Changes in surfactant-associated protein mRNA profile in growth-restricted fetal sheep

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Gagnon, Robert, J ohnathan Langridge, Kevin Inchley, J un Murotsuki, and Fred Possmayer. Changes in surfactant-associated protein mRNA profile in growth-restricted fetal sheep. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L459–L465, 1999.—To test the hypothesis that chronic placental insufficiency resulting in fetal growth restriction causes an increase in fetal lung surfactant-associated protein (SP) gene expression, we embolized chronically catheterized fetal sheep (n = 6) daily using nonradioactive microspheres in the abdominal aorta for 21 days (between 0.74 and 0.88 of gestation) until fetal arterial oxygen content was reduced by ~40–50%. Control animals (n = 7) received saline only. Basal fetal plasma cortisol concentration was monitored. At the end of the experiment, fetal lung tissues were collected, and ratios of tissue levels of SP-A, SP-B, and SP-C mRNA to 18S rRNA were determined by standard Northern blot analysis. Total DNA content of fetal lungs was reduced by 30% in the embolized group compared with control group (P = 0.01). There was a 2.7-fold increase in fetal lung SP-A mRNA (P < 0.05) and a 3.2-fold increase in SP-B mRNA (P < 0.01) in the chronically embolized group compared with those in the control group. SP-A and SP-B mRNA tissue levels were highly correlated with the mean fetal plasma cortisol levels on days 20–21 (r = 0.90, P < 0.01 for SP-A mRNA and r = 0.94, P < 0.01 for SP-B mRNA). SP-C mRNA tissue levels were not significantly affected by placental insufficiency. We conclude that fetal growth restriction due to placental insufficiency is associated with alterations in fetal lung SP, suggesting enhanced lung maturation that was highly dependent on the degree of increase in fetal plasma cortisol levels.

fetal growth retardation; placental insufficiency; glucocorticoids; surfactant apoprotein expression

THE QUESTION OF WHETHER fetal growth restriction (FGR) is associated with an enhancement in fetal lung maturation is still controversial. In a recent multivariate analysis, Tyson et al. (31) suggested that growth-restricted premature infants are at similar risk of respiratory distress syndrome compared with normal infants. However, on the basis of human and animal observations, it is not known whether placental insufficiency and chronic hypoxemia, as possible etiologies for FGR, are associated with an increase in lung maturation.

Pulmonary surfactant is composed of phospholipids, including dipalmitoylphosphatidylcholine (DPPC), and four surfactant-associated proteins (SPs) (21, 22). SP-A and SP-D are hydrophilic oligomeric glycoproteins. Although SP-A binds DPPC and enhances the surface activity of lipid mixtures in the presence of SP-B, it has little surface activity by itself. SP-D binds phosphatidylcholine, but no biophysical activities have been reported. SP-A and SP-D have critical roles in the innate immune defense system (6, 12, 14) and in the modification of the immune response in the lung (4). SP-B and SP-C are low-molecular-weight hydrophobic proteins that enhance surfactant lipid adsorption (9, 12, 14, 22). SP-B promotes selective DPPC adsorption from phospholipid mixtures, whereas SP-C is responsible for the formation of a surface-associated surfactant reservoir (22, 33). Combined deficiency of SP-B and mature SP-C is incompatible with life (34). Numerous studies have shown that the production of surfactant lipids and proteins is developmentally and hormonally regulated and increases near term (22, 24, 34).

The primary purpose of this study was to test the hypothesis that chronic fetal hypoxemia resulting in FGR in the ovine fetus is associated with changes in fetal lung SP in a manner consistent with enhanced lung maturation. It is well established that stress can lead to maturation of fetal organs, including the lung (1–3). Stress-induced maturation could be mediated, at least in part, by an increase in fetal cortisol. Maternal administration of glucocorticoids can lead to an increase in surfactant production in the human and sheep, as well as in other species, depending on the gestational age (1, 3, 15). Therefore, a second objective of the present study was to determine whether the potential increase in surfactant apoprotein gene expression with FGR was related to alterations in fetal cortisol levels in late gestation (approximately day 130 of the 147-day term).

