Neuronal NO modulates spontaneous and ANG II-stimulated fetal swallowing behavior in the near-term ovine fetus

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El-Haddad, Mostafa A., Conrad R. Chao, Sheng-Xing Ma, and Michael G. Ross. Neuronal NO modulates spontaneous and ANG II-stimulated fetal swallowing behavior in the near-term ovine fetus. Am J Physiol Regulatory Integrative Comp Physiol 282: R1521–R1527, 2002; 10.1152/ajpregu.00229.2001.—Spontaneous fetal swallowing occurs at a markedly higher rate compared with spontaneous adult drinking activity. This high rate of fetal swallowing is critical for amniotic fluid volume regulation. Central NO is critical for maintaining the normal rate of fetal swallowing, as nonselective inhibition of NO (with central N\textsuperscript{G}-nitro-L-arginine methyl ester) suppresses spontaneous and angiotensin II (ANG II)–stimulated swallowing. We sought to differentiate the contributions of central endothelial vs. neuronal NO in the regulation of spontaneous and stimulated fetal swallowing, using a selective neuronal NO synthase (nNOS) inhibitor. Six time-dated pregnant ewes and fetuses were chronically prepared with fetal vascular and intracerebroventricular (icv) catheters and electrocorticogram (ECoG) and esophageal electromyogram electrodes and studied at 130 ± 1 days of gestation. After an initial 2-h baseline period (0–2 h), the selective nNOS inhibitor \textit{N}-propyl-L-arginine (NPLA) was injected icv (2–4 h). At 4 h, the dose of NPLA was repeated, together with ANG II, and fetal swallowing was monitored for a final 2 h. Four fetuses also received an identical control study (on an alternate day) in which NPLA was replaced with artificial cerebrospinal fluid (aCSF). Suppression of nNOS by icv NPLA significantly reduced mean (± SE) spontaneous fetal swallowing (1.35 ± 0.12 to 0.50 ± 0.07 swallows/min; \(P < 0.001\)). Injection of ANG II in the presence of NPLA had no diposogenic effect on fetal swallowing (0.68 ± 0.09 swallows/min). In the aCSF study, icv aCSF did not change fetal swallowing (0.93 ± 0.10 vs. 0.95 ± 0.09 swallows/min), whereas icv ANG II resulted in a significant increase in the rate of fetal swallowing (2.0 ± 0.04 swallows/min; \(P = 0.001\)). We speculate that the suppressive diposogenic effects of central NPLA indicate that spontaneous and ANG II–stimulated fetal swallowing is dependent on central nNOS activity.

amniotic fluid; angiotensin II

**THIRST AND APPETITE-MEDIATED**

Ingestive behavior develop and are likely imprinted in utero, thus preparing for newborn and adult ingestive behavior. Fetal swallowing activity is markedly different from that of the adult, as basal fetal swallowing occurs at a markedly (6-fold) higher rate compared with basal adult drinking activity after body weight differences are adjusted for (35). This high rate of basal fetal swallowing is critical for the regulation of amniotic fluid volume (30) and the development of the fetal gastrointestinal tract. Disordered fetal swallowing has been associated with both a decrease (oligohydramnios) and an increase (polyhydramnios) in amniotic fluid volume (11, 18). Both conditions are associated with a significant increase in perinatal morbidity and mortality, and limited treatment modalities are currently available. However, the mechanisms underlying the high rate of basal human fetal swallowing are poorly understood.

Behavioral studies in the adult rat have demonstrated important roles for the central ANG II receptor subtype 1 (AT1), glutamate N-methyl-D-aspartate (NMDA) receptor subtype 1 (NMDA-NR1), and central NO synthase (NOS) in the regulation of stimulated, but not basal, adult drinking (1, 40, 43). Our studies in the ovine fetus have similarly demonstrated an important role for ANG II, glutamate NMDA receptors, and NO in regulating both basal and stimulated fetal swallowing (7–9). Therefore, it appears that the high rate of basal fetal swallowing is regulated similarly to stimulated adult drinking, both requiring an upregulation of ANG II, glutamate, and NO pathways within the diposogenic neurons.

