Glucocorticoid-Mediated Repression of REDD1 mRNA Expression in Rat Fetal Distal Lung Epithelial Cells

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ABSTRACT: REDD1 (Regulated in Development and DNA Damage-1) is a stress-response gene that represses mammalian target of rapamycin (mTOR) thus decreasing protein synthesis. In contrast to studies using cell lines and adult alveolar type II (ATII) cells, we find that REDD1 mRNA levels did not increase in rat fetal distal lung epithelia (FDLE) or fetal lung fibroblasts grown in primary cultures and then exposed to 3% O₂. REDD1 mRNA expression was repressed by dexamethasone (DEX) in FDLE and ATII, but induced by DEX in fibroblasts. Lung epithelial cell lines, A549 and MLE-15, showed increases in REDD1 mRNA in response to hypoxia and DEX. The effect of DEX on REDD1 mRNA and protein in FDLE and fibroblasts was dose- and time-dependent. Inhibitor studies support repression of REDD1 mRNA by DEX in FDLE was mediated via glucocorticoid receptor and not by nongenomic effects of glucocorticoids via MAPK pathways. The half-life of REDD1 mRNA was shorter in DEX-exposed FDLE compared with hormone-free media suggesting that DEX reduced REDD1 mRNA stability in FDLE. These studies indicate that REDD1 expression in response to hypoxia and DEX is cell-type specific and that physiologically appropriate levels of PO₂ should be used when investigating fetal lung development. (*Pediatr Res* 65: 514–519, 2009)

Glucocorticoid (GC) hormones are catabolic and affect both protein synthesis and degradation in many mammalian tissues, particularly in lymphoid tissues and skeletal muscle (1). In the lung, dexamethasone (DEX) has been shown to decrease protein synthetic rates by attenuating mRNA translation at two levels: translational efficiency (*i.e.* translation initiation) and translational capacity (*i.e.* ribosome biogenesis) (2). Because GC are used extensively to promote fetal lung maturation in women in preterm labor (3) and to treat a variety of acute and chronic lung conditions such as asthma, chronic obstructive pulmonary disease (4), and respiratory distress syndrome (5), it is essential that we develop an improved understanding of how individual lung cell types respond to GC exposure.

GC exposure attenuates mRNA translation by a variety of signal transduction mechanisms (6), including decreased phosphorylation of the ribosomal protein S6 kinase (p70S6 K) and of the eukaryotic initiation factor (eIF)-4E-binding protein (4E-BP) (7). The serine-threonine kinase, mammalian target of rapamycin (mTOR) is a central integrator of environmental signals, including nutrients, growth factors, hormones, and hypoxia, and mTOR promotes mRNA translation by phosphorylating p70S6 K and 4E-BP (8). Recent publications have provided evidence that the protein encoded by REDD1 (Regulated in Development and DNA damage responses) may play a critical role in linking a variety of cues, including hypoxia and GC treatment, to inhibition of translation via the mTOR pathway (9–11). DEX-mediated induction of REDD1 transcription has been demonstrated in lymphoid cells (12) and in skeletal muscle *in vivo* and L6 myoblasts (10). In contrast, numerous publications have shown REDD1 mRNA is induced via hypoxia inducible factor-1 (HIF-1) (13) in a variety of cultured cell lines (11,14).

During gestation, the developing fetus is exposed to increases in circulating cortisol which are important for maturation of the lung (15). In addition, the fetal lung experiences rather low O₂ tension (3% O₂) relative to the postnatal lung (21% O₂). Both PO₂ and GC have been demonstrated to be important regulators of fetal lung maturation (16). We recently showed that a combination of fetal O₂ levels and DEX inhibited mTOR signaling in primary cultures of rat fetal distal lung epithelia (FDLE), yet this was not associated with increased expression of REDD1 in response to hypoxia and GC as would be predicted (17). Surprisingly, REDD1 was unresponsive to PO₂ and was repressed by 50 nM DEX.

