Mild chronic hypoxemia modifies expression of brain stem angiotensin peptide receptors and reflex responses in fetal sheep

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Pulgar VM, Hong JK, Jessup JA, Massmann AG, Diz DI, Figueroa JP. Mild chronic hypoxemia modifies expression of brain stem angiotensin peptide receptors and reflex responses in fetal sheep. Am J Physiol Regul Integr Comp Physiol 297: R446–R452, 2009. First published June 10, 2009; doi:10.1152/ajpregu.00023.2009.—The effects of chronic mild hypoxemia on the binding of angiotensin receptors in selected brain stem nuclei and reflex responses were studied in fetal sheep. Fetal and maternal catheters were placed at 120 days’ gestation, and animals received intratracheal maternal administration of nitrogen (n = 16) or compressed air in controls (n = 19). Nitrogen infusion was adjusted to reduce fetal brachial artery PO2 by 25% during 5 days. Spontaneous baroreflex sensitivity and spectral analysis of the pulse interval were analyzed during the 5 days hypoxemia period using 90 min of daily recording.Brains of control and hypoxic animals were collected, and brain stem angiotensin receptor binding was studied by in vitro autoradiography at 130 days of gestation. After 5 days of hypoxia, some animals in each group were submitted to one complete umbilical cord occlusion during 5 min. Intrastriatal binding showed that chronic mild hypoxemia significantly increases angiotensin type 1 receptor, angiotensin type 2 receptor, and ANG-(1–7) angiotensin receptor binding sites in the nuclei tractus solitarius and dorsal motor nucleus of the vagus (P < 0.05). Hypoxemia induced lower baroreflex sensitivity and a higher low frequency-to-high frequency ratio in the fetus, consistent with a shift from vagal to sympathetic autonomic cardiac regulation. Cord occlusion to elicit a chemoreflex response induced a greater bradycardic response in hypoxic fetuses (slope of the initial fall in heart rate; 11.3 ± 1.9 vs. 6.4 ± 1.2 beats·min⁻¹·s⁻¹, P < 0.05). In summary, chronic mild hypoxemia increased binding of angiotensin receptors in brain stem nuclei, decreased spontaneous baroreflex gain, and increased chemoreflex responses to asphyxia in the fetus. These results suggest hypoxemia-induced alterations in brain stem mechanisms for cardiovascular control.

fetus; hypoxemia; baroreflex

OXYGEN DEPRIVATION IMPOSES diverse challenges to fetal development, with changes in the autonomic cardiovascular control via baroreflex and chemoreflex pathways playing a major role as a survival strategy (16). In fetal sheep, acute episodes of hypoxemia induce a redistribution of cardiac output to maintain blood flow and oxygen delivery to the heart and brain (14). This response is initiated by stimulation of peripheral chemoreceptors and maintained by endocrine mechanisms involving secretion of hormones such as catecholamines, neuropeptide Y, arginine vasopressin, and cortisol (11). During acute severe hypoxia, i.e., complete occlusion of the umbilical cord, fetuses respond with a fall in heart rate (FHR) and an increase in blood pressure. This acute FHR during hypoxia represents a key fetal adaptation, believed to help reduce myocardial work and oxygen requirements (11). This initial bradycardia, which is mediated by chemoreflex vagal pathways (5, 17, 23), occurs before the increase in blood pressure and is an indication of the severity of the hypoxic insult (5). Recently, it was reported that the slope of the initial bradycardia increases during short repeated cord occlusions associated with severe acidosis, indicating an increase in chemoreflex responses with fetal metabolic deterioration (3).

Experiments aimed to identify central neurons activated by hypoxia in the fetus have shown increased neuronal activation in several brain stem nuclei of fetal sheep, including those involved in cardiovascular regulation, specifically the nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (dmnX) (6, 27). These nuclei express angiotensin II (ANG II) receptors and are associated with sensory and motor pathways involved in the reflex control of circulation (9). In addition, the NTS is responsible for ANG II actions related to the integration of signals modulating chemo- and baroreflex cardiovascular responses, with a net effect of an overall suppression of vagal baroreflex function (2, 9). In sheep, angiotensin type 1 (AT1) and angiotensin type 2 (AT2) receptors in brain stem are developmentally regulated, with a greater expression observed in fetal brain areas including the NTS and area postrema in term [141 ± 4 days gestational age (dGA)] compared with preterm animals (109 ± 4 dGA) (18).