NO is a reactive gas molecule formed in both adult and fetal brains in response to activation of two important constitutional NOS enzymes, 1) neuronal (nNOS) and 2) endothelial NOS (eNOS) (3). We have previously demonstrated a critical role of NO in the regulation of ovine fetal swallowing. Central \(\textit{N}^\text{G}\)-nitro-L-arginine methyl ester (L-NAME), a nonspecific inhibitor of both nNOS and eNOS, decreases both spontaneous and ANG II–stimulated fetal drinking (8).

In this experiment, we hypothesized that neuronal NO, rather than the endothelial NO, contributes to the regulatory mechanism of NO-mediated fetal swallowing. The aim of this study was to determine the relative contribution of nNOS to the heightened level of spontaneous and ANG II–stimulated fetal swallowing. We utilized the highly selective nNOS inhibitor \(\textit{N}\)-propyl-
l-arginine (NPLA), which has maximal nNOS inhibitory effects with minimal eNOS inhibition.

MATERIALS AND METHODS

Six time-dated pregnant ewes with singleton fetuses (130 days’ gestation on the first study day) were studied. Animals were housed indoors in individual steel study cages and acclimated to a 12:12-h light-dark cycle. Surgical procedures and studies were approved by the Harbor-University of California at Los Angeles Animal Use Committee. Food (alfalfa pellets) and water were provided ad libitum, except for the witholding of food for 24 h before surgery.

Surgical Preparation

Anesthesia was induced with ketamine hydrochloride (15–20 mg/kg im). General anesthesia was maintained with 1–2% isoflurane and 1 l/min of O2. The uterus was exposed by a midline abdominal incision, and a small hysterotomy was performed to expose the fetal hindlimb. Polyethylene catheters with inner diameter (ID) 0.040 in. and outer diameter (OD) 0.070 in. were placed in the fetal femoral vein and artery and threaded to the inferior vena cava and abdominal aorta, respectively. Surgical placement of bipolar electromyography (EMG) electrodes (thyrohyoid muscle, upper and lower nuchal esophagus) for determination of swallowing activity was performed as previously described (36). Electrodes were also implanted on the parietal dura through two drilled burr holes (5 mm above the bregma and 10 mm from each side of the sagittal suture) for the determination of fetal electrocortical activity (ECoG). An 18-gauge needle connected to a polyethylene catheter (0.02 in. ID, 0.04 in. OD) was inserted into the lateral ventricle that was identified 20 mm above the lambdoidal suture and 5 mm lateral to the sagittal suture. The lateral ventricle needle and the dural electrodes were immobilized with dental cement with the assistance of two stainless steel screws fixed in the skull. An intrauterine catheter (0.125 in. ID, 0.25 in. OD; Corometrics Medical System, Wallingford, CT) was inserted for amniotic fluid pressure measurement. Uterine and maternal incisions were repaired and all catheters and electrodes externalized to the maternal flank and placed in a cloth pouch. Catheters also were placed in the maternal femoral vein and artery. Animals were allowed ≥5 days for postoperative recovery, which included catheter maintenance and antibiotic administration (11).

Study Protocol

The study protocol was conducted over 2 days. The 1st day of the study (n = 4), the effects of central artificial cerebrospinal fluid (aCSF) and ANG II on fetal swallowing were examined, whereas the 2nd day (n = 6), the effect of central injection of NPLA (selective nNOS inhibitor) on spontaneous and ANG II-stimulated fetal swallowing was examined. Studies were initiated with the ewes standing in the same individual cages in which they were maintained before the study. An equilibration period included preparation of the animals for the experiment and confirmation of starting criteria (fetal arterial pH > 7.30). All doses were injected slowly over 5 min. The same animals were used on alternate days to examine the effects of ANG II receptor blockade and NMDA antagonist on fetal swallowing. The 1st study day (aCSF and ANG II) was also utilized as a control day for these experiments. Results of these experiments are reported separately.

