Increased lung prolyl hydroxylase and decreased glucocorticoid receptor are related to decreased surfactant protein in the growth-restricted sheep fetus

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Orgeig S, McGillick EV, Botting KJ, Zhang S, McMillen IC, Morrison JL. Increased lung prolyl hydroxylase and decreased glucocorticoid receptor are related to decreased surfactant protein in the growth-restricted sheep fetus. Am J Physiol Lung Cell Mol Physiol 309: L84–L97, 2015. First published May 1, 2015; doi:10.1152/ajplung.00275.2014.—Experimental placental restriction (PR) by carunclectomy in fetal sheep results in intrauterine growth restriction (IUGR), chronic hypoxemia, increased plasma cortisol, and decreased lung surfactant protein (SP) expression. The mechanisms responsible for decreased SP expression are unknown but may involve decreased glucocorticoid (GC) action or changes in hypoxia signaling. Endometrial caruncles were removed from non-pregnant ewes to induce PR. Lungs were collected from control and PR fetuses at 130–135 (n = 19) and 139–145 (n = 28) days of gestation. qRT-PCR and Western blotting were used to quantify lung mRNA and protein expression, respectively, of molecular regulators and downstream targets of the GC and hypoxia-signaling pathways. We confirmed a decrease in SP-A, -B, and -C, but not SP-D, mRNA expression in PR fetuses at both ages. There was a net downregulation of GC signaling with a reduction in GC receptor (GR)-α and -β protein expression and a decrease in the cofactor, GATA-6. GC-responsive genes including transforming growth factor-β1, IL-1β, and β2-adrenergic receptor were not stimulated. Prolyl hydroxylase domain (PHD)2 mRNA and protein and PHD3 mRNA expression increased with a concomitant increase in hypoxia-inducible factor-1α (HIF-1α) and HIF-1β mRNA expression. There was an increase in mRNA expression of several, but not all, hypoxia-responsive genes. Hence, both GC and hypoxia signaling may contribute to reduced SP expression. Although acute hypoxia normally inactivates PHDs, chronic hypoxemia in the PR fetus increased PHD abundance, which normally prevents HIF signaling. This may represent a mechanism by which chronic hypoxemia contributes to the decrease in SP production in the IUGR fetal lung.

chronic hypoxia; prolyl hydroxylase; surfactant; fetus

IN DEVELOPED COUNTRIES, 8–12% of babies are born intrauterine growth restricted (IUGR), defined as a body weight less than the 10th percentile for their gestational age (47). These babies are at increased risk of preterm birth (30, 31). Moreover, there is controversy surrounding the risk of respiratory distress syndrome (RDS) in this obstetric subgroup (12), which may be in part attributable to the heterogeneous causes of IUGR (maternal, fetal, and/or placental factors; reviewed in Refs. 21, 38, and 56). These causes may contribute to the clinical findings of an increased (5, 53), decreased (72, 78, 85, 86), or no overall change (46, 78) in the risk of RDS. The underlying cause of neonatal RDS is an immaturity of the pulmonary surfactant system (1), an important lipoprotein complex secreted to the air-liquid interface lining the alveoli that prevents alveolar collapse during expiration. The heterogeneous causes of IUGR may well impact on the expression of surfactant proteins (SPs) and thus determine susceptibility to RDS at birth (1).

Studies in sheep models of IUGR further emphasize the controversy, as they have reported either an increase (27), a decrease (66), or no change (18, 82) in the expression of SPs, including SP-A, -B, -C, and -D in the lung of IUGR fetuses. The umbilicalplacental embolization model of IUGR causes a daily episode of hypoxemia for 20 days in late gestation resulting in increased SP-B and -C mRNA expression in the lung (27). This increase in SPs was attributed to a rise in plasma cortisol concentrations in the last 2 days of the protocol. In contrast, the carunclectomy model of IUGR causes chronic hypoxemia and hypercortisolemia in late gestation (71) and a decrease in SP-A, -B, and -C protein and mRNA expression in the lung (66). Despite this finding, the mechanisms regulating this decrease in SP expression are not clear. Potential mediators may include alterations in glucocorticoid (GC) or hypoxia-dependent signaling in the lung, two vital processes regulating fetal lung maturation.