We have previously shown that, in the near-term fetal sheep, a 5-day period of mild hypoxia has significant effects on electrocorticogram activity and cardiovascular responses (34). A potential explanation for the higher fetal heart rate despite the significant higher blood pressure observed in the last 2 days of the hypoxic period is attenuation in the sensitivity of the baroreflex. Therefore, the aim of the present study was to determine if chronic mild hypoxemia affects fetal baro- and chemoreflex responses and if prolonged mild hypoxia is associated with changes in ANG II receptor binding in the fetal brain stem.

MATERIALS AND METHODS

Animal Preparation and Postoperative Care

All procedures for housing, handling, surgical implantation of catheters, and postoperative management were approved by Wake Forest University’s Institutional Animal Care and Use Committee and have been described previously (34, 35). Thirty five date-mated sheep at 118–120 dGA (0.83 gestation, term 144 ± 5 days) were operated under halothane general anesthesia. Briefly, maternal vascular (carotid artery, jugular vein, and femoral artery) and a tracheal catheter were inserted. Fetal vascular (femoral and brachial arteries) and an amniotic

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catheter were placed. A vascular occluder (24 mm in diameter, 6 ml volume) was placed at the base of the umbilical cord. Catheters and occluder were exteriorized through the flank of the ewe. Animals were allowed to eat and drink ad libitum. Throughout the entire study period, sheep were housed in individual metabolism cages to which they had been acclimatized. Nineteen animals were allocated to the control group (male 11, female 8; twins 4) and sixteen (male 11, female 5; twins 3) to the hypoxemia group. After 5 days of hypoxemia, seven animals from the control group and four from the hypoxemic group were killed, and brains were collected for the binding studies. Of the remaining animals, 7 in the control and 11 in the hypoxic group had a complete data set for umbilical cord occlusion while in hypoxemia. In each group, recordings from 12 animals were used to obtain baroreflex and heart variability data during the 5-day hypoxemia period. Fetuses were killed by exsanguination under general anesthesia after cesarean section delivery either after 5 days of hypoxemia (for binding studies, at 130 dGA) or 3 days after the cord occlusion.

**Animal Studies**

**Chronic hypoxemia.** The protocol used to induce chronic mild hypoxemia in the fetus as previously described was conducted by maternal intratracheal administration of nitrogen gas (PGS45, purity-99.96%; National Welders, Charlotte, NC) to induce maternal hypoxemia and reduce fetal brachial artery PO2 by 25% (n = 16). Compressed air (medical grade; National Welders) was administered in the control animals (n = 19) (34, 35). All studies began at least 4 days after surgery in animals in which fetal arterial PO2, pH, PCO2, and bicarbonate were within the normal range for our laboratory. Maternal and fetal PCO2 were maintained at normal levels by using intratracheal infusion of CO2 (0.5–1 l/min, purity 99.95%; National Welders).

**Umbilical cord occlusion protocol.** In a subset of animals, a 5-min complete cord occlusion was applied to control (n = 7) and hypoxemic (n = 11) fetuses after 5 days of hypoxic or normoxic conditions. In the case of twins, cord occlusion was performed just in one fetus. Occlusion was achieved by complete inflation of the occluder cuff. Blood samples were obtained during the last minutes of the occlusion (blood draw started 4 min into the occlusion). After the occlusion (3 days), fetuses were delivered by cesarean section and killed by exsanguination under halothane general anesthesia.

**Measurements of baroreflex sensitivity.** Fetal and maternal blood pressure were recorded using solid-state pressure transducers (World Precision Instruments, Sarasota, FL) connected to preamplifiers in a custom-built data acquisition system (CWE, Ardmore, PA). Blood pressure signals were analog-filtered and sampled at 150 Hz before being processed by the 12-bit analog-to-digital converter. Pressure recorded from either a fluid-filled catheter tied to the back of the animal or from the amniotic catheter was electronically subtracted from either a fluid-filled catheter tied to the back of the sheep and fetal arterial pressure catheters to remove differences determined by the sheep changing position and uterine contractions.

