Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus

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Ducsay CA, Hyatt K, Mlynarczyk M, Root BK, Kaushal KM, Myers DA. Long-term hypoxia modulates expression of key genes regulating adrenomedullary function in the late gestation ovine fetus. Am J Physiol Regul Integr Comp Physiol 293: R1997–R2005, 2007. First published August 15, 2007; doi:10.1152/ajpregu.00313.2007.—We previously communicated that long-term hypoxia (LTH) resulted in a selective reduction in plasma epinephrine following acute stress in fetal sheep. The present study tested the hypothesis that LTH selectively reduces adrenomedullary expression of phenylethanolamine-N-methyltransferase (PNMT), the rate-limiting enzyme for epinephrine synthesis. We also examined the effect of LTH on adrenomedullary nicotinic, muscarinic, and glucocorticoid receptor (GR) expression. Ewes were maintained at high altitude (3,820 m) from 30 to 138 days gestation (dGA); adrenomedullary tissue was collected from LTH and age-matched, normoxic control fetuses at 139–141 dGA. Contrary to our hypothesis, in addition to PNMT, adrenomedullary expression (mRNA, protein) of tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DBH) were reduced in the LTH fetus. Immunocytochemistry indicated that TH and DBH expression was lower throughout the medulla, while PNMT appeared to reflect a reduction in PNMT-expressing cells. Nicotinic receptor alpha 1, 2, 3, 5, 6, 7, beta 1, 2, and 4 subunits were expressed in the medulla of LTH and control fetuses. Messenger RNA for alpha 1 and 7 and beta 1 and 2 subunits was lower in LTH fetuses. Muscarinic receptors M1, M2, and M3 as well as the GR were also expressed, and no differences were noted between groups. In summary, LTH in fetal sheep has a profound effect on expression of key enzymes mediating adrenomedullary catecholamine synthesis. Further, LTH impacts nicotinic receptor subunit expression potentially altering cholinergic neurotransmission within the medulla. These findings have important implications regarding fetal cardiovascular and metabolic responses to stress in the LTH fetus.

Adrenal; ovine; catecholamine


The expression of the enzymes responsible for the biosynthesis of catecholamines in the adrenal medulla has been examined during gestation in fetal sheep (3). Levels of mRNA for tyrosine hydroxylase (TH) in the medulla increase coincident with the onset of splanchic innervation (~100–125 DGA) and subsequently decline after ~140 DGA. Phenylethanolamine N-methyltransferase (PNMT) mRNA exhibits a slightly different pattern of expression compared with TH, with a progressive increase slightly earlier in gestation at ~80–100 DGA and an additional larger increase near term (~140–146 DGA) (3). The noted increase in PNMT near term in the adrenal medulla occurs coincident with increased adrenal epinephrine content and increased basal epinephrine release from perfused adrenal glands (9, 12). Higher basal plasma epinephrine concentrations are also noted after ~130–135 DGA in the ovine fetus (9, 37). As glucocorticoids have been shown to stimulate PNMT expression in bovine adrenomedullary cells in vitro, the prepartum increase in adrenomedullary PNMT and apparent enhanced epinephrine production may be directly related to the exponential increase in fetal glucocorticoid production that occurs between ~135 DGA and term (28, 39, 43).

Cholinergic stimulation of rat PC12 cells via nicotinic receptor activation has been shown to stimulate expression of TH, dopamine beta hydroxylase (DBH) and PNMT (15, 16, 19, 39, 40). Nicotinic receptors likely represent the major receptor mediating cholinergic regulation of catecholamine biosynthesis and release. Consistent with pharmacological studies, α3, α5, α7 and β2 nicotinic receptor subunits are expressed in adult bovine adrenal medullary cells (10, 29). In addition to cholinergic regulation of catecholamine biosynthesis and release via nicotinic receptors, activation of muscarinic receptors potentiates nicotinic receptor-mediated catecholamine release in bovine adrenomedullary cells (5, 42). Pharmacological studies support the expression of multiple muscarinic receptor subtypes in both bovine and rat adrenal medulla, while other studies using RT-PCR indicate that type 3 and 4 muscarinic receptors are expressed in the adrenal medulla (6, 17, 36). The coincident onset of the increase in expression of TH and PNMT with functional/structural innervation in the late-gestation sheep fetus supports a role for cholinergic mechanisms in...
the regulation of expression of these key enzymes mediating catecholamine biosynthesis. The type and subclass of cholinergic receptors expressed in, and regulating, adrenal medullary cells in the ovine fetus is presently unknown.

