Intrauterine growth restriction delays surfactant protein maturation in the sheep fetus

Sandra Orgeig, Tamara A. Crittenden, Ceilidh Marchant, I. Caroline McMillen, and Janna L. Morrison


Pulmonary surfactant is synthesized by type II alveolar epithelial cells to regulate the surface tension at the air-liquid interface of the air-breathing lung. Developmental maturation of the surfactant system is controlled by many factors including oxygen, glucose, catecholamines, and cortisol. The intrauterine growth-restricted (IUGR) fetus is hypoxicemic and hypoglycemic, with elevated plasma catecholamine and cortisol concentrations. The impact of IUGR on surfactant maturation is unclear. Here we investigate the expression of surfactant protein (SP) A, B, and C in lung tissue of fetal sheep at 133 and 141 days of gestation (term 150 ± 3 days) from control and carunclectomized Merino ewes. Placentally restricted (PR) fetuses had a body weight <2 SD from the mean of control fetuses and a mean gestational PaO₂ <17 mmHg. PR fetuses had reduced absolute, but not relative, lung weight, decreased plasma glucose concentration, and increased plasma cortisol concentration. Lung SP-A, -B, and -C protein and mRNA expression was reduced in PR compared with control fetuses at both ages. SP-B and -C but not SP-A mRNA expression and SP-A but not SP-B or -C protein expression increased with gestational age. Mean gestational PaO₂ was positively correlated with SP-A, -B, and -C protein and SP-B and -C mRNA expression in the younger cohort. SP-A and -B gene expression was inversely related to plasma cortisol concentration. Placental restriction, leading to chronic hypoxemia and hypercortisolism in the caruncleplacentoma model, results in significant inhibition of surfactant maturation. These data suggest that IUGR fetuses are at significant risk of lung complications, especially if born prematurely.

Lung development must occur during prenatal life in preparation for the transition to air breathing in extrauterine life. The timing of this development is critical because the capacity to produce pulmonary surfactant to facilitate lung function is required for a successful transition to the extrauterine environment. Pulmonary surfactant is produced by type II alveolar epithelial cells (AECs), which form after 16 wk of gestation in humans (20). Surfactant is a lipid-protein mixture that regulates surface tension at the air-liquid interface of the alveoli. Of the four surfactant proteins (SP), SP-B and SP-C are directly involved in the surface active functions of surfactant, whereas SP-A and SP-D are primarily involved in the innate pulmonary host defense system (18). Surfactant production is regulated by substrate supply (oxygen and glucose) and hormones such as glucocorticoids and growth factors (36). Hypoxia reduces SP-A expression in the fetal lung (2). Exposure to 48 h of hypoxia increased SP-A and SP-B mRNA expression in the lung of the sheep fetus after, but not before, 134 days of gestation (term 150 ± 3 days) (4). Starvation decreased total surfactant phospholipids in neonatal guinea pig lung tissue (24) resulting in reduced alveolar and capillary surface areas (23).

Interestingly, glucose infusion blocks the late gestation rise in disaturated phosphatidylcholine in sheep lung liquid (46) predisposing to respiratory distress syndrome (RDS). Glucocorticoids mobilize glucose but also have direct effects on the lung by increasing differentiation to type II AEC (12) resulting in increased SP production.

The complexity of interaction between the factors that regulate surfactant protein synthesis is exemplified in the case of the intrauterine growth-restricted (IUGR) fetus. IUGR is defined as a birth weight less than the 10th centile and occurs in 8% of babies (22). IUGR is most commonly caused by placental insufficiency leading to chronic hypoxemia and hypoglycemia (9, 11). The fetus responds to the reduced substrate supply with a range of adaptations. Neuroendocrine adaptations include a reduction in plasma IGF-1 concentration (37) and an augmentation of the normal prepartum surge in cortisol (10, 32, 38). There are also changes in lung structure in the IUGR fetus, which include structural deficits to both the alveolar cells and the lung parenchyma (25, 39).