In addition to direct transcriptional activation of target genes by binding of the activated glucocorticoid receptor (GR) to its response element, GC signaling can also reduce expression of specific genes either by direct binding to negative glucocorticoid response elements, by modulating the effects of other transcription factors such as NF- κ B, or via post-transcriptional and nongenomic actions including pathways controlling mRNA stability (6,18).

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Abbreviations: ATII, adult type II alveolar epithelial cells; DEX, dexamethasone; FDLE, fetal distal lung epithelium; GC, glucocorticoid; GR, glucocorticoid receptor; HIF-1, hypoxia inducible factor-1; mTOR, mammalian target of rapamycin; REDD1, regulated in development and DNA damage-1

Figure 1. Expression of REDD1 mRNA in response to PO_2 and DEX. (A) Representative Northern blots and quantitation from rat primary cultures of FDLE, fetal lung fibroblasts and ATII cells are shown. (B) Representative Northern blots and quantitation from A549 and MLE-15 are shown. Data are presented as mean $\pm$ SEM, $n = 5-8$ samples from three independent experiments. □: hormone-free media. ■: media + 50 nM DEX. * $p < 0.05$ relative to hormone-free condition; § $p < 0.05$ relative to 21% O_2 .

We hypothesized that regulation of REDD1 mRNA expression in response to PO_2 and DEX was distinct in the developing lung. We conducted experiments in primary cultures of rat FDLE and their adjacent lung fibroblasts, in adult rat alveolar type II epithelial cells (ATII) and in lung epithelial cell lines.

MATERIALS AND METHODS

Cell isolation and culture. Primary cultures of FDLE and their adjacent fibroblasts were obtained from 20-d timed gestation rat fetuses by enzymatic digestion, centrifugation, and differential adherence as previously described (19). ATII cells were isolated from 100–200 g male Sprague Dawley rats by enzymatic digestion and purified based on differential adherence of cells to dishes coated with rat IgG (20). All animal procedures were approved by the Hospital for Sick Children Animal Care committee. Cells were grown in DMEM-glucose (4.5 g/L glucose with 2 mM L-glutamine and 110 mg/L sodium pyruvate) supplemented with 10% FCS (FCS, Cansera, Rexdale, Ontario), 100 U/mL penicillin G sodium and 100 $\mu\text{g/mL}$ streptomycin sulfate. Culture media was replaced 24 h after seeding of FDLE (48 h for ATII) to remove unattached cells, at which time media containing hormone-depleted FCS (stripped with charcoal and ion exchange resin) was used, supplemented as indicated with DEX, RU486 (Sigma Chemical, Oakville, ON, Canada), or SB203580 (Calbiochem, San Diego, CA) and cells were placed in incubators containing either 3% O_2 –5% CO_2 –balance N_2 (“fetal” atmosphere) or 5% CO_2 –balance room air (“postnatal” atmosphere) for up to 48 h.

Lung epithelial cell lines (A549 and MLE-15) were obtained from the American Type Culture Collection and grown to confluence in “postnatal” atmosphere, after which media was changed as above for FDLE and cells were allocated to “fetal” or “postnatal” incubators for 48 h.

RNA isolation and northern blot hybridization. Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Burlington, ON, Canada). Gel electrophoresis was performed using 10 μg of RNA per sample on a 1% agarose 2.2 M formaldehyde gel in 1 $\times$ MOPS (3-morpholinopropanesulfonic acid) buffer (20 mM MOPS pH7.0, 5 mM sodium acetate, 1 mM EDTA) and transferred to Hybond- N^+ membrane. Membranes were hybridized in Expresshyb solution (Clontech, Palo Alto, CA) at 65°C using ^{32}P -labeled random primed cDNA probes for REDD1. Hybridized membranes were washed at high stringency and analyzed using a STORM PhosphorImager equipped with ImageQuant software (Molecular Dynamics, GE Healthcare, Baie d’Urfe, QC, Canada). The mRNA levels were normalized to 18S ribosomal RNA.