aCSF/ANG II study. After a 2-h baseline period (0–2 h), aCSF (1ml; Na 145, K 3.3, Ca 121, Mg 2.2, Cl 121, and HCO3 25 meq/l; osmolality 297 mOsmol/kg) was injected into the intracerebroventricular space, and the fetuses were monitored for 2 h. At 4 h, ANG II (1 ml, 6.4 μg) was injected intracerebroventricularly and fetuses monitored for a final 2 h.

nNOS study. After a 2-h baseline period (0–2 h), the selective nNOS inhibitor (NPLA; 6 μg, 1 ml) was injected intracerebroventricularly, followed 2 h later with an intracerebroventricular injection of 1 ml of both NPLA (6 μg) and ANG II (6.4 μg), and fetuses were monitored for a final 2 h. The dose of NPLA was picked up on the basis of a comparison of the inhibition constant (the dose required to block 50% of the enzyme activity in vitro) for both NPLA (57 nM) and l-NAME (70 μM). NPLA is an ~800-fold more potent inhibitor of nNOS than l-NAME. We have previously demonstrated that intracerebroventricular l-NAME in a dose of 1 mg/kg was sufficient to block central NOS activity. Considering the potency and the selectivity of NPLA as nNOS inhibitor compared with l-NAME, we have estimated that 2 μg/kg of NPLA would achieve adequate suppression of central nNOS.

Throughout the experiments, fetal swallowing and ECoG activities, fetal and maternal arterial blood pressures and heart rates, and amniotic fluid pressure were monitored continuously. Maternal and fetal blood samples were collected at 60 and 120 min of the control and study periods for measurement of hematocrit, blood gases, osmolality, electrolytes, and arginine vasopressin (AVP).

Analytical Methods

Fetal body weight was estimated by the formula of Robillard et al. (29). Maternal and fetal blood samples were collected into iced tubes containing 10 U/ml lithium heparin. Blood aliquots were assessed for hematocrit, pH, PO2, and PCO2, the remaining blood was centrifuged, and plasma osmolality and sodium, chloride, and potassium concentrations were measured. Maternal and fetal plasma aliquots were stored at −20°C until assayed for AVP. Fetal blood samples were replaced with an equivalent volume of heparinized maternal blood withdrawn before the study, and maternal blood samples were replaced with an equivalent volume of 0.15 M saline.

Blood PO2, PCO2, and pH were measured at 39°C with a NOVA Stat 3 blood gas analyzer system (Nova Biomedical, Waltham, MA). Plasma osmolality was measured by freezing-point depression on an Advanced Digimatic osmometer (model 3 MO, Advanced Instruments, Needham Heights, MA). Plasma sodium, potassium, and chloride concentrations were determined by a NOVA 5 electrolyte analyzer (Nova Biomedical). Fetal and maternal blood pressures and amniotic pressures were monitored continuously by means of World Precision Instruments (Sarasota, FL) signal conditioners and transducers. Fetal blood pressure was corrected for amniotic cavity pressure. One-minute segments of fetal blood pressure monitor were analyzed at 1 and 2 h (control period) and 5, 10, 15, 30, 60, and 120 min after drug injections. For the analysis of plasma AVP, the samples were collected into an ice-chilled glass tube containing 10 IU of heparin and 500 IU of aprotinin per milliliter of blood. Samples were extracted by a modification of the procedure of La Rochelle et al. (19), and AVP levels were determined by radioimmunoassay (37). Plasma AVP recoveries in our laboratory averaged 70%.
Digitization of all signals was performed at a rate of 75 Hz. EMG and ECoG signals were directed into a Grass physiological recorder and directed into a Windaq (Dataq Instruments, Akron, OH) analog-digital system. 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. The time at the onset of each swallow was stored for later analysis, and swallowing activity was expressed as swallows per minute.

Fetal ECoG 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 clearly belong to either LV or HV activities were considered intermediate ECoG activity. Intermediate ECoG activity constituted <5% of the total ECoG activity and was not considered in the analysis of the data. Total swallowing activity was calculated as defined above and expressed as swallows per minute and swallows per minute ECoG state.