There is controversy around the impact of IUGR on lung maturation. Some authors suggest that the lungs of IUGR fetuses are more mature (17) because of the elevated plasma glucocorticoid concentrations resulting in a reduced risk of RDS (21, 44, 45), whereas others suggest that IUGR fetuses are at greater risk of RDS (27). Different responses to reduced substrate supply have been measured in the sheep fetus (33). Repeated umbilical cord occlusion over 4 days did not change fetal weight but reduced SP-A, -B, and -C mRNA expression at 130 days but not 112 days of gestation (35). In contrast, uteroplacental embolization (UPE) for 21 days from 109 to 130 days of gestation resulting in hypoxemia, hypoglycemia, and IUGR led to an increase in SP-A and -B mRNA expression, but SP-C remained unchanged (13). In the UPE model of IUGR, fetal cortisol is elevated during the last 2 days of the protocol and correlates strongly with SP-A and -B mRNA expression. Hence, the increase in SP may be due to an acute rise in cortisol (13). The fact that SP-C, a specific marker of type II AECs, does not change suggests that this model may result in a qualitatively different response that is different from that observed in a chronically hypoxemic, hypoglycemic fetus with chronically elevated cortisol concentrations. Therefore, we investigated the effect of carunclectomy, a model of placental

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insufficiency that results in chronic hypoxemia, hypoglycemia, and hypercortisolemia, on SP production by measuring SP-A, -B, and -C mRNA and protein expression in lung tissue as well as the key factors regulating their production including arterial PO2, plasma glucose, and plasma cortisol. Our initial hypothesis was that placental restriction (PR) resulting in reduced fetal substrate supply and fetal growth restriction would result in an increase in the gene and protein expression of SP-A, SP-B, and SP-C.

MATERIALS AND METHODS

All experiments were performed according to guidelines of and approved by the University of South Australia Animal Ethics Committee. Tissues used in this study were available from a series of placently restricted and control fetal sheep, and data on cardiovascular and neuroendocrine function from some of these animals have previously been published (11, 14, 32).

Animals and Surgery

The majority of the endometrial caruncles were removed in 20 Merino ewes before conception as previously described (3, 7, 11, 32). The ewes' recovery from surgery was observed for 4–7 days. After a minimum 10-wk recovery, these 20 plus 24 control ewes entered a mating program. At 110–125 days, surgery was performed under aseptic conditions with general anesthesia induced by sodium thiopentate (1.25 g; Pentothal, Rhone Merieux, Pinkenba, Australia) and maintained by inhalation of halothane (2.5–4%) in oxygen. Briefly, vascular catheters (Critchley Electrical Products, Silverwater, Australia) were inserted as previously described in the maternal jugular vein, the fetal femoral and carotid arteries, jugular vein, and the amniotic cavity (32, 43). Fetal catheters were exteriorized through a small incision in the ewe's flank. At surgery, antibiotics were administered intramuscularly to the ewe (153.5 mg Procaine penicillin, 393 mg benzathine penicillin, 500 mg dihydrostreptomycin; Lyppards, Adelaide, Australia) and fetus (150 mg Procaine penicillin, 112.5 mg benzathine penicillin, 250 mg dihydrostreptomycin; Lyppards). Antibiotics were administered intramuscularly to each ewe for 3 days after surgery and to each fetus intra-amniotically (500 mg ampicillin; Lyppards) for 4 days. Antibiotics were administered intramuscularly to each fetus intra-amniotically (500 mg ampicillin; Lyppards) for 4 days after surgery and to each fetus intra-amniotically (500 mg ampicillin; Lyppards) for 4 days after surgery.

Arterial Blood Measurements

Fetal carotid arterial blood gas samples (0.5 ml) were collected daily for the measurement of PaO2, PaCO2, pH, oxygen saturation (SaO2), and hemoglobin (Hb) and calculated at 39°C with an ABL 520 analyzer (Radiometer, Copenhagen, Denmark).