Cortisol, the endogenous GC, is an important regulator of lung and surfactant maturation (2, 10, 26, 79, 83) although the regulatory pathway is not straightforward. Experimental studies have yielded many conflicting findings attributable to differences in the relative timing during gestation, dosage, route of administration, and the species investigated (56, 68). Moreover, there are many other transcriptional and hormonal factors that influence lung and surfactant maturation (8, 35), which may mask the importance of endogenous cortisol or may compensate in cases of experimental GC deficiency. For example, GC receptor (GR) or corticotrophin-releasing hormone-null mice have reduced neonatal viability attributable to respiratory failure, resulting predominantly from structural immaturity characterized by thickened alveolar septa and a reduced air space-to-tissue ratio (19, 59, 61). However, they are still capable of producing surfactant, suggesting that GC signaling is not essential for surfactant production (19). Therefore, there are significant reductions or delays in SP gene expression in these animals (19, 59). Endogenous activity of cortisol may also be influenced within lung tissue by the presence of two isoforms of the 11β-hydroxysteroid dehydrogenase (11βHSD)
Cells demonstrate increased hypoxia-inducible factor-1 reduction in expression of all four SPs. Concomitantly, these cultured type II alveolar epithelial cells (AECs) leads to a by hypoxia signaling. For example, exposure to hypoxia of gestation was correlated with the degree of hypoxemia expe-

Vation of the thyroid transcription factor (TTF)-1 and by other transcription factors and cofactors such as GATA-6 (28, 54, 58). GC action within the fetal lung is also mediated through the action of the mineralocorticoid receptor (MR), which is known to play a role in normal lung development (44). Thus an increase in plasma cortisol concentration would be expected to increase SP expression in the lung, but, despite higher plasma cortisol concentrations in late gestation, the IUGR fetus in the corticlectomy model has reduced SP expression (66). In contrast, it was shown that the decrease in SP mRNA and protein expression in the lung of the IUGR fetus in late gestation was correlated with the degree of hypoxemia experienced (66).

Surfactant maturation is also regulated at the molecular level by hypoxia signaling. For example, exposure to hypoxia of cultured type II alveolar epithelial cells (AECs) leads to a reduction in expression of all four SPs. Comconitantly, these cells demonstrate increased hypoxia-inducible factor-1α (HIF-1α) and HIF-2α mRNA expression and that of genes with a hypoxia-response element (HRE), including VEGF, glucose transporter 1 (GLUT1), and pyruvate dehydrogenase kinase, isozyme 1 (PDK1) (40). These changes are completely reversible, following reoxygenation (40). The hypoxia-induced increase in VEGF expression is also able to promote surfactant maturation and protects against neonatal RDS (20). Hence, hypoxia signaling is a key regulator of lung maturation and promotes the successful transition to air breathing in extrauterine life (77). Hypoxia causes stabilization of the HIF-α subunits, allowing dimerization with the constitutively expressed HIF-1β subunit, leading to binding to the HRE and upregulation of target genes (4, 88). Additional downstream effects of hypoxia on lung development may involve molecular regula-

Table 1. qRT-PCR primer sequences and final primer concentrations for newly designed target genes

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<th>Primer Concentration, μM</th>
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Accession numbers refer to the published cDNA sequences from which the primer sequences were designed. **PDK1**, pyruvate dehydrogenase kinase, isozyme 1; **ADRB2**, β2-adrenergic receptor.
querie Park, New South Wales, Australia) and maintained with 2–4% halothane in oxygen. At surgery, antibiotics were administered to the ewe (153.5 mg procaine penicillin, 393 mg benzathine penicillin, 500 mg dihydrostreptomycin; Lyppards, Beverley, South Australia, Australia). After 10–12 wk, ewes entered a mating program. Pregnancy was confirmed by ultrasound at 60 days of gestation.

Vascular surgery was performed in 20 of 25 control fetuses and in 21 of 22 placentally restricted (PR) fetuses at 105–115 days of gestation as previously described (25). Briefly, under anesthesia (as above), catheters were implanted in the fetal carotid artery and jugular vein. A catheter was also inserted into the amniotic cavity. At surgery, antibiotics were administered to the ewe (as above) 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 intraamniotically (500 mg ampicillin; Lyppards) for 4 days after surgery. Animals were allowed to recover from surgery for at least 4 days before experimentation. Fetal arterial blood samples (0.5 ml) were collected daily to monitor pH, PO2, PCO2, oxygen saturation (SO2), and hemoglobin (Hb) content using an ABL520 analyzer (Radiometer, Copenhagen, Denmark). The mean gestational arterial PO2 was calculated for each fetus as the mean of all arterial PO2 values collected from 4 days postsurgery until 137–144 days. Fetuses were included in the control group if the ewe did not undergo carunclectomy surgery and they had a mean gestational PO2 < 17 mmHg and in the PR group if they had a mean gestational PO2 < 17 mmHg and their body weight was less than the 10th percentile of the control group (24, 29, 57). Fetal blood gas and body weights and changes in SP gene and protein expression in a similar cohort of these animals have previously been published (66). SP gene expression data have been repeated in the current cohort of animals using advanced normalization methods (37, 87) and are presented in Table 2.