MATERIALS AND METHODS

Surgical procedures. Thirteen pregnant sheep of Western crossbreed, each with a singleton fetus and all of the same flock, were surgically prepared between 104 and 106 days of gestation. Twelve of the thirteen sheep are the same as those described in previous publications (17, 18). Briefly, under general anesthesia, the uterus was exposed and a hindlimb was exteriorized through a uterine incision. Polyvinyl catheters (V4; Bolab, Lake Havasu City, AZ) were implanted via the femoral artery and vein as previously described (18). After surgery, the sheep were housed in individual metabolic cages,
Northern blotting with $^{32}$P-labeled probes from cDNAs for the manufacturer’s directions. The mRNAs were assayed by nylon membrane using the TURBOBLOTTER rapid down-
gases and plasma cortisol levels. Fetal and maternal plasma paired maternal and fetal femoral arterial blood samples same duration with the vehicle diluted in sterile saline. Control fetuses were injected over the same period for the content by 40–50% of the injected was adjusted to decrease the fetal arterial oxygen noon) for a total of 21 days. The number of microspheres were injected into the experimental fetuses through the abdominal aorta every 15 min over a 2-h period (10 AM to noon) for a total of 21 days. The number of microspheres injected was adjusted to decrease the fetal arterial oxygen content by 40–50% of the day 1 preembolization value. Control fetuses were injected over the same period for the same duration with the vehicle diluted in sterile saline. Paired maternal and fetal femoral arterial blood samples were taken daily at 9 AM for measurement of arterial blood gases and plasma cortisol levels. Fetal and maternal plasma cortisol and prostaglandin E$_2$ (PGE$_2$) levels were measured with a specific radioimmunoassay in 12 of the 13 sheep as previously described (18). Because of technical difficulties, fetal plasma cortisol and PGE$_2$ could not be analyzed in one fetus.

The ewes were killed on day 21 (approximately day 130 of gestation) at 4 PM, the fetuses were delivered, and their organs were dissected rapidly. Right lower lobe lung tissues were snap-frozen in liquid nitrogen and stored at $-70^\circ$C until analysis.

Determination of fetal tissue DNA concentration. Fetal lung tissues (0.5 g) were homogenized in phosphate buffer (2 mol/l sodium chloride and 50 mmol/l EDTA, pH 7.4) and centrifuged at 2,500 rpm for 5 min, and the supernatant was assayed for DNA. The concentration of DNA was assayed in triplicate with the fluorometric method (5) and is expressed as milligrams of DNA per gram of tissue wet weight. The intra-assay coefficient of variation was <3%. To determine total lung DNA content, we multiplied the concentration of DNA by total lung wet weight and corrected for fetal body weight.

Isolation of lung RNA. Total RNA was isolated from these tissue samples with the TRIZol Reagent (GIBCO BRL) according to the manufacturer’s protocol. We quantified the isolated RNA in diethyl pyrocarbonate-treated water by measuring absorbance at 260 or 280 nm (25).

Northern blot analysis. RNA was electrophoretically separated on 1.0% agarose-formaldehyde gels. Twenty-microgram denatured samples of total RNA were electrophoresed on the gel at 100 V for $\sim$1.5 h and then transferred to a Zeta-Probe nylon membrane using the TURBOBLOTTER rapid downward transfer system (Schleicher and Schuell) according to the manufacturer’s directions. The mRNAs were assayed by Northern blotting with $^{32}$P-labeled probes from cDNAs for ovine SP-A, SP-B, and SP-C isolated in our laboratory (unpublished observations).

The SP-A probe employed for the Northern blotting experiments was a 322-bp Sac I-Kpn I fragment of clone ovine SP-A2 (oSPl-A2; nucleotides 201–516). This fragment corresponds to amino acids 112–219, which are found in the neck and globular carbohydrate recognition domains of SP-A (12, 14). The SP-B probe used for the Northern blotting experiments was a 280-bp DdeI fragment of clone oSP-B1 (nucleotides 144–476), which contains the full-length coding region for the mature SP-B peptide. This 280-bp cDNA fragment was removed from the original 610-bp ovine clone by DdeI, blunted, and ligated into the Sma I site of Bluescript with a BamHI 1-Pst I restriction digest. The SP-C probe used in Northern blotting was a 900-bp fragment generated from ovine clone oSP-C1 with EcoR I. This is the complete oSP-C1 clone, which corresponds to amino acids 4–188 of the SP-C proprotein and the 3’-untranslated end of the ovine cDNA. A cDNA for rabbit 18S RNA was used for normalization (35).

