Central angiotensin II AT1 receptors mediate fetal swallowing and pressor responses in the near-term ovine fetus

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El-Haddad, Mostafa A., Yaser Ismail, Dave Gayle, and Michael G. Ross. Central angiotensin II AT1 receptors mediate fetal swallowing and pressor responses in the near-term ovine fetus. *Am J Physiol Regul Integr Comp Physiol* 288: R1014–R1020, 2005. First published November 18, 2004; doi:10.1152/ajpregu.00479.2003.—Swallowed volumes in the fetus are greater than adult values (per body weight) and serve to regulate amniotic fluid volume. Central ANG II stimulates swallowing, and nonspecific ANG II receptor antagonists inhibit both spontaneous and ANG II-stimulated swallowing. In the adult rat, AT1 receptors mediate both stimulated drinking and pressor activities. Five pregnant ewes with singleton fetuses (130 ± 1 days) were prepared with fetal vascular and lateral ventricle (LV) catheters and electrocorticogram and esophageal electromyogram electrodes and received three studies over 5 days. On *day 1* (ANG II), following a 2-h basal period, 1 ml artificial cerebrospinal fluid (aCSF) was injected in the LV. At *time 4 h*, ANG II (6.4 μg) was injected in the LV, and the fetus was monitored for a final 2 h. On *day 3*, AT1 receptor blocker (losartan 0.5 mg) was administered at 2 h, and ANG II plus losartan was administered at 4 h. On *day 5*, AT2 receptor blocker (PD-123319; 0.8mg) was administered at 2 h and ANG II plus PD-123319 at 4 h. In the ANG II study, LV injection of ANG II significantly increased fetal swallowing (0.9 ± 0.1 to 1.4 ± 0.1 swallows/min; *P* < 0.05). In the losartan study, basal fetal swallowing significantly decreased in response to blockade of AT1 receptors (0.9 ± 0.1 to 0.4 ± 0.1 swallows/min; *P* < 0.05), while central injection of ANG II in the presence of AT1 receptor antagonism did not increase fetal swallowing (0.6 ± 0.1 swallows/min). In the PD-123319 study, basal fetal swallowing did not change in response to blockade of AT2 receptor (0.9 ± 0.1 swallows/min), while central injection of ANG II in the presence of AT2 blockade significantly increased fetal swallowing (1.5 ± 0.1 swallows/min; *P* < 0.05). ANG II caused significant pressor responses in the control and PD-123319 studies but no pressor response in the presence of AT1 blockade. These data demonstrate that in the near-term ovine fetus, AT1 receptor but not AT2 receptors are involved in mediating spontaneous and ANG II-mediated fetal swallowing as well as cardiovascular regulation.

MATERIAL AND METHODS

Five time-dated pregnant ewes with singleton fetuses (130 ± 1 days’ gestation on the first study day) were studied. Western mixed-breed sheep were obtained from a local farm (Nebeek Farm, Palmdale, CA). 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 Los Angeles Animal Use Committee. Food (alfalfa pellets) and water were provided ad libitum, except that food was withheld for 24 h before surgery (water was available to the animal until the time of surgery).

Surgical preparation. Anesthesia was induced with ketamine hydrochloride (15–20 mg/kg im), and general anesthesia was maintained with 1% to 2% isoflurane and 1 l/min of oxygen. The uterus was opened in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

IN THE NEAR-TERM OVINE FETUS, fetal swallowing contributes importantly to amniotic fluid volume regulation and gastrointestinal tract development (20, 27, 47). The near-term ovine fetus demonstrates an intact swallowing response to central dypsogenic stimuli, including hypertonicity (39) and ANG II (40). Furthermore, tonic central ANG II activity contributes to spontaneous swallowing activity, as nonselective blockade of central ANG II receptors attenuated both basal as well as ANG II-mediated fetal swallowing activities (16).

In adult animals, brain-derived ANG II is a potent stimulant of water and sodium intake and an important regulator of blood pressure (21, 37, 51). The recent development of specific, highly selective, nonpeptide ANG II receptor antagonists have identified two major ANG II-receptor subtypes, AT1 and AT2. In adult animals of several species, neuronal AT1 receptors are found in the lamina terminalis, hypothalamic paraventricular nucleus, and the nucleus tractus solitarius mediate the dypsogenic, pressor, and arginine vasopressin (AVP)-releasing actions of ANG II (13, 19, 30). The AT2 receptor is expressed differently from AT1 receptor, as it is found mainly in the cerebellum and thalamus (1–3). AT2 receptors may contribute importantly to fetal development, they are highly expressed during fetal development, and expression declines rapidly after birth (29, 48). Although little is known of the function of AT2 receptors, several (22, 23) although not all studies (8, 49) have suggested that AT2 and AT1 receptors act synergistically to augment water intake. Notably, the AT2 receptor also has been demonstrated to oppose the pressor effect of AT1 receptor stimulation (31, 44).

