Central cholinergic mechanisms mediate swallowing, renal excretion, and c-fos expression in the ovine fetus near term

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1Department of Human Sport Science, Beijing Sport University, Beijing, China 2Perinatal Research Laboratory, Soochow University School of Medicine, Suzhou, China; and 3Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, California

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Shi L, Mao C, Zeng F, Zhu L, Xu Z. Central cholinergic mechanisms mediate swallowing, renal excretion, and c-fos expression in the ovine fetus near term. Am J Physiol Regul Integr Comp Physiol 296: R318–R325, 2009. First published September 10, 2008; doi:10.1152/ajpregu.90632.2008.—Fetal swallowing and renal metabolism contribute importantly to amniotic and body fluid homeostasis. To determine central cholinergic modulation of swallowing activity and renal excretion associated with neural activity, we examined the effects of intracerebroventricular injection of carbachol, a cholinergic agonist, in ovine fetuses at 0.9 gestation. Fetuses were chronically prepared with thyrohyoid, nuchal and thoracic esophagus, and diaphragm electromyogram electrodes, as well as lateral ventricle and vascular catheters. Electrodes were also implanted on the parietal dura for determination of fetal electrocorticogram (ECoG). After 5 days of recovery, fetal swallowing, ECoG, and urine output were monitored during basal period and the experimental period following intracerebroventricular injection of 0.9% NaCl as the control (n = 5) or carbachol (3 μg/kg, n = 5). Central carbachol did not significantly change fetal low voltage (LV) and high voltage (HV) ECoG temporal distributions. However, swallowing activity during LV ECoG was elevated significantly after intracerebroventricular carbachol. Associated with the swallowing activation, c-fos immunoreactivity in the putative dipsogenic center, subfornical organ, was enhanced significantly. The fetal urine flow rate and renal Na+, K+, and Cl− excretion were markedly increased following intracerebroventricular carbachol and sustained at the high level for at least 2 h. The results indicate that the central cholinergic mechanism is established and functional in regulation of fetal behavior and renal excretion at least 0.9 gestation, which plays an important role in maintenance of fetal body fluid homeostasis.

fetus; carbachol; thirst

STIMULATED WATER INTAKE, in concert with renal water and electrolyte excretion, is the fundamental physiological mechanism that maintains body fluid homeostasis. It is well established that in adult animals, central cholinergic mechanisms play an important role in the control of cardiovascular and body fluid homeostasis (12, 15). Central application of cholinergic agonist, carbachol, elicits a variety of physiological responses, including natriuretic, dipsogenic, and pressor responses, as well as vasopressin secretion (2, 23, 44). Our recent studies demonstrated that intracerebroventricular injection of carbachol also produced pressor responses (42) in the ovine fetus near term, indicating that the central cholinergic mechanism-mediated cardiovascular regulation is functional before birth. However, the role of central cholinergic mechanisms in fetal fluid balance and renal excretion is largely unknown.

Progress has been made in demonstrating that swallowing activity can be regulated by hyperosmotic, hypovolemic, and other stimuli such as ANG II and neuropeptide Y in late gestation (31, 33, 48, 49). Fetal renal fluid and electrolyte excretion also can be controlled by hemorrhage, osmotic, and vasopressin mechanisms (6, 10, 11, 15, 29). Nijland et al. found anticholinergic suppression of ovine fetal swallowing (28). However, study on the fetal brain cholinergic activation-mediated swallowing and renal regulation in utero has been limited. In fact, there have been no data regarding cholinergic effects on fetal swallowing and renal excretion by direct application of cholinergic agonists in fetal brain intracerebroventricularly.