Inserts were electrophoresed on agarose, isolated with a Sephaglas Bandprep kit, and labeled with $[^{32}$P]dCTP with an oligolabeling kit (Pharmacia Biochemicals). Hybridizations to complementary RNAs were conducted in a HYBAID Micro-Y hybridization oven overnight at 43°C as indicated by the manufacturer’s directions. The filters were washed with standard conditions and exposed to Kodak XAR-2 film in the presence of an intensifying screen (35). Autoradiographs were scanned by laser densitometry with an Ultrascan densitometer. Only densitometric values within the linear range were included in the analysis. Northern blots were washed for reprobing as previously described (35).

Data analysis. To determine the relative abundance of SP-A, SP-B, and SP-C mRNAs, as well as 18S rRNA, all samples were isolated, hybridized, and scanned on a single blot for comparison. For each RNA sample, the ratio of SP-A, SP-B, and SP-C to 18S rRNA densitometry was calculated, and the grouped mean was obtained. We determined significance for the SP-A, SP-B, and SP-C mRNAs using unpaired t-tests for small samples with variances not assumed to be equal. Simple linear regression was also used when appropriate. Data are expressed as grouped means ± SE.

RESULTS

Fetal lung weight and total DNA content. The mean fetal body weight was 3.7 ± 0.4 kg in the control group (n = 7) compared with 2.6 ± 0.3 kg in embolized fetuses (n = 6; P < 0.05), and the mean of combined fetal lung weight was 31 ± 3 g/kg body wt in the embolized group, which was not significantly different from the control group (30 ± 2 g/kg body wt). However, the mean total fetal lung DNA content was significantly reduced to 103 ± 6 mg/kg body wt in the embolized fetuses compared with that in control fetuses (148 ± 12 mg/kg body wt; P = 0.01). Each fetus survived throughout the experiments and was born alive without significant metabolic acidosis. The mean fetal arterial oxygen content decreased significantly (36% decrease) from 9.2 ± 0.12 mmol/l before embolization to 1.88 ± 0.35 mmol/l (P < 0.001) on day 21 of embolization but remained unchanged in the control group. None of the animals showed evidence of preterm labor as reflected by uterine electromgram activity and amniotic pressure recordings.

SP-A, SP-B, and SP-C mRNAs in fetal lungs. When total RNA samples extracted from lung tissues were subjected to Northern blot analysis with the ovine cDNA probes, transcripts were detected at 2.2, 1.9, and 0.9 kb for SP-A, SP-B, and SP-C, respectively, in all samples from both control and embolized groups of fetuses (Figs. 1–3). There was a 2.7-fold increase in the relative abundance of SP-A mRNA (Fig. 1) and a
3.2-fold increase in the relative abundance of SP-B mRNA (Fig. 2) in the embolized groups compared with control groups ($P < 0.05$ and $P < 0.01$, respectively, for SP-A and SP-B mRNA). However, no significant change in SP-C mRNA was observed after 21 days of fetal placental embolization (Fig. 3). There was a strong positive correlation between fetal lung tissue SP-A and SP-B mRNAs (Fig. 4). In contrast, the correlation was poor between either SP-A or SP-B and SP-C ($r = 0.68$, $P < 0.05$, and $r = 0.59$, not significant, respectively).

Relationship between SP-A, SP-B, and SP-C mRNAs and fetal plasma cortisol. Because it has been previously shown that maternal administration of glucocorticoids increases fetal lung SP-A and SP-B mRNAs in rats (19, 27, 28), rabbits (8), and sheep (15, 20) and because fetal plasma cortisol levels were elevated during the last 48 h (days 20–21) of fetal placental embolization in the present study (18), we determined whether a correlation existed between the mean fetal plasma cortisol levels during the last 48 h of the experiments (days 20–21) and SP-A, SP-B, and SP-C mRNA abundance in the 12 fetuses using paired samples.