Hypoxia is a potent fetal stressor, and the effect of experimentally induced, acute (min to hours), prolonged (several hours to days), or chronic (a few weeks) hypoxia on fetal plasma catecholamine concentrations and catecholamine synthesis has been examined (2, 20, 38). In addition to the duration, the severity has varied greatly among the various paradigms employed to induce hypoxia. Unfortunately, models of chronic hypoxia often result in a mixed phenotype with effects on such parameters as blood pH and/or fetal growth and metabolism. Our laboratory has developed a model of long-term hypoxia (LTH), in which pregnant ewes are maintained at high altitude (3,820 m) resulting in a sustained, moderate hypoxic state from day 30 of gestation to term without associated fetal growth retardation or fetal acidemia (1, 22, 23).

Using this model, we previously communicated that fetal plasma epinephrine concentrations were significantly attenuated in response to a secondary stressor (superimposed acute hypoxia) compared with control fetuses (26) despite exhibiting similar basal plasma catecholamine levels. In contrast to epinephrine, the norepinephrine response to the secondary stress was unaffected by LTH.

The purpose of the following study was to determine the effect of LTH on expression of genes involved in the production of catecholamines in the fetal ovine adrenal medulla. Specifically, we examined the expression of TH, DBH, and PNMT using real-time PCR and Western blot analysis. We also examined the effect of development under conditions of LTH on nicotinic and muscarinic receptor mRNA levels and glucocorticoid receptor (GR) protein expression in the developing adrenal medulla.

**METHODS**

**Animals**

All procedures were conducted with approval of the Institutional Animal Care and Use Committees (Loma Linda University School of Medicine, Loma Linda, CA). Pregnant ewes were maintained at the Barcroft Laboratory White Mountain Research Station (elevation 3,820 m) beginning at day 30 of gestation (term = 146 days). After spending ~105–110 days of gestation at high altitude, the animals were transported to Loma Linda University Medical Center Animal Research Facility (elevation: 346 m), where they were implanted with a nonocclusive tracheal catheter (4.0 mm OD) and an arterial catheter. Maternal Po2 for LTH group was maintained at ~60 mmHg (mean Po2 measured in animals at altitude) by adjusting humidified nitrogen (N2) gas flow through the tracheal catheter as previously described (1, 21, 23). Normoxic, age-matched pregnant ewes were used as controls. Between days 139 and 141 of gestation, both control and LTH ewes were sedated with pentobarbital sodium, intubated, and maintained under general anesthesia with 1.5–2% halothane in oxygen. Fetuses (for this study, all fetuses were from twin pregnancies) were then delivered through a midline laparotomy, and the fetal adrenal glands were collected, frozen rapidly in liquid nitrogen, and stored at ~80°C until analyzed.

**Quantitative Real-Time PCR**

Messenger RNA for TH, DBH, and PNMT, selected muscarinic receptor subtypes and nicotinic receptor subunits was quantified using real-time PCR (quantitative RT-PCR, or qRT-PCR); we have previously described and validated the methods for qRT-PCR for a variety of genes in our laboratory (14, 32, 33). Total RNA was prepared from fetal adrenal glands (n = 7 or 8 for control and LTH groups) with an RNA preparation kit as per manufacturer’s instructions (Qiagen, Valencia, CA). Before reverse transcription, residual genomic DNA was removed from total RNA with DNase I (1 Unit, 60 min at 37°C; Ambion, Austin, TX). The DNase I was subsequently removed from the RNA samples via PCR clean-up columns (Qiagen). An initial denaturation step was performed for 5 min at 95°C before first-strand synthesis at 42°C for 50 min. Reverse transcription was then performed using 1 μg total RNA, with oligo dT as the primer, and Superscript II as reverse transcriptase; the reaction was terminated by heating to 70°C for 15 min.