Western blots. Tissue culture dishes on ice were washed twice with ice-cold PBS and lysed by direct addition of RIPA buffer (150 mM NaCl; 20 mM Tris pH 8; 0.1% SDS; 1% TritonX-100; 0.5% sodium deoxycholate; 1 $\times$ Roche complete protease inhibitor cocktail (Roche Applied Science, Laval, QC, Canada). Proteins (50 μg) were size fractionated on SDS–PAGE gels and transferred to nitrocellulose. After blocking using 5% nonfat dry milk in PBS supplemented with 0.05% (wt/vol) TritonX-100, membranes were incubated 1 h with 1:1000 dilution of rabbit polyclonal antibody against REDD1 (ProteinTech Group, Chicago, IL) and washed several times in PBS. Second-

ary antibody was diluted 1:40,000 and incubated for 1 h at room temperature. All further washes were in PBS with 0.05% TritonX-100. ECL detection reagents were from GE Healthcare (Baie d’Urfe, QC, Canada). Blots were blocked and probed for actin, according to standard protocols.

RNA stability assay. FDLE or fibroblasts were seeded and cultured as described in the presence or absence of 50 nM DEX for 48 h. Transcription was stopped by addition of 5 $\mu\text{g/mL}$ actinomycin D (VWR Canlab, Mississauga, ON, Canada) and cells were harvested at different time points (0, 0.5, 1, 2, and 3 h). RNA was isolated and analyzed by Northern blot hybridization. REDD1 mRNA half-life was calculated using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA) using a one-phase exponential decay model.

Statistical analysis. Data are presented as mean $\pm$ SEM. Statistical significances were calculated using one-way ANOVA followed by Tukey post test with $p < 0.05$ being considered as statistically significant. Two-way ANOVA followed by Bonferroni post-test was used to compare effects of DEX over time in the time course experiments. Statistical analysis was performed using GraphPad Instat version 3.01 and GraphPad Prism version 4.0.

RESULTS

Effects of oxygen and DEX on REDD1 mRNA expression in distal lung cells and cell lines. Our previous study (17) showed that when primary cultures of FDLE were exposed to different PO_2 levels, there was no significant effect on REDD1 mRNA or protein, and that 50 nM DEX treatment decreased, rather than increased, the REDD1 expression. This study (Fig. 1A) confirmed these results and showed that primary cultures of the adjacent fetal lung fibroblasts also failed to respond to changes in PO_2 . Primary cultures of ATII cells, in contrast, showed a statistically significant increase in REDD1 mRNA expression under 3% O_2 . Fetal lung fibroblast cultures responded differently from the two epithelial cultures to DEX, showing a significant increase in REDD1 mRNA after a 48-h treatment with 50 nM DEX, in both fetal (3%) and postnatal (21%) O_2 atmospheres (Fig. 1A). Although published studies reported that hypoxia induced REDD1, we noted that the experimental protocols used severe hypoxia ($\leq 1\%$ O_2) and usually used transformed or cancer-derived cell lines. Accordingly, we examined whether 3% O_2 was sufficiently hypoxic to induce REDD1 in the A549 cell line (human lung carcinoma), and the murine transformed cell line, MLE-15 (Fig. 1B). Steady-state levels of REDD1 mRNA were significantly

Figure 2. Dose response of DEX-mediated regulation of REDD1 in primary cultures of fetal lung cells. Representative Northern blots and quantitation of REDD1 mRNA in Northern blots from rat FDLE (A) or fibroblasts (B, FIB) are shown. Western blots and quantitation of REDD1 protein in lysates from FDLE (C) and fibroblasts (D) treated with various concentrations of DEX confirm that protein levels correlate with changes induced in mRNA levels. Results are expressed as mean $\pm$ SEM relative to expression in hormone-free cultures, $n = 5-7$. * $p < 0.05$ relative to control (0 nM DEX).

increased in 3% O_2 in both A549 and MLE-15 epithelia. Both cell lines also exhibited increased REDD1 mRNA levels in response to 50 nM DEX with 3% O_2 and DEX showing additive effects. Because neither FDLE nor primary lung fibroblasts showed changes in REDD1 mRNA in response to changes in PO_2 , the remaining experiments were carried out in primary fetal cells cultured under 21% O_2 .