The subfornical organ (SFO), an area rich in cholinergic binding sites and projections (35, 46, 50), has been demonstrated to play an important role in body fluid regulation (8). Although the early work has shown that an active cholinergic system is present in fetuses (19, 20), it is unknown whether the SFO is able to respond to cholinergic signals in the fetal period. Therefore, we hypothesized that fetal brain cholinergic mechanisms-related body fluid regulation is established before birth. To test this hypothesis, we determined the fetal swallowing activity and renal excretion associated with SFO activation induced by intracerebroventricular carbachol. The information gained will add knowledge in the functional development of cholinergic mechanisms-mediated regulations of fetal body fluids.

MATERIALS AND METHODS

Animals and surgical preparation. Ten time-dated pregnant ewes (gestational age 125 ± 5, term ~145 days, singleton) were used. Animals (~50 kg Hu sheep) were housed indoors in individual steel cages (125 × 88 × 135 cm) and acclimated to a 12:12-h light-dark cycle. Both food and water were provided ad libitum, except for the last 20 h before surgery, when food was withheld. All protocols and ethical issues in use of animals in this study were approved by the institutional animal care committee of Soochow University School of Medicine (Suzhou, China).

Anesthesia was induced by an intramuscular injection of ketamine hydrochloride (20 mg/kg; Hengrui Medicine, JiangSu, China) and atropine sulfate (50 μg/kg; LintRui Pharmaceutical, ZhengZhou, China) and was maintained by maternal endotracheal ventilation with 1 l/min oxygen and 3% isoflurane. Polyethylene catheters (ID = 1.8 mm, OD = 2.3 mm) were inserted into a maternal femoral vein and
artery and advanced into the inferior vena cava and abdominal aorta, respectively. The uterus was exposed by a midline abdominal incision, and a small hysterotomy was performed to provide access to fetal hindlimbs. Polyethylene catheters (ID = 1.0 mm, OD = 1.8 mm) were inserted into the fetal femoral vein and artery. Bipolar electromyography (EMG) electrodes were placed on the fetal thyrhythmoid muscle and upper and lower esophagus for determination of swallowing activity as previously reported (21, 48). Electrodes were also implanted on the parietal dura through two Burr holes for the determination of fetal electrocortic activity (ECOG) (49). An intracranial cannula (18 gauge) was placed in the lateral ventricle and immobilized with dental cement with the assistance of two stainless-steel screws affixed in the fetus skull. The coordinates for cannula placement were anterior-posterior: +0.1 cm in the front of the bregma; medial-lateral: 0.8 cm from the middle line; and ventral: 1.8 cm below the dura (41). Patency of the catheter at insertion was assessed by free flow of cerebrospinal fluid via gravity drainage. An intraperitoneal catheter was inserted for measuring amniotic fluid pressure. The fetal bladder was catheterized (ID = 1.3 mm; OD = 2.3 mm) via cystotomy, and the fetal urachus suture was ligated to eliminate urine flow to the allantoic cavity (25). The uterine incisions were closed in layers. All catheters were passed through a subcutaneous tunnel and exteriorized through a small incision on the ewe’s flank and placed in a cloth pouch.

Five days of postoperative recovery were allowed before experimental studies. Antibiotics were administered intravenously daily to the ewe (70 mg gentamicin and 1 g oxacillin; North China Pharmaceutical, Shijiazhuang, China) and to the fetus (5 mg gentamicin and 30 mg oxacillin) for 3–4 days postsurgery.