Real-time PCR was performed using 25 to 100 ng of cDNA (equal to input RNA) per PCR. All PCR were performed in triplicate. Initial qRT-PCRs were performed using serial dilutions of cDNA ranging from 250 to 15,625 ng (250, 125, 62.5, 31.25, 15.625 ng) to determine that the quantity of cDNA used for analysis of each specific mRNA was within the linear range of amplification for each primer. For each mRNA, the starting amount of cDNA for qRT-PCR used was within the linear amplification range. For each primer set, the amplicon was directly sequenced by Sanger dyeoxysequencing (Oklahoma Medical Research Foundation Sequencing Core, Oklahoma City, OK) to confirm amplicon identity. SYBRGreen (1× SYBRGreen master mix; Bio-Rad, Hercules, CA) was used as the fluorophore, and PCR was performed using a Bio-Rad iCycler equipped with the real-time optical fluorescent detection system. The primer sequences were derived from bovine sequences obtained from the National Center for Biotechnology Information (NCBI) either as cDNA sequences or were derived from the bovine genome resource at NCBI (http://www.ncbi.nlm.nih.gov/genome/guide/cow/) based on the human sequence for that gene. The NCBI accession numbers used are listed in Table 1. A three-step PCR was used: 95°C for 45 s, annealing (primer specific but typically 55–60°C) for 30 s and 72°C extension for 30 s. A total of 35 cycles was performed. A melt curve analysis was conducted on each sample after the final cycle to ensure that a single product was obtained, and agarose gel electrophoresis confirmed that the single PCR product was of the expected size. We used cyclophilin as a “house keeping” mRNA, using the identical first-strand cDNA used for quantification of specific mRNAs of interest and in the same PCR run as for the gene of interest to circumvent any discrepancy between run variation. As previously reported (14, 32, 33), after analysis of several candidate mRNAs cyclophilin demonstrated no gestational age or LTH modulation of expression; we have also found that cyclophilin and GAPDH were equally efficacious when used as internal housekeeping mRNAs in our real-time PCR applications (14). Control PCR for each primer pair and RNA source included 1) elimination of reverse transcriptase during first-strand cDNA synthesis (ensures that PCR product depends upon RNA) and 2) no RNA/ cDNA in reverse-transcription reaction (assures that no amplicon contamination has occurred). Primers were utilized that provided 1) a single PCR product (identity confirmed by sequencing), 2) dilution curve of cDNA exhibited a slope of 100% ± 10% “efficiency” where 100% = Δ3 Ct/log cDNA input (Ct is the threshold PCR cycle at which fluorescence is detected above baseline), and 3) the melt curve analysis post-PCR must demonstrate one product. For quantification purposes, a synthetic single-stranded DNA standard was used to generate a standard curve (100, 10, 1, 0.1, 0.01, and 0.001 pg of standard DNA) for extrapolation of starting cDNA concentrations per reaction. Each standard point was run in duplicate and in the same PCR block as the unknowns. Linear regression was used to quantify starting RNA (cDNA) based on Ct values as extrapolated from the standard curve. The efficiency of the standard and primers was 100% based on the above criteria.
Table 1. NCBI accession numbers

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FW, forward; RV, reverse; CYCLO, cyclophilin; GAPDH, glyceraldehyde phosphate dehydrogenase; TH, tyrosine hydroxylase; DBH, dopamine β-hydroxylase; PNMT, phenylethanolamine N-methyltransferase; NR, nicotinic receptor; M, muscarinic; Bt, Bos taurus; Hs, Homo sapiens; NCBI, National Center for Biotechnology Information.

Western Blot Analysis

Western blot analysis was performed similarly to what has been described for our laboratory (32, 33). Adrenal glands (for analysis of TH, DBH, and PNMT) or isolated medulla (for analysis of GR) were homogenized (4°C, 1 ml, 0.1 M acetic acid, 100 mM sodium chloride, pH 5.0, containing 1 mM pepstatin, 0.4 mM pefablock, 1 μg/ml leupeptin), centrifuged at 12,000 g for 2 min, and the supernatant was subsequently electrophoretically transferred to nitrocellulose membranes and subjected to Western blot analysis. The membranes were blocked for 1 h with TBS (10 mM Tris·HCl, pH 7.2, 100 mM saline) containing 0.1% Tween 20 (TTBS) and 10% nonfat dry milk. Membranes were then washed twice in TTBS and incubated with primary antibodies (Chemicon for TH, DBH, and PNMT; and Affinity Bioreagents CA1–511A for GR) prepared in TTBS-5% nonfat dry milk overnight at 4°C. A chemiluminescent detection system (Pierce) was used, and blots were exposed to film (Hypermex; Kodak, Rochester, NY) for varying lengths of time. For controls, we omitted the primary antiserum. Films were quantified by densitometry. To ensure that equal protein amounts were loaded for each sample, identical SDS-PAGE was performed with the same protein samples from control and LTH adrenal glands and subsequently stained with Coomassie blue.