Postmortem. All animals were humanely killed with an overdose of pentobarbital sodium (Lyppards) at 133 (control, n = 11; PR, n = 8) to 140 (control, n = 14; PR, n = 14) days of gestation.
Fig. 2. There was no change in lung glucocorticoid (GC) receptor (GR) mRNA expression (A) or the 74-kDa GR band (D) and 38-kDa GR band (E) protein abundance with either treatment or gestational age. There was reduced lung 94-kDa GRα (B) and 91-kDa GRβ (C) band protein abundance in the PR (solid bars) group compared with the control (open bars) group at both 133 and 140 days of gestation. There was no effect of treatment or gestational age on the ratio of the 94-kDa/91-kDa GR band protein abundance ratio (G) or mRNA expression of the mineralocorticoid receptor (MR) (H). Representative Western blot from animals in each treatment group at each gestational age for GR bands, 94 kDa, 91 kDa, 74 kDa (133 d, control/PR; 140 d, control/PR), and 38 kDa (133 d, control/PR; 140 d, control/PR), are presented (F). *Treatment; #gestational age; \( P \leq 0.05 \).
by the presence of one double-stranded DNA product of the correct size and sequence. Controls containing no cDNA were included for each primer set on each plate to test for reagent contamination. Melt curves and/or 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. Amplification efficiencies were determined from the slope of a plot of C_v (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration. The amplification efficiency was close to 100%. Each sample was run in triplicate for target and reference genes. The reactions were quantified by setting the threshold within the exponential growth phase of the amplification curve and obtaining corresponding C_v values. The abundance of each transcript relative to the abundance of the three stable reference genes was calculated using DataAssist Software v3.0 (Applied Biosystems) (37) and expressed as mean normalized expression (MNE).

Protein extraction and Western blotting. In a subset of animals (133-day gestation, control, n = 7 and PR, n = 5; 140-day gestation, control, n = 6 and PR, n = 6), proteins were extracted from lung tissue by sonication in lysis buffer [50 mmol/l Tris-HCl, pH 8, 150 mmol/l NaCl, 1% NP-40, 1 mmol/l Na orthovanadate, 30 mmol/l Na fluoride, 10 mmol/l Na pyrophosphat, 10 mmol/l EDTA, and a protease inhibitor tablet (complite Mini; Roche, Indianapolis, IN)]. Protein content of extracts was determined by bicinchoninic acid protein assay as previously described (91). Before Western blot analysis, protein samples were bulk diluted (5 mg/ml) in 1× SDS sample buffer (containing 75 mmol/l Dl-dithiothreitol), and samples (20 μg protein) were subjected to SDS-PAGE and stained with Coomassie blue protein (Thermo Fisher Scientific, Rockford, IL) to ensure equal loading of the proteins. The amount of protein in each well and the linearity of the density measures were tested as previously described (11, 60, 89, 90). Proteins were transferred onto a nitrocellulose membrane (Amersham Hybond-C extra; GE Healthcare, Silverwater, NSW, Australia) and stained with Ponceau S to assess the efficacy of the transfer. The membranes were incubated with primary antibody overnight with agitation at 4°C and subsequently with secondary mouse horseradish peroxidase antibodies (Cell Signaling Technology, Beverly, MA) for 1 h at room tempera-

