Ovine surfactant protein cDNAs: use in studies on fetal lung growth and maturation after prolonged hypoxemia

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Departments of 1Obstetrics and Gynaecology, 2Biochemistry, and 6Pediatrics, 2Medical Research Council Group in Fetal and Neonatal Health and Development, 3Lawson Research Institute, and 4London Health Sciences Centre, University of Western Ontario, London, Ontario N6A 5A5; and 7Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Braems, Geert A., Li-Juan Yao, Kevin Inchley, Anne Brickenden, Victor K. M. Han, Allen Grolla, John R. G. Challis, and Fred Possmayer. Ovine surfactant protein cDNAs: use in studies on fetal lung growth and maturation after prolonged hypoxemia. Am J Physiol Lung Cell Mol Physiol 278: L754–L764, 2000.—cDNAs for ovine surfactant-associated protein (SP) A, SP-B, and SP-C have been done and shown to possess strong similarity to cDNAs for surfactant apoproteins in other species. These reagents were employed to examine the effect of fetal hypoxia on the induction of surfactant apoprotein expression in the fetal lamb. Postnatal lung function is dependent on adequate growth and maturation during fetal development. Insulin-like growth factor (IGF) I and IGF-II, which are present in all fetal tissues studied, possess potent mitogenic and proliferative actions, and their effects can be modulated in either a positive or a negative fashion by IGF-specific binding proteins (IGFBPs). Hypoxia can lead to increases in circulating cortisol and catecholamines that can influence lung maturation. Therefore, the effects of mild hypoxia in chronically catheterized fetal lambs at gestational days 126–130 and 134–136 (term 145 days) on the expression of pulmonary surfactant apoproteins and IGFBPs were examined. Mild hypoxia for 48 h resulted in an increase in plasma cortisol that was more pronounced at later gestation, and in these animals, there was a twofold increase in SP-A mRNA. SP-B mRNA levels also increased twofold, but this was not significant. SP-C mRNA was not altered. No significant changes in apoprotein mRNA were observed with the younger fetuses. However, these younger animals selectively exhibited reduced IGFBP-5 mRNA levels. IGF-I mRNA was also reduced at 126–130 days, although this conclusion is tentative due to low abundance. IGF-II levels were not affected at either gestational age. We conclude that these data suggest that mild prolonged fetal hypoxia produces alterations that could affect fetal cellular differentiation early in gestation and can induce changes consistent with lung maturation closer to term.

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also influence expression of some surfactant apoprotein mRNAs. Because homologous molecular probes were unavailable, we cloned the cDNAs encoding ovine SP-A, SP-B, and SP-C from an ovine lung cDNA library. Using these cDNAs as molecular probes, we examined the impact of prolonged (48-h) hypoxemia on the levels of mRNAs for SP-A, SP-B, and SP-C. In addition, we determined the effects of sustained hypoxia on the expression of IGF-I, IGF-II, and IGFBP-5 mRNAs in the fetal ovine lung. Because the effects of hypoxia could be dependent on the maturational state of the HPA axis, these studies were performed at two different gestational ages.

MATERIALS AND METHODS

Screening of sheep lung cDNA library. An adult sheep lung cDNA library (Clontech Laboratories) constructed in the bacteriophage λgt10 vector was used for screening. Plaques were screened with duplicate filter lifts of phages plated at 5 × 10⁴ plaque-forming units/150-mm plate and probed with rabbit SP-A cDNA (47), human SP-B cDNA (16), and rabbit SP-C cDNA (9). The filter lifts of phages were prehybridized for 2 h at 42°C in 50% formamide, 5× saline-sodium phosphate-EDTA (SSPE; 1× SSPE is 150 mM NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH 7.4), 5× Denhardt's solution (1× Denhardt’s solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), and 100 µg/ml of salmon sperm DNA. Hybridization was performed in the same solution containing the appropriate [³²P]dCTP-labeled cDNA probe at 42°C overnight. The cDNA probes were labeled by random priming with [³²P]dCTP (specific activity 3,000 Ci/mmol; Dupont NEN, Mississauga, ON). The filters were washed twice for 15 min each in 1× saline-sodium-citrate (SSC; 1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 6.8)-0.1% SDS at room temperature followed by a single 15-min wash in 0.1× SSC-0.1% SDS at 45°C and exposed to XAR film (Eastman Kodak, Rochester, NY) for 48 h at ~70°C. Phage DNA was isolated by a modified liquid lysate method (39). To confirm cDNA content, phage stocks were subjected to Southern blot analysis with the hybridization conditions described above. The inserts were further subcloned into the EcoRI site of the multiple cloning region of Bluescript SK(+) (Strategene Cloning Systems) and sequenced by the Sanger dideoxy method with a T7 sequencing kit (Pharmacia Biotech) and the M13 "universal" primer or appropriate synthetic oligonucleotide primers synthesized on a Pharmacia gene assembler at the Medical Research Council Group Core Facility (University of Western Ontario, London, ON).