Postmortem

Ewes were killed with an overdose of sodium pentobarbitone (8 g; Vibrac Australia, Peakhurst) at 133 (n = 15) or 141 days of (n = 29) gestation. The uterus was removed by hysterectomy, and the fetus was removed. Fetal organs including the lung were dissected and weighed. Samples of lung (larger lobe, right lung) were snap frozen and stored at –80°C for gene and protein analysis.

Quantification of Gene Expression

Total RNA extraction from fetal tissues. Total RNA was extracted from all fetal lung samples using TR Reagent Solution (Molecular Research Centre) as per the manufacturer’s guidelines, and 10 μg of total RNA was DNase treated with TURBO DNA-free kit (Ambion) in a final volume of 10 μl. Total RNA was quantified by spectrophotometric measurements at 260 and 280 nm. Complementary DNA (cDNA) was synthesized with Superscript III First-Strand Synthesis System (Invitrogen) (14, 15) using 3 μg of DNase-treated total RNA and random hexamers in a final volume of 20 μl as per the manufacturer’s guidelines. Controls containing either no RNA transcript or no Superscript III were used to test for reagent contamination and genomic DNA contamination, respectively.

Quantitative real-time RT-PCR. The relative abundance of SP-A, -B, and -C mRNA transcripts in all fetal lung samples were measured by qRT-PCR using Fast SYBR Green Master Mix (Applied Biosystems) in a final volume of 20 μl on a Corbett Rotor-Gene 3000 instrument (Corbett Life Science). Gene-specific primer sets for Sheep SP-A, SP-B, and SP-C and for the housekeeping genes Cyclophilin and ribosomal protein P0 (Rpp0) (Table 1) for real-time PCR were designed based on cDNA sequences obtained from GenBank (NCBI). Primers were validated to generate a single transcript as confirmed by the abundance of a single double-stranded DNA product of the correct size and sequence (BigDye Terminator v3.1 Cycle Sequencing IMVS). Optimal final primer concentrations were 300 nM for primer sets SP-A, SP-B, and SP-C, and 450 nM for Rpp0 and Cyclophilin. Each qRT-PCR well contained 10 μl of Fast SYBR Green Master Mix (2×) (Applied Biosystems), 1 μl each of forward and reverse primer (Sigma, Genosys) for the appropriate gene (Table 1), and 1 μl of diluted relevant cDNA brought to a volume of 20 μl with sterile water. Thermal cycling parameters for the Corbett Rotor-Gene 3000 instrument were 95°C for 2 min for enzyme activation and then 40 cycles of denaturation (95°C for 15 s) and annealing/extension (60°C for 30 s). Controls for each primer set containing no cDNA were included on each plate to test for reagent contamination. Melting curve/ dissociation curves were also run to check for nonspecific product formation. Amplification efficiency reactions were performed on five triplicate serial dilutions of cDNA template for each primer set and each treatment at each gestational age. Amplification efficiencies were determined from the slope of a plot of Ct, (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (ranging from 1 to 100 ng). Ct values were in the linear amplification range for all genes. Each sample was run in triplicate for target genes SP-A, SP-B, and SP-C and also for the endogenous control gene Rpp0. The reactions were quantitated by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding Ct values. The abundance of each transcript relative to the abundance of the reference gene Rpp0 was calculated using Q-Gene analysis software (34).

Quantification of Protein Expression

Protein extraction. Total protein was extracted from lung tissue of 14 control and 11 PR fetuses with minor modifications to a published protocol (34).