Fig. 3. There was reduced lung thyroid transcription factor 1 (TTF-1) mRNA (A) and protein abundance (C) in addition to GATA-6 mRNA expression (B) at 140 days compared with 133 days of gestation. There was an increased TTF-1 (C) protein abundance and decreased GATA-6 (D) protein abundance in the lung of PR (solid bars) fetuses at 133 and 140 days of gestation compared with the control (open bars) group. Representative Western blots from animals in each treatment group at each gestational age for TTF-1 and GATA-6 are presented (133 d, control = 6 and PR = 5; 140 d, control = 5 and PR = 5). *Treatment; #gestational age; P < 0.05.
ture. The following primary and secondary antibody combinations were used for the following proteins: 11B/HSD-1, 1:500 rabbit anti-human polyclonal antibody (Cayman Chemical, Ann Arbor, MI), 1:1,000 secondary anti-rabbit; 11B/HSD-2, 1:250 rabbit anti-human polyclonal antibody (Cayman Chemical), 1:500 secondary anti-rabbit; GR, 1:2,000 rabbit anti-mouse polyclonal antibody (Bethyl Laboratories, Montgomery, TX), 1:2,000 secondary anti-rabbit; TTF-1, 1:500 rabbit anti-human polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2,000 secondary anti-rabbit; GATA-6, 1:500 goat anti-human polyclonal antibody (Santa Cruz Biotechnology), 1:2,000 secondary anti-rabbit; PHD-1, 1:500 rabbit anti-human polyclonal antibody (Novus Biologicals, Littleton, CO), 1:2,000 secondary anti-rabbit; PHD-2, 1:1,000 rabbit anti-human polyclonal antibody (Novus Biologicals), 1:2,000 secondary anti-rabbit. Antigens were detected by enhanced chemiluminescence and quantified by densitometry as previously described (11, 60, 89, 90).

Statistical analysis. The effects of treatment (control vs. PR) and age (133 vs. 140 days) on the relative expression of target genes and/or proteins were determined using a two-way ANOVA. When there was an interaction between the effects of treatment and age, the data were split by age and the effect of treatment was reanalyzed by t-test at either 133 or 140 days of gestation. All data are presented as means ± SE. All analyses were performed using SPSS 21 for Windows (Statistical Package for Social Scientists, Chicago, IL). A probability of <5% (P < 0.05) was taken as significant.

RESULTS

Lung SP mRNA expression. Although we have previously described decreased expression of SP-A, -B, and -C mRNA in growth-restricted fetuses, gene expression at that time was normalized to a single housekeeper gene, RPP0 (66). We confirm here that SP-A, -B, and -C mRNA expression was decreased in PR compared with control fetuses at both gestational ages (Table 2). In addition, there was an effect of gestational age, with all three genes being more highly expressed at 140 compared with 133 days of gestation (Table 2). However, there was no effect of age or treatment on SP-D mRNA expression (Table 2).

Lung mRNA expression and protein abundance of cofactors involved in GC signaling. There was no effect of PR on mRNA expression of TTF-1 and GATA-6, which are cofactors involved in GC signaling, and expression of both TTF-1 and GATA-6 mRNA was decreased with advancing age (P < 0.05, Fig. 3, A and B). However, there was an opposing PR effect on TTF-1 and GATA-6 protein abundance, with TTF-1 increasing in PR fetuses and GATA-6 decreasing in PR fetuses at both gestational ages (P < 0.05, Fig. 3, C and D).

Lung mRNA expression of molecules regulating SP expression. There was no effect of PR or age on mRNA expression of factors involved in the regulation of SPs in the lung, including IL-1β, TGF-β1, and ADRB2 (Fig. 4).

Lung mRNA expression and protein abundance of PHDs. There was no effect of PR or gestational age on lung PHD1 mRNA expression and PHD1 protein abundance (Fig. 5, A and D). Lung PHD2 mRNA expression and PHD2 protein abundance were significantly higher in the PR fetuses compared with controls at both 133 and 140 days of gestation (P < 0.05, Fig. 5, B, C, and E).
There was an increase in PHD3 mRNA expression in the PR compared with control fetuses at both gestational ages \((P < 0.05\), Fig. 5C).

Lung mRNA expression of HIFs, their downstream targets, and receptors of VEGF. Lung HIF-1α and HIF-1β mRNA expression was significantly higher in PR fetuses compared with controls at both 133 and 140 days of gestation \((P < 0.05\), Fig. 6). Lung HIF-2α mRNA expression was increased in both control and PR fetuses at 140 compared with 133 days of gestation \((P < 0.05\), Fig. 6).