Baroreflex sensitivity (BRS) was calculated by the sequence method. Heart rate variability was analyzed by spectral analysis and evaluated as the power of two regions of the frequency spectrum [low frequency (LF); high frequency (HF); see below].

**SEQUENCE METHOD.** The sequence method takes advantage of spontaneous changes in arterial blood pressure as reported by others (29, 30). We used a software package for BRS analysis in humans (Nevrokard BRS, Medistar, Ljubljana, Slovenia) as previously described (41). For each fetus (12 in control and 12 in hypoxic group), a 90-min period of continuous blood pressure recording (4:00 to 7:00 A.M.) was used to extract systolic blood pressure (SBP) and the pulse interval using the peak-peak routine in Advanced Codas (DataQ Instruments, Akron, OH). The latter is considered an indirect estimate of the R-R interval (RRI) (29). The sequence values containing of SBP with their respective RRI values was then presented to the Nevrokard BRS software. As previously described (51), BRS calculated by the sequence method is based on the determination of sequences at least three beats long during which SBP consecutively increases (up) or decreases (down), which are accompanied by changes in the same direction of the RRI of the subsequent beats. The parameters used in the present analysis included sequences with a minimal RRI change of 3 ms, a minimal SBP change of 1 mmHg, a minimal number of 3 beats in the sequence, and a minimal correlation coefficient of 0.85. The computational scanning of RRI and SBP recordings identifies the sequences and calculates the correlation coefficient for each sequence. For sequences with correlation coefficients >0.85, the slope is calculated and accepted. Next, the mean of all the individual slopes, which is a measure of BRS, is calculated. We used SBP vs. RRI total (including up and down sequences) to evaluate the spontaneous BRS.

**SPECTRAL ANALYSIS OF HEART RATE.** Frequency domain analysis was also carried out analyzing spontaneous changes in heart rate using the Nevrokard package (29). Studies in animals and humans suggest that variability of pulse interval at a frequency ~0.25 Hz (HF) can be used as an indicator of vagal cardiac control, whereas LF variability (~0.1 Hz) reflects sympathetic and parasympathetic modulation (4). These studies have also suggested that the LF-to-HF power ratio would reflect sympathetic cardiac modulation (28, 31). Power spectral analysis of RRI variability was performed on data from the same file used for the sequence method using fast-Fourier transform and the data integrated over a specified frequency range. The frequency ranges used have been validated for the fetal sheep to represent sympathetic and parasympathetic cardiac modulation (LF, 0.025–0.31 Hz; HF, 0.5–1.25 Hz) (21). Power of RRI spectra in the LF (LFRRI) and HF (HFRRI) range were calculated in normalized units, and the ratio of LFRRI/HFRRI was used as an estimation of sympathovagal balance (31).

**Binding Measurements**

Angiotensin receptors were measured by binding of [125I-sarco-containing]ANG II ([125I-Sar1-Thr8]ANG II) in seven control and four hypoxic fetuses. At necropsy, brains were removed and brain stem frozen on dry ice before transferring to ~80°C for storage. Series of adjacent coronal sections (15 μm) were cut and mounted on Superfrost/plus slides (Fisher Scientific, Pittsburgh, PA). Sections were incubated for 30 min in 10 mM HEPES buffer with 125 mM NaCl, 5 mM MgCl2, 1 mM EGTA, and 0.5% BSA, pH 7.4, at room temperature and then for 45 min in the same buffer with [125I-Sar1-Thr8]ANG II (2,200 Ci/mmol) with protease inhibitors to protect the ligand. Total binding was determined in the absence of competitors, and non-specific binding was determined using excess unlabeled [Sar1-Thr8]ANG II (3 μM). The following two concentrations of [125I-Sar1-Thr8]ANG II were used: a low concentration (0.2 nM) for the competition study and a higher concentration (0.6 nM) to provide an estimate of maximal binding. Angiotensin receptor antagonists [AT1 antagonist losartan (3 M), AT2 antagonist PD-123319 (3 M), and ANG-(1-7) antagonist d-Ala (3 μM)] were used as competitors as described (8). After exposure to films, images were quantified using a computerized densitometric system (MCID; InterFocus Imaging, Cambridge, UK).