Table 1. Details of primer sequences for qRT-PCR

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′-3′)</th>
<th>Final Primer Concentration, μM</th>
<th>Accession No. or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP-A</td>
<td>Forward AGCTCCAGGGCAAGCTCATG</td>
<td>0.3</td>
<td>AF211856</td>
</tr>
<tr>
<td></td>
<td>Reverse CTTGCACTTCCACGATGGA</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>SP-B</td>
<td>Forward GGCCCCAGATCCGTGGT</td>
<td>0.3</td>
<td>AF107544</td>
</tr>
<tr>
<td></td>
<td>Reverse CCTTGGCACCTTCTGGAGG</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>SP-C</td>
<td>Forward GCAAGAGCTTCTGAGGAG</td>
<td>0.3</td>
<td>AF076634</td>
</tr>
<tr>
<td></td>
<td>Reverse CAGGGCGCTCTACAGAG</td>
<td>0.3</td>
<td>DQ386893</td>
</tr>
<tr>
<td>Rpp0</td>
<td>Forward CAAACCAGGATCTGACTACGAT</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse AGGACAGATTGACGAG</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>Forward GTCTGGTCGAGAAGATATCCAG</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse CCTTGGCCATGGCAACGATGCCC</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

Accession numbers refer to the published cDNA sequences from which primer sequences were designed.
method (28). One hundred milligrams of lung tissue was snipped with scissors in 1 ml of homogenization buffer (0.1 M potassium phosphate buffer, Roche complete proteinase inhibitor tablet) and then minced with a polytron PT 3100 (Kinematica, Lucerne, Switzerland) for 10 s at high speed. The homogenate was centrifuged at 1,450 g. The pellet was resuspended in 100 μl of homogenization buffer.

Protein expression. Protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce, Thermo Fisher Scientific, Rockford, IL), and 5 μg of total lung protein was analyzed on a precast 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Gels were run, reduced, and transferred to pSQ-PVDF (0.2 μm) with a semidry blotter (BioRad Laboratories, Hercules, CA). Membranes were blocked with 5% skim milk TBS-T (10 mM Tris, 0.9% NaCl, pH 7.4, with 0.1% Tween 20) and incubated with primary antibody (mouse anti-human monoclonal antibody to SP-B, produced by Dr Y. Suzuki, Kyoto University, Japan and kindly donated by F. Possmayer, Univ. of Western Ontario, Canada, 1:1,000; rabbit anti-human SP-A polyclonal antibody, Chemicon International, Millipore, Billerica, MA, 1:2,000; rabbit anti-human recombinant SP-C polyclonal antibody, a generous gift from Byk Gulden, 1:500) overnight at 4°C. α-Actin polyclonal antibody (Sigma-Aldrich, St. Louis, MO) was used at 1:2,000 as the internal loading control standard. Blots were incubated with secondary antibody, 1:10,000 for 1 h at room temperature. Immunoreactive bands were visualized with chemiluminescence on FluorChem 8900 (San Leandro, CA). Samples were randomized before loading onto gels and were quantified using densitometry. Intra-gel expression was normalized to a specific quality control sample run on every gel. Inter-gel expression was normalized to α-actin. α-Actin expression did not alter between age groups. The average of three separate experiments for each sample was used for analysis.

Quantification of Plasma Hormone Concentrations

Cortisol radioimmunoassay. Total plasma cortisol concentration was measured in extracts, using an 125I radioimmunoassay kit (GE Healthcare, Sydney, Australia) as previously described (47). The average efficiency of recovery of 125I cortisol using dichloromethane extraction was 90%. The sensitivity of the assay was 0.39 nmol/l. The rabbit anti-cortisol antibody cross-reacted <1% with cortisone and 17-hydroxyprogesterone and <0.01% with aldosterone, pregnenolone, estradiol, and progesterone. The inter- and intra-assay coefficients of variation (CV) were less than 10%.

Plasma glucose. Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (Konelab 20, Program Version 6.0 automated analysis system, Thermo Fisher Scientific) (14). The sensitivity of the assay was 0.5 mmol/l, and the intra- and interassay CVs were both <5%.