Animals and surgery. Sheep of a mixed breed with a single insemination date underwent surgery on days 118–122 or 126–128 of gestation (term is 145 days). The surgical and experimental procedures have been previously described (3, 4). The recovery period was at least 5 days. The animal protocols were approved by the Animal Care Committees of the Lawson Research Institute and the University of Western Ontario according to the guidelines of the Canadian Council on Animal Care. Hypoxemia was induced by lowering the fraction of maternal inspired oxygen over 48 h. The fetal arterial PO₂ dropped by 6.9 ± 1.1 and 6.8 ± 1.1 mmHg in the younger and older hypoxic fetuses, respectively. There were no changes in fetal arterial pH and arterial PCO₂. At the end of the 48-h experimental period, fetal tissues (hypoxemia, 8 fetuses at 126–130 days and 5 fetuses at 134–136 days gestation; normoxemia, 10 fetuses at 126–130 days and 5 fetuses at 134–136 days gestation) were collected, frozen, and stored at ~80°C until analyzed (for further details, see Ref. 4). Blood samples were collected from mother and fetus during the episodes of hypoxemia, and plasma was collected for measurements of ACTH and cortisol as previously reported (4).

Northern blot analysis. Total RNA was isolated with the lithium chloride-urea method followed by Northern blot analysis as previously described (3, 4). Samples of RNA (20 µg) were electrophoresed under denaturing conditions on 1% agarose-2.2 M formaldehyde gels. RNA was transferred to a nylon membrane (Zetaprobe, Bio-Rad, Mississauga, ON) by capillary blotting and immobilized by covalent cross-linking (GS Gene Linker, Bio-Rad). The membrane was prehybridized overnight at 42°C in a buffer containing 50% (vol/vol) formamide, 7% (wt/vol) SDS, autoclaved 4× SSPE, and 0.5 mg/ml of denatured salmon sperm (Lofstrand Laboratories, Gaithersburg, MD). Specific cDNA probes for ovine SP-A, SP-B, and SP-C were prepared as described in Screening of sheep lung cDNA library. The IGF-I and IGF-II probes have been previously described (3). A 0.5-kb Pst I-Sac II fragment from human IGFBP-5 cDNA isolated by our group (23) was used to probe for IGFBP-5 mRNA. This cDNA, cloned from an adult sheep lung cDNA library with a human IGFBP-5 cDNA, was 92% homologous to human IGFBP-5 (23).

The growth factor Northern blots were probed sequentially for IGF-I, IGFBP-5, IGF-I, and 18S rRNA. The surfactant apoprotein mRNAs were probed in the sequence SP-A, SP-B, and SP-C. The cDNA probes were labeled by random priming with deoxy-α-³²PdCTP (specific activity 3,000 Ci/mmol; Dupont NEN, Mississauga, ON). The filters were washed twice for 15 min each in 1× saline-sodium-citrate (SSC; 1× SSC is 150 mM NaCl and 15 mM sodium citrate, pH 6.8)-0.1% SDS at room temperature followed by a single 15-min wash in 0.1× SSC-0.1% SDS at 45°C and exposed to XAR film (Eastman Kodak, Rochester, NY) for 48 h at ~70°C. Phage DNA was isolated by a modified liquid lysate method (39). To confirm cDNA content, phage stocks were subjected to Southern blot analysis with the hybridization conditions described above. The inserts were further subcloned into the EcoRI site of the multiple cloning region of Bluescript SK(+) (Strategene Cloning Systems) and sequenced by the Sanger dideoxy method with a T7 sequencing kit (Pharmacia Biotech) and the M13 "universal" primer or appropriate synthetic oligonucleotide primers synthesized on a Pharmacia gene assembler at the Medical Research Council Group Core Facility (University of Western Ontario, London, ON).