The role of ANG II receptor subtypes in mediating fetal swallowing and cardiovascular regulation has not been previously explored. In view of the fundamental differences in expression and localization of AT2 receptors in the fetal compared with adult brain, and the evidence that AT2 receptors may synergistically augment AT1-mediated adult drinking behavior, we sought to determine the specific roles of central AT1 and AT2 receptors in mediating spontaneous and ANG II-mediated fetal swallowing as well as cardiovascular regulation.
exposed by a midline abdominal incision, and a small hysterotomy was performed to expose the fetal hindlimb. Polyethylene catheters with inner diameters of 0.040 in. and outer diameters of 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 and upper and lower nuchal esophagus) for determination of swallowing activity was performed. 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 (ECOG) activity. An 18-gauge needle connected to polyethylene catheter (inner diameter, 0.02 in.; outer diameter, 0.04 in.) was inserted into the lateral ventricle that was identified 20 mm above the lambdoid suture and 5 mm lateral to the sagittal suture. The lateral ventricle needle and the dural electrodes were immobilized with dental cement and two stainless steel screws fixed in the skull. An intrapartum catheter (inner diameter, 0.125 in.; outer diameter, 0.25 in.; Corometrics Medical Systems, Wallingford, CT) was inserted for measurement of amniotic fluid pressure. Uterine and amniotic pressures were monitored continuously. Maternal and fetal blood samples were collected into chilled iced tubes that contained 10 U/ml of lithium heparin and 500 IU aprotinin per milliliter of blood. Samples were extracted by use of a modification of the procedure of LaRochelle et al. (28), and AVP levels were determined with radioimmunoassay. Plasma AVP recoveries in our laboratory average 70%.

Data analysis. Digitization of all signals was performed at a rate of 75 Hz. EMG and ECoG activity signals were directed to a Grass physiological recorder and to a Windaq analog digital system (Dataq Instruments, Akron, OH). 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 activity was assessed by visual analysis and was divided into periods of low voltage and high voltage. Periods of ECoG activity that did not clearly belong to either low- or high-voltage activities were considered to be 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 earlier and was expressed as swallows per minute.

Statistical analysis. For each animal, swallowing rate per minute, percentage of time spent in each ECoG activity state, and percentage of swallow duration in each state were calculated for the control and drug exposure periods. Data of fetal swallowing and all other fetal and maternal parameters were analyzed using one-way repeated measures ANOVA with the Dunnett’s test for post hoc analysis. All data are presented as mean values ± SE, and P < 0.05 was considered to be statistically significant.

RESULTS

Control aCSF-ANG II study. ICV injection of aCSF did not change total fetal swallowing (0.9 ± 0.2 to 1.0 ± 0.2 swallow/min), whereas ICV injection of ANG II significantly increased swallowing activity (1.4 ± 0.2 swallow/min; P < 0.05; Fig 1A). Fetal swallowing occurred primarily during low-voltage ECoG activity (control 1.2 ± 0.2; aCSF 1.2 ± 0.1; ANG II 1.9 ± 0.4 swallow/min during low-voltage ECoG activity; P < 0.05). Fetal swallowing during high-voltage ECoG activities were as follows: control 0.6 ± 0.1, aCSF 0.6 ± 0.1, and ANG II 0.8 ± 0.1 swallow/min (P < 0.01).

The percentage of time spent in low-voltage ECoG activity was stable during the control period (52 ± 5%), after injection of aCSF (52 ± 1%), and after injection of ANG II (56 ± 5%; P = NS). Fetal mean blood pressure did not change after injection of aCSF, but it significantly increased at 15 min after ICV injection of ANG II (53 ± 4 to 58 ± 3 mmHg; P < 0.003; Fig 2A). Fetal heart rate increased at 5 min after ICV injection
of ANG II; however, it achieved significance only at 30 min (170 ± 4 to 208 ± 18 beats/min; P < 0.01). Fetal plasma AVP concentration increased significantly after ICV injection of ANG II (0.9 ± 0.2 to 5.6 ± 2.3 pg/ml; P < 0.03).