Statistical Analyses

All data are presented as means ± SE. P < 0.05 was considered significant.

Experimental groups. Animals were divided into treatment (control vs. PR) and age (133 vs. 141 days) groups (Table 2). All control fetuses had a mean gestational PaO2 below 17 mmHg (29). All PR fetuses had a mean gestational PaO2 of greater than 17 mmHg and were therefore defined as chronically hypoxic (7, 32), and, in addition, were growth restricted. Table 2 shows fetal number, the proportion of singleton vs. twin fetuses, and the sex ratio in each group. In the case of twins, only one fetus was included in the analysis.

Data analysis. Mean gestational arterial PO2, O2 saturation (SO2), Hb, PCO2, and pH were calculated as the mean of the values for all samples collected between surgery and postmortem. A two-way ANOVA (SPSS, Chicago, IL) was used to determine differences in the mean fetal blood gases, pH, as well as normalized mRNA and protein expression between treatment (control vs. PR fetuses) and age (133 vs. 141 days) groups. Plasma glucose and cortisol concentrations were determined during late gestation from a sample taken on the day of postmortem in a subset of animals in the study for which plasma samples were available (control, n = 8; PR, n = 9). An unpaired Student’s t-test was used to determine differences between plasma glucose and cortisol concentration in the older fetuses between the control and PR fetuses. Regression analysis was performed to investigate relationships between mean gestational PaO2, plasma glucose, and plasma cortisol concentrations and surfactant protein using SigmaPlot (Systat Software).

RESULTS

Fetal Growth and Blood Gas Status

PR resulted in decreased fetal body weight and increased relative brain weight at 133 and 141 days of gestation (Table 3). There was also a decrease in absolute lung weight but no change in relative lung weight in the PR compared with the control fetuses (Table 3). They were also mildly hypercapnic but not acidotic (Table 4). Both control and PR fetuses at the older gestational age had elevated hemoglobin compared with the younger fetuses (Table 4). PR fetuses had lower plasma glucose concentrations (control, 1.05 ± 0.33, n = 5; PR, 0.56 ± 0.19, n = 6; P < 0.05) and higher plasma cortisol concentra-

| Table 2. Number of fetuses in each group at each gestational age that were singleton or twin and male or female |
|---------------------------------|------------------|-----------------|------------------|------------------|
| 133 Days | 141 Days | 133 Days | 141 Days |
| Sample size | 9 | 15 | 6 | 14 |
| Singleton:twin | 1:8 | 5:10 | 3:3 | 7:7 |
| Male:female | 6:3 | 5:9 | 4:2 | 7:6 |

PR, placental restriction; *sex was not recorded in 1 fetus.

Table 3. Fetal body and organ weights

| Table 3. Fetal body and organ weights |
|---------------------------------|------------------|------------------|------------------|------------------|
| 133 Days | 141 Days | 133 Days | 141 Days |
| Gestational Age | 134 ± 1 | 132 ± 1 | 141 ± 1 | 141 ± 1 |
| Body weight, kg | 3.6 ± 0.2 | 3.6 ± 0.2 | 1.9 ± 0.1* | 4.7 ± 0.2† |
| Relative brain weight, g/kg | 15.1 ± 0.56 | 15.1 ± 0.56 | 26.0 ± 4.8* | 12.0 ± 0.5† |
| Lung weight, g | 106.7 ± 3.0 | 106.7 ± 3.0 | 59.3 ± 11.3* | 123.6 ± 4.5 |
| Relative lung weight, g/kg | 30.0 ± 1.16 | 30.0 ± 1.16 | 29.5 ± 1.8 | 26.2 ± 1.0† |

*P < 0.05 from control fetuses (i.e., treatment effect); †P < 0.05 from 133 days of gestation (i.e., age effect).
Fetal Lung SP Gene and Protein Expression

Lung SP-A, SP-B, and SP-C mRNA expression as well as SP-A, SP-B, and SP-C protein expression was lower in PR than control fetuses in both gestational age groups (Fig. 1). There was an increase in SP-B and SP-C, but not SP-A, mRNA expression with increasing gestational age. In contrast, there was an increase in SP-A, but not SP-B or SP-C, protein expression with increasing gestational age.