Dose-dependent effect of DEX on REDD1 mRNA expression.

To determine whether higher pharmacological doses of DEX could increase REDD1 mRNA levels in FDLE, we exposed FDLE cells to increasing DEX concentrations (Fig. 2A). DEX at low doses depressed basal expression of REDD1 reaching minimum expression at 50 nM, after which the REDD1 level had a nonsignificant trend to increase, which did not return to basal levels. In contrast, treatment of primary lung fibroblasts with increasing concentrations of DEX (Fig. 2B) showed a statistically significant increase in REDD1 when fibroblasts were exposed to doses of DEX greater than 1 nM. This effect of DEX reached a plateau at concentrations ≥ 50 nM. Western blots to detect REDD1 protein levels in FDLE (Fig. 2C) and fibroblasts (Fig. 2D) indicated that changes in REDD1 protein closely followed the shapes of the mRNA dose response curves, although the magnitude of the changes appears larger at the protein level (note the log scale on the y-axes in parts C and D).

Time course of DEX effects on REDD1 mRNA expression.

REDD1 expression has been reported to be influenced by cell density in cultured cells, with increased REDD1 in HeLa and

Figure 3. Time course of DEX-mediated effects on REDD1 in primary cultures of fetal lung cells. Representative Northern blots and quantitation from (A) FDLE and (B) fibroblasts. $\square$: hormone-free media. $\blacktriangle$: media + 50 nM DEX. * indicates $p < 0.05$ when comparing DEX vs hormone-free. Quantitative data are presented as mean $\pm$ SEM, $n = 6-8$ samples from 3-4 independent experiments. Representative Western blots of REDD1 protein from FDLE (C) and fibroblasts (D). Both time ($p < 0.0001$) and DEX ($p = 0.0334$) were significant effects in FDLE by quantitated Western data. Only time ($p < 0.001$) effect reached significance in fibroblasts, under hormone-free and DEX condition. Data are presented as mean $\pm$ SEM, $n = 3$.

293T cells at high compared with low cell density, and this effect, like hypoxia, appeared to be mediated transcriptionally via HIF-1 (21). Because GC can decrease cell proliferation, we questioned whether cell density could be a confounding factor in our results. We examined the time course of REDD1 expression in both FDLE and fibroblasts after treatment with DEX. Cells were visually 95-100% confluent when DEX treatment was initiated and fully confluent by 24 h. FDLE are contact inhibited and do not proliferate after the monolayer reaches confluence. In FDLE cultured in hormone-free media (Fig. 3A), time in culture had a significant effect on REDD1 expression with increased levels at 24 and 48 h compared with $t = 0$ h. However, DEX-mediated repression of REDD1 was apparent earlier, within 2 h of exposure to agonist, and was maintained throughout the time course. In fibroblast cultures (Fig. 3B), the DEX-induced increase in REDD1 expression was similarly apparent within 2 h of initiating agonist, and levels of REDD1 remained higher in DEX-treated than in hormone-free fibroblast cultures throughout the time course. REDD1 protein levels were assessed by Western blot (Fig. 3C and D) over parallel time courses, demonstrating comparable changes in protein and mRNA, with the exception that REDD1 protein persisted in fibroblasts at 48 h when REDD1 mRNA decreased.