Similarly, lung GLUT1, JMJD1A, ACE, and FLT1 mRNA expression was higher in the PR fetuses compared with controls at both 133 and 140 days of gestation \((P < 0.05\), Fig. 7, A, D, F, and G). There was an effect of gestational age on lung expression with a decrease in JMJD1A and an increase in ACE mRNA expression at 140 days of gestation \((P < 0.05\), Fig. 7, D and F). There was an interaction effect between PR and gestational age on lung PDK1 and VEGF mRNA expression \((P < 0.05\), Fig. 7, B and C). Lung VEGF mRNA expression was higher in PR fetuses compared with controls at 140 days but not at 133 days of gestation \((P < 0.05\), Fig. 7C), whereas PDK1 mRNA expression was higher in PR fetuses compared with controls at 133 but not 140 days of gestation \((P < 0.05\), Fig. 7B). There was no effect of PR or gestational age on lung ADM and FLT1 mRNA expression \((P < 0.05\), Fig. 7, E and H).

Lung mRNA expression of markers of cell-cycle progression, proliferation, and type II AEC differentiation. Lung p27 mRNA expression was higher in PR fetuses compared with controls at both 133 and 140 days of gestation \((P < 0.05\), Fig. 8A). There was an interaction between treatment group and age on lung CCND1 mRNA expression \((P < 0.05\), Fig. 8B) in that it was lower in the PR fetuses compared with controls at 140 but not 133 days of gestation \((P < 0.05\). Lung CDC25A mRNA expression was lower in PR fetuses compared with controls at both 133 and 140 days of gestation \((P < 0.05\), Fig. 8C) and also decreased with gestational age \((P < 0.05\). Lung PCNA mRNA expression decreased with gestational age, but there was no treatment effect \((P < 0.05\); Fig. 9A), whereas KI-67 mRNA expression was lower in PR fetuses compared with controls at both 133 and 140 days of gestation \((P < 0.05\), Fig. 9B). Lung LAMP3 mRNA expression was not different either between treatments or gestational ages \((P < 0.05\).

**DISCUSSION**

We have previously shown that the carunclectomy model of IUGR results in fetuses that are chronically hypoxemic, hyperglycemic, and hypercortisolemic in late gestation (71) and also inhibits pulmonary surfactant development, leading to reduced mRNA and protein expression of SP-A, -B, and -C in the lung.
compared with 133 days of gestation; however, there was no effect of PR on plasma cortisol concentrations, may be related to alterations in that the inhibition of surfactant maturation, despite elevated plasma cortisol concentration and SP gene expression in the lung of the PR sheep fetus (66). In this study, we hypothesized the inhibition of surfactant maturation, despite elevated plasma cortisol concentrations, may be related to alterations in the prereceptor metabolism of cortisol, which may differentially affect cortisol availability in the fetal lung (66). However, in the present study, we found no change in mRNA or protein expression of 11βHSD-1, the enzyme isoform that generates cortisol from inactive cortisone (84). Although we found a significant decrease in mRNA expression in 140-day PR fetuses of 11βHSD-2, the isoform that deactivates cortisol, there was no change in its protein expression. Furthermore, there was no difference in the ratio of 11βHSD-1 to 11βHSD-2 mRNA or protein. These results suggest that lung tissue availability of cortisol in PR fetuses is not likely to be affected near term. However, there is a complex interplay between hypoxia signaling and GC signaling at the cellular level, which among several mechanisms also involves alterations in the expression and activation of the GR (92). For example, in human AECs, 48 h of hypoxia downregulates the GRα subtype (39). In the present study, we found no change in mRNA expression of GR, but we did find a decrease in PR fetuses in the 94- and 91-kDa forms of GR, which correspond to known GR isoforms, GRα and GRβ, respectively (76). Interestingly, we found a 3.5–5.0-fold greater expression of GRβ compared with GRα in the lung. GRα is the predominant and widespread isoform, including in lung epithelial cells (73, 74), and is responsible for the induction and repression of target genes via binding with cortisol (63). On the other hand, protein expression of GRβ is generally lower than that of GRα, but it is relatively highly expressed in some immune and epithelial cells, including the epithelial lining of terminal bronchioles (63, 65). GRβ does not bind cortisol but instead can act as a dominant-negative inhibitor of GRα-mediated transactivation and transrepression. Specifically, it exerts its antagonistic effect by binding with greater affinity to DNA, thereby blocking the action of GRα, or by forming heterodimers with GRα (63, 64). Hence, the balance between GRα and GRβ expression is important in determining GC signaling in tissues, and an imbalance has been shown to underlie the pathogenesis and the GC resistance of several inflammatory diseases, including asthma (33, 62, 75). However, in our study, we observed no significant effect of treatment or gestational age on the ratio of the GRβ to GRα isoform. Finally, there was no change in the 74-kDa (GRP) or 38-kDa isoform, which has been described recently in placenta as possibly placental specific (76) but was a hitherto unknown form. We found no change in MR mRNA expression in PR fetuses but a developmental downregulation in the older control and PR fetuses. It is possible that the decrease in GR protein expression may contribute to reduced cortisol-mediated gene transcriptional effects in the lung, leading to reduced SP expression in PR fetuses.

