Expression of nitric oxide synthase isoforms is reduced in late-gestation ovine fetal brainstem

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Wood, Charles E., Gin-Fu Chen, and Maureen Keller-Wood. Expression of nitric oxide synthase isoforms is reduced in late-gestation ovine fetal brainstem. Am J Physiol Regul Integr Comp Physiol 289: R613–R619, 2005; doi:10.1152/ajpregu.00722.2004.—Fetal baroreflex responsiveness increases in late gestation. An important modulator of baroreflex activity is the generation of nitric oxide in the brainstem nuclei that integrate afferent and efferent reflex activity. The present study was designed to test the hypothesis that nitric oxide synthase (NOS) isoforms are expressed in the fetal brainstem and that the expression of one or more of these enzymes is reduced in late gestation. Brainstem tissue was rapidly collected from fetal sheep of known gestational ages (80, 100, 120, 130, 145 days gestation and 1 day and 1 wk postnatal). Neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) mRNA was measured using real-time PCR methodology specific for ovine NOS isoforms. The three enzymes were measured at the protein level using Western blot methodology. In tissue prepared for histology separately, the cellular pattern of immunostaining was identified in medullae from late-gestation fetal sheep. Fetal brainstem contained mRNA and protein of all three NOS isoforms, with nNOS the most abundant, followed by iNOS and eNOS, respectively. nNOS and iNOS mRNA abundances were highest at 80 days gestation, with statistically significant decreases in abundance in more mature fetuses and postnatal animals. nNOS and eNOS protein abundance also decreased as a function of developmental age. nNOS and eNOS were expressed in neurons, iNOS was expressed in glia, and eNOS was expressed in vascular endothelial cells. We conclude that all three isoforms of NOS are constitutively expressed within the fetal brainstem, and the expression of all three forms is reduced with advancing gestation. We speculate that the reduced expression of NOS in this brain region plays a role in the increased fetal baroreflex activity in late gestation.

In addition to its role as an endothelial cell-derived vascular smooth muscle relaxant, the role of nitric oxide as a neurotransmitter is well established (11). In the medullary nuclei of the adult animal, nitric oxide is synthesized by neurons that express the neuronal form of nitric oxide synthase (nNOS or NOSIII) (3, 23). One of the physiological roles of nNOS in medullary neurons is the local generation of NO that, in turn, acts as an inhibitory neurotransmitter with regard to baroreflex responsiveness (13). Numerous studies in this laboratory have helped to establish the importance of baroreceptor and chemoreceptor reflexes in the minute-to-minute control of blood pressure and umbilical-placental blood flow in the fetus in utero (24, 27). Various studies have demonstrated that the reflex mechanisms governing cardiovascular function in the fetus mature as a function of gestational age (18, 19, 22).

This maturation of reflex responsiveness augments responses to hypotension and hypoxia, both more likely in the periparturient period, and readies the fetus for extrauterine life.

We designed the present experiments to investigate one possible mechanism by which reflex responsiveness might increase in late-gestation fetal sheep. Little is known about the relative abundance or cellular site of expression of the three isoforms of NOS in the fetal brain. We hypothesized that nNOS is expressed in fetal brainstem and that the level of expression of this enzyme is reduced in late gestation, providing one possible explanation for increased baroreflex responsiveness in late gestation. Because little is known about the level of expression of the other two known forms of NOS: endothelial NOS and inducible NOS (eNOS and iNOS, respectively) in the fetal brain, we included in our study an investigation of both of these isoforms of the enzyme. We hypothesized that both eNOS and iNOS would be expressed at lower levels than nNOS and that there would be no ontogenetic pattern of expression of these two enzymes.

Materials and Methods

Tissues used in this study were obtained from newborn (n = 4) and 1-wk-old (n = 4) lambs, and 24 fetal sheep of the following ages: 80 (n = 5), 100 (n = 4), 120 (n = 4), 130 (n = 4), 145 (n = 4) days gestation. The animals were humanely euthanized using an overdose of pentobarbital sodium intravenously. Brains were rapidly isolated, dissected, and snap-frozen in liquid nitrogen, and then stored at −80°C until studied. Brainstem tissue consisted of medulla and caudal pons. The neural tissue was sectioned at the caudal pons-rostral spinal cord border and ~1 mm rostral to the obex. These experiments were approved by the University of Florida Institutional Animal Care and Use Committee.