Statistical Analysis

For each animal, swallowing rates per minute, percent time spent in each ECoG state, and percent swallowing in each state were calculated for the control and drug exposure periods. Mean blood pressure and heart rate values were similarly determined for each period. Comparisons were made using one-way repeated-measures ANOVA with Tukey's or Dunnett's test for post hoc analysis. All values are presented as means ± SE, and P < 0.05 was considered statistically significant.

RESULTS

aCSF/ANG II Study

Fetal swallowing. In the aCSF/ANG II study (n = 4), intracerebroventricular aCSF did not change total fetal swallowing (0.93 ± 0.10 vs. 0.95 ± 0.09 swallows/min), whereas intracerebroventricular ANG II resulted in a significant increase in the rate of fetal swallowing (2.0 ± 0.04 swallows/min; P = 0.001, Fig. 1). Fetal swallowing occurred primarily during LV ECoG activity (control, 1.2 ± 0.1; aCSF, 1.3 ± 0.1; ANG II, 2.9 ± 0.1 swallows/min LV ECoG; P = 0.0002). Fetal swallowing also increased during HV ECoG activity, yet not significantly, after intracerebroventricular ANG II (control, 0.28 ± 0.06; aCSF, 0.28 ± 0.07; ANG II, 0.65 ± 0.09 swallows/min HV ECoG).

Fetal ECoG and cardiovascular and blood changes. Fetal ECoG activity did not significantly change during the aCSF/ANG II study. The percent time spent in LV ECoG was stable during the control period (62 ± 1%) and after aCSF (55 ± 2%) and ANG II (57 ± 4%) injections (P = nonsignificant (NS)). Fetal mean blood pressure (BP) did not significantly change after intracerebroventricular aCSF (49 ± 1 vs. 53 ± 2 mmHg), whereas intracerebroventricular ANG II significantly increased mean fetal BP (60 ± 6 mmHg; P < 0.05 vs. control, Fig. 2). Fetal heart rate (HR) significantly decreased after intracerebroventricular ANG II injection (from 180 ± 8 to 158 ± 5 beats/min; P = 0.02).

Fetal hematocrit (30 ± 1%), pH (7.37 ± 0.01), PO2 (20 ± 2 mmHg), Pco2 (46 ± 2 mmHg), plasma osmolality (300 ± 2 mOsmol/kg), and electrolyte concentrations did not change during the aCSF/ANG II study. AVP concentration did not change after intracerebroventricular aCSF (3 ± 0.5 vs. 5 ± 1.5 pg/ml), whereas it significantly increased after intracerebroventricular ANG II (from 3.2 ± 0.4 to 34.9 ± 13.7 pg/ml; P = 0.03). Maternal values did not significantly change from basal values throughout the aCSF/ANG II study (mean BP, 105 ± 2 mmHg; HR, 118 ± 5 beats/min; hematocrit, 29 ± 1%; pH, 7.47 ± 0.01; Pco2, 33 ± 2 mmHg; plasma osmolality, 305 ± 2 mOsmol/kg; sodium, 147 ± 2 meq/l; chloride, 112 ± 2 meq/l; potassium, 4.4 ± 0.2 meq/l; AVP, 3.7 ± 1.0 pg/ml).

NPLA Study

Fetal swallowing. Suppression of nNOS by intracerebroventricular NPLA significantly reduced mean spontaneous fetal swallowing (from 1.35 ± 0.12 to 0.50 ± 0.07 swallows/min; P = 0.001, Fig. 3). Intracerebroventricular injection of ANG II in the presence of NPLA had no dipsogenic effect on fetal swallowing (0.68 ± 0.09 swallows/min, Fig. 3). Fetal swallowing occurred primarily during LV ECoG activity (control, 1.6 ± 0.1; NPLA, 0.7 ± 0.1; ANG II, 0.9 ± 0.1 swallows/
Fetal swallowing develops in utero and occurs at a much higher rate compared with adult spontaneous drinking activities. The high rate of fetal swallowing is critical for maintaining a normal volume of amniotic fluid (30) and thus normal pregnancy outcomes. Various neurotransmitter systems likely develop in utero and contribute to the regulation of fetal drinking activity. NO, a reactive gas molecule, acts in the brain as a neuromodulator rather than as a conventional neurotransmitter. NO is formed in both adult and fetal brains in response to activation of two important NOS enzymes, nNOS and eNOS. nNOS and eNOS have been localized to discrete areas in the adult brain, including the dipsogenic centers (2, 39). In human fetal brain, the distribution of nNOS neurons is similar to that found in the adult brain; however, the overall density is higher in the fetus (26). Thus nNOS in fetal life may contribute to neuronal maturation and play an important role in the development of human brain functions, including the high rate of fetal swallowing activity.

