagonists may suppress dehydration-induced thirst and vasopressin responses (4).

In view of the demonstrated functionality of fetal AVP secretory systems, we sought to examine the near-term ovine fetal plasma osmolality threshold and sensitivity for stimulation of AVP secretion. We further sought to correlate plasma AVP and ANG II levels, central neuronal responsiveness, and the activity of AVP-containing neurons in the SON and PVN.

MATERIALS AND METHODS

Ten time-dated pregnant ewes with singleton fetuses (130 ± 2 days of gestation on the study day) were used. Animals were housed in individual study cages in a light-controlled room (12:12-h light-dark cycle), with food and water provided ad libitum.

Surgery. Anesthesia was induced with ketamine hydrochloride (20 mg/kg im), and general anesthesia was maintained with 1–2% isoflurane and 1 l/min oxygen. An intrauterine catheter (Corometrics Medical System, Wallingford, CT) was inserted for measuring amniotic fluid pressure. Polyethylene catheters were placed in the femoral artery and vein and threaded to the inferior vena cava and abdominal aorta, respectively, of both the fetus and the ewe. Bipolar electromyography electrodes (model AS632, Cooner Wire, Chatsworth, CA) were placed on the fetal thyrohyoid muscle and the nuchal and thoracic esophaagus for determination of swallowing activity, as previously reported (17, 29). All catheters were externalized to the maternal flank and placed in a cloth pouch. For 5–6 days, animals were allowed postoperative recovery, which included catheter maintenance and antibiotic administration.

Experimental design. Animals were studied from 8:00 AM to 1:00 PM, and only if the basal fetal arterial pH was >7.3. Studies began with a baseline period (−120 to 0 min) followed by the study period (0–160 min). There were two groups of animals (control, n = 5; experimental, n = 5). For the control animals, isotonic (0.15 M) NaCl was infused intravenously to the maternal ewe and fetus (2.5 and 0.5 ml/min, respectively) for 80 min.

To minimize osmotically mediated transplacental fluid alterations, the experimental study sought to increase both fetal and maternal plasma osmolality in parallel. Beginning at time 0, hypertonic saline (0.85 M NaCl) was infused intravenously to the maternal ewe and fetus (2.5 and 0.5 ml/min, respectively) for 60 min, after which the infusate was changed to 4 M NaCl for an additional 20 min (to achieve a further increase in plasma osmolality). After infusions, animals were continually observed for an additional 80 min. The results for fetal swallowing and circumventricular organ and hindbrain Fos immunoreactivity (FOS-ir) in this protocol have been submitted as a separate report.

Throughout the study, maternal and fetal blood pressures, heart rates, and amniotic fluid pressure were measured continuously. Maternal and fetal blood samples were collected at −60 and −30 min of the baseline period and at 15, 30, 60, and 100 min after initiation of the infusion. Fetal and maternal blood pressures and amniotic fluid pressures were measured by means of a Beckman R612 (Beckman Instruments, Fullerton, CA) physiological recorder with Statham (Garret, Oxnard, CA) P23 transducers. Fetal blood pressure was corrected for amniotic cavity pressure. Maternal and fetal blood samples were collected into iced tubes containing lithium heparin. Blood aliquots were assessed for hematocrit, pH, PO2, and PCO2; remaining blood was centrifuged, and plasma osmolality, potassium, sodium, chloride, and potassium concentrations were measured. Fetal blood samples were replaced with an equivalent volume of heparinized maternal blood withdrawn before the study.

Blood PO2, PO2, and pH were measured at 39°C with a Radiometer BM 33 MK2-PH M2 MKS acid-base analyzer system (Radiometer, Copenhagen, Denmark). Plasma osmolality was measured by freezing point depression on an Advanced Digimatic osmometer (model 3MO, Advanced Instruments, Needham Heights, MA). Plasma sodium, potassium, and chloride concentrations were determined by a Nova 5 electrolyte analyzer (Nova Biomedical, Waltham, MA).