The mean fetal plasma cortisol increased from $1.4 \pm 0.2$ ng/ml on day 1 before embolization to $12.4 \pm 3.1$ ng/ml (ANOVA, time effect, $P < 0.0001$) during the last 48 h of the experiment (days 20–21), which was significantly higher than the control level of $2.1 \pm 0.6$ ng/ml during the last 48 h of the experiment (ANOVA,
group effect, \( P = 0.013 \)). Figure 5 shows a significant, strong positive correlation between fetal lung tissue SP-A and the mean fetal plasma cortisol levels during the last 48 h of the experiments. A similar relationship was observed between SP-B and fetal plasma cortisol levels (Fig. 6). No significant correlation was observed between SP-C and fetal plasma cortisol levels (\( r = 0.43 \)) or between fetal body weight and fetal plasma cortisol levels. In addition, there was no significant relationship between fetal arterial \( \text{PO}_2 \) or arterial oxygen content and SP-A, SP-B, or SP-C mRNA.

Relationship between SP-A, SP-B, and SP-C mRNA and fetal plasma PGE\(_2\). The mean fetal plasma PGE\(_2\) was 629 ± 27 pg/ml in the embolized group during the last 48 h of the experiments (days 20–21), which was significantly higher than 435 ± 42 pg/ml in the control group (\( P < 0.005 \)). There was no significant correlation between SP-A, SP-B, or SP-C mRNA and fetal plasma PGE\(_2\) (\( r = 0.33, 0.47, \) and \(-0.09, \) respectively). Multiple linear regression analysis adding fetal plasma PGE\(_2\) to fetal plasma cortisol levels did not improve the correlation between fetal plasma cortisol and the gene expression for any of the SPs.

**DISCUSSION**

Progressive hypoxemia and FGR induced by repeated fetal placental embolism for 21 days resulted in a 30% decrease in fetal body weight on day 130 of gestation but caused no alteration in lung-to-body weight ratio. However, total lung DNA content in the embolized fetal lambs was depressed by 30%. This decreased cellularity is consistent with enhanced maturity and/or increased lung water secondary to less fluid absorption and clearance, a mechanism that would be less consistent with enhanced maturation. In addition, the present study has demonstrated that there is an increased SP-A and SP-B gene expression in fetal sheep lung after progressive hypoxemia and FGR induced by fetal placental embolization. The degree of increase in SP-A and SP-B mRNAs was highly dependent on the fetal plasma cortisol levels attained during days 20–21 of embolization. In contrast, SP-C mRNA remained unchanged and was relatively independent from fetal plasma cortisol levels. Likewise, fetal carcass or lung weights were not related to cortisol levels.

A major difficulty in understanding fetal development is the number of potential factors that can influence maturation (1, 2, 11, 16, 23, 26). Fetal lung maturation can be accelerated by a large number of hormones and factors, including glucocorticoids, thyroxine, estrogens, prolactin, and adrenocorticotropic hormone (ACTH), as well as by a novel mesenchyme factor, the fibroblast-pneumocyte factor (29). These factors can influence gross lung morphology, the appearance of lamellar bodies, enhanced phospholipid synthesis and/or accumulation in lung tissue, and changes in phospholipid levels and composition in alveolar spaces and amniotic fluid. Whereas early investigations focused on surfactant lipid synthesis, with the control of phosphatidylcholine as the principal aim, more recently, emphasis has shifted to the control of the production of SP (1, 2, 3, 16, 24, 34). Whereas SP-A-knockout mice lacking SP-A develop normal breathing at birth, SP-B and possibly SP-C are essential for the transition to extrauterine life (13, 34).

The ontogeny of SP gene expression in fetal sheep has not been reported. However, lambs delivered before 130 days gestation usually develop respiratory distress and severe hyaline membranes, whereas virtually all lambs delivered after 135 days gestation maintain spontaneous breathing (7), suggesting an increase in SP in the fetal sheep late in gestation. Therefore, as demonstrated in our control group of sheep fetuses at ~130 days gestation, the relative abundance in fetal lung SP-A and SP-B mRNAs would be anticipated to be inadequate under normal conditions. Moreover, the
close relationship observed between SP-A and SP-B (Fig. 4) suggests parallel changes in SP-A and SP-B mRNAs under both normoxic and chronically hypoxic conditions.