**Data Analysis**

BRS data were analyzed by two-way analysis of variance and the Newman-Keul’s test for post hoc comparisons. For the binding experiments, data are expressed as means ± SE for each nucleus, with measurements taken at the intermediate rostrocaudal level of the dorsal medulla. The bradycardic response to umbilical cord occlusion was calculated as the slope of the acceleration in RRI and used as an index of chemoreflex responses (1). Results of binding experiments and chemoreflex response were analyzed by r-test. Statistical significance was accepted with P < 0.05.
RESULTS

Fetal arterial pH, PCO₂, PO₂, oxygen content (O₂ct), and hemoglobin concentration values before hypoxemia, during hypoxemia (average of the 5-day period), and during umbilical cord occlusion are shown in Table 1.

Fetal Brain Stem Angiotensin Binding

In the group of animals whose brains were collected after 5 days of chronic hypoxemia, angiotensin peptide receptor binding was analyzed in the ventral (Fig. 1A) and in the dorsal medulla (Fig. 1C) medulla. In control animals, the AT₁ receptor was the most abundant subtype in NTS and dmX, and the AT₂ predominated in the inferior olivary nuclei (ION). Chronic hypoxemia significantly increased total ANG II binding sites in NTS and dmX (Fig. 2). Although no significant difference in total ANG II binding was observed in the area postrema, there was a trend for increased total binding in the ION. The use of specific antagonists revealed that, in NTS and dmX, chronic hypoxemia significantly increased binding for all three receptor subtypes [AT₁, AT₂, and ANG-(1-7)] (Fig. 2, P < 0.01). In ION, the differences observed were significant only for ANG-(1-7) receptors (P < 0.05).

Spontaneous BRS During Mild Chronic Hypoxemia

Before the hypoxic challenge, the spontaneous BRS (SBP vs. RRI using all sequences) showed no differences between hypoxic and control (9.0 ± 0.4 vs. 8.7 ± 0.2 ms/mmHg) groups. The temporal progression of BRS during the hypoxemia period is shown in Fig. 3A. A statistically significant treatment effect was observed for the 5-hypoxemia period, with the hypoxic group showing lower spontaneous baroreceptor sensitivity compared with controls (F = 14.88, P < 0.001). No effects of chronic hypoxemia were observed in maternal BRS at day 5 (control 22.8 ± 5.7 vs. hypoxic 20.5 ± 4.9 ms/mmHg). These maternal values were statistically higher than fetal values in both groups (P < 0.05).

Spectral Analysis of the Fetal Heart Rate During Mild Chronic Hypoxemia

The 5-day hypoxic challenge was associated with a significant change in the relative power of LF and HF for RRI, yielding increased values of the RRI LF to HF ratio in the hypoxic group. As shown in Fig. 3B, a statistically significant treatment effect (F = 32.63, P < 0.001) was observed in the temporal progression of RRI LF-to-HF ratio during the 5-day hypoxemia period.

Fetal Heart Rate Responses to Asphyxia

The 5-min complete umbilical cord occlusion resulted in pH, PCO₂, PO₂, and O₂ct values that were not different between control and hypoxic animals (Table 1). The analysis of the 1-s average of the heart rate deceleration showed an initial rapid fall (Fig. 4A) in both groups. As shown in Fig. 4B, the calculated slope of the FHR was steeper in the hypoxic group (hypoxic 11.3 ± 1.9 vs. 6.4 ± 1.2 beats·min⁻¹·s⁻¹, P < 0.05). Consequently, the time to achieve the minimal heart rate was also different between both groups, with the hypoxic group showing a faster response (hypoxic 24 ± 2 s vs. control 35 ± 2 s; P < 0.05). The ratio between FHR and the difference in PO₂ (ΔFHRΔPO₂) during the occlusion, another estimate of chemoreflex activation (15), was higher in hypoxic fetuses (hypoxic 10.6 ± 0.5 beats·min⁻¹·mmHg⁻¹, control 6.2 ± 0.5 beats·min⁻¹·mmHg⁻¹, P < 0.001).