It is believed that drinking behavior is triggered by accumulation of presynaptic ANG II, releasing presynaptic glutamate, which binds to postsynaptic glutamate NMDA receptors with subsequent calcium influx, activation of nNOS, and NO release (13). Neuronal NO diffuses readily through cell membranes in a retrograde pattern (34), affecting large numbers of neighboring synaptic functions (12). Once inside the target neurons, NO may potentiate the release of fast-signalizing, excitatory neurotransmitters, including glutamate, from the presynaptic nerve endings (22, 28, 34), thus potentiating synaptic activity. Although the mechanism of the dipsogenic action of central NO is likely not mediated by soluble guanylyl cyclase, other signaling pathways such as cyclic adenosine diphosphate ribosyl cyclase (33, 38) may be responsible for the dipsogenic action.

We have previously demonstrated that central L-NAME decreases both spontaneous and ANG II-stimulated fetal drinking (8, 9). As a nonselective NOS inhibitor, L-NAME blocks both cerebral eNOS and nNOS. Through eNOS-mediated actions, L-NAME may increase cerebrovascular resistance and reduce local cerebral blood flow (10), potentially altering swallowing behavior. Although an important role of central NOS in fetal swallowing has been demonstrated, there was little information as to whether this was primarily an nNOS or an eNOS action. In the present study, we specifically investigated the role of nNOS in the regul-
loration of spontaneous and ANG II-stimulated fetal swallowing. We sought to remove potential tonic-stimulatory effects of nNOS on spontaneous fetal swallowing by utilizing the highly selective nNOS inhibitor NPLA. We chose NPLA as a selective nNOS inhibitor because of its highly selective nNOS inhibitory activity (42). Zhang et al. (42) recently identified water-soluble NPLA as a highly selective nNOS inhibitor with a potency of nNOS (bovine brain) inhibition 3,158 times that of iNOS and 149-fold that of eNOS. Other N\textsuperscript{ω}-substituted L-arginine analogs have far less nNOS inhibition selectivity (42). Although 7-nitroindazole is the most commonly used nNOS inhibitor in experimental animals, it has not been used centrally. Furthermore, 7-nitroindazole does not dissolve in water but requires dimethyl sulfoxide (DMSO), which has anti-inflammatory, antioxidant and local anesthetic biological effects. These effects of DMSO may likely alter cellular responses if injected intracerebroventricularly.

The results of the present study indicate that the nNOS, rather than the eNOS, is responsible for mediating spontaneous and ANG II-stimulated fetal swallowing. Although fetal electrocortical activity and systemic blood pressure are possible confounders that may indirectly influence fetal swallowing, there was no significant effect of NPLA on fetal ECoG or blood pressure. The stimulatory role of brain-derived NO on fetal swallowing activity is consistent with our previous data in the fetal sheep (8). However, studies in the adult rat have been conflicting. Liu et al. (21) and Zhu and Herbert (43) demonstrated that intracerebroventricular injection of l-NAME inhibits water intake in response to systemic hypertonicity and central ANG II, respectively. A stimulatory role of NO on adult rat drinking behavior was further supported by Kadecarlo et al. (16) and Kannan et al. (17). Conversely, Roth and Rowland (31) and Calapai and Caputi (5) have suggested that increased central NO activity by intracerebroventricular l-arginine inhibits water intake induced by dehydration and central ANG II. The reason for the discrepancies in NOS effects on adult rat water intake is unknown. However, NO demonstrates both stimulatory (23) and inhibitory (24) actions on nerve synapses. Thus local neuronal activation, sites of action, dosage, and species differences may contribute to response variation in the adult.