Losartan study. ICV injection of the AT1 receptor antagonist (losartan) significantly reduced total mean spontaneous fetal swallowing (0.9 ± 0.1 to 0.4 ± 0.1 swallow/min; P < 0.001; Fig. 1B). ICV ANG II in the presence of losartan did not increase swallowing activity (0.6 ± 0.1 swallow/min; Fig. 1B). Fetal swallowing occurred primarily during low-voltage ECoG activity (control 1.4 ± 0.2; losartan 0.5 ± 0.1; losartan plus ANG II 0.8 ± 0.1 swallow/min low-voltage ECoG activity; P < 0.003). Fetal swallowing during high-voltage ECoG activities were as follows: control 0.7 ± 0.1, losartan 0.4 ± 0.1, and losartan + ANG II 0.5 ± 0.1 swallow/min (P < 0.001).

The percentage of time spent in low-voltage ECoG activity was stable during the control period (51 ± 5%), after injection of losartan (49 ± 4%), and after injection of losartan and ANG II (53 ± 6%; P = NS). Neither fetal blood pressure (49 ± 2 mmHg; Fig. 2B), heart rate (166 ± 3 beats/min), nor plasma AVP (2 ± 1 pg/ml) changed significantly during the study.

PD-123319 study. ICV injection of AT2 antagonist (PD-123319) did not change total mean spontaneous fetal swallowing (0.9 ± 0.1 swallow/min; Fig. 1C). ICV ANG II in the presence of PD-123319 significantly increased swallowing activity (1.5 ± 0.1 swallow/min; P < 0.001; Fig. 1C) Fetal swallowing occurred primarily during low-voltage ECoG activity (control 1.1 ± 0.2; PD-123319 1.1 ± 0.2; PD-123319 plus ANG II 1.8 ± 0.1 swallow/min low-voltage ECoG activity; P < 0.003). Fetal swallowing during high-voltage ECoG activities were as follows: control 0.7 ± 0.1, PD-123319 0.7 ± 0.1, and PD-123319 + ANG II 1.2 ± 0.1 swallow/min (P < 0.001).

The percentage of time spent in low-voltage ECoG activity was stable during the control period (56 ± 5%), after injection of PD-123319 (52 ± 2%), and after injection of PD-123319 and ANG II (57 ± 2%; P = NS). Fetal blood pressure did not change in response to PD-123319 but significantly increased at 5 min after ICV injection of ANG II (control 51 ± 3 mmHg; ANG II 58 ± 3 mmHg; P < 0.02; Fig. 2C). Fetal heart rate did not change in response to P-123319 or ICV ANG II. Fetal plasma AVP concentration increased significantly after ICV ANG II (1.1 ± 0.5 to 5.5 ± 2.8 pg/ml; P < 0.03).

In all study protocols, fetal hematocrit, pH, PO2, PCO2, plasma osmolality, and sodium, chloride, and potassium concentrations values drawn at the end of the 1st hour of the baseline period did not change (Table 1). Similarly, maternal arterial parameters did not change from control values.

DISCUSSION

This study examined the roles of central ANG II receptor subtypes in the regulation of fetal swallowing and systemic blood pressure with the use of selective ANG II receptor antagonists. ICV injection of ANG II increased both fetal swallowing activities and systemic blood pressure, consistent with previous reports (17). Selective blockade of the central AT1 but not AT2 receptor resulted in a significant reduction in basal fetal swallowing and eliminated the dipsogenic effect of central ANG II. These data demonstrate that the high rate of basal as well as ANG II-mediated fetal swallowing is regulated via central AT1 receptors. Our results also demonstrated that central injection of AT2 receptor antagonist (PD-123319) which blocked CSF accessible AT2 receptor affected neither dipsogenic nor pressor responses of central ANG II.

The ANG II dose used in our experiment was 6.4 μg diluted in 1 ml of aCSF. The CSF volume in the near-term ovine fetus is ~7 ml (18); this resulted in a final concentration of ANG II in the CSF of 0.8 μg/ml. Higher concentration of ANG II is expected at the site of injection (lateral ventricle). Although