Messenger RNA (mRNA) was isolated using Trizol (Gibco, Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. After isolation, mRNA was stored in RNA Secure (Ambion, Austin, TX) at −80°C until use. Total mRNA in each sample (4 μg) was converted to cDNA using a high-capacity cDNA archive kit using methodology recommended by the kit manufacturer (Applied Biosystems, Foster City, CA). The newly synthesized cDNA, stored at −20°C, was used for assay of mRNA for the three NOS isoforms by real-time PCR. Real-time RT-PCR reactions were run using AmpliTaq Gold DNA Polymerase (Applied Biosystems), and primers and probe (Geno-Mechanix, Alachua, FL) were specifically designed using Primer Express software (Applied Biosystems). Probes for all three NOS isoforms were labeled with 6-carboxyfluorescein (6-FAM) in the 5’ position and TAMRA in the 3’ position. Sequences of primers and probes are reported in Table 1. In each sample, 18S ribosomal RNA was also measured using real-time RT-PCR method-
ology, with probe, primers, and reagents purchased from Applied Biosystems. All mRNA abundances for the NOS isoforms were normalized to the abundance of 18S rRNA, using the relative cycle threshold (ΔCt) method. All reactions for NOS isoforms were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems), using 100-ng cDNA, 100-nM primers, and 200-nM probe. For 18S rRNA, the reaction was run on 1-ng cDNA. For all of the genes of interest, dilution of the cDNA resulted in linear and quantitative shifts in the cycle threshold. Reactions were amplified using the following conditions: 48°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

NOS isoform protein was measured in the same tissues using Western blot analysis. Homogenization of tissue for protein analysis was carried out using boiling lysis buffer containing 1% SDS, 1.0 mM sodium orthovanadate, 10 mM Tris 7.4. Homogenized samples were boiled for 5 min and centrifuged at 11,000 rpm at 4°C for 20 min to remove particulate matter. Supernatants were aliquoted into microcentrifuge tubes and stored at −80°C until Western blot analysis was performed. Samples, (15 μg protein/lane for nNOS, 60 μg protein/lane for iNOS and eNOS) assayed using the Bradford method, were loaded onto 7.5% Criterion Tris-HCl gel (Bio-Rad, San Rafael, CA) for SDS-PAGE. Each gel accommodates 16 lanes of unknowns, one lane of molecular weight markers (Rainbow markers, Amersham, San Francisco, CA) and one lane of a positive control, for statistical comparison. Each NOS isoform was therefore measured on two gels, with two samples from each age group on each gel. Both blots were simultaneously treated with ECL reagent and exposed on the same film, to reduce between-gel variability. After electrophoresis the proteins were transferred onto nitrocellulose membranes overnight at 22 volts (BioRad). After transfer, the membranes were blocked, and then exposed to appropriate primary and horseradish peroxidase-conjugated secondary antibodies. For nNOS, we used Transduction Laboratories N31020 monoclonal anti-nNOS at a final dilution of 1:2,500. For eNOS, we used Transduction Laboratories N30020 monoclonal anti-eNOS at a final dilution of 1:500. For iNOS, we used Affinity Bioreagents PA3-030A polyclonal anti-iNOS at a final dilution of 1:2,000. Secondary antibodies were Sigma goat anti-mouse IgG A-2554 (Peroxidase Conjugated) at a final dilution of 1:10,000 or Sigma goat anti-rabbit IgG A-0545 (Peroxidase Conjugated) at a final dilution of 1:10,000. Immunoreactive proteins were visualized using ECL chemiluminescence reagents (Amersham). Blots were analyzed using Quantity One densitometric analysis software (Bio-Rad). The results of the densitometry were expressed as relative optical density units.

The cellular distribution of NOS proteins was measured using immunohistochemistry. Fetal (n = 4) and neonatal (n = 3) brains were perfusion fixed with 4% buffered paraformaldehyde, dissected into gross tissue regions. Tissue was processed for embedding by dehydration with progressively increasing concentrations of reagent alcohol, followed by xylene. All tissues were embedded in paraffin and cut in 5-μm-thick sections on a Zeiss rotary microtome. Sections were mounted on poly-L-lysine-coated slides, deparaffinized in xylene, and rehydrated in decreasing concentrations of alcohol in water.