DISCUSSION

We have shown that chronic mild hypoxemia decreases spontaneous BRS and increases chemoreflex activation in response to asphyxia, and that these functional changes are associated with increased angiotensin peptide receptors in brain stem regions involved in cardiovascular regulation.

The long-term effects of fetal chronic hypoxemia on cardiovascular regulation are not completely understood. BRS plays a fundamental role in the modulation of sympathetic and vagal efferent discharge during hypoxemia. In rats, chronic intermittent hypoxemia decreases BRS and increases sympathetic activation (22). A decrease in BRS has also been shown in humans exposed to acute hypoxia (37) and in patients with central sleep apnea, a condition known to cause hypoxic episodes (38). Conversely, the chemoreceptor response to acute hypoxemia has been shown to be inversely related to the oxygenation level previous to the acute hypoxic challenge (5, 20).

Angiotensin receptors are found in several nuclei on the dorsal medulla, such as the NTS, dmX, area postrema, and ventrolateral medulla among others (46). AT₁ receptors in the NTS are thought to mediate the effects of ANG II to reduce baroreflex responses (48). Both plasma and neuronal angiotensin peptides can mediate the effects on cardiovascular reflexes. Circulating ANG II acting on structures outside the blood barrier, such as the area postrema, and neuronal ANG II acting as a neurotransmitter (9) have been proposed as mechanisms to explain the effects of ANG II. Injection of ANG II in the NTS decreases baroreceptor-mediated reflex bradycardia (32), whereas injection of ANG II antagonists blocks the effects of stimulation of the hypothalamic defence area (36). Activation of AT₁ and ANG-(1-7) receptors in the NTS has been shown to
have opposite effects on baroreflex control in the adult rat (39). In the fetal sheep, AT$_1$ receptors are found in the medulla oblongata (10), and injection of ANG II in the lateral ventricle causes a mild increase in blood pressure with no changes in fetal heart rate (42). Considering that the central effects of angiotensin antagonists have been shown to be age dependent in adult rats, studies using selective antagonist are needed to uncover the relative contribution of each receptor subtype to resting BRS during fetal life. Thus the functional relevance of the increased expression of angiotensin receptors in NTS and dmnX with regards to the changes in BRS in the hypoxemic fetus cannot be established at the present time. However, the impairment of BRS in the fetal sheep exposed to chronic hypoxia would suggest a predominance of AT$_1$ receptor function since effects at the ANG-(1-7) (39) receptor would be expected to have the opposite effects on BRS.

Traditionally, sensitivity of the baroreceptor-mediated reflex has been determined using infusions of pressor drugs, the “Oxford method,” to obtain pairs of RRI and SBP data points over an extended range of values (44). An alternative “noninvasive” method has been used extensively to derive indexes of BRS from analysis in the time and frequency domains. The advantage of the drug infusion method is that it allows exploration of the effects of various receptor subtypes on baroreflex control in the hypoxemic fetus.
ing the entire range of the sigmoid relationship of the stimulus-response baroreceptor curve, from threshold to saturation. On the other hand, the noninvasive method provides a major advantage for repeated assessment in the same individual and for assessing BRS in humans. Notwithstanding, the noninvasive method is not universally accepted because of reports showing lack of correlation when both methods have been compared (25). Supporters of the noninvasive method claim methodological and interpretation flaws as the main source of the discrepancy (29, 31).

We are confident that we have applied the sequential method for evaluating BRS as it was intended, and as such it provides an accurate representation of the relationship between the changes in RRI and the changes in arterial blood pressure. The values we obtained for BRS in the control group (8.9 ± 0.2 ms/mmHg) are in good agreement with previously reported values for BRS in fetal sheep using pressor drugs [7.2 ± 0.9 (7), 8.44 ± 1.43 (19), and 7.81 ± 3.54 (43)]. Also in support of the validity of our data is the consistent observation of higher values of BRS in the pregnant adult sheep compared with the fetus (19).