Fig. 6. There was higher normalized mRNA expression of hypoxia-inducible factor 1α (HIF-1α) (A) and HIF-1β (B) in the lung of the PR (solid bars) group compared with the control (open bars) group at 133 and 140 days of gestation. There was higher HIF-2α (C) mRNA expression at 140 days of gestation compared with 133 days of gestation; however, there was no effect of PR on lung HIF-2α mRNA expression. *Treatment; #gestational age; P < 0.05.

(66). Here we confirm these findings and show that SP-D mRNA expression is differentially regulated because it does not change in response to IUGR induced by carunclectomy. As the IUGR in this model is accompanied by hypercortisolemia as well as chronic hypoxemia, we have investigated both the GC and hypoxia signaling pathways to determine the molecular regulatory pathway that may be altering surfactant maturation. It appears that both pathways may contribute to the observed inhibition in surfactant maturation. While the exact mechanism of the surfactant inhibition is still unclear, we have observed both an increase in the mRNA expression of molecules that inhibit cell proliferation and a decrease in molecules that stimulate proliferation or cell-cycle progression in the lung of the PR fetus. This may suggest that the chronic hypoxemia and hypercortisolemia affect cellular proliferation of type II AECs and hence the functional capacity of the lung to produce surfactant.

We have previously shown an inverse relationship between plasma cortisol concentration and SP gene expression in the lung of the PR sheep fetus (66). In this study, we hypothesized that the inhibition of surfactant maturation, despite elevated plasma cortisol concentrations, may be related to alterations in

The 38-kDa isoform, which has been described recently in placenta as possibly placental specific (76) but was a hitherto unknown form. We found no change in MR mRNA expression in PR fetuses but a developmental downregulation in the older control and PR fetuses. It is possible that the decrease in GR protein expression may contribute to reduced cortisol-mediated gene transcriptional effects in the lung, leading to reduced SP expression in PR fetuses.

As the SP promoter regions do not possess a GRE, their expression is regulated indirectly by cortisol through TTF-1. The SP gene promoters in turn possess a TTF-1-binding element (54), which requires both TTF-1 and various cofactors such as GATA-6 to activate transcription (8, 9, 54). Although we found no change in TTF-1 or GATA-6 mRNA expression in response to PR, we did find opposing changes in protein abundance, with a modest increase in TTF-1 and a decrease in GATA-6 in PR fetuses. We also found a downregulation of TTF-1 mRNA and protein and GATA-6 mRNA expression in the 140-day compared with the 133-day fetuses in both control and PR groups. Hence, despite the modest upregulation of TTF-1, there appears to be a net downregulation of key factors

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regulating the GC signaling pathway, which may contribute in part to the observed inhibition of SPs in this chronic model of nutrient and oxygen restriction.

As the PR model of IUGR is accompanied by chronic hypoxemia (71), we also investigated the hypoxia signaling pathways to determine the likely molecular regulatory pathway of reduced SP expression. Given the variable responses of the surfactant system in different models of IUGR and the correlational relationship between SP expression and the degree of hypoxemia in the carunclectomy model (66), we have previously proposed that the impact of IUGR on lung SP production is dependent on the frequency, degree, and duration of hypoxemia induced by the experimental protocol (56, 67). In the present study, both HIF-1α and HIF-1β mRNA expression increased in PR fetuses at 133 and 140 days of gestation. There is significant evidence that physiological hypoxia alters HIF-α signaling in the lung and that this affects pulmonary surfactant. Specifically, HIF-1α and HIF-2α are expressed in AECs, and both are regulated by hypoxia (34, 40). It follows then that the fetus, which is normally hypoxemic compared with the neonate or adult, has elevated HIF-1α expression relative to the term neonate (36). Acute hypoxia in isolated type II AECs causes an increase in the expression of HIF-1/2α and a concomitant decrease in SPs, which is rapidly reversed when cells are exposed to oxygen (40). Further evidence of the importance of HIF-α signaling for normal surfactant development is demonstrated in Hif-2α knockout mice, which demonstrate impaired AEC differentiation, an almost complete loss of SP expression, and death within hours of parturition (20). In the present study, there was no treatment effect on HIF-2α mRNA expression, but there was an increase with gestational age.