Despite extensive investigation, the precise mechanisms by which glucocorticoids influence surfactant apoprotein expression remain unidentified. Nevertheless, it is clear that the genes of the surfactant apoproteins are regulated independently from each other and from the genes responsible for regulating phospholipid synthesis (1, 16, 24, 34). Surfactant apoprotein regulation has been studied in vivo and in vitro. Maternal treatment of pregnant rabbits with glucocorticoids at 0.77 of term gestation also results in increases in SP-A and SP-B mRNAs but a reduction in SP-C mRNA (8). Glucocorticoid treatment of pregnant rats leads to increased SP-A, SP-B, and SP-C mRNA expression (19, 28, 30). The effects on SP-A mRNA were gestation dependent, with the glucocorticoid induction disappearing near term. In contrast, glucocorticoid effects on SP-B and SP-C mRNAs remained evident late in gestation.

Polk et al. (20) observed that a single direct injection with betamethasone to the pregnant ewe of 127 days gestation led to an increase in levels of mRNA for SP-A (3-fold), SP-B (2.5-fold), and SP-C (2.5-fold) 24 h later. The marked increase observed in SP-A and SP-B mRNAs after chronic fetal placental embolization was similar to the two- to threefold increase in SP-A and SP-B mRNAs after maternal administration of betamethasone for 24 h (20). These observations, as well as our observations in this fetal sheep model of placental insufficiency, a variable increase in fetal plasma cortisol during the last 48 h before delivery, implied a causative relationship between fetal plasma glucocorticoid levels and lung maturation as assessed by changes in SP-A and SP-B mRNAs.

In view of the marked differences in the regulation of SP-A and SP-B expression, the apparent close relationship between mRNA levels for these two surfactant apoproteins and ovine fetal cortisol levels, both with and without FGR, is surprising. A potential explanation is that cortisol influences differentiation of fetal terminal epithelial cells to pretype II or type II cells, which gain the capacity for SP-A and SP-B expression. Cortisol could be involved in promoting differentiation of bronchiolar Clara cells that express SP-A and SP-B.

SP-C mRNA expression is limited to the distal respiratory epithelium in early gestation and becomes confined to type II cells as development proceeds (16). In our study, in contrast to Polk et al. (20), glucocorticoid levels did not appear to influence SP-C gene expression. The basis for the difference between their results and those presented here is not understood but could be related to differences in dose and exposure. These investigators reported that SP-A and SP-C were significantly elevated when the glucocorticoid was injected on day 121. However, we observed a weak but significant correlation between SP-A and SP-C mRNAs, suggesting that their regulation might be interrelated. Interestingly, double injection on days 121 and 127 augmented the increase in SP-B mRNA but abolished the increases in the levels of the mRNAs for SP-A and SP-C by day 128 (20). Although SP-A and SP-B mRNA levels display a strong relationship with endogenous fetal plasma cortisol levels in the current experiments, the study by Polk et al. (20) clearly demonstrated that exogenous glucocorticoids can have distinct effects on the mRNA expression of these two proteins. Whether other species show a close coordination between fetal endogenous cortisol levels and SP-A and SP-B expression in vivo is not known.

In vitro studies using explants and type II or type II-like cells demonstrated that glucocorticoids can influence expression of all three surfactant apoproteins in the rat, rabbit, and human (1, 2, 3, 16, 24, 34). In the human, glucocorticoids produce dose- and time-dependent stimulatory and inhibitory effects on SP-A mRNA levels due to enhanced transcription but decreased message stability. Glucocorticoids also produce variable effects on SP-A mRNA with fetal rabbit explants. Stimulatory effects of glucocorticoids have been observed with rat, rabbit, and human SP-B gene transcription in vitro, but glucocorticoids can also promote SP-B mRNA stability (24, 32, 34, 35). Glucocorticoids can also enhance SP-C transcription and stability (32, 36). In general, the effects on SP-C mRNA require longer incubation times. This may explain the absence of significant increases in SP-C levels in vivo with rabbit and lamb fetuses. Cytokines inhibit glucocorticoid-induced increases in SP-C but not in SP-B mRNA, indicating that the SP-B gene effects involved primary responses to the cytokine. Putative glucocorticoid response elements have now been identified in SP-B and SP-C promoter regions but not in the SP-A promoter region (24). These observations further emphasize the complexity of surfactant apoprotein regulation.