Blood samples for AVP assays were collected in chilled heparinized glass tubes. Plasma samples for AVP were extracted by a modification of the procedure (17), and AVP levels were determined by radioimmunoassay. Sensitivity of our AVP antiserum is 0.8 pg of AVP/tube, with intra-assay and interassay coefficients of variation of 6 and 9%, respectively. Plasma AVP recoveries average 70%. Blood samples for ANG II assays were collected in chilled plastic tubes containing EDTA (1 mg/ml) and aprotinin (250 kalikrein-inhibitor units/ml). Samples for ANG II extraction were acidified with 1 N HCl and extracted using a modification of the method of Cain et al. (6). Columns were washed with methanol (10 ml) followed by distilled water (10 ml) before use. Acidified plasma samples were added slowly to the columns, and the columns were washed with 0.1% trifluoroacetic acid (TFA). The absorbed ANG II was eluted with 80% methanol and 0.1% TFA, and the eluates were dried in a Speed-Vac concentrator. ANG II radioimmunoassay was performed with antiserum (Amersham, Arlington Heights, IL). The sensitivity of the assay was 2 pg/tube. The intra- and interassay coefficients of variation were 7 and 9%, respectively. The Amersham antiserum (raised against [L-Ile5]ANG II) is cross-reactive 100% with [Val5]ANG II and des-Asp1-ANG II (angiotensin III) but shows minimal cross-reactivity with ANG I (<2%). Immunoradioassay data were treated in a double-blind manner.

FOS-ir staining. Fetal brains were perfused and collected at the end of study for FOS-ir analysis: at the end of the study, maternal ewes were anesthetized with ketamine anesthesia (20 mg/kg iv) and ventilated with a mixture of isoflurane and oxygen. A middle abdominal incision was made, and the fetal head and neck were exposed. A 16-gauge needle was inserted into one side of the fetal carotid artery for perfusion. The fetuses were perfused via carotid artery with 0.01 M PBS followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer. Postfixation was performed in PFA solution for 12–24 h, after which brains were placed in 20% sucrose in 0.01 M phosphate overnight. Thirty-micrometer coronal sections of fetal brain were cut through the forebrain and hindbrain on a cryostat. Every other section of the SON...
and PVN and every third section of other parts of the forebrain were used for FOS-ir staining with the avidin-biotin-peroxidase technique. The primary antibody (Santa Cruz Biotech, Santa Cruz, CA) was raised from rabbits against Fos protein in cells. The tissue sections were incubated for 1 h at room temperature on a gentle shaker and then overnight at 4°C in the primary antibody (Fos antibody with 0.3% Triton X-100). The sections were further incubated in goat antirabbit serum (1:500) and then processed using the Vectastain ABC kit (Vector Labs, “Elite” ABC reagent, Burlingame, CA). The sections were treated with 1 mg/ml diaminobenzidine tetrahydrochloride (Sigma) (0.02% hydrogen peroxide). All nonspecific background staining, sections were incubated for 20 min in blocking serum. The selected sections were incubated in vasopressin antibody (1:1,000; Incstar, Stillwater, MN) overnight, after which anti-rabbit antibody (Vector Labs) was applied. The sections were dried overnight, dehydrated in alcohol, and then were coverslipped.

To identify colocalization of positive FOS-ir and AVP in the SON and PVN neurons, hypothalamic sections were first taken and processed using the c-Fos procedure. To reduce endogenous peroxidase activity, sections were rinsed for 5 min in a 0.5% solution of H2O2 in 0.01 M PBS, followed by two washes in 0.01 M PBS (each for 5 min). To reduce nonspecific background staining, sections were incubated for 20 min in blocking serum. The selected sections were incubated in vasopressin antibody (1:1,000; Incstar, Stillwater, MN) overnight, after which anti-rabbit antibody (Vector Labs) was applied. The sections were dried overnight, dehydrated, and then coverslipped with Depex.

Data analysis. The number of positive FOS-ir cells in fetal brain sections was evaluated in a qualitative manner by microscopy analysis, as reported before (28). The regions counted were the SON and PVN in the forebrain. Fetal blood pressure was adjusted for amniotic fluid pressure. Repeated-measures ANOVA was used to determine differences over time and effects of treatments. Comparison of before and after treatments was determined with one-way ANOVA for time and effects of treatments. Comparison of before and after treatments was determined with one-way ANOVA for time and effects of treatments.

The threshold value was solved for the threshold value using the basal (time 0) mean plasma AVP value. All data are expressed as means ± SE.