To our knowledge, this is the first report on sequential assessment of BRS under chronic hypoxic conditions in the fetus. Our data show a reduction in BRS in the chronically hypoxicemic group. In contrast, no change in BRS was observed in animals exposed to an intermittent stimuli, i.e., 1 h acute hypoxemia during 14 days (47) or intermittent umbilical cord occlusions during 3 wk (15). This suggests that a prolonged hypoxicemic challenge is necessary to induce detectable changes in the sensitivity of the baroreceptor reflex in the fetus.

On the basis of the well-established role of brain stem ANG II in cardiovascular regulation in the adult, we propose that the changes in BRS may be mediated by an increase in the effects of angiotensin peptides resulting from the increase in AT1 receptors in NTS. The decrease in BRS is evident before the increase in blood pressure (34), indicating that altered BRS is not a consequence of the higher blood pressure that is observed on days 4 and 5 of the hypoxemic period (34). This diminished baroreflex response may be involved in the increase in blood pressure observed; however, no clear role has been established for the baroreflex in the long-term control of blood pressure (49). In the fetal sheep, data suggest that stimulation of carotid chemoreceptors prevents the expected baroreceptor-mediated heart rate response to carotid occlusion (40). Therefore, we cannot rule out the possibility that the decrease in the BRS is a consequence of an increased chemoreceptor activity.

Alterations in sympathetic outflow have been demonstrated in diverse models of fetal hypoxemia. Using the analysis of spontaneous variation in heart rate, we aimed to assess the balance between sympathetic and vagal systems. The spectral analysis showed an increase in the relative power of low frequencies and a decrease in the relative power of high frequencies of the RRI spectrum. The ranges for LF and HF we used have been validated for the analysis of autonomic influences in heart rate variability in fetal sheep by Kimura et al. (21). Although these authors used cycles/beat in their analysis, the frequency ranges for LF and HF are equivalent when expressed in Hertz. Moreover, a narrower range of frequencies (LF 0.04–0.15 Hz; HF 0.15–0.4 Hz) like the ones used by Li et al. (24) and Min et al. (26) yielded similar results. Thus our findings are in keeping with the redistribution in the power spectrum of heart rate variability during acute hypoxemia in late-gestation fetal sheep (24, 26). This later frequency range is also similar to that used in the human fetus where frequencies ranging 0.03–0.31 Hz and 0.13–1.0 Hz have been used for LF and HF, respectively (50). The changes in the LF to HF ratio have been interpreted to reflect the balance between sympathetic and parasympathetic input on the heart, with an increased sympathetic activity favoring the LF component and consequently a higher ratio (31). Thus the increased LF to HF ratio we observed in hypoxicemic animals does suggest an increase in sympathetic outflow starting on day 1 of hypoxemia. An increased sympathetic drive is consistent with the higher heart rate observed from day 1 in hypoxicemic animals and the subsequent increase in blood pressure (34). Although we do not have evidence to conclusively link the changes in receptors to the impairment in cardiovascular regulation and there is the possibility that they are not causally related, the latter seems less likely. Central actions of ANG-II through the AT1 receptor would be consistent with greater sympathetic and lower parasympathetic control of heart rate. In fact, in the spontaneously hypertensive rat, elevated AT1 receptors in the NTS accompany the hypertension and impaired baroreflex function in those animals (45).

Our finding of a faster bradycardic response in hypoxicemic animals following cord occlusion is consistent with reports indicating an increased chemoreflex response in hypoxicemic or otherwise compromised animals (1, 3, 13). The enhanced chemoreflex response to asphyxia may also be mediated by the increase in binding sites for angiotensin peptides. Injection of ANG II in NTS potentiates the cardiac component of the chemoreceptor reflex (32), and losartan injection in the NTS blocks the effects of chemoreflex activation on baroreflex bradycardia (12).

**Perspectives and Significance**

Cardiovascular reflex responses represent key mechanisms of adaptation and survival. The effects of prenatal hypoxemia on brain stem angiotensin binding may represent another mechanism of fetal adaptation to chronic hypoxemia. Whether these functional and anatomical changes are causally related, are maintained after birth, and have a negative impact on cardiovascular regulation require further investigation. Interestingly, in adult rats exposed prenatally to hypoxemia, arterial
blood pressure and heart rate were not affected under resting conditions, but blood pressure, blood pressure variability, and heart rate under stress conditions were increased (33).

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