Importantly, it has been demonstrated that chronic, as opposed to acute, hypoxia actually causes a destabilization of the HIF-1α subunit via an increase in the activity of PHD 1, 2, and 3 enzymes. Under oxygen-rich (normoxic) conditions, prolyl-hydroxylation by the PHDs leads to destruction of the HIF-α subunit, thereby limiting downstream HIF signaling.

Fig. 7. There was higher normalized mRNA expression of glucose transporter 1 (GLUT1) (A), JMJD1A (D), angiotensin-converting enzyme (ACE) (F), and FLT1 (G) in the lung of the PR (solid bars) group compared with the control (open bars) group at 133 and 140 days of gestation. Lung pyruvate dehydrogenase kinase, isozyme 1 (PDK1) (B) mRNA expression was higher in the PR fetuses compared with controls at 133 but not 140 days of gestation, whereas VEGF (C) mRNA expression was higher in the PR fetuses compared with controls at 140 but not 133 days of gestation. There was an effect of gestational age on lung expression with a decrease in JMJD1A (D) and an increase in ACE (F) mRNA expression at 140 days of gestation. There was no change of adrenomedullin (E) and FLK1 (H) mRNA expression. *Treatment; #gestational age; P < 0.05.
Under acute hypoxic conditions, the PHDs are inactivated, and hence HIF-α subunits are stabilized, thus promoting hypoxic regulation of lung maturation. However, during chronic hypoxia, the PHDs become overactive, thereby reducing HIF-α subunit stability and hence decreasing the transcriptional activity normally stimulated by HIF signaling (32). This may be regulated by changes in local mitochondrial oxygen consumption at the cellular level (32). Interestingly, PHD3 is upregulated in response to increasing duration of hypoxia, and its expression correlates with increased HIF-1α protein expression (17, 51). This finding highlights the complex regulatory network relating HIF-1α and PHD expression, and interestingly this feedback has been proposed to play a protective role by regulating HIF-α destabilization following exposure to reoxygenation (51).

In this study, we observed an increase in PHD2 mRNA expression and PHD2 protein abundance and PHD3 mRNA expression in the lung of the PR fetus. As the PR fetus is chronically hypoxic in late gestation, this increased PHD expression represents a potential mechanism regulating HIF signaling and the observed delay in surfactant system maturation (66). Despite the limitation of being unable to measure protein expression of the HIF-1α subunit in fetal lung tissue because of a combination of the short half-life of this protein (5–8 min) (6) and the methods used for tissue sample collection under consideration of animal ethics, we have provided evidence for downstream effects on HIF signaling in the lung of the PR fetus with upregulation of expression of a series of genes with HREs. Hence, despite the increase in PHD2 and 3 mRNA expression, there is evidence of hypoxic signaling in the lung, with an increase in mRNA expression of HIF-1α and -1β subunits as well as genes with HREs, including VEGF, GLUT1, PDK1, JMJD1A, ACE, and FLT1. Although some genes with an HRE can be increased by other signaling pathways [for example VEGF mRNA can be increased by...
IGF1 (13)), this does not appear to be the case here because, in this model, the chronic hypoxemia did not increase IGF1 mRNA. However, an increase in IGF1 mRNA was found in the lung of mice that were exposed to hypoxia and then hyperoxia in the neonatal period (55). Interestingly, these mice also had a decrease in SP-B and -C mRNA expression and no change in VEGF or TGF-β mRNA expression but a decrease in MTOR mRNA expression, which is downstream of the IGF1R (55). While there was no change in IGF1 or IGF2 in the lung of PR fetuses, we did find higher IGF2 and IGF1R mRNA expression. Increased IGF1R expression has been associated with an increased risk of RDS in neonates at birth (15).

Despite a lack of change in IGF1 mRNA expression, there was an increase in VEGF expression in the PR fetal lung but only at 140 days of gestation despite an increase in some genes with HRE at both gestational ages. This increase may be mediated directly by hypoxia, which has been shown to increase gene expression of lung VEGF in vitro and in vivo (3, 50, 70). Its expression plays a vital role in normal vascularization, branching morphogenesis, and alveolarization in the lung (77). Hif-2α knockout mice develop RDS, and this is associated with reduced VEGF expression (20). VEGF administration in vivo increases gene expression of SP-A, -B, and -D in the lung of the fetal sheep (81) and rat (14). Furthermore, intrauterine delivery or postnatal intratracheal instillation of VEGF stimulates surfactant production and protects preterm mice against RDS (20). However, in our model of IUGR, there is an inhibition of SP production (66) despite an increase in mRNA expression of VEGF and its receptor, FLT1, in the lung.