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RESULTS

Sheep cDNA clones. The sheep lung cDNA library in λgt10 was screened with ³²P-labeled rabbit SP-A, human SP-B, and rabbit SP-C cDNAs. Phage DNA was digested with EcoRI and examined by Southern hybridization. The SP-A clone SLSPA-2 of 810 bp includes 624 bases of the -untranslated region and the sequence for the first 41 amino acids missing in the SLSPA-2 clone (Fig. 1).
Amino acid sequence comparison between sheep, human, dog, rat, mouse, and rabbit SP-As shows considerable sequence identity (Fig. 1).

The SP-B clone of 816 bp includes 729 bases of coding sequence, with an in-frame stop codon at bases 730–732, and 84 bases of 3'-untranslated region without the poly(A) tail or signal. This clone lacks the 5'-untranslated region and 375–393 bp of coding sequence (Fig. 2A). The coding sequence contained in this clone begins at amino acid 132 of the human SP-B sequence and amino acid 125 of the canine sequence (Fig. 2B). The ovine clone predicts a protein 243 amino acids long and includes the entire 79-amino acid mature active SP-B, corresponding to Phe201 to Ser279 of the human sequence. Comparison of the mature peptide amino acid sequence of sheep with other known mammalian species shows excellent homology, particularly in the mature SP-B sequence. No longer SP-B clones were isolated despite extensive library screening.

SP-C cDNA was sequenced in both directions with two clones, SLSPC-1 and SLSPC-2, and was found to contain 802 bp including residues of the poly(A) tail, but it lacks the 5'-untranslated region and 9 bp of coding sequence for 3 amino acids compared with the sequence of the published human SP-C (Fig. 2A). The ovine SP-C cDNA sequence shows excellent homology, particularly in the mature SP-C sequence. No longer SP-C cDNA was isolated despite extensive library screening.

Effect of hypoxia on expression of pulmonary surfactant apoproteins. RNA prepared from the different groups of sheep was size-fractionated on agarose-formaldehyde gels, transferred to nylon membranes, and examined with cDNAs for the SPs. The level of the 1.9-kb SP-A transcript increased twofold in the lungs of the hypoxemic fetuses on days 134–136 compared with that in control animals (P < 0.05; Fig. 4, Table 1). The corresponding SP-B mRNA levels also showed a twofold increase but failed to reach significance (P = 0.10, 0.5; Fig. 4). At least three transcripts (4.6, 2.8, and 1.7 kb) were detected for SP-B by Northern blot analysis, all of which increased to about the same extent. Hypoxemia did not influence SP-C mRNA levels (0.9 kb). Hypoxemia did not influence any of the pulmonary SP mRNA levels in the younger 126- to 130-day-old fetuses.

Plotting SP mRNA levels for all fetuses against each other revealed that SP mRNA levels at these gestational ages were correlated [SP-A mRNA vs. SP-B mRNA: r = 0.875, P < 0.0001 (Fig. 5); SP-A mRNA vs. SP-C mRNA: r = 0.74, P < 0.0001 (Fig. 6); SP-B mRNA vs. SP-C mRNA: r = 0.81, P < 0.0001 (Fig. 7)]. These observations indicate that a coordinated pulmonary maturation occurs within individual lambs over these gestational ages, with all animals being considered together. Thus it appears that although hypoxia specifically affected the steady-state levels of the mRNAs for SP-A and SP-B, the levels of SP-C mRNA are correlated with the levels of the mRNAs for the other two surfactant apoproteins in both normoxic and hypoxic animals.
A previous study (3) on these animals has shown that mild hypoxia for 48 h affects fetal plasma cortisol levels, probably secondary to transient elevations in ACTH. With the younger fetuses (124–126 days), plasma cortisol levels increased rapidly from a baseline of 4–10 ng/ml for at least 12 h (significant) but fell to 7 ng/ml by 24 h (not significant). In the older fetuses (134–136 days), plasma cortisol increased from 10 ng/ml and remained in the 20–30 ng/ml range during the 48-h hypoxic period (significant). Because other studies (8, 17, 24, 45) have suggested that glucocorticoids can promote fetal lung maturity in a number of species, the levels of the mRNAs for the SPs were plotted against fetal plasma cortisol levels where available (Fig. 8). A low but significant correlation was observed between SP-A \( r = 0.578 \), P < 0.005) or SP-B \( r = 0.598 \), P < 0.005; Fig. 8) mRNA levels and plasma cortisol when all animals were considered. SP-C mRNA levels did not display a clear relationship with cortisol.