Immunocytochemistry

For TH, adrenal glands from LTH and control fetuses were fixed overnight at 4°C in paraformaldehyde (4%) in PBS, paraffin-embedded, sectioned (20 μm), affixed to Microfrost Plus glass slides, and stored at room temperature. For DBH and PNMT, adrenal glands from both LTH and control fetal sheep (n = 3/group) were cryosectioned (15°C; 20-μm sections), affixed to Microfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA), and stored at −80°C. The procedures used for immunostaining were optimized for each antiserum used.
Tyrosine Hydroxylase

Unless otherwise specified, all procedures were performed at room temperature. For TH immunostaining, slides (one slide per fetus containing sections representing the midsection of the adrenal gland) were heated for 30 min at 59°C and subsequently deparaffinized by sequential treatment with xylene (10 min, twice) followed by ethanol (twice for 3 min with 100%, 95%, 70%) with a final rinse (30 s) in deionized water. Antigen retrieval was accomplished by treating sections with Proteinase K (DAKO, Glostrup, Denmark) for 3 min. Endogenous peroxidase activity was quenched by treating sections with 3% H2O2 (15 min). Following three, 5-min washes in 0.1M PBS (pH 7.4), sections were incubated for 1 h at room temperature in blocking serum [5% nonfat dry milk (NFDM), 5% normal goat serum (NGS), 0.3% Triton X-100, 50 mM Tris, pH 7.6]. Tyrosine hydroxylase rabbit polyclonal Ab (Chemicon, Temecula, CA) diluted 1:1,000 in primary antibody solution (5% NFDM, 3% NGS, 0.1% Triton X-100, 20 mM Tris, pH 7.6) was applied, and the sections were subsequently incubated overnight at 4°C in a humidified chamber. After three, 5-min washes in 0.1 M PBS (pH 7.4), sections were incubated for 1 h with biotinylated goat anti-rabbit secondary antibody diluted 1:200 in secondary antibody solution (2% NGS, 0.1% Triton X-100, 20 mM Tris, pH 7.6). Sections were then washed three times, 5-min each, in 0.1 M PBS (pH 7.4) and then incubated in ABC Complex for 30 min in a humidified chamber, washed in 0.1M PBS (pH 7.4) and diaminobenzidine (DAB) substrate applied dropwise. Color development was allowed to proceed for ~7 min followed by quenching with three successive 5-min washes in 0.1 M PBS (pH 7.4). Sections were dehydrated by sequential dips into dilutions of ethanol and then xylene and allowed to dry overnight.

PNMT and DBH

Frozen tissue sections were placed at room temperature overnight. After an initial wash in PBS (pH 7.4), sections were fixed in either 4% paraformaldehyde (10 min; PNMT) or in 10% formalin in 0.1 M sodium cacodylate, pH 7.4, with 25 mM CaCl2 (30 min; DBH), and then washed six times in PBS (pH 7.4). For DBH, sections were washed with PBS, 20 mM Tris·HCl (pH 9.0), then submersed into 20 mM Tris·HCl, at pH 9.0 (heated to boiling just prior to use), for antigen retrieval. For DBH, after cooling to room temperature, sections were washed in PBS, deionized water, and again in PBS. For PNMT, after fixation, sections were washed four times in PBS. For both PNMT and DBH, sections were treated with 0.1% H2O2 containing 50% methanol for 10 min to quench endogenous peroxidase activity. Sections were washed three times in PBS (pH 7.4) and blocked (5% NFDM, 5% NGS, 0.3% Triton X-100 in PBS, pH 7.4) for 1 h. Sections were washed three times with PBS (pH 7.4) and incubated with either a rabbit polyclonal anti-PNMT antibody (Chemicon; 1:1,000 in primary antibody solution) or a rabbit polyclonal anti-DBH antisera (Chemicon; 1:2,000 in primary antibody solution) overnight at 4°C. Following six washes in PBS (pH 7.4), sections were incubated for 1 h in biotinylated goat anti-rabbit secondary antibody diluted 1:200 in secondary antibody solution. After eight washes in PBS, sections were incubated in ABC Complex for 30 min, then washed three times in 0.1 M Tris·HCl, 0.15 M NaCl (pH 7.5). DAB substrate was applied and left on for ~3–5 min before quenching with 0.1 M Tris, 0.15 M NaCl (pH 7.5), then in PBS. Tissues were dehydrated by sequential dips in dilutions of ethanol, followed by isopropanol and xylene, and allowed to dry at RT overnight.