Effect of DEX on REDD1 mRNA stability. Decreased steady-state levels of REDD1 mRNA in FDLE exposed to DEX could be due to either decreased transcription or increased degradation. We determined the stability of REDD1

Figure 4. DEX decreases the stability of REDD1 mRNA in FDLE but not in fibroblasts. REDD1 mRNA decay after actinomycin D treatment in FDLE (A and C) and fibroblasts (B and D, FIB). Half-life ($T_{1/2}$) was calculated by nonlinear curve-fit using an equation for one-phase exponential decay. Data are presented as mean $\pm$ SEM, $n = 6$ samples from three independent experiments. $\square$: hormone-free media. $\blacktriangle$: 50-nM DEX.

mRNA in FDLE and lung fibroblasts that were cultured with or without 50 nM DEX and then treated with actinomycinD. The disappearance of REDD1 mRNA over time was quantified relative to 18S rRNA and fitted to a one-phase exponential decay model. In FDLE, REDD1 mRNA was significantly less stable in the presence of DEX ($T_{1/2} = 0.86 \pm 0.13$ versus 1.4 ± 0.05 h in hormone free media, $p < 0.01$, Fig. 4A and C). This represents an $\sim 40\%$ decrease in mRNA stability, comparable to the decrease in steady-state REDD1 mRNA level. In contrast, the $T_{1/2}$ of REDD1 mRNA was significantly less in fibroblasts than in FDLE under either condition ($T_{1/2} = 0.32 \pm 0.017$ in DEX and 0.34 ± 0.019 h in hormone free media, $p < 0.01$ versus FDLE, $n = 6$ samples from three experiments) and was not affected by DEX treatment.

Effects of inhibitors of glucocorticoid receptor versus p38 MAPK. The effects of DEX on REDD1 mRNA expression in FDLE could be blocked by the specific GR antagonist RU486 at both moderate (50 nM) and high (1000 nM) DEX doses (Fig. 5A), demonstrating that the down-regulation of REDD1 is mediated exclusively via the GR even at extremely high agonist concentrations. Because GC have been shown to induce MAPK phosphatase to block MAPK activity (22), it was hypothesized that MAPK signaling may be involved in the phenomenon. Although GC can inhibit both p38 MAPK and ERK1/2, ERK signaling is up-regulated during cellular proliferation. Our experiments used cultures very near or at confluence at the beginning of the GC treatment. Proliferation in FDLE ceases at confluence, as they are contact inhibited; hence, a role for ERK1/2 seems unlikely. In addition, p38 MAPK is involved in the regulation of mRNA stability via AU-rich elements in mRNA's 3'UTRs, whereas there is little evidence suggesting that ERK plays such a role except in combination with p38MAPK (23). Accordingly, we treated FDLE with either DEX or the p38 MAPK inhibitor SB203580 and assessed REDD1 mRNA levels after 4 and 24 h. Inhibition of p38 MAPK over this time period neither altered REDD1 levels compared with control condition, nor mimicked the effects of DEX treatment.

Figure 5. DEX effect on REDD1 in FDLE is mediated via the glucocorticoid receptor, not MAPK pathways. (A) DEX-mediated repression of REDD1 mRNA in FDLE is blocked by the GR antagonist RU486. White bar: control. Black bar: 20 μ M RU486. $*p < 0.05$ relative to control (0 nM DEX). In (B), inhibition of MAPK with SB203580 does not mimic the effect of DEX on REDD1 mRNA expression in FDLE. $\square$: hormone-free media. $\blacktriangle$: media + 50 nM DEX. $\bullet$: 2.5 μ M SB203580. $*p < 0.05$ when comparing DEX vs hormone-free.

DISCUSSION

REDD1 is a transducer of the cellular response to a variety of stresses through the mTOR pathway, generally resulting in a decrease in translation initiation and protein synthesis. Our results demonstrate that modulation of REDD1 expression in response to PO_2 and DEX is cell-type specific. Although REDD1 mRNA has been shown to be up-regulated by hypoxia in a HIF-1 dependent manner (14), we show that such up-regulation does not occur in primary cultures of FDLE or fetal lung fibroblasts cultured in 3% O_2 versus 21% O_2 . Although the physiologic "fetal" atmosphere we used has a higher PO_2 than published reports studying the effects of hypoxia on the induction of REDD1 (11,13,14), it was sufficient to up-regulate REDD1 in human (A549) and mouse (MLE-15) lung epithelial cell lines and in primary cultures of adult ATII cells. It has also been previously demonstrated that this physiologic fetal O_2 atmosphere is sufficient to up-regulate HIF-1 in cultured FDLE (24).