Table 1. Primer and probe sequences used to measure ovine nNOS, iNOS, and eNOS mRNA abundance by real-time PCR

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>nNOS</td>
<td>AAAACCTTCAAGAACATCCAAATCCA</td>
<td>AGCTCTGCTTTCCGCGTTGGT</td>
<td>TCGATCGAGGATTCGTGGCTGCC</td>
</tr>
<tr>
<td>iNOS</td>
<td>TGATGCAGAAGCCAGATGCTCA</td>
<td>TCTCCCTGTGCTGGTGAAGAAG</td>
<td>CGCGGTCAAGGAGGAGGAATCAG</td>
</tr>
<tr>
<td>eNOS</td>
<td>TTTACGGCCAGCAGTCTGCTCA</td>
<td>AGGCGGACCGAGATGCTG</td>
<td>TGGAAAACCTGCAAAGCAGGAATGCC</td>
</tr>
</tbody>
</table>

Fig. 1. Abundance of iNOS and nNOS mRNA compared with the abundance of eNOS mRNA. Data are presented as means ± SE. The letter “a” represents a statistically significant higher value than eNOS, and “b” represents statistically significantly higher value than iNOS.

Fig. 2. nNOS mRNA (top) and protein (bottom) abundance in brainstem tissue from 80-, 100-, 120-, 130-, and 145-day gestation fetal sheep and 1- and 7-day postnatal lambs (n = 4–5 per group). Data are expressed as a fold change relative to 80-day fetal sheep. Data are represented as mean values ± 1 SE. The letter “a” represents a statistically significant difference from 80-day fetal sheep, and “b” represents statistically significant difference from 100-day fetal sheep. A representative Western blot of nNOS is superimposed on the bar graph.
Immunohistochemistry was performed using the same commercial antibodies for all three NOS isoforms that were used in the Western blot experiments. The antibodies were used at a final concentration of 1:500 for nNOS and iNOS and 1:200 for eNOS. Glial acidic fibrillary protein (GFAP) and neuron-specific enolase (NSE) were stained using prediluted antibodies from Zymed, Inc (South San Francisco, CA: catalog number 08–0021 and 08–0042, respectively). Visualization was accomplished using goat anti-rabbit and goat anti-mouse IgG conjugated to red (Alexa Fluor594: Catalog #A11037 Molecular Probes Eugene, OR) and green (Alexa Fluor488: Catalog #A11034 Molecular Probes) fluorophores. Alternatively, immunostaining was visualized using biotinylated anti-rabbit and anti-mouse IgG and streptavidin-conjugated horseradish peroxidase from Zymed (South San Francisco, CA), and metal-enhanced diaminobenzidine (DAB) from Pierce (Rockford, IL). Some sections stained with DAB were counterstained using hematoxylin (Fisher Scientific). Both fluorescent and brightfield immunostaining were visualized and photographed using a Zeiss Axioplan 2 microscope and a SPOT digital imaging system (McKnight Brain Institute, University of Florida). In several sections, fluorescent immunostaining was visualized using a BioRad 1024ES confocal microscope.

The expression of nNOS, iNOS, and eNOS mRNA was normalized to 18S mRNA and reported graphically as the change relative to the mean concentration at 80 days of gestation. The calculation of relative expression was performed using the ΔΔCt method, as described previously. Statistical analysis, on the other hand, was performed on the values of ΔCt, because these values are normally distributed (26). Values of ΔCt (for mRNA) and relative optical density (for protein) were analyzed using two-way ANOVA (Sigma Stat, SPSS, Chicago, IL), and pairwise comparisons of group means were performed using Duncan's multiple range test (7). Protein data (optical densities) were analyzed by one-way ANOVA, followed by Duncan's multiple range test. The null hypothesis (i.e., all groups are similar) was rejected when P < 0.05.

RESULTS

All three isoforms of NOS were expressed in the fetal brainstem. The relative abundance of nNOS was greatest, followed by iNOS and eNOS, respectively, in the 80-day fetal brainstem (Fig. 1).

nNOS mRNA abundance was highest at 80 days' gestation, then decreased significantly (P = 0.015 by one-way ANOVA) as a function of developmental age (Fig. 2). Post hoc analysis revealed that the abundance at 145 days and postnatal ages were significantly lower than at 80 and 100 days (P < 0.05 by Duncan’s multiple range test). nNOS protein abundance was also decreased significantly as a function of developmental age (P < 0.01 by one-way ANOVA). Post hoc analysis of the protein data revealed a similar trend compared with the mRNA data. The nNOS abundance in the more mature animals (130- and 145-day fetuses, and 1- and 7-day lambs) was significantly less than in the 80-, 100-, and 120-day fetuses (P < 0.05 by Duncan’s multiple range test).