Effect of hypoxia on expression of IGF and IGFBP mRNAs. Examination of Northern blots probed with IGF-I and IGF-II mRNAs revealed that several IGF-I transcripts, with a major band at 7.3 kb, were present in low abundance in fetal lung tissue (Table 2). IGF-I mRNA levels were reduced 40% by hypoxemia in the 126- to 130-day-old fetuses \( P = 0.01 \) but were unchanged at 134–136 days. However, due to the low abundance of the mRNA, the signal was extremely weak and the observed decrease must be considered tentative. The levels of the multiple pulmonary IGF-II transcripts (1.2–6.2 kb) remained unaffected by hypoxemia (Fig. 9; Table 2). IGFBP-5 mRNA, which was detected as a 6.4-kb transcript, was significantly reduced by hypoxemia at 126–130 days \( P = 0.0001 \). Other IGFBP mRNAs were either not detected (IGFBP-1) or too low (IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-6) at the ages studied. The relative levels of other IGFBP mRNAs were therefore not determined. IGFBP-5 mRNA showed a negative correlation with cortisol levels (Fig. 10). A significant correlation was also observed between the levels of IGF-I and IGF-BP-5 \( r = 0.596 \), P = 0.001; data not shown).
DISCUSSION

Surfactant apoprotein cDNAs. The amino acid sequences predicted for ovine SP-A, SP-B, and SP-C from their cDNAs (Figs. 1–3) show high sequence homology with other species, indicating retention of functionality throughout evolution. SP-A is a water-soluble glycoprotein of 26–36 kDa depending on the extent of glycosylation. SP-A cooperates with the hydrophobic proteins to influence the morphological and physicochemical properties of surfactant phospholipids. SP-A also has a role in the innate host defense system (10, 27, 30, 33). Ovine SP-A possesses a similar overall structure to SP-A from other species. After a 21-amino acid signal peptide, there are four distinct domains including 1) an amino-terminal domain involved in inter- and intra-chain disulfide bridging (Asp22 to Leu28), 2) a collagen-like triple helical domain (Gly29 to Pro101, including 24 Gly-X-Y repeats, 12 of which have Pro at the Y position and possess a kink introduced by a 4-amino acid insert between repeats 13 and 14), 3) a short “neck” region (Ala102 to Val135) containing hydrophobic residues thought to be involved in initiating folding and helical stabilization, and 4) a carboxy-terminal globular segment (Gly136 to Phe249) that acts as a calcium-dependent carbohydrate recognition domain (10, 27, 30, 33).

Fig. 4. Effect of prolonged hypoxia on level of mRNAs for SP-A, SP-B, and SP-C. Northern blot analyses on total RNA were conducted as indicated in text. 18S rRNA was used to correct for differences in gel loading and transfer efficiency. d, Day.

Surfactant Protein mRNAs

<table>
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<th>Fetuses 134-136 d</th>
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<td><strong>SP-C mRNA</strong></td>
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nus and extending through the collagen-like region. Six of these triple helices combine to form a bouquet-shaped octadecamer.

Comparison of the amino acid sequences of ovine SP-A with those of SP-A from other species revealed identities of 60–70% with the other SP-As listed (Fig. 1B). Particularly high amino acid sequence homology is evident at domain junctions. Two Cys residues within the signal peptide and five Cys residues in the mature protein are present in all of the species shown, although human SP-A has two extra Cys residues located within the collagen-like region. Most of the unique alterations (i.e., alterations not observed in other species) result in conserved changes such as substitution between hydrophobic amino acids, Gly-Ala exchanges, and charge-conserving substitutions.

Three exceptions were observed. Sheep SP-A contains a Val-Ala-Ser-Gly sequence initiating with Val15 that was truncated in all other species. Human SP-A lacks Val15, whereas canine SP-A lacks Ala16 (ovine numbers are used for convenience). Rodent SP-As do not have Ser18, whereas rabbit SP-A lacks both Val15 and Gly18. The effect of these deletions on SP-A structure or function is unknown.

A second exception was the introduction of a Glu134 residue for various uncharged residues near the neck-carbohydrate recognition domain junction. In addition, Met228 observed in all other species in the COOH-terminal region was replaced by Ile228. This Ile lies within a highly conserved loop generated by disulfide interactions between Cys225 and Cys239. This loop is thought to be involved in calcium and carbohydrate binding (33).