In the present study, there was no significant effect of NPLA on basal fetal blood pressure. Although there have been no previous studies in the fetus examining the effect of central inhibition of nNOS on systemic blood pressure, we previously demonstrated that central administration of the nonselective central NOS inhibitor (l-NAME) does not alter fetal blood pressure (8). Under physiological conditions, NO inhibits sympathetic vasoconstrictor influences both by reducing the release of noradrenaline from postganglionic sympathetic fibers and by attenuation of neuronal sympathetic excitability within the medullary areas that regulate sympathetic outflow from the brain stem (41). In the adult rat, central injection of the nonselective NOS blocker (l-NAME) has been reported to increase (25) or not change (39) arterial blood pressure. In humans, NO does not appear to be involved in the tonic restraint of central sympathetic outflow (14). The difference between our results and those in adult rats may be explained, in part, by differences between fetal and adult central control of basal blood pressure; an endogenous excitatory pathway that is permissive for sympathoexcitation after NO inhibition may be lacking in the fetus. Furthermore, interspecies developmental differences in nNOS expression may exist. Intracerebroventricular injection of aCSF produced a nonsignificant, transient increase in fetal blood pressure. Although the injection was performed very slowly over 5 min, it remains possible that the intracerebroventricular injection of aCSF may have been responsible for this nonsignificant increase in fetal blood pressure. As expected, central injection of ANG II significantly increased fetal blood pressure. Central injection of ANG II in the presence of blocked nNOS continued to have a similar pressor effect to that in the control study, suggesting that central nNOS does not contribute to the neural pathway regulating the pressor effect of central ANG II.

Evidence implicates NO as a modulator of AVP secretion. NOS proteins and mRNA have been identified in the neural circuitry for regulation of AVP secretion (15). A primary function of centrally released AVP is to regulate arterial blood pressure (6). In our aCSF/ANG II study, ANG II significantly increased fetal plasma AVP concentration, consistent with our previous data (9). In the experimental study, central nNOS blockade did not influence the basal plasma AVP levels, whereas ANG II injection in the presence of blocked nNOS failed to produce the same AVP-releasing effect shown in the control study. Our data indicate that brain-derived NO does not contribute to the basal release of AVP, whereas the neuronal pathway implicated in the ANG II-releasing effect of AVP does require NO-induced synaptic potentiation. Previous data regarding the role of central NO in regulating the release of AVP have been conflicting, with the central nonselective NOS inhibitor (l-NAME) causing both stimulation (27) and suppression (20) of AVP secretion. As noted, these discrepancies may perhaps be explained by dose differences and specificity of the site of action of l-NAME.

In conclusion, the present results demonstrate that the high rate of spontaneous and ANG II-mediated fetal swallowing is dependent on central nNOS activity. Furthermore, the AVP response to central ANG II is also mediated via nNOS activity. On the contrary, basal control of fetal blood pressure, ANG II pressor effect, and basal AVP release do not appear to be mediated via nNOS activity.

**Perspectives**

Spontaneous fetal swallowing occurs at a markedly higher rate compared with spontaneous adult drinking activity. This high rate of fetal swallowing...
is critical for amniotic fluid volume regulation and fetal gastrointestinal tract development. Our laboratory has explored the neuronal mechanisms responsible for regulating spontaneous fetal swallowing. Collectively, we have demonstrated that nNOS, ANG II, and glutamate NMDA receptors are essential components in the pathway(s) mediating the high rate of fetal swallowing. It is also likely that other neurotransmitters (i.e., cholinergic and adrenergic) contribute in parallel or in continuation with these factors in regulating spontaneous fetal swallowing. Fetal relative overexpression of nNOS, ANG II, and glutamate NMDA receptors may explain, in part, the high rate of fetal swallowing.

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REFERENCES