The regulation of mRNA translation when cells are exposed to changes in ambient PO_2 is critical to our understanding of how cells prevent loss of energy homeostasis under hypoxic conditions. Severe hypoxia (*i.e.* 1% O_2) may be relevant to understanding cancers; however, to understand normal fetal lung growth and development, it is important to use the physiologically relevant level of PO_2 on lung cells grown in primary culture. Although precise roles for oxygen-sensing mechanisms in the fetus have not been clearly defined, fetal and perinatal oxygen tensions influence both lung morphogenesis and the perinatal transition from fluid secretion to fluid absorption, essential for a successful transition of the infant to an independent, air-breathing existence (25). It is understood that acute versus prolonged hypoxia results in changes in mRNA translation regulated by distinct mechanisms of translational control, only some of which are HIF-1 dependent (26). It is possible that the lack of up-regulation of REDD1 in FDLE in response to 3% O_2 represents an important characteristic of these cells to permit the very

high rates of protein synthesis required for normal fetal growth in the uterine environment. Our observation that ATII cells up-regulate REDD1 mRNA in response to fetal O₂ concentration is consistent with such an adaptation being specific to fetal cells to prevent growth retardation under the developmentally appropriate 3% O₂ environment.

DEX produced opposite effects on REDD1 expression in FDLE and lung fibroblasts even though both were obtained from the same fetuses and grown in primary culture. Although DEX induced REDD1 in fibroblasts, similar to results published in lymphoid cells and myoblasts, in FDLE DEX exerted a repressive effect. Kimball *et al.* (27) recently demonstrated rapid protein degradation as a regulatory mechanism controlling REDD1 expression when translation elongation and mTOR signaling are inhibited by cycloheximide. However, we found that DEX-mediated changes in REDD1 mRNA and protein levels are well correlated indicating that DEX effects were mediated at the RNA level.

The expression of REDD1 has been reported to be affected by cell density (21). Both FDLE and fibroblasts were fully confluent at the time of harvest for REDD1 mRNA analysis; however, we also examined REDD1 mRNA steady-state levels during the 48 h leading up to harvest. FDLE cultured under hormone-free conditions showed increases in REDD1 after 24 and 48 h, which may have been because of cells achieving full confluence or because of the establishment of a fully polarized monolayer. This increase in REDD1 expression after 24 h was not mediated via MAPK pathways as REDD1 expression was not affected by the MAPK inhibitor SB203580 over the time course. Repression of REDD1 levels by DEX were apparent already after 2 h of treatment and maintained throughout the time course, indicating that DEX effects were not due to slowing of confluence. Fibroblast cultures showed little increase in REDD1 over time in culture under hormone-free media and in fact tended to decrease after 48 h suggesting that high cell density did not induce REDD1 in these cells. In contrast to FDLE, fetal lung fibroblasts do not exhibit contact inhibition, with multilayered growth creating thick stacks of alternate layers of cell. If up-regulation of REDD1 at high cell density is associated with contact inhibition, this might explain the differences in REDD1 mRNA time course in FDLE *versus* fibroblasts under hormone-free conditions. Similar to FDLE, DEX effects on REDD1 were significant within 2 h of initiating treatment and were maintained over 48 h.

REDD1 mRNA stability in FDLE was significantly decreased by 50 nM DEX treatment and this decrease of ~40% is similar in magnitude to the drop in steady-state REDD1 mRNA level. This indicates that DEX-mediated regulation of REDD1 mRNA was post-transcriptional in these cells. Steroid hormones have been shown to regulate gene expression post-transcriptionally by altering mRNA stability of numerous targets, including GC-mediated regulation of growth hormone, fatty acid synthetase, inflammatory response proteins, collagenase, and cyclin D3 (28). The stability of REDD1 mRNA in fibroblasts was unchanged by DEX, confirming a transcriptional up-regulation as initially reported in lymphoid cells (12).

The lack of induction of REDD1 by DEX in both FDLE and ATII cells is