SP-B is a low-molecular-mass hydrophobic protein secreted with surfactant lipids in lamellar bodies. SP-B promotes surfactant lipid adsorption and surface monolayer enrichment in disaturated phosphatidylcholine (10, 27, 38). In those species studied, preSP-B is produced as a 41-kDa preproprotein that can be glycosylated before being proteolytically processed within multivesicular bodies to yield the 79-amino acid hydrophobic mature protein (46). It is thought that mature SP-B is a surface membrane protein containing two or three amphipathic \( \alpha \)-helices.

The ovine SP-B clone initiated at an Asn corresponding to Asp132 in the human sequence and contained the entire mature peptide (Phe58 to Ser126). High homologies were observed for the ovine segment Asn1 to Phe243 with the corresponding segments in SP-Bs from human (74.8%), dog (73.6%), rat (68.7%), mouse (70.7%), and rabbit (73.6%). The mature ovine SP-B protein shows amino acid homology of ~90% with the other species studied (Fig. 2). This protein is stabilized by three intra- and one interdisulfide bridge forming a homodimer of 17 kDa. The Cys residues are entirely conserved (Fig. 2B) both within the mature protein and in the remaining predicted sequence. The amino acids within the mature protein show very conserved changes, and the ovine sequence does not exhibit any residues unique to this species. This is consistent with an apparent importance of the three-dimensional structure of mature SP-B.

| Table 1. Effect of hypoxia on expression of SP mRNAs in fetal lamb lungs |
|-----------------|-----------------|-----------------|
| Gestational Age | Condition | mRNA Level |
| 126–130 days   | Normoxemia    | SP-A: 1.21 ± 0.24 (9) |
|                 | Hypoxemia     | SP-B: 1.46 ± 0.50 (8) |
| 134–136 days   | Normoxemia    | SP-A: 1.88 ± 0.21 (5) |
|                 | Hypoxemia     | SP-B: 3.80 ± 1.32 (5) |

Values are means ± SE expressed as ratio of 18S rRNA signal; nos. in parentheses, no. of fetuses. SP, surfactant protein. *P < 0.05 compared with corresponding normoxic fetuses. †P < 0.10 > 0.05 compared with corresponding normoxic fetuses.

Fig. 5. Relationship between levels of mRNAs for SP-B and SP-A in normoxic (open symbols) and hypoxic (solid symbols) lamb lungs at 126–130 (triangles) and 134–136 (circles) days gestation. Results for each mRNA are expressed as ratio of 18S rRNA signal.

Fig. 6. Relationship between levels of mRNAs for SP-C and SP-A in normoxic (open symbols) and hypoxic (solid symbols) lambs at 126–130 (triangles) and 134–136 (circles) days gestation. Results for each mRNA are expressed as ratio of 18S rRNA signal.
SP-C is produced as a preproprotein of 21 kDa that is acylated at Cys28 to Cys29, which are located near the NH2 terminus of the mature lipoprotein. SP-C is proteolytically processed at least partially in the lamellar bodies, a process that requires SP-B (46). SP-C promotes surfactant lipid adsorption and appears to be involved in the formation of a surface-associated surfactant reservoir (40).

The amino acid sequence for the SP-C proprotein demonstrated high amino acid sequence similarity ranging from 77.5% in the rabbit to 84% in the human (Fig. 3B). The first residue in our ovine sequence is designated Arg4 to conform to the other sequences. Only 12 of the 190 amino acid residues predicted by our sequence were not present in at least one of the other species. The bulk of the unique residue alterations were conservative. The exception was ovine Arg176, which replaced uncharged and/or hydrophobic amino acids in the other SP-Cs. Nine of the twelve unique amino acid residues were localized in the second half of the protein chain, and six of these were localized between Pro148 and Val178. Only two of the unique ovine residues occur in positions where any of the other four sequences possess identical amino acids. This indicates that most of these alterations do not involve critical amino acids.

None of the unique alterations occurred within the mature peptide Leu241 to Leu5835 (numbers in parentheses refer to mature peptide). The active peptide region exhibited extremely high homology, particularly in the last 25 amino acids that possess only one difference: the human has an Ile for Val4623. This region contains Lys141 to Arg3012, thought to anchor the active peptide to the phospholipid bilayer surface, and an extraordinarily hydrophobic 23-amino acid segment with characteristics of α-helical transmembrane domains. The putative transmembrane domain of SP-C is unique in containing 11 Val residues in 4 of the 5 sequences and 10 Val residues in the remaining sequence. The regions flanking the active peptide region also show high homology, possibly indicating their importance in proteolytic processing (27).