The abundance of iNOS mRNA was also significantly (P = 0.002 by one-way ANOVA) decreased as a function of devel-

![iNOS mRNA](image1.png)

![iNOS Protein](image2.png)

![eNOS mRNA](image3.png)

![eNOS Protein](image4.png)

Fig. 3. iNOS mRNA (top) and protein (bottom) abundance in brainstem tissue from 80-, 100-, 120-, 130-, and 145-day gestation fetal sheep and 1- and 7-day postnatal lambs (n = 4–5 per group). Data are expressed as fold change relative to 80-day fetal sheep. Data are presented as means ± SE. The letter “a” represents a statistically significant difference from 80-day fetal sheep, and “b” represents a statistically significant difference from 100-day fetal sheep. A representative Western blot of iNOS is superimposed on the bar graph.

![eNOS mRNA](image5.png)

![eNOS Protein](image6.png)

Fig. 4. eNOS mRNA (top) and protein (bottom) abundance in brainstem tissue from 80-, 100-, 120-, 130-, and 145-day gestation fetal sheep and 1- and 7-day postnatal lambs (n = 4–5 per group). Data are expressed as fold change relative to 80-day fetal sheep. Data are presented as means ± SE. A representative Western blot of eNOS is superimposed on the bar graph.
opmental age (Fig. 3). As for nNOS, iNOS mRNA was significantly lower at 145 days and postnatal ages compared with 100 days (*P* < 0.05 by Duncan’s multiple range test). In contrast, there was no statistically significant change in the abundance of iNOS protein as a function of developmental age (*P* > 0.05 by one-way ANOVA).

The abundance of eNOS mRNA was not significantly altered as a function of fetal gestational age, although the general pattern of expression appeared similar to both nNOS and iNOS (*P* = NS by one-way ANOVA; Fig. 4). eNOS protein, on the other hand, was significantly decreased (*P* = 0.002 by one-way ANOVA). Post hoc analysis of the data using Duncan’s test revealed that eNOS protein abundance in postnatal ages was significantly reduced compared with 80-day fetuses and that its abundance in 120 day fetuses was significantly greater than in all subsequent ages (*P* < 0.05).

In immunofluorescent images, nNOS was clearly localized in neurons (Fig. 5A). iNOS immunostaining was widespread, although localized to glia (Fig. 5, B, C, and F). We did not observe costaining of iNOS and NSE, suggesting that there is little or no expression of iNOS in neurons (Fig. 5E). eNOS was visible in vascular endothelial cells (Fig. 5I) but was also visible in both neurons (Fig. 5G) and glia (Fig. 5H). Representative brightfield images for eNOS (Fig. 6, A, D, E), iNOS (Fig. 6B) and nNOS (Fig. 6C) reveal patterns of staining that are consistent with the immunofluorescence data (Fig. 5). Brightfield images of eNOS immunostaining reveal that eNOS is expressed in medullary cardiovascular reflexogenic areas, including nucleus tractus solitarius (Fig. 6D) and ventrolateral medulla (Fig. 6E).

**DISCUSSION**

The results of this study demonstrate that all three isoforms of NOS are expressed in the fetal brainstem and that the expression of the three NOS isoforms is developmentally...
regulated. The decreasing abundance of NOS suggests the possibility that the increased baroreceptor and chemoreceptor reflex responsiveness in relatively mature fetuses might be related to a decrease in the production of nitric oxide.

It is likely that all three isoforms of NOS are involved in neuronal signaling in many pathways traversing the brainstem. Our interest in studying these enzymes is the possibility that nitric oxide generation within the fetal brainstem might be inhibitory to the baroreceptor and chemoreceptor pathways. This possibility is supported by work published by Chikada and colleagues (4), demonstrating that intracerebroventricular injection of l-NAME, a nonselective inhibitor of NOS isoforms, into conscious rats increased blood pressure and heart rate. More recently, Lo and colleagues (13) demonstrated that microinjection of l-arginine into the nucleus tractus solitarius in rats decreased heart rate and that this effect was blocked by injection of l-NAME. Chan et al. (3) have reported pharmacologic data demonstrating roles for both iNOS and nNOS as modulators of neuronal mechanisms controlling cardiovascular function. Ma and colleagues (14) have presented data that suggest that exogenous glucocorticoids alter baroreflex and chemoreflex responsiveness in the fetal sheep. It is possible that glucocorticoid alters cardiovascular function in the fetus. Results of experiments by Wood and colleagues (25) and later by Wintour and colleagues (6, 21) demonstrated that physiological increases in fetal plasma cortisol increase fetal blood pressure. Fletcher and colleagues (8, 9) have demonstrated that exogenous glucocorticoids alter baroreflex and chemoreflex responsiveness in the fetal sheep.

The quantitatively most important form of NOS in the brainstem is nNOS, expressed in 158 (± 7 SE)-fold higher abundance than iNOS and 4.9 (± 1 SE)-fold higher abundance than eNOS at 80 days’ gestation. The decrease in the abundance of nNOS mRNA in older fetuses suggests the possibility that NOS as an inhibitory neurotransmitter has a reduced importance quantitatively at the end of gestation.