Murotsuki et al. (18) recently demonstrated that chronic fetal placental embolization conducted for 21 days in sheep causes progressive fetal hypoxia, asymmetric FGR, and adrenal hypertrophy in addition to a large increase in basal fetal plasma ACTH and cortisol levels during the last 48 h of embolization. However, in two fetuses, the fetal plasma cortisol level reached was relatively low and overlapped with the control group (Figs. 5 and 6), indicating that the fetal cortisol response to chronic hypoxemia may be variable. Similarly, Goland et al. (10) reported a significant increase in corticotropin-releasing hormone and ACTH and a variable increase in cortisol in the umbilical cord blood at delivery in growth-retarded human fetuses. Because our data demonstrate a close relationship between the absolute fetal plasma cortisol level and fetal lung tissue SP-A and SP-B mRNAs, it is possible to speculate that when FGR is the result of placental insufficiency, a variable increase in fetal plasma cortisol levels could lead to a variable enhancement in fetal lung maturation in response to chronic hypoxemia. This variability in fetal glucocorticoid response to chronic hypoxemia suggests that maternal administration of glucocorticoid in pregnancy complicated with FGR might be beneficial in enhancing fetal lung maturation in some cases.
The possibility exists that the observed increases in SP-A and SP-B mRNAs are related to other factors besides cortisol. The previous study by Murotsuki et al. (18) revealed that long-term hypoxia and FGR elicited increases in serum PGE2 levels by day 10. Studies in a number of species (1, 3, 16) have established that PGE2 can increase pulmonary cAMP levels and that this is associated with increases in SP-A mRNA. cAMP response elements have been identified in the known SP-A promoter region. The effects of cAMP on gene expression can interact with those elicited by glucocorticoids. cAMP can also elicit SP-B and SP-C gene expression, but the effects are limited compared with those observed with SP-A (16, 24, 34). However, no significant correlation was observed between the levels of mRNA for the surfactant apoproteins and fetal plasma PGE2. It should also be noted that Polk et al. (20) observed that injection of betamethasone elicited increased thyroxine levels that could affect surfactant production (1, 3, 16).

The current studies were not designed to assess neonatal ventilatory needs postdelivery, and, therefore, we do not know whether an increase in fetal lung SP-A and SP-B gene expression after FGR would result in improved lung function either on day 130 or closer to term. Polk et al. (20) observed that glucocorticoid treatment of fetal lambs on gestational day 121, day 127, or both led to similar improved lung compliances on day 128. Lavage phospholipid levels were elevated with the 7-day and repeated-treatment protocols only. Only SP-B mRNA was elevated with the double injection, suggesting that the increase in SP-B mRNA could be critical, but because surfactant apoprotein levels were not determined, this conclusion remains unproven. Nevertheless, it appears possible that the two- to threefold increase in fetal lung SP-A and SP-B mRNAs shown in the current study could be associated with improved neonatal lung function. The lack of relationship between SP-C mRNA and endogenous fetal plasma cortisol levels, in contrast to the close relationship between SP-A and SP-B mRNAs and fetal plasma cortisol, provides further evidence of a differential regulation of the SPs by glucocorticoids in vivo.

In summary, asymmetric FGR and chronic hypoxia in fetal sheep caused by chronic placental damage were associated with a significant increase in fetal lung SP-A and SP-B mRNAs shown in the current study could be associated with improved neonatal lung function. The lack of relationship between SP-C mRNA and endogenous fetal plasma cortisol levels, in contrast to the close relationship between SP-A and SP-B mRNAs and fetal plasma cortisol, provides further evidence of a differential regulation of the SPs by glucocorticoids in vivo.

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