For all antigens (TH, DBH, and PNMT), a control staining was performed in which primary antisera was omitted.

Statistical Analysis

Differences between normoxic control and LTH fetuses in mRNA and protein for each gene of interest were compared using Student’s two tailed t-test. Significance was set at the 0.05 level; all data are presented as means ± SE.

RESULTS

There were no differences in fetal weight between LTH (3.68 ± 0.14 kg) and control fetuses (3.45 ± 0.23 kg). LTH resulted in a significant decrease in mRNA for all three major enzymes mediating catecholamine synthesis, TH, DBH, and PNMT (Fig. 1). Western blot analysis confirmed a decrease in expression of these three enzymes at the protein level (Fig. 1B). Similarly, immunocytochemistry staining for TH and DBH appeared to be reduced in the adrenal medulla of the LTH fetal sheep (Fig. 2) consistent with Western blot analysis and qRT-PCR. For PNMT, the number of medullary cells expressing PNMT appeared reduced compared with control fetuses (Fig. 2), while the intensity of the staining did not appear different between the two groups. PNMT staining was localized to the peripheral region of the medulla in both control and LTH, with the LTH fetuses exhibiting PNMT-positive cells in the region adjacent to the cortex. Overall, neither the medulla nor the cortex was grossly reduced in size as a result of LTH compared with control fetuses.

To further explore the effect of LTH on adrenomedullary function, we examined nicotinic and muscarinic receptor mRNA to identify potential mechanisms through which LTH decreases expression of the enzymes mediating catecholamine synthesis. As shown in Figs. 3 and 4, not only are the anticipators α3, α5, α7, and β2 nicotinic receptor subunits expressed in the ovine fetal adrenal, but also α1, α2, α6, β1, and β2 subunits. We did not observe any appreciable mRNA for α9, or β3 subunits using several primer sets. We further used qRT-PCR to quantify α1, α5, α6, α7, β1, β2, and β4 mRNA levels. As shown in Fig. 3, mRNA for α1 and α7 was decreased in the LTH adrenal; α6 demonstrated a trend for being lower in the LTH adrenal, while α2, α5, α6 remained unchanged. Fig. 4 illustrates the significant reduction in β1 and β2 mRNA in the LTH fetuses with no difference in β3 between the two groups. Muscarinic receptor subtype M1, M2, and M3 were expressed to a similar extent; although M4 or M5 was not detected in either group (Fig. 5). To ascertain that the lack of RT-PCR product for nicotinic receptor subunits and the M4 and M5 receptors reflected a lack of expression and not simply a primer failure, we performed PCR using ovine genomic DNA (since the primers used did not span introns) to ensure that all primer sets tested yielded a PCR product. Further, because the primers were within introns, specificity of the PCR for reverse transcription was confirmed for each primer and RNA sample by omission of the RT as a control.

Western blot analysis for medullary glucocorticoid receptor protein expression revealed a band at 95 kDa, consistent with the full-length, active form of the receptor in both groups (Fig. 6). However, there was no difference in the mean relative optical densities of bands between the control and LTH groups.

DISCUSSION

We previously reported that the adrenal cortex in the late-gestation sheep fetus undergoes a distinct adaptation to development under conditions of long-term hypoxia (33). Complementary changes were also noted at the level of the anterior pituitary in the LTH fetus (32). These adaptive changes cul-
terminate in the capacity of the LTH fetus to maintain normal basal plasma cortisol concentrations in the face of the stressful hypoxic environment while enhancing cortisol production in response to a secondary stressor. We hypothesized that the adaptation observed in the fetal hypothalamic-pituitary-adrenal axis was likely beneficial, allowing the fetus to achieve optimal growth and to survive acute secondary stressors under oxygen-compromised conditions.

In addition to adaptation to LTH at the level of the adrenal cortex, we previously communicated (26) that secondary stress-induced secretion of epinephrine was severely blunted in LTH fetal sheep, while norepinephrine achieved plasma levels similar to the normoxic control fetuses. Basal plasma concentrations of epinephrine and norepinephrine were similar in the LTH fetal sheep compared with control fetuses. On the basis of these earlier observations, we hypothesized that expression of PNMT, the rate-limiting enzyme for epinephrine synthesis, would be lower in the medulla of the LTH fetal adrenal compared with control, while TH and DBH, fundamental for norepinephrine production, would remain unaffected. Our present results substantiate that the expression of PNMT is indeed reduced in the adenomedulla of LTH fetal sheep, thus contributing to the significantly blunted epinephrine response observed postsecondary stress in these fetuses. Immunocytochemical analysis indicated that the reduced mRNA and protein levels for PNMT were more likely due to a decreased number of medullary cells expressing this enzyme rather than a generalized decrease in expression in the medulla. This...