Effect of prolonged hypoxia on maturation of the surfactant system. cDNAs for the surfactant apoproteins were used to examine the effects of prolonged hypoxia (48 h) on fetal pulmonary development. The transition to extrauterine life requires a functional lung with a well-developed capillary system in close proximity to the future air spaces. Neonatal adaptation also requires the presence of sufficient pulmonary surfactant. By lowering the surface tension to low values, surfactant reduces the work of breathing during lung expansion and prevents collapse of the terminal air spaces (alveoli, air sacs, and terminal bronchioles) during expiration. Surfactant may also facilitate removal of fetal lung liquid after birth (38).

These studies examined pulmonary status during the early (126–130 days) and late (134–136 days) stages of the transitional period for lung maturation in the fetal lamb (8). Prolonged hypoxia led to changes in the levels of a number of mRNAs for proteins related to lung growth and surfactant maturation. Hypoxia for 48 h led to a significant increase in SP-A mRNA but only at the later gestational age (Fig. 4, Table 1). In addition, an elevation in SP-B mRNA levels was observed with hypoxia in the late-gestation group, although this failed to achieve significance (P < 0.10 > 0.05). In other
animal species, such as the rabbit and rat, SP-A and SP-B mRNAs tend to increase rapidly near the transition period associated with the development of a viable lung (8). SP-C mRNA is detected earlier but increases more steadily during fetal and neonatal development. This could explain the lack of an effect of hypoxia on SP-C mRNA levels.

Although the mechanisms responsible for the increase in SP-A and SP-B mRNA levels with hypoxia are unknown, it appears possible that the maturing effects of glucocorticoids could be involved. Prolonged fetal hypoxia was accompanied by a transient doubling of fetal cortisol levels in the 126- to 130-day fetuses, and a prolonged larger increase (from ∼10 to 25 ng/ml) in the later 134- to 136-day group (3). During a study aimed at evaluating the ability of glucocorticoids to promote parturition, Liggins (31) observed that prematurely delivered lambs of 120 days gestation possessed partially inflated lungs. More recently, it has been shown that glucocorticoids administered in late gestation not only improved lung function but also led to an increase in alveolar SP-A (17, 25, 26). These observations are consistent with the ability of glucocorticoids to promote surfactant maturation; Liggins (31) observed that prematurely delivered lambs of 120 days gestation possessed partially inflated lungs. More recently, it has been shown that glucocorticoids administered in late gestation not only improved lung function but also led to an increase in alveolar SP-A (17, 25, 26). These observations are consistent with the ability of glucocorticoids to promote surfactant maturation.

The suggestion that glucocorticoids could be involved in maturation of the surfactant system in these studies is supported by another investigation (15) involving prolonged placental insufficiency for 15 days, which showed significant increases in SP-A and SP-B mRNAs and that these increases correlated with fetal cortisol levels. These findings taken together with the present study indicate that elevations in endogenous glucocorticoids suffice to induce pulmonary maturation around 130 days gestation. Recent investigations (26, 37) have demonstrated that betamethasone maternally administered between 104 and 128 days gestation is considerably more effective in promoting lung maturation than betamethasone administered directly into the fetus. This difference occurred despite the presence of higher fetal glucocorticoid levels with direct administration. However, maternal betamethasone administration was also associated with a significant decrease in fetal size (37). Thus although the mechanisms are not understood, it is clear glucocorticoids can influence lung growth and maturation of the surfactant system.

Although the elevations in surfactant apoprotein mRNA at the later gestational age in the present study could be explained in part through activation of the fetal pituitary-adrenal axis, it should be noted that other factors related to prolonged hypoxia could be functioning in vivo. Prolonged hypoxia can lead to increased circulating catecholamines, and with some forms of hypoxia, prostaglandin E2 is released (6, 14), leading to increased cAMP formation in the lungs (35). Studies with rat and human tissues in culture have demonstrated that β-adrenergic agonists and cAMP analogs can stimulate SP-A mRNA transcription, lead-