Fig. 1. A: total fetal total swallows per minute during the baseline period (control), after artificial cerebrospinal fluid (aCSF) injection at 2 h, and after ANG II injection at 4 h into the intracerebroventricular space (ICV) (*P < 0.05 vs. control). B: total fetal total swallows per minute during the baseline period (control), after selective ANG II AT1 receptor antagonist (losartan), and after combined injection of losartan and ANG II into the ICV space (*P < 0.05 vs. control). C: total fetal total swallows per minute during the baseline period (control), after AT2 receptor antagonist (PD-123319), and after combined injection of PD-123319 and ANG II and into the ICV space (*P < 0.05 vs. control).
there are no previous studies that measured the basal level of ANG II in the ovine fetus CSF, the basal ANG II concentration in the CSF of adult rats is 0.6 μg/ml (7, 38). Thus the dose administered is an appropriate physiological stimulant dose. Although we have previously used lower doses of central ANG II (40), we have found that such a low dose of ANG II (0.5 μg/kg) does not consistently produce dipsogenic and pressor responses. Therefore, we have gradually increased the dose of central ANG II to 2.0 μg/kg, which totals 6.4 μg in an approximately 2.5- to 3-kg ovine fetus. This central ANG II dose consistently and reliably produces dipsogenic and pressor responses.

Losartan is highly selective for AT1 receptors with 30,000 times more selectivity to AT1 than AT2 receptors (10, 11), while PD-123319 is characterized by its high affinity for AT2 receptors with very low affinity for AT1 receptors (50). The dose of the selective AT1 and AT2 blockers were calculated based on rat studies (41, 50), after adjustments for the differences in CSF volume between adult rat (0.3 to 0.4 ml) (34) and near-term fetal sheep (~7 ml) (18). The chemical purity of both losartan and PD-123319 was checked by the manufacturers (Merck and Sigma, respectively) utilizing high-performance liquid chromatography.

We have used ICV PD-123319 in a dose of 0.8 mg. Converting this dose to a nanomolar concentration, the concentration of PD-123319 in the CSF would be 1,551 nM. In a study by McMullen et al. (33) in pregnant ewes, PD-123319 competition curve demonstrated that PD-123319 achieved half-maximal displacement (IC50) of ANG II from AT2 receptor in the uterine arteries at a concentration of 14.5 nM. In the brain tissue the IC50 of PD-123319 is 210 nM (9). Therefore our dose of PD-123319 used centrally was sufficient to block >50% of periventricular AT2 receptors. In agreement with our study, Weisinger et al. utilizing water-deprived adult sheep found that PD-123319 ICV infusion (5,000 pg/h) did not block the dipsogenic effect of ICV infusion of ANG II (3.8 pg/h) (49). As PD-123319 was dissolved in normal saline and we utilized aCSF in the control study (aCSF/ANG II), we have utilized three additional control normal saline studies in which 1 ml of normal saline was injected ICV after 2-h basal periods. ICV normal saline caused no hemodynamic or biochemical changes as fetal blood pressure, heart rate, blood gases,
osmolality, and chemistry did not change from basal values. Similarly, fetal swallowing did not change from basal values (0.8 swallow/min).

In the adult rat (21) central AT1 blockade completely prevented drinking and AVP responses to central ANG II, while central AT2 blockade potentiated the ANG II-induced drinking and AVP release (24), suggesting an inhibitory effect of AT2 receptors on AT1-mediated responses. In the adult sheep, ICV injection of AT1 receptor antagonism blocked the water intake caused by ICV hypertonic NaCl in sodium-depleted animals, while ICV PD-123319 did not alter AT1-mediated water intake (49, 51). Similarly, central AT1 antagonism blocked postprandial and systemic ANG II-induced drinking in adult sheep (32). These reports indicate that in the adult rat and sheep, AT1 but not AT2 receptors mediate stimulated drinking behavior.

In contrast to the above data, several studies in the adult rat have indicated that central blockade of AT2 receptors significantly reduced drinking activities induced by central ANG II and hypertonic saline (23, 42). Furthermore, AT2 knockout mice have an impaired drinking response to water deprivation (22, 31), indicating a role for AT2 receptors in mediating osmotic drinking. Other studies have demonstrated that both AT1 and AT2 blockers inhibit central ANG II-induced drinking (6) and AT2 blockers (PD-123319) inhibit ANG II mediated drinking only in rats, which are fed high-salt diets (10). Thus AT2 receptors may synergistically enhance AT1-mediated water intake only under special circumstances.