In addition to VEGF and FLT1, there was also increased mRNA expression of other genes with HREs, including GLUT1, PDK1, ACE, and the histone demethylase JMJDA in the lung of the PR fetus. There was, however, no change in ADM expression, a target of JMJDA1 signaling (45). Despite an increase in HIF-α and upregulation of some genes with an HRE in the PR fetal lung, there is increased PHD expression and reduced SP expression. This suggests that altered regulation of hypoxia signaling may limit responsiveness of the fetal lung to produce surfactant following exposure to chronically hypoxic conditions. Thus this biochemical regulation or further alterations to cellular proliferation may play a dominant role in regulating surfactant maturation in response to chronic hypoxemia in this model.

It is possible that hypoxemia inhibits maturation and proliferation of type II AECs, which directly contributes to the functional capacity of the fetal lung to produce surfactant. The structural architecture of the PR fetal lung differs from control animals with smaller and more densely packed alveoli in instillation-fixed tissue samples and an overall reduction in total gas exchange surface density in late gestation (49). However, the surfactant-producing capability of the PR fetal lung has not been previously evaluated. A limitation of the current study is that we were unable to determine the numerical density of type II AECs present in the fetal lungs because tissue samples were not fixed. However, in this study, we have shown changes in the expression of genes involved in proliferation (PCNA and KI-67) and in regulating cell-cycle progression at the molecular level (p27, CCND1, CDC25A), which may contribute to a change in the number of type II AECs present in the fetal lung tissue. Although PCNA did not change specifically in response to PR, KI-67 gene expression was significantly reduced in lung tissue of PR fetuses at both 133 and 140 days of gestation, supporting the possibility that type II AEC proliferation is reduced. Keratinocyte growth factor promotes proliferation of type II AECs in culture with increased protein expression of CCND1 and CDC25A and decreased expression of p27 (93). The increase in p27 and decrease in CCND1 and CDC25A observed in PR fetuses also support the possibility of a decrease in lung cell proliferation in PR fetuses. Hence, in the present study, the downregulation of gene expression in the lung of the PR fetus of direct regulators of movement through G phase (CCND1) and S phase (CDC25A) of the cell cycle and the concomitant upregulation of the cell cycle inhibitor p27 at both 133 days and 140 days of gestation provide evidence for
repression of cell-cycle regulation (93). Moreover, GC signaling via GR promotes differentiation and inhibits proliferation, as demonstrated in GR-null mice that exhibit an increase in expression of cell-cycle stimulators and a decrease in the expression of cell-cycle inhibitors (7). Hence, in our model, the hypercortisolemia may be partly responsible for the increased expression of the cell-cycle inhibitor p27 and the reduced expression of cell-cycle stimulators, CDC25A and CCND1, which may lead to a decrease in AEC proliferation. Hence, it is possible that, in addition to the altered stimulus for hypoxia regulation of lung maturation in the PR fetal lung following the response to chronic hypoxia, there is also a limited cellular biochemical capacity of the PR lung to produce surfactant.

In this study, we have demonstrated that, despite increased plasma cortisol concentrations in the PR fetus, there are changes in the expression of genes and proteins regulating GC signaling, which are consistent with a net downregulation of this pathway. This may partly explain the inhibition in SP expression seen in the carunclectomy model of IUGR and suggests a failure of the PR fetal lung to respond to the ordinarily stimulatory effects of GCs. In addition, we provide evidence for increased HIF-1α gene expression and further downstream effects on hypoxia signaling leading to increased expression of genes with HREs in the lung of the chronically hypoxic PR fetus. However, this is coupled with increased PHD expression, which normally functions to limit HIF-α signaling. We have also demonstrated a concomitant reduction in cell-cycle stimulators and an increase in cell-cycle inhibitors that may decrease cellular proliferation and hence the capacity to produce surfactant within the PR lung. Taken together, these findings represent a potential hypoxia-mediated mechanism contributing to delayed surfactant maturation observed in the lung of the PR fetus in late gestation. The limited responsiveness to GCs and altered regulation of hypoxia signaling by PHDs have implications for surfactant maturation in the lung of the PR fetus and may contribute to an increased risk of respiratory complications at birth.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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