### Table 2. Effect of mild hypoxia for 48 h on IGF-I, IGF-II, and IGFBP-5 mRNA levels in fetal lamb lungs

<table>
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<tr>
<th>Gestational Age</th>
<th>Condition</th>
<th>IGF-I</th>
<th>IGF-II</th>
<th>IGFBP-5</th>
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<td>126–130 days</td>
<td>Normoxemia</td>
<td>0.260 ± 0.025 (9)</td>
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<td>Hypoxemia</td>
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<td>134–136 days</td>
<td>Normoxemia</td>
<td>0.144 ± 0.009 (5)</td>
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<td>Hypoxemia</td>
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Values are means ± SE expressed as ratio of 18S rRNA signal; nos. in parentheses, no. of fetuses. IGF-I and IGF-II, insulin-like growth factor I and II, respectively; IGFBP-5, IGF binding protein-5. It should be noted that, because of low abundance, the levels of the IGF-I mRNA listed must be considered tentative. *P < 0.01 compared with corresponding normoxemic fetuses. †P < 0.0001 compared with corresponding normoxemic fetuses.

### Pulmonary IGF-II and IGFBP-5 mRNA

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Fig. 9. Northern blot showing effects of prolonged fetal hypoxia on mRNAs for insulin-like growth factor (IGF) II and IGF binding protein (IGFBP)-5 at 126-130 and 134–136 days gestation.
Effect of prolonged hypoxia on growth factors. IGF-I and IGF-II are peptides of ~7.5 kDa with structural homology to proinsulin that can influence cellular proliferation and differentiation. IGF-I is produced in a number of fetal tissues including the lung. Infusion of anti-IGF-I antibodies into rats reduced the growth of embryos transplanted under the kidney capsule (32). IGF-I levels increase in fetal lambs during development (5). Infusion of IGF-I into fetal lambs resulted in anabolic effects on fetal placental protein and carbohydrate metabolism (19). These observations suggest a role for IGF-I during fetal lung development and differentiation (36, 42). In a previous study, our group (21) has observed that fetal hypoxia for a 24-h duration at ~118 days gestation results in a reduction in the relative rate of DNA synthesis as assessed by [3H]thymidine incorporation during the last 8 h by a limited number of tissues, including the lung. Furthermore, fetal hypoxia for 24 h in lambs at this gestational age leads to a nonsignificant reduction in fetal plasma IGF-I and IGF-II and a significant lowering of hepatic IGF-I mRNA levels (34). In the present study, prolonged hypoxia for 48 h resulted in a reduction in lung levels of the mRNAs for IGF-I and IGFBP-5 at the earlier (126–130 days) but not at the later (134–136 days) gestational ages examined (Table 2), although, due to low abundance, the IGF-I mRNA data must be considered tentative. Nevertheless, when taken in concert with the previous reports, it appears that cellular proliferation may be affected, at least potentially. On the basis of our previous experience with this species, we anticipate that such an alteration would not be sufficient to produce frank decreases in fetal lung weight. Rather, we suggest that because these factors can alter cell division or differentiation depending on the cell type, the potential alterations in the IGF-I-to-IGF-II ratio and IGFBP-5 levels might generate differences in lung cell complement, leading to altered lung differentiation. Theoretically, this reduced proliferation could be related to enhanced maturation, although there is no direct evidence for this in the present study.

IGF-II levels are high in blood and a number of tissues during fetal development and decline postnatally (18, 36, 42). Ablation of the genes encoding IGF-II by gene targeting results in growth-retarded animals in utero, but there is little effect after birth (11, 12). IGF-II supplementation stimulated growth of transplanted rat embryos (32). IGF-II levels in the lamb increase in early and mid gestation and then decline near term (5). Alterations in pulmonary IGF-II expression during development in the fetal lamb have been observed after manipulation of intraluminal lung volumes where IGF-II mRNA accumulation and DNA synthesis were increased after tracheal obstruction, whereas the opposite effects were produced by continuous draining of lung liquid (22). In a variation on this experiment, abolishment of fetal breathing movements by high spinal cord transaction led to decreases in IGF-II gene expression, lung liquid volume, and total lung DNA in late-gestation fetal lambs (20). In the studies reported here, hypoxia did not have a significant impact on IGF-II mRNA accumulation at either gestational age. This indicates that at least some of the factors that regulate pulmonary IGF-I are different from those influencing IGF-II.