Fig. 1. Expression of tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine-N-methyltransferase (PNMT) in the adrenal medulla of normoxic control (CONT) and long-term hypoxic (LTH) fetal sheep. A: TH, DBH, and PNMT mRNA concentrations (pg mRNA/100 ng cDNA) as determined by quantitative RT-PCR as described in METHODS. All values represent means ± SE for control (n = 7) and LTH (n = 8) fetal sheep. B: Western blot analysis for TH, DBH, and PNMT as described in METHODS. Representative samples for control (lanes 1 and 2) and LTH (lanes 4 and 5) fetuses. C: densitometric analysis of TH, DBH, and PNMT protein levels for control (n = 5) and LTH (n = 5) fetal adrenals as determined by Western blot analysis. Data are expressed in arbitrary units, means ± SE. (*P < 0.01, compared with control).

Fig. 2. Representative immunocytochemistry staining for TH (A and B), DBH (C and D), and PNMT (E and F) in control and LTH fetal adrenals. PNMT-positive cells in the region adjacent to the cortex are indicated by the arrows. M, medulla; Ctx, cortex.
pattern of PNMT expression is consistent with the findings of Adams et al. (3) using a placental restriction model, which results in both fetal growth retardation and hypoxia in the ovine fetus. In these studies, fetal hypoxia was in a range similar to that observed in our LTH model (1, 23) These investigators observed a decrease in PNMT expression, in which PNMT-expressing cells were restricted to the region of the medulla adjacent to the cortex and noted that the extent of PNMT-positive cells were considerably less compared with control fetuses. Because unstressed plasma concentrations of epinephrine were not different between LTH and control fetuses (26), the amount of PNMT expressed or the number of PNMT-expressing cells must be sufficient to maintain resting plasma concentrations of epinephrine yet limiting in producing sufficient stores of this catecholamine for release upon stress. It will be of interest to further examine how target organs have adapted in these fetal sheep in response to the limited epinephrine component of the catecholamine response to stress.

The findings of the present study, that both TH and DBH were suppressed in the adrenal medulla of LTH fetal sheep, in spite of normal resting and secondary stress induced plasma norepinephrine concentrations, implicates the sympathetic ganglia as compensating for the reduced capacity for adrenomedullary norepinephrine synthesis and secretion. Because we previously found that bilateral section of the splanchnic nerve resulted in a loss in capacity for release of both epinephrine and norepinephrine in response to hypotension (34), the changes in the LTH model may reflect a unique adaptation of the sympathetic system to development under conditions of LTH. Alternatively, metabolism and clearance catecholamines from fetal plasma may also be reduced in response to LTH.

A previous study by Adams et al. (3) found that a placental restriction model of fetal hypoxia resulted in decreased adrenomedullary PNMT expression also found no effect on the expression of TH in the medulla of late-gestation fetal sheep. However, using the same model, Coulter et al. (13) found that at 90 days of gestation, immunostaining for both DBH and TH was lower in the placental restricted fetuses. Thus, in this model of experimentally induced hypoxia, the adrenal medulla is adapting differently than in the LTH model used in the present study. The placental restriction paradigm, which results in fetal growth restriction may represent a more potent stressor compared with altitude-induced LTH.

Fig. 3. A: ethidium bromide-stained agarose gel of RT-PCR for nicotinic receptor α subunits (lanes 1–7, 9) from a representative fetus from both CONT and LTH fetal sheep. Reverse transcription was performed on 100 ng cDNA for each primer set. B: quantitative RT-PCR was used to determine the amount of mRNA (expressed as fg mRNA/100 ng cDNA) for the nicotinic receptor subunits expressed in the ovine fetal adrenal from control (n = 7) and LTH (n = 8) fetal sheep, as described in METHODS. All values represent means ± SE (*P < 0.05, compared with control).