Relationship Between Fetal Hypoxemia, Hypoglycemia, and Plasma Cortisol with SP Gene and Protein Expression

Regression analyses between SP or SP mRNA expression and either fetal mean gestational PaO2, plasma cortisol, or plasma glucose concentration are given in Table 5. For mean gestational PaO2, separate analyses were performed for the two age groups as well as for the total population (Table 5). The strongest relationships were evident between SP-A, -B, and -C normalized protein expression and mean gestational PaO2 at the younger gestational age (see Fig. 2), with $r^2$ values of 0.87, 0.66, and 0.78, respectively. There were also significant cor-
relations ($r^2$ of 0.56 and 0.66, respectively) between SP-B and -C mRNA expression and mean gestational PaO$_2$ in the younger cohort. The relationship between SP-A mRNA expression and mean gestational PaO$_2$ in the younger cohort just failed significance at $P = 0.0548$ ($r^2 = 0.49$). The regression analysis was limited by the number of samples for which mean PaO$_2$ values were available. There was no significant relationship between SP-A and -B mRNA and mean gestational PaO$_2$ in the older cohort, and SP-C mRNA demonstrated a very weak significant relationship ($r^2 = 0.1991$).

There was no relationship between plasma cortisol concentration and SP-A, -B, or -C protein expression in the older cohort (no cortisol measures were available for the younger cohort). SP-A and SP-B gene expression, however, were related to plasma cortisol concentration via an inverse exponential regression, whereas SP-C did not show a relationship (Table 5).

There was no relationship between plasma glucose concentration and SP-A, -B, or -C mRNA expression or with SP-A, -B, or -C protein expression (Table 5). A summary figure of the relationships between SP and SP mRNA expression and mean gestational PaO$_2$, plasma cortisol and plasma glucose concentration is given for SP-B in Fig. 3.

**DISCUSSION**

We have shown that IUGR induced by carunclectomy results in a significant reduction in tissue SP and SP mRNA expression but no change in relative lung weight at both 133 days, the earliest age when lambs may survive without ventilation (5), and 141 days of gestation, near term. These biochemical and molecular alterations are partially accounted for by changes in mean gestational PaO$_2$ and plasma cortisol concentration, but not plasma glucose concentration. Hence,
the chronic hypoxemia and the hypercortisolemia associated with the carunclectomy model of IUGR play a role in the inhibition of surfactant maturation, suggesting that preterm IUGR fetuses may be at increased risk of RDS, particularly the younger fetuses. This is of specific importance to IUGR babies at risk of preterm delivery.

IUGR fetuses are hypoxemic, hypoglycemic, and have higher circulating plasma cortisol and noradrenaline concentrations compared with appropriately grown fetuses. To survive chronic periods of poor placental substrate supply, the fetus adapts by altering the growth trajectory of specific organs (31). In the present study, we show a reduction in absolute lung weight but no change in relative lung weight (i.e., lung-to-body weight ratio) (Table 3). Others have also found no change in relative lung weight but have nevertheless shown changes in lung structure as a result of IUGR, indicative of delayed maturation associated with the trachea and its mucosal and submucosal surfaces (39). While lung ultrastructure was not significantly altered, IUGR fetuses did demonstrate predominantly smaller AECs, although there appeared to be no change in type II cell number or in the number and size of the lamellar bodies they possessed. However, there was a reduction in lung liquid and associated phospholipid content in IUGR fetuses (39). Moreover, in a cohort of IUGR postnatal lambs of the UPE model, there was evidence of increased blood-air barrier thickness at 8 wk of age, which was likely due to edema and/or deposition of increased amounts of extracellular matrix (26). This thickening of the alveolar septa could result in a permanent increase in lung tissue volume density and a reduction in lung compliance (26). In a recent detailed morphometric study of a cohort of carunclectomized sheep, IUGR fetuses at ~140 days of gestation showed very significant lung structure changes. This cohort demonstrated lung growth sparing based on the ratio of lung volume-to-body weight, a highly significant increase in the number of air spaces, and a concomitant decrease in the gas exchange surface density in response to IUGR (25). Hence, there is growing evidence that IUGR leads to developmental alterations in lung structure, which are likely to impair lung function at birth and possibly postnatally.