**RESULTS**

**Cardiovascular and blood values.** Maternal and fetal arterial blood pressures [systolic and diastolic pressure, mean arterial pressure (MAP) and adjusted fetal MAP] or heart rate did not change significantly in response to either isotonic or hypertonic saline infusions (Table 1). There was a significant difference in fetal plasma osmolality and Na+ concentration between the control and treated groups (\( F_{8,1} = 52.02 \) and 12.3, respectively, \( P < 0.01 \)). Fetal plasma osmolality increased, although not significantly, at 30 min during infusion of 0.85 M NaCl. However, a significant increase in fetal plasma osmolality (315 ± 5 mosmol/kg) occurred at 60 min, and a further increase occurred in response to infusion of 4 M NaCl (332 ± 4 mosmol/kg; \( F_{19,4} = 15.10, P < 0.01 \); Table 2). Fetal plasma sodium significantly increased at 60 min and after infusion of 4 M NaCl (\( F_{19,4} = 34.09, P < 0.01 \)). Consistent with the study objectives, maternal plasma osmolality and Na+ concentration also significantly increased at 60 and 100 min of the study (\( F_{19,4} = 11.7 \) and 17.62, respectively, \( P < 0.01 \); Table 2). Both maternal and fetal blood pH, Po2, Pco2, hemoglobin, and hematocrit did not change in response to the hypertonic saline infusions (Table 2).

**Plasma AVP and ANG II.** There was a significant difference in plasma AVP levels between isotonic and hypertonic saline-infused maternal ewes (\( F_{8,1} = 7.44, P < 0.05 \)) and fetuses (\( F_{8,1} = 5.54, P < 0.05 \)). In control groups, intravenous infusion of isotonic saline did not change maternal or fetal plasma AVP levels (\( F_{19,4} = 0.05 \) and 1.41, respectively, nonsignificant (NS)). However, infusion of hypertonic NaCl increased both maternal (\( F_{18,4} = 8.70, P < 0.001 \)) and fetal plasma AVP levels (\( F_{19,4} = 8.54, P < 0.001 \)). There was a small but insignificant increase in maternal plasma AVP at 15 and 30 min of the hypertonic saline infusion. Maternal

**Table 1. Fetal and maternal arterial blood pressure and heart rate measures**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>100 min</th>
</tr>
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<tr>
<td><strong>Fetal measures before and after isotonic NaCl infusion</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
<td>65 ± 5</td>
<td>72 ± 7</td>
<td>72 ± 7</td>
<td>75 ± 7</td>
<td>71 ± 10</td>
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<td>Diastolic pressure, mmHg</td>
<td>49 ± 7</td>
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<td>MAP, mmHg</td>
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<td>62 ± 6</td>
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<td>60 ± 7</td>
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<td>Adjusted MAP, mmHg</td>
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<td>48 ± 4</td>
<td>49 ± 4</td>
<td>49 ± 2</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>164 ± 7</td>
<td>166 ± 4</td>
<td>174 ± 23</td>
<td>176 ± 11</td>
<td>163 ± 6</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic pressure, mmHg</td>
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<td>68 ± 2</td>
<td>66 ± 1</td>
<td>64 ± 1</td>
<td>72 ± 2</td>
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<tr>
<td>Diastolic pressure, mmHg</td>
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<tr>
<td>MAP, mmHg</td>
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<td>57 ± 2</td>
<td>56 ± 1</td>
<td>54 ± 1</td>
<td>59 ± 1</td>
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<tr>
<td>Adjusted MAP, mmHg</td>
<td>47 ± 2</td>
<td>47 ± 1</td>
<td>46 ± 0</td>
<td>46 ± 1</td>
<td>49 ± 1</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>161 ± 8</td>
<td>168 ± 5</td>
<td>160 ± 5</td>
<td>173 ± 8</td>
<td>171 ± 3</td>
</tr>
<tr>
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<tr>
<td>Systolic pressure, mmHg</td>
<td>123 ± 14</td>
<td>119 ± 13</td>
<td>120 ± 14</td>
<td>121 ± 16</td>
<td>119 ± 15</td>
</tr>
<tr>
<td>Diastolic pressure, mmHg</td>
<td>80 ± 9</td>
<td>76 ± 8</td>
<td>76 ± 7</td>
<td>77 ± 8</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>102 ± 12</td>
<td>98 ± 11</td>
<td>98 ± 11</td>
<td>99 ± 12</td>
<td>97 ± 10</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>106 ± 11</td>
<td>103 ± 16</td>
<td>104 ± 12</td>
<td>108 ± 11</td>
<td>103 ± 15</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 5 \). MAP, mean arterial pressure.
isotonic or hypertonic saline (osmolality (315 ± 2 to 332 ± 4 mosmol/kg). Fetal plasma AVP significantly increased (5-fold; 0.8 ± 0.1 to 4.6 ± 1.0 pg/ml) after the 4 M NaCl infusion, in association with a further increase in fetal plasma osmolality (299 ± 2 to 315 ± 5 mosmol/kg). Fetal plasma AVP did not change significantly at 15, 30, or 60 min of 0.85 M NaCl infusion, despite an ~5% increase in fetal plasma osmolality (299 ± 2 to 315 ± 5 mosmol/kg).

Neither maternal nor fetal plasma ANG II levels changed in response to intravenous infusion of either isotonic or hypertonic saline (F_{S,1} = 0.34 and 1.43, respectively; NS).

Linear regression of plasma AVP vs. plasma osmolalities demonstrated a greater slope in maternal ewes compared with fetuses (maternal 0.23 ± 0.05, r^2 = 0.49, P < 0.05 vs. fetal 0.06 ± 0.02, r^2 = 0.37, P < 0.05; Fig. 2). The plasma osmolality threshold was similar for ewes and fetuses (302 vs. 304 mosmol/kg, respectively).

**FOS-ir and AVP immunoreactivity.** In the control fetuses infused with isotonic saline, there was little FOS-ir in fetal basal forebrain structures. However, intravenous hypertonic saline produced intense FOS-ir in the fetal forebrain. The areas of strongest FOS-ir included the SON and PVN in the hypothalamus. There were significant differences of FOS-ir between isotonic and hypertonic NaCl-infused fetuses (SON: t = 12.75, PVN: t = 9.77, both P < 0.01; Figs. 3 and 4). FOS-ir was also induced by hypertonic saline in the

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**Table 2. Fetal and maternal arterial values before and after hypertonic NaCl infusion**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
<th>100 min</th>
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</thead>
<tbody>
<tr>
<td><strong>Fetal values</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hct, %</td>
<td>29 ± 1</td>
<td>28 ± 2</td>
<td>27 ± 3</td>
<td>28 ± 2</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>8.8 ± 1.7</td>
<td>8.8 ± 2</td>
<td>9.0 ± 1.1</td>
<td>9.0 ± 1.5</td>
<td>9.0 ± 1.2</td>
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<tr>
<td>pH</td>
<td>7.3 ± 0.2</td>
<td>7.4 ± 0.1</td>
<td>7.3 ± 0.1</td>
<td>7.3 ± 0.2</td>
<td>7.3 ± 0.1</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>21 ± 5</td>
<td>21 ± 2</td>
<td>19 ± 3</td>
<td>20 ± 3</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>PCO2, Torr</td>
<td>52 ± 2</td>
<td>50 ± 4</td>
<td>52 ± 1</td>
<td>52 ± 1</td>
<td>48 ± 10</td>
</tr>
<tr>
<td>Osmolality, mosmol/kg</td>
<td>299 ± 2</td>
<td>307 ± 1</td>
<td>311 ± 3</td>
<td>315 ± 5*</td>
<td>332 ± 4*</td>
</tr>
<tr>
<td>Na, meq/l</td>
<td>141 ± 1</td>
<td>145 ± 1</td>
<td>146 ± 1</td>
<td>147 ± 1*</td>
<td>160 ± 2*</td>
</tr>
<tr>
<td>K, meq/l</td>
<td>4.0 ± 0.9</td>
<td>3.9 ± 0.6</td>
<td>3.8 ± 0.7</td>
<td>3.6 ± 0.3</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>Cl, meq/l</td>
<td>107 ± 1</td>
<td>107 ± 1</td>
<td>114 ± 1*</td>
<td>115 ± 2*</td>
<td>128 ± 2*</td>
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<td><strong>Maternal values</strong></td>
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<td></td>
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<tr>
<td>Hct, %</td>
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<td>26 ± 2</td>
<td>25 ± 1</td>
<td>24 ± 2</td>
<td>25 ± 2</td>
</tr>
<tr>
<td>Hb, g/dl</td>
<td>8.3 ± 2</td>
<td>7.2 ± 2</td>
<td>7.2 ± 1</td>
<td>7.1 ± 1</td>
<td>7.2 ± 1</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>PO2, Torr</td>
<td>116 ± 13</td>
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<td>120 ± 12</td>
<td>118 ± 11</td>
<td>111 ± 7</td>
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<tr>
<td>PCO2, Torr</td>
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<td>39 ± 7</td>
<td>36 ± 2</td>
<td>38 ± 2</td>
<td>37 ± 3</td>
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<tr>
<td>Osmolality, mosmol/kg</td>
<td>301 ± 4</td>
<td>305 ± 4</td>
<td>311 ± 7</td>
<td>313 ± 2*</td>
<td>321 ± 5*</td>
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<tr>
<td>Na, meq/l</td>
<td>147 ± 1</td>
<td>148 ± 1</td>
<td>150 ± 1</td>
<td>152 ± 2*</td>
<td>156 ± 2*</td>
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<td>K, meq/l</td>
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<td>4.5 ± 1.3</td>
<td>3.9 ± 0.2</td>
<td>4.3 ± 1.0</td>
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<tr>
<td>Cl, meq/l</td>
<td>111 ± 1</td>
<td>112 ± 1</td>
<td>114 ± 2</td>
<td>117 ± 3</td>
<td>120 ± 3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5. Hct, hematocrit; Hb, hemoglobin; PO2 and PCO2, oxygen and carbon dioxide pressures, respectively.

Fig. 1. Maternal and fetal plasma arginine vasopressin (AVP) levels before and after iv infusion of hypertonic NaCl. Values are means ± SE; N = 5. *P < 0.01.
median preoptic nucleus (MePO), the OVLT, and the SFO (reported separately).

AVP-containing neurons were observed in both SON and PVN. The FOS-ir in the SON was distributed throughout the nuclei. In the PVN, FOS-ir was observed in both dorsal and lateral parts. There was no difference in the total counting of AVP immunoreactivity (AVP-ir) in SON and PVN between control and hypertonic saline-infused fetuses ($t = 1.23$ and 1.20, respectively, NS). Dual immunoreactivity for FOS and AVP in the SON and PVN was much higher in hypertonic NaCl-infused fetuses than in isotonic NaCl treated animals ($t = 15.05$ and 12.05, respectively, $P < 0.001$; Fig. 3). Not all of the AVP-ir-positive neurons in the SON and PVN were double-stained with FOS-ir: 64% and 56% of AVP-containing neurons colocalized with positive FOS-ir in the PVN and SON, respectively. Among all positive FOS-ir neurons in the SON and PVN, 40% were not AVP-ir-stained (Fig. 5).

**DISCUSSION**

The present study was designed to assess the responsiveness of the hypothalamic neurohypophysial system in the near-term fetus, utilizing intravenous saline infusions to compare fetal and adult AVP responses to a progressive elevation of plasma osmolality. The results demonstrate that in 90%-gestation ovine fetuses, elevation of plasma osmolality due to the infusion of hypertonic NaCl produced a graded increase in AVP release. However, fetal plasma AVP did not significantly change until there was a plasma osmolality increase of 6% or greater, whereas maternal AVP increased in response to a 3% increase of osmolality. Confirming the stimulation of AVP secretion, AVP-containing neurons in the neurosecretory parts of the fetal hypothalamus were activated after this osmotic stimulation.

The contribution of both plasma hyperosmolality and volume in the control of AVP secretion and dipsongenic responses is recognized (i.e., the double depletion hypothesis of thirst) (9, 10). Hypertonic saline infusions to maternal ewes increase both maternal and fetal plasma osmolalities but may reduce fetal extracellular fluid volume (because of fetal-to-maternal osmotically mediated water flow) and thus potentiate fetal AVP secretion. Conversely, hypertonic saline infusions to the fetus may expand fetal extracellular fluid volume and partially suppress the osmotically induced fetal AVP release (11, 22). The present study utilized simultaneous infusions of hypertonic fluids into both fetus and ewe to minimize transplacental water flow. The study design did not induce significant alterations in maternal or fetal systolic, diastolic, or mean blood pressures, whereas plasma osmolality was gradually elevated. Fetal arterial hematocrit values did not change significantly, suggesting relatively minor changes in plasma volume. These results indicate that AVP release and c-Fos expression in the fetal hypothalamus primarily resulted from osmotic stimulation, without significant volume or baroreceptor-mediated influence.

Intravenous hypertonic saline lowers hematocrit and expands blood volume in some (35) but not all.
(3) studies. For example, Bathgate and Sernia (3) reported that intravenous hypertonic NaCl infusion over 2 h increased plasma osmolality from 304 to 404 mosmol/kg and plasma Na\(^+\) from 149 to 204 meq/l, whereas hematocrit remained unchanged. In our experiments, the duration of the hypertonic saline infusion likely permitted osmolar equilibration with the interstitial compartment, minimizing intravascular volume alterations. In addition, an increased permeability due to a relative immaturity of fetal blood vessels and the use of dual maternal and fetal infusions may have contributed to the lack of fetal hematocrit change.

Comparison of plasma osmolality thresholds for AVP release revealed a similar plasma osmolality threshold between the maternal ewe and the near-term ovine fetus. Several earlier studies (8, 24) calculated plasma osmolality threshold by use of the x-axis intercept of the regression line, thus assuming a basal plasma AVP level of zero. However, our data and those of others indicate that basal plasma AVP levels in the euhydrated state are actually greater than zero. Therefore, the mean basal plasma AVP level is an appropriate value to use for the regression (8, 24). Contrary to the similarity of the osmotic thresholds, the sensitivity of the AVP-osmolality relationship (as exhibited by the regression slope) demonstrated a reduced fetal sensitivity. Thus maternal plasma AVP levels increased significantly, with a 2–4% plasma osmolality increase, consistent with previous reports of adult animals (11, 21, 27). In comparison, a 6% or greater increase in fetal plasma osmolality was required to significantly increase plasma AVP. The reduced sensitivity of the fetus may be due to immature osmoregulation mechanisms, a lack of integrative osmoreceptor-to-hypothalamus neural mechanisms, or the presence of enhanced suppressive mechanisms.

Thus the plasma osmolality sensitivity (i.e., regression slope) for AVP release is reduced in the ovine fetus in utero, compared with the maternal ewe. These results suggest that fetal neuroendocrine mechanisms to cellular dehydration are not completely developed, although fetal physiological responses to osmotic challenge are present at this near-term stage. Teleologically, this response may be of importance for the unique in utero fluid dynamics. Although fetal AVP

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**Fig. 4.** c-Fos expression induced by intravenous hypertonic NaCl in the SON and PVN. A and C: control fetus infused with 0.15 M NaCl. B and D: fetus infused with hypertonic NaCl. A and B: SON (×10); C and D: PVN (×20). OC, optic chiasma; 3v, the third ventricle.
secretion and dipsogenic responses may reduce the production of fetal urine and lung fluid (~250 ml·kg⁻¹·day⁻¹ near term) and increase the absorption of amniotic fluid by fetal swallowing, it is essential that developmental mechanisms serve to maintain an adequate volume of amniotic fluid for fetal growth and development. Thus, in response to maternal dehydration, it may be beneficial for the maternal ewe to respond first with maternal antidiuresis and thirst before fetal fluid-altering responses are evoked that would reduce amniotic fluid volume.

Previous studies in our laboratory have demonstrated that fetal intraperitoneal hypertonic saline injections produced FOS-ir in ovine fetal brain areas including the SON (7). However, intraperitoneal hypertonic saline may potentially evoke a stress and/or pain response (31). Consistent with this hypothesis, intraperitoneal hypertonic saline increased fetal c-Fos expression in both the SON and PVN (31), the areas believed to respond to stress in addition to AVP secretion. In the present study, the use of intravenous hypertonic infusions permitted the assessment of plasma hypertonicity effects on the hypothalamic-neurohypophysial system without the sensory effects of intraperitoneal injections. These results are the first, to our knowledge, to demonstrate that intravenous hypertonic infusion can induce c-Fos expression in AVP neurons in the fetal hypothalamus. On gross inspection, all regions of the SON and PVN, including parvicellular and magnocellular parts, expressed positive c-Fos after intravenous hypertonic NaCl, and a majority of FOS-ir cells localized to AVP-containing neurons. Detailed analysis revealed that two-thirds of AVP cells in the fetal SON and PVN were double labeled with FOS-ir and AVP. Furthermore, 56–64% of FOS-ir in these two nuclei were located in the AVP-containing neurons. This cellular phenomenon in the fetal hypothalamus can be interpreted as suggesting that two-thirds of AVP neurons in the fetal SON and PVN are functionally activated by hypertonicity due to intravenous hypertonic infusions. The remaining one-third of AVP neurons (without Fos expression) may require

Fig. 5. Double labeling of FOS-ir and AVP-ir in the PVN. A and C: control fetus infused with iv 0.15 M NaCl; B and D: fetus infused with hypertonic NaCl. A and B: ×100; C and D: ×40. Arrows show colocalization of FOS-ir and AVP-ir.
greater osmotic stimulation, may be functionally immature or may be activated in response to alternative stimuli (e.g., volume, pain, emesis). As noted above, it is unlikely that other factors (e.g., increase in extracellular fluid volume) suppressed AVP secretion. In addition to the FOS-ir in double-labeled neurons, ~40% of induced FOS-ir located in non-AVP cells. This demonstrates that a large portion of non-AVP-containing neurons in the SON and PVN can also respond to osmotic stimulation in the near-term ovine fetus. Previous studies have demonstrated that c-fos induced by dipsogenic stimulation in the adult rat hypothalamus can occur in oxytocin neurons (13, 36). For example, in the rat SON and PVN, colocalization of oxytocin and Fos induced by intraperitoneal hypertonic saline was 80 and 60%, respectively (13). Controversy persists as to whether plasma hypertonicity stimulates oxytocin release in sheep (26, 33). The distribution of FOS-ir throughout the SON and dorsal PVN in the present study suggests that both oxytocin and corticotropin-releasing hormone may be candidates for activated non-AVP cells. Further studies are needed to characterize these fetal osmotically activated cells during early development.

In the present study, intravenous hypertonic saline induced c-Fos expression not only in the hypothalamus but also in the fetal MePO, SFO, and OVLT (quantitative results reported separately). Mason (25) demonstrated that supraoptic neurons themselves are osmotically sensitive, because dissociated SON neurons can be excited by hypertonic solution; thus osmoreceptors are distributed in the hypothalamus. However, other researchers (27, 34) argued that the circumventricular organs are the main location for central osmoreceptors on the basis of central lesions (15, 23, 37). Anatomic connections between the circumventricular structures, MePO, and the hypothalamus have been confirmed, and destruction of these structures in adult animals impairs AVP release (15, 20, 23). The present results indicating FOS-ir in the fetal MePO, SFO, and OVLT, as well as in the SON and PVN, support the hypothesis that the pathways from the SFO, OVLT, and MePO to the hypothalamus are important for AVP-mediated body fluid regulation in the developing fetus.

The mechanism mediating hypertonicity-induced endocrine responses has been intensely investigated in adults (11, 15, 21, 27). Angiotensin or its precursors may be increased under condition of dehydration (10), and AVP release may be mediated by both central and peripheral ANG II stimulation (11, 14, 21). Peripherally, plasma ANG II acts primarily on angiotensin receptors in the SFO, OVLT, and AP (16, 30), influencing activity of the SON and PVN in the hypothalamus. In the present study, fetal plasma ANG II levels did not change in response to plasma hypertonicity. It appears that c-Fos expression in the circumventricular structures and the hypothalamus, as well as the release of AVP by cellular dehydration, is independent of peripheral angiotensin. However, it remains possible that central renin-angiotensin mechanisms, developed during the last one-third of the gestation, may mediate osmotically stimulated AVP release (32, 38).

In summary, this study provides important information for understanding the development of central neuroendocrine responses to cellular dehydration in utero. The results demonstrate that AVP neurons in the SON and PVN were activated in association with increased plasma AVP but not plasma ANG II levels. Analysis of threshold responses between the maternal ewe and fetus revealed that the near-term ovine fetus has a similar plasma osmolality threshold but a reduced sensitivity for AVP secretion, compared with adult animals. This reduced sensitivity may serve to preserve fetal urine and lung liquid production, and thus amniotic fluid volume, during periods of maternal dehydration.

We thank Jim Humme and Dr. Jeong Jae Lee for their assistance in sheep surgery and physiological studies. This study was supported by National Institutes of Health Grants DK-43311 and HL-40899 to M. G. Ross.

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