Surprisingly, iNOS is expressed in the fetal brainstem, not in response to any inflammatory insult. Several investigators have reported that iNOS is expressed in both glia and neurons in response to cytokine exposure or other proinflammatory conditions (2, 10, 16). Similarly, iNOS is expressed in the cerebellum in adult rats (17). The apparently constitutive expression of iNOS in the fetal brainstem suggests that nitric oxide is synthesized in a calcium-independent manner. The expression of iNOS suggests a possible link between glucocorticoids and baroreflex function in the fetus. Results of experiments by Wood and colleagues (25) and later by Wintour and colleagues (6, 21) demonstrated that physiological increases in fetal plasma cortisol increase fetal blood pressure. Fletcher and colleagues (8, 9) have demonstrated that exogenous glucocorticoids alter baroreflex and chemoreflex responsiveness in the fetal sheep. It is possible that glucocorticoid alters cardiovascular function in the fetus. Results of experiments by Wood and colleagues (25) and later by Wintour and colleagues (6, 21) demonstrated that physiological increases in fetal plasma cortisol increase fetal blood pressure. Fletcher and colleagues (8, 9) have demonstrated that exogenous glucocorticoids alter baroreflex and chemoreflex responsiveness in the fetal sheep.

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cular reflex control acutely in the fetal sheep by altering the level of expression of iNOS in the fetal brainstem. The influence of ontogenetic increases in fetal plasma cortisol is less clear. iNOS mRNA is decreased late in gestation when circulating cortisol concentrations are elevated, while iNOS protein is relatively constant.

The expression of eNOS is most common in vascular endothelial cells; however, previous studies in other laboratories have reported eNOS expression in both neurons and glia (1, 12). For example, eNOS is expressed in the cerebral cortex of rats in low levels (12, 29). The expression of the eNOS is upregulated in neurons within the penumbra after middle cerebral artery occlusion (12). eNOS is highly expressed in hippocampal pyramidal cells (5). There is a substantial nonendothelial expression of eNOS in the suprachiasmatic nucleus, although the expression appears to be localized to astrocytes (1).

nNOS expression in the medulla has been reported by numerous laboratories and is widely regarded as the major nitric oxide-producing enzyme in medullary neurons (14). As such, nNOS expression is well characterized in the nucleus tractus solitarius, as well as other medullary regions important for baroreceptor and chemoreceptor reflex function (20). Massman and colleagues have reported the expression of nNOS in cerebral cortex, cerebellum, striatum, and hippocampus in fetal sheep, as well as the ontogenetic pattern of expression in these brain regions (15). Interestingly, they reported multiple transcripts for nNOS in these tissues, mostly corresponding to the alternatively spliced transcripts identified in tissues from adult animals of various species (28). Although the present study is the first to report the expression of nNOS in the medulla of the fetus, a potential drawback of the experimental design is the lack of information about the expression of splice variants. Nevertheless, there is limited information about splice variants of nNOS in the sheep, and it is possible that several splice variants are present in the fetal brainstem. A comprehensive investigation of all the potential splice variants in this tissue is beyond the scope of this study; however, the real-time PCR probe and primers were designed to anneal with a portion of the molecule that would be expected to appear in all of the reported variants. As a result, the ontogenetic pattern for nNOS expression in this study represents the theoretically total expression of nNOS.

For all three forms of NOS, we observed development-related decreases in the abundance at either the mRNA (iNOS), protein (eNOS), or mRNA and protein (nNOS) levels. We cannot fully explain the differences between the mRNA and protein for eNOS or iNOS. We suspect that there is a true decrease in abundance of both mRNA and protein for eNOS and that our failure to demonstrate statistical significance at the protein level might have been the result of a type II statistical error (30). For iNOS, there was a statistically significant decrease in mRNA abundance but no trend for decrease at the protein level. It is possible that this difference might have been caused by differential regulation of iNOS expression in neurons in other brain regions that contribute to brainstem iNOS immunoreactivity via axoplasmic transport. On the other hand, we cannot rule out a type II statistical error in the iNOS Western blot experiments.

We conclude that there is an ontogenetic decrease in the expression of nNOS, iNOS, and eNOS in the brainstem of the ovine fetus throughout the latter half of gestation. Furthermore, we conclude that all three forms of NOS were found in neurons. We speculate that the decreased abundance of one or more of these enzymes is related to the increased baroreflex and chemoreflex responsiveness in late gestation.

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