**Behavioral and physiological experiments.** All experiments were performed on conscious animals standing in their cages, with food and water provided ad libitum. Studies began with a basal period (60 to 0 min) followed by an experimental period (0 to 120 min) in two groups (control: n = 5; experimental: n = 5). Fetal arterial blood pH was assessed and studies were undertaken only if the fetal arterial pH were ≥7.3. Beginning at time 0, carbachol (3 μg/kg; Sigma-Aldrich, St. Louis, MO) in isotonic saline (1 ml) was injected intracerebroventricularly into the fetus over 5 min. Drug doses were based on estimated fetal body weight (32). To the control animals, isotonic saline (vehicle) was injected. Throughout the basal and experimental periods, maternal and fetal arterial blood was withdrawn at timed intervals for measurement of pH, blood gases, hematocrit, plasma electrolyte composition, and osmolality. Fetal blood samples were replaced with an equivalent volume of heparinized maternal blood withdrawn before the study. In addition, fetal urine was collected into glass tubes (10 min/each tube) for determination of urine osmolality, Na+, K+, and Cl− concentrations. Blood PO2, Pco2, and pH were measured with a Nova analyzer (Nova Biochemical, Model pHOx Plus L, Waltham, MA), adjusted to sheep internal temperature (39°C). Plasma and urinary osmolality was measured by an Advanced Digmatic osmometer. Throughout the study, fetal swallowing and ECOG were measured continuously. Maternal and fetal systolic and diastolic pressure and heart rate were monitored using a Power-Lab Physiological Record with chart 5 software (AD Instruments, Sydney, Australia).

**Immunostaining experiments.** At the conclusion of the study, the animals were anesthetized and ventilated with a mixture of isoflurane and oxygen as described. A middle abdominal incision was made and the fetal head and neck were exposed. A 16-gauge needle was inserted into the fetal carotid artery for perfusion with 0.1 M phosphate-buffered saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer under anesthesia. The brain was removed immediately following perfusion. Twenty-micrometer coronal sections were cut through the fetal brain on a cryostat. Every section of the SFO was used for immunostaining (c-fos-ir) using the avidin-biotin-peroxidase technique. The tissue sections were incubated overnight at 4°C in the primary antibody (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA). The sections were further incubated in a goat anti-rabbit serum (1:1,000) for 1 h and then processed using the Vectastain ABC kit for 1 h (Vector Laboratories, Burlingame, CA). The sections were then treated with 1 mg/ml diamonobenzidine tetrahydrochloride (0.02% hydrogen peroxide; Sigma-Aldrich). All sections were mounted on slides, dehydrated in alcohol, and then coverslipped.

### Table 1. Fetal and maternal arterial blood and cardiovascular values before and after fetal intracerebroventricular injection of carbachol

<table>
<thead>
<tr>
<th></th>
<th>Babasal</th>
<th>15 min</th>
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<td>Hct, %</td>
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Carbachol, 3 μg/kg, MAP, mean arterial pressure; HR, heart rate; Hct, hematocrit; Hb, hemoglobin. Values are expressed as means ± SE; n = 5. *P < 0.01 compared to the basal level.
Data analysis. All signals were determined by computer analysis of waveforms by using a PowerLab system with Chart 5 software (AD Instruments). An EMG-propagated swallow, representing a coordinated laryngeal-esophageal contraction, was defined by a time sequence of integrated EMG signals from the thyrohyoid muscle to the upper and lower nuchal esophagus (39). Fetal ECoG activity was assessed by visual analysis and was divided into periods of low voltage (LV) and high voltage (HV). Periods of ECoG activity that did not belong to either LV or HV activity were considered to be intermediate ECoG (<5% of total ECoG). Intermediate ECoG constituted less than 5% of the total electrocortical activity and was not considered. Total swallowing activity was calculated as defined above and expressed as swallows per minute. The percentage of swallows associated with each electrocortical state was then calculated.

The number of c-fos-ir-positive cells in the brain was evaluated quantitatively using National Institutes of Health imaging analysis software and in a blinded manner (48). SPSS software was used for statistical analysis. A repeated-measures ANOVA was used to determine differences over time and effects of the treatments. Comparison before and after the treatments was determined with one-way ANOVA followed by the Tukey post hoc test. All data were expressed as means ± SE.

RESULTS

Blood values. Histological analysis confirmed that all intracerebroventricular cannulae were inserted into the fetal lateral ventricle. For both the control and experimental animals, intracerebroventricular injection of carbachol (3 µg/kg) or vehicle had no effect on maternal or fetal plasma osmolality, Na⁺, K⁺, Cl⁻ concentrations, and arterial blood pH, P[subscript O2], P[subscript CO2]. All arterial values were within normal ranges and were not different between the control and experimental groups (Table 1). There was no difference in either maternal mean arterial pressure or heart rate between the control and experimental groups. However, intracerebroventricular injection of carbachol significantly increased fetal mean arterial pressure and decreased fetal heart rate (Table 1).