There has been significant controversy about the impact of IUGR on surfactant maturation (33). Early studies indicated the possibility that IUGR infants demonstrate accelerated surfactant maturation, due to an increase in phosphatidylcholine in amniotic fluid. There was speculation that this accelerated development may afford some protection to IUGR infants and that they may be at lower risk of neonatal respiratory distress (21, 44, 45). Others show an increased risk of respiratory distress in IUGR babies (27).

Our data show that uterine carunclectomy leading to placental restriction and IUGR results in decreased SP expression. Other studies (summarized in Table 6) indicate that there is significant conflicting information on the impact of IUGR on SP expression, reflecting the complex, multifactorial nature of both IUGR and the regulation of surfactant maturation.

In a study utilizing maternal hypoxemia, only older fetuses responded to hypoxemia with an increase in SP mRNA expression correlating with a cortisol surge (4), a result very similar to that observed with the UPE model by Gagnon et al. (13). On the other hand, in an umbilical cord occlusion model (35) (Table 6), there was a significant reduction in SP-A, -B, and -C mRNA expression between 130 and 133 days (35). This decrease in SP mRNA expression occurred despite an increase in cortisol at the end of the 4-day experimental period. The authors argue that their model causes a much more severe
hypoxemia (PaO₂ decreased by 70% as opposed to 30–50% in other studies) and is associated with both hypercapnia and a transient acidosis. It is likely that the chronic hypoxemia in our model represents a “steady state” that elicits a different cellular response leading to inhibition as opposed to the stimulation of SP and SP mRNA expression seen either with the UPE model (13) or after 48 h of maternal hypoxemia (4), which represent

Table 6. Summary of studies investigating the effect of hypoxia/IUGR on surfactant

<table>
<thead>
<tr>
<th>Hypoxia/IUGR Induction</th>
<th>Surfactant Effect</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal hypoxia (48 h at 126–130 days or 134–136 days)</td>
<td>SP-A, and -B mRNA increased in older group; no change in SP-C mRNA</td>
<td>Correlated with elevated plasma cortisol, especially later in gestation</td>
<td>(4)</td>
</tr>
<tr>
<td>Uteroplacental embolization (109–130 days)</td>
<td>SP-A and -B mRNA increased; no change in SP-C mRNA</td>
<td>Correlated with cortisol surge in last 48 h</td>
<td>(13)</td>
</tr>
<tr>
<td>Uteroplacental embolization (120–140 days)</td>
<td>No change in SP-A, -B, and -C mRNA or protein</td>
<td>Cortisol increased; no correlation with SPs</td>
<td>(6)</td>
</tr>
<tr>
<td>Repeated umbilical cord occlusions for 4 days at 112–115 days and 130–133 days</td>
<td>No change in SP-A, -B, and -C mRNA at 112–115 days; 85% reduction in SP-A and -B mRNA and a 66% reduction in SP-C mRNA at 130–133 days</td>
<td>Plasma cortisol increased on day 4 particularly in older group</td>
<td>(35)</td>
</tr>
</tbody>
</table>

IUGR, intrauterine growth restriction.
models in which there are more acute or intermittent dynamic changes in \( P_{aO_2} \). Clearly, the timing of the hypoxemic insult relative to gestational age, the duration of hypoxemia, and the magnitude and timing of the cortisol response, are all crucial in eliciting the SP or SP mRNA expression response.