In addition to alterations in IGF-I and IGF-II, lung growth and differentiation can be influenced by IGFBPs. IGFBPs associate with IGFs with high affinity and can either inhibit or potentiate IGF activity depending on the IGFBP involved, the cellular system, and the particular conditions (28). In rats, the mRNA for IGFBP-2 increases in the fetal lung between days 16 and 18 of gestation but falls toward term (22 days) (44). IGFBP-3, IGFBP-5, and IGFBP-6 mRNAs all increase slowly during fetal rat development. IGFBP-4 mRNA shows no change, and IGFBP-1 mRNA was not detected. In this species, glucocorticoids tend to decrease mRNA expression of IGFBP-2, IGFBP-3, IGFBP-4, and IGFBP-5 but increase the levels of pulmonary IGFBP-6 mRNA. In the lamb, IGFBP-2 increases in fetal lung during early and mid gestation and declines near term (5, 13). IGFBP-1, IGFBP-3, and, to a lesser extent, IGFBP-4 increase in lamb plasma during gestation. Hypoxia for 24 h around 118 days does not affect IGF-I or IGF-II but leads to a decrease in IGFBP-2 (34). In fetal lambs, IGFBP-2 is expressed in the lung before 75 days gestation but becomes very low in abundance at later gestational ages. Except for IGFBP-5, the remaining IGFBP mRNA levels are very low in the fetal ovine lung at the dates examined.

IGFBP-5 sequence is highly conserved among different species, although the significance is unknown. It has been observed that IGFBP-5 can undergo relatively specific degradation by serine and metalloproteases, leading to a greater availability of the growth factors (43). Proteases also release IGF-I and IGF-II from the extracellular matrix during remodeling, thus further complicating interpretation of IGF-I and IGF-II interac-

![Fetal Mean Cortisol (in ng/ml)](image_url)
tions in tissues. The effect of lowering pulmonary IGFBP-5 in prolonged fetal hypoxia is not known, although it would presumably lead to higher levels of free IGFs. IGF-I and IGFBP-5 mRNA levels were both decreased, making it difficult to judge the net effect. Interestingly, IGF-I mRNA and IGFBP-5 mRNA levels were significantly correlated throughout gestation, suggesting the possibility of coordinate control. It has been reported that in fetal rat lung explants, glucocorticoid treatment reduces IGFBP-5 mRNA levels (44). As a result, the increased cortisol levels at 126–130 days gestation could produce the lowering of IGF-I and IGFBP-5 observed in the present studies. However, other details regarding the regulation of IGFBP gene expression in the lung are not currently available. To our knowledge, this is the initial report on specific changes in IGFBPs in the fetal ovine lung in response to external stimuli.

Due to low abundance, the apparent reduction in pulmonary IGF-I mRNA with prolonged hypoxia at 126–130 days must be considered tentative, even though IGF-I and IGFBP-5 mRNA levels were significantly correlated with each other over both gestational ages with and without hypoxia. However, although the levels of IGF-I and IGFBP-5 mRNAs (which are both decreased by hypoxia) were correlated, there was no correlation with either SP-A or SP-B mRNAs (which are also affected by hypoxia). This suggests that the effects of development with and without hypoxia impact separately on certain elements of the growth factor and surfactant apoprotein systems. The basis for the separate relationships is unknown. It appears that the growth factor system is susceptible to alteration at 126–130 days gestation, whereas the surfactant system is more prone to stimulation at 134–136 days gestation. Therefore, even though both systems may be affected by increased cortisol levels, there is a spatial and temporal distinction.

In summary, maternal hypoxia resulted in alterations in the expression of a number of mRNAs related to lung growth and maturation of the surfactant system of the lung. These alterations were dependent on gestational age. With hypoxic fetuses at 126–130 days gestation, there was a decrease in the mRNAs for IGF-I and IGFBP-5, but no alterations were detected in the mRNAs for surfactant apoproteins. In contrast, hypoxic episodes 6–10 days later had no effect on the mRNAs for growth-related factors but resulted in a marked concomitant increase in SP-A. A concomitant increase in SP-B mRNA was observed with hypoxia, but this did not achieve significance (P < 0.10 > 0.05). The lack of any change in mRNA for SP-C shows that hypoxia induces specific changes in lung mRNA expression, even within the surfactant apoprotein mRNAs that are highly correlated with one another. The present observations suggest that the gestational age-dependent alterations in IGF-I, IGFBP-5, and surfactant apoprotein mRNAs could possibly represent regulation of pulmonary maturation processes during fetal hypoxia, which is mediated in part by cortisol, although other factors such as circulating catecholamines must also be considered.

During the preparation of this manuscript, we became aware of the work of S. M. Pletschmann and U. Pison, who have also cloned the CDNAs for the surfactant apoproteins. We thank these investigators for generously sharing their findings with us. Our two groups have agreed that it would be appropriate to publish both studies together.

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