Swallowing activity. During the basal period in the control and experimental fetal groups, normal swallowing rates in LV and HV ECoG were not changed (Fig. 1, A and B). However, in the first 15 min (0–15 min) after carbachol injection, the fetal swallowing significantly increased to 4.2 ± 0.5/min LV ECoG (P < 0.05, Fig. 1A). During the first hour (0–1 h)
following intracerebroventricular carbachol, the swallowing increased to 4.9 ± 0.8 swallows/min LV ECoG (Fig. 1A and Fig. 2). In the second hour after intracerebroventricular injection, the increased swallowing activity still maintained at a high level (3.7 ± 0.6/min LV ECoG). Swallowing activity during HV ECoG was not significantly changed (Fig. 1B). In addition, central carbachol administration had no significant change on the percentage of LV and HV ECoG (Fig. 3).

Fetal urine flow and electrolyte excretion. There was no significant difference in urine flow rate, osmolality, Na⁺, K⁺, and Cl⁻ concentrations between the control and the experimental fetuses prior to intracerebroventricular injections of vehicle or carbachol. In the control group, no change of the urine values was observed before and after the intracerebroventricular injection. However, in the experimental group, the urine flow rate significantly increased after intracerebroventricular carbachol. It reached the peak at the 60 min after intracerebroventricular injection and then gradually returned to the basal level by 120 min (Fig. 4). The urine osmolality, Na⁺, K⁺, and Cl⁻ concentrations were all significantly elevated following the central administration of carbachol and were sustained at the high level even at 120 min after the injection (Fig. 4B and Fig. 5). We observed that the Na⁺, K⁺, and Cl⁻ excretion was increased in the first hour and gradually decreased in the second hour after intracerebroventricular carbachol. However, they were still significantly higher at 120 min after central administration of carbachol than the basal level before intracerebroventricular injection.

c-fos-immunoreactivity. In the control fetuses, there was none or few c-fos-ir in SFO. However, intracerebroventricular carbachol produced intense c-fos-ir in the SFO (Fig. 6). There was significant difference of c-fos-ir in the SFO between the intracerebroventricular vehicle and the intracerebroventricular carbachol injected fetuses (P < 0.01).

DISCUSSION

The present study demonstrated four important and novel observations. First, intracerebroventricular injection of carbachol induced a significant increase of swallowing activity in the fetus near term. Second, the swallowing activity occurred in LV ECoG was increased significantly; Third, central carbachol stimulated diuresis, natriuresis, and kaliuresis in the near-term fetus. Fourth, the neural activity labeled with c-fos-ir in the putative dipsogenic center SFO was significantly enhanced. Although numerous studies showed central cholinergic activity plays an important role in the control of body fluid homeostasis in adults, as far as we know, this was the first study to investigate the link between central carbachol and the fetal behavioral activity (swallowing) and renal excretion in utero. These results indicate that central cholinergic mechanisms become functional in the ovine fetus serving the regulation of body fluid homeostasis at least at near term. The information added knowledge in the functional development of cholinergic mechanisms-mediated fetal renal regulations and swallowing responses.