In determining the likely drivers for the changes in surfactant mRNA and protein expression in the present study, we found that SP expression demonstrated the strongest relationship with mean gestational \( P_{aO_2} \), particularly in the younger cohort. This suggests that a low mean gestational \( P_{aO_2} \), as is present in IUGR fetuses, inhibits both SP and SP mRNA expression. Oxygen is crucial for the spontaneous differentiation of human fetal lung and SP-A gene expression in vitro (1). As described above, short-term hypoxemia in late gestation can stimulate SP mRNA expression (4). Hypoxia stabilizes a potent transcription factor, hypoxia-inducible factor 1α (HIF-1α), which is essential for differentiation of type II AECs and SP expression (41). However, it appears that chronic hypoxia, as opposed to acute hypoxia, has different effects on the stability of the HIF-1 complex (16).

We also found that there was a significant exponential inverse correlation between SP-A and -B mRNA, but not protein expression and plasma cortisol, in the older fetuses, suggesting that the elevated cortisol in IUGR fetuses inhibits SP mRNA expression. The role of glucocorticoids on SP gene expression is complicated, species specific, and gestational age dependent. For example, dexamethasone induces SP-A mRNA transcription in a dose-dependent manner in midgestation human fetal lung explants, but at the same time reduces SP-A mRNA and protein levels due to posttranscriptional degradation (30). While glucocorticoids enhance SP-B and -C transcription (48), the effect is modest and likely mediated indirectly rather than by direct binding of the glucocorticoid receptor (GR) to the SP genes (48). This is further supported by the fact that GR−/− mice demonstrate normal levels of SP gene expression in the perinatal and postnatal periods (48). This variability in SP gene expression response to glucocorticoids is borne out by the various studies described in Table 6 of acute or prolonged/sustained as well as chronic hypoxemia, which are all accompanied by elevated cortisol concentrations, yet nevertheless show marked differences in the effect on SP and SP gene expression.

We found that there was no correlation between either SP mRNA or protein expression and plasma glucose concentrations suggesting that in this model, glucose status is not involved in driving SP metabolism. However, in various animal models or in vitro systems, both glucose and insulin have an effect on surfactant lipid and protein metabolism. For example, glucose infusion in the fetal sheep with a concomitant infusion during late gestation in pregnant rabbits resulted in fetal hyperinsulinemia and hyperglycemia and no detectable difference in alveolar lavage phospholipids (19). As fetal hyperinsulinemia associated with maternal diabetes is associated with a reduction in SP-A protein in amniotic fluid (42) and an increased risk of developing RDS in the newborn, it has been hypothesized that insulin inhibits type II cell differentiation. Subsequently, it was shown that insulin administration to human fetal lung tissue explants inhibits both SP-A and SP-B gene expression (8). As the IUGR fetus is characterized by hypoglycemia and concomitant hypoinsulineaemia (40), this pathway is unlikely to be the mechanism for the observed inhibition of SP mRNA or protein expression in the present study.

In conclusion, although short-to-medium term hypoxemia of the order of hours to days can be a potent stimulus for surfactant maturation, the chronic hypoxemia associated with the caruncleectomy model of IUGR appears to play a role in the inhibition of surfactant maturation. The molecular mechanisms responsible for this inhibition are likely related to the chronic nature of the hypoxemia itself or to the associated changes in cortisol and the various proteins related to its prereceptor metabolism. However, at this stage it is not possible to exclude the involvement of other potential mediators of surfactant maturation, such as thyroid hormones, autonomic neurotransmitters, prolactin, numerous growth factors, as well as physical forces of stretch and expansion (36). The data in this current study suggest that the chronically hypoxic IUGR fetus is at significant risk of lung complications, especially if born prematurely.

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GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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