Fig. 4. A: ethidium bromide-stained agarose gel of RT-PCR for nicotinic receptor β subunits (lanes 1–4; G, GAPDH; C, cyclophilin) from a representative fetus from both CONT and LTH fetal sheep. Reverse transcription was performed on 100 ng cDNA for each primer set. B: concentration of mRNA for nicotinic receptor β1, β2, and β4 subunits in control and LTH fetal sheep as determined by quantitative RT-PCR as described in METHODS. C: concentration of mRNA for cyclophilin mRNA, as determined by quantitative RT-PCR in control and LTH fetal adrenals, as described in METHODS. All values represent means ± SE of n = 7 control and n = 8 LTH fetal sheep (*P < 0.05, compared with control).
The mechanism by which long-term hypoxia mediates the decreased expression of the key catecholamine biosynthetic enzymes is unknown. We addressed whether LTH altered expression of nicotinic receptor subunits and muscarinic receptors in the adrenal gland as a potential means for regulating medullary cell function. Previous studies using bovine and rodent adrenomedullary cells indicated that ACh regulated both expression of the enzymes mediating catecholamine synthesis and catecholamine release primarily via nicotinic receptors composed of α3, α5, α7, and β4 subunits (10, 29, 31). In addition to the predicted expression of α3, α5, α7 subunits, α1, α2, and α6 were expressed at similar levels of mRNA. In the LTH fetal adrenal, mRNA for α1 and α7 was significantly reduced with a trend for a decrease in α5 mRNA. Of interest was our observation that, in addition to β4, β1, and β2 mRNAs were detected; however, while β1 and β2 mRNA were lower in the LTH fetal adrenal, β4 mRNA levels were not different from control. The presence of α and β subunits not previously reported to be expressed in adrenal medullary tissue could reflect a unique fetal pattern of expression. As a word of caution in interpreting these findings, the presence of vascular components such as endothelial cells, vascular smooth muscle, and cortex in the tissue preparation could contribute to the detection of these subunits (30). The decreased expression of nicotinic receptor subunits in the LTH fetal adrenal implicates a decreased capacity to respond to cholinergic stimuli as a potential mechanism leading to the decreased expression of all three enzymes mediating catecholamine synthesis.

Because muscarinic receptors have been implicated in potentiating nicotinic receptor mediated cholinergic regulation of catecholamine biosynthesis, we also addressed expression of this G-protein-coupled class of ACh receptors. Although prior studies indicated M3 and M4 as the subtypes of muscarinic receptor expressed in the medulla (17), we observed M1, M2, and M3, but not M4 or M5 mRNA. Unlike the effects of LTH on nicotinic receptor subunit mRNA, we did not observe an effect on muscarinic receptor expression in the adrenal gland. Thus, it is doubtful whether deficiencies in muscarinic signaling are contributing to the decreased expression of TH, DBH, and PNMT noted in the LTH fetus. Further, these data would indicate that muscarinic receptor-mediated cholinergic signaling is incapable of compensating for decreases in nicotinic receptor-mediated neurotransmission in the LTH fetus.

Glucocorticoids are classically thought to play a major role in regulating PNMT expression in most species (7, 41, 44). Although we previously reported that basal plasma levels of cortisol are not different between LTH and control fetuses (1, 23), the potential does exist for lower intra-adrenal concentrations of cortisol in the LTH fetus, as we previously found that the enzymes responsible for cortisol synthesis are lower in the cortex of LTH fetuses (33). It also remained possible that expression of the GR was suppressed in the medulla of the LTH adrenal, thus decreasing the sensitivity to cortisol. Glucocorticoid receptors have been shown to be regulated by hypoxia in other tissues like the kidney (24, 27). However, adrenal medullary glucocorticoid receptor expression was not different in the LTH fetuses. Although there were no differences in glucocorticoid receptor protein between groups, to our knowledge, these are the first data to demonstrate the presence of glucocorticoid receptors in the ovine fetal adrenal medulla.

In conclusion, we report that LTH in the developing sheep fetus results in major deficits in catecholamine biosynthesis in the adrenal medulla. These effects likely have broad term implications for response of these fetuses to a wide variety of stressors. From a clinical perspective, these apparent deficiencies in catecholamine biosynthesis could also have a deleterious effect in the transition from fetus to newborn, with impact at the level of both metabolism and cardiovascular function. A potential role for modulation of nicotinic receptor subunit...
expression in the decreased expression of TH, DBH, and PNMT was also implicated by our studies. How this may affect expression in the decreased expression of TH, DBH, and PNMT was also implicated by our studies.

REFERENCES