Fetal swallowing activity induced by intracerebroventricular injection of carbachol. Swallowing is an intrinsic fetal behavior that develops in utero in ovine or human fetuses in preparation for neonatal food and fluid ingestion. Swallowing of amniotic fluid serves to regulate amniotic fluid volume and promotes fetal gastrointestinal growth and development (27, 30). In sheep and perhaps human fetuses, dipsogenic-mediated swallowing responses develop during the last third of gestation (34). In utero swallowing could be stimulated by intravenous or intracerebroventricular hypertonic saline (33) and by intracerebroventricular ANG II (48). However, the central cholinergic-mediated fetal swallowing and renal function is not clear. Carbachol, a cholinergic agonist, is also a putative stimulator of water intake. When centrally administered in mature rats, in addition to pressor responses and vasopressin secretion, carbachol has been repeatedly shown to produce a reliable dipsogenic and natriuretic response (2, 7, 23, 36, 44). Central injection of atropine in rats, a cholinergic muscarinic receptor antagonist, completely inhibits carbachol-induced drinking (9, 22). In fetuses, a previous study has shown that cholinergic

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Thus, we focused on LV and HV ECoG only. Although there was a small percentage of intermediate ECoG activity and that alterations in fetal electrocortical activity may affect fetal swallowing by using intravenous injection of cholinergic antagonist atropine sulfate in sheep (28). To our knowledge, the present study was the first to examine the fetal swallowing and renal responses in response to direct central cholinergic stimulation signals in utero. Central mechanisms play an important role in dipsogenic responses and renal regulation (1, 8). Thus, the data provide new information in the control of swallowing and renal regulation in fetal neurophysiology.

In the present study, fetal swallowing values were similar to those observed in previously reported data in near-term fetuses (48, 49). Previous studies also have demonstrated that fetal swallowing activity occurred in association with electrocortical activity and that alterations in fetal electrocortical activity may affect fetal swallowing activity by using intravenous injection of cholinergic antagonist atropine sulfate passing the blood-brain barrier vs. atropine methyl nitrate acting peripherally only in fetal sheep (28). Because fetal swallowing occurs predominantly during LV ECoG periods (14), change of the relative duration of LV periods may influence the swallowing rate. Although there was a small percentage of intermediate ECoG status in the fetus, which was not changed following the treatment. Thus, we focused on LV and HV ECoG only.

Fetal swallowing may be regulated by multiple factors, including dipsogenic stimulation, appetite, amniotic fluid availability, and behavioral status (3, 14, 21). In the present study, we used c-fos mapping technique to determine fetal central nervous activity that correlated to stimulated swallowing responses. C-fos expression has been well described as a marker of neuronal activation in the brain nuclei such as rats (13). A number of studies confirmed dipsogenic areas in the adult brain following stimulation by carbachol (23, 37). This provided a reference for comparisons in examining fetal nuclei activity after administration of carbachol. The SFO is demonstrated to be a key receptor site for intracerebroventricular injected cholinergic agonists (36) and is replete with cholinergic receptors. For example, microinjection of carbachol into the SFO of the rat results in fluid conservation responses, including increased water intake (4). The induced intense c-fos-ir in the SFO in the present study is evidence that this area was activated by intracerebroventricular carbachol. Associated with the stimulated swallowing activity in the fetus, the c-fos results suggest that the critical area in the central nervous system for cholinergic-mediated dipsogenic mechanisms is functional near term. Martin (24) reported that microinjection of carbachol directly into the posterior hypothalamic nucleus of conscious rats evokes a dose-dependent increase in mean arterial pressure that may involve neuropeptide Y (NPY). Their finding showed that cholinergic activity occurred in the adult brain in cardiovascular regulation possibly via NPY. Whether the central activity marked by c-fos in the present study was related to NPY needs further investigation.

Previous functional experiments in the near-term ovine fetus have shown that several dipsogens can stimulate fetal swallowing activity. For example, fetal swallowing activity could be stimulated by systemic hyperosmolality (33, 49) in the ovine fetus. However, in the present study, the fetal physiological status remained stable as arterial values (particularly plasma osmolality and Na⁺ concentrations) did not change in response to intracerebroventricular injection of carbachol, thereby excluding the possibility of contribution of systemic
sodium/osmolality to the stimulated-swallowing activity. The unchanged arterial PO$_2$ and PCO$_2$ also can exclude the possibility of hypoxia that might stimulate fetal swallowing as previously reported (40).