In accord with the high density of AT1 receptors in various brain regions involved in cardiovascular regulation in several species (2, 4, 5, 35), the AT1 receptor contributes to adult blood pressure control. The AT1 receptor maintains resting blood pressure in anaesthetized rats on a low-salt diet, suggesting a role in maintaining resting blood pressure under conditions of increased central ANG II levels (15). In the conscious adult rat, chronic lateral ventricle infusion of AT1 antagonist significantly decreased basal blood pressure (53), suggesting that AT1 receptor in the brain plays a significant physiological role in the regulation of basal blood pressure. In conscious newborn lambs, both systemic and lateral ventricle administration of AT1 antagonist (losartan) but not AT2 antagonist (PD-123319) significantly decreased resting mean arterial blood pressure (43). Similarly, there is a marked attenuation of pressor and AVP-releasing effects of central ANG II in AT1a knockout and in wild-type mice treated with central AT1 receptor antagonist (31). Furthermore, AT1a knockout mice demonstrate reduced basal blood pressures compared with wild-type mice (31, 46).

The AT2 receptor has been repeatedly shown to oppose the pressor and AVP-releasing effects of AT1 receptors in the adult animals. AT2 knockout mice as well as mice treated with central AT2 blocker (PD-123319) demonstrate exaggerated pressor and AVP-releasing responses to central ANG II (31). Similarly, AT2 knockout mice had increased sensitivity to the pressor effect of peripheral injection of ANG II (22). Our results in the ovine fetus demonstrated that AT1 but not AT2 receptors mediate both the pressor and AVP-releasing effects of central ANG II. Although AT2 blockade resulted in small rise in basal blood pressure, the variance was large, and to detect a significant increase in basal blood pressure in response to AT2 blockade with 80% confidence, a sample size of 13 would be required.

ANG II receptors situated within the dipsogenic neurons are easily accessible from the CSF compartment. In fact, dipsogenic neurons from the subfornical organ (SFO) and organ vasculosum lamina terminalis (OVLT) are in direct contact with CSF. As detected with electron microscopy of neuronal composition of the SFO, axonal terminals and dendrites making direct contact with ependymal cells and CSF (14). Further studies revealed that direct ICV injection of ANG II was as effective as direct tissue injection into dipsogenic sensitive sites close to the ventricles (26). Conversely deep sites away from the ventricles were insensitive to direct injection of ANG II not crossing the ventricles, supporting the hypothesis of periventricular receptor site for angiotensin (26).

Few studies have been published describing the ontogeny of ANG II receptors (AT) in the fetal rat brain. Utilizing in situ hybridization technique, AT1 receptor mRNA subtype appears in late gestation at embryonic day 19 (E19) in forebrain areas involved in fluid homeostasis and cardiovascular regulation (35). AT2 receptor mRNA appears earlier at E13 and is strongly but transiently expressed in certain structures involved mainly in motor functions and sensory integration (36).

Although the precise signaling pathways and the functional roles are unclear, AT2 receptors may antagonize, under physiological conditions, several AT1-mediated actions (1, 11, 22, 25, 36). The mechanism by which AT2 receptors inhibit AT1 function is not clear. However, stimulation of the AT1 receptor increases neuronal depolarization and firing rate (12, 45), while AT2 stimulation results in neuronal hyperpolarization and reduced neuronal excitability (52). Although AT2 receptors are highly expressed in fetal brain (48), they do not appear to exert antagonistic effects to either AT1-mediated drinking or pressor effects. Teleologically, the high expression of AT2 receptors in early fetal life may facilitate rapid cell turnover and differentiation by opposing the growth-promoting effect of AT1 receptor (29).

In conclusion, we report an important role for AT1 receptors in maintaining ANG II-stimulated ingestive behavior in the near-term ovine fetus. We have also demonstrated that ANG II-mediated AVP-releasing effects are mediated via AT1 receptors. Despite the relative overexpression of AT2 receptors in the fetal compared with the adult brain, the role for AT2 receptors remains uncertain.
Perspectives

Near-term ovine fetus has functional central dipsogenic mechanism as demonstrated by increased drinking activities in response to central ANG II. Similar to the adult, ovine fetus utilizes AT1 receptors to mediate dipsogenic response. Our data demonstrate that AT2 receptors, in regions accessible to the CSF, do not contribute to fetal drinking, pressor, or AVP-releasing effects of central ANG II in ovine fetus. In light of the controversial role of AT2 receptor in mediating drinking activities in the adult rat, we must interpret with caution our findings regarding the role of AT2 receptor in the fetus. We emphasize that our studies were physiological in nature and utilized ICV route to access periventricular ANG II receptors, and to rule out with certainty any role of AT2 receptors in drinking and cardiovascular regulation it would be necessary to carry out other studies at hypothalamic tissue level including binding, molecular and electrophysiological studies.

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