Besides osmolality and hypoxia, change of blood pressure also can influence water intake. It is well known that hypotension facilitates drinking behavior, whereas hypertension inhibits it (18). In the present and our recent studies (42), fetal mean arterial pressure was increased by intracerebroventricular application of carbachol, as has been demonstrated in adult animals (15, 16). However, fetal swallowing after central administration of carbachol was significantly increased despite an increased fetal blood pressure. This suggests that ovine fetal dipsogenic mechanisms in response to carbachol stimulation are well developed near term. Despite the potential inhibitory effects of increased blood pressure, the fetal central dipsogenic mechanisms still functioned, as evidenced by increased fetal swallowing.

**Fetal urine flow and electrolyte excretion induced by intracerebroventricular injection of carbachol.** The present study demonstrates that central administration of carbachol increases fetal urine production and alters sodium, potassium, and chloride excretion. In fetuses, several investigations have shown that fetal renal functions (such as urinary flow rate, free water clearance, and urine osmolality) can be affected by hemorrhage and hyperosmotic mechanisms (6, 11, 29), indicating that volume and osmotic mechanisms in the control of the renal fluid-electrolyte excretion develop in utero.

Central administration of carbachol induces diuretic, natriuretic, and kaliuretic responses in adults (43–45). An increase of blood pressure may affect renal excretion (5). This also was possible in the fetus in the present study since intracerebroventricular carbachol-increased fetal urine was accompanied with an increase of blood pressure. Fetal urine flow rate, Na$^+$, K$^+$, and Cl$^-$ excretions were markedly increased after intracerebroventricular carbachol, and they reached the maxima about 1 h after the injection and gradually decreased in the second hour. It is interesting to note that 120 min after intracerebroventricular carbachol injection, accelerated urine flow almost returned to the basal level, while electrolyte excretions were still higher than that during the basal period. It seems the diuretic response was more transient than the promotion of the electrolyte excretion. The results

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**Fig. 5.** Fetal urine electrolyte concentrations and renal excretion before and after i.c.v. injection of carbachol into the ovine fetuses. *A–C.* urine sodium, potassium, chloride concentrations, respectively. *D–F.* renal sodium, potassium, chloride excretion. *P* < 0.05, **P** < 0.01 compared with the basal level. Carbachol, 3 μg/kg. For each group, *n* = 5.
showed a relatively longer period of sustained high level of fetal urine osmolality and Na⁺ concentrations, indicating there could be an increase of solute-free water reabsorption during this period.

Although it was argued that the blood-brain barrier (BBB) in the developing brain may be different in important respects from those in the adult brain, it is noted that the underlying morphological feature of the BBB is the presence of tight junctions, which locate between cerebral endothelial cells and between choroid plexus epithelial cells. These junctions are present in blood vessels in the fetal brain. They are effective in restricting entry of proteins from cerebrospinal fluid into blood at 90% gestational age in rats (38). The previous study has demonstrated that the BBB is relatively impermeable to low-molecular weight amino acids even at 60% of gestation in sheep (47). Thus, the swallowing activity and renal responses following intracerebroventricular carbachol were unlikely to be caused by possible leakage of carbachol from the fetal brain.

**Perspectives and Significance**

The present study demonstrated that intracerebroventricular injection of cholinergic agonist carbachol can produce a pronounced swallowing activity, diuretic, natriuretic, and kaliuretic responses associated with neural activation in the SFO in the near-term fetus. The results are important to increase our understanding of the functional developmental level of brain cholinergic mechanisms mediating drinking behavior and renal functions at near term. The data not only provide additional evidence of central cholinergic signal-induced neuronal activity in the SFO in utero, but also offer an opportunity for further investigation of mechanisms that may influence the development of fetal central cholinergic and neuroendocrine systems in light of programming of diseases in later life. Information gained aids in understanding the development of central cholinergic mechanisms in the control of body fluid homeostasis, which is important not only in prenatal life but also in postnatal health.

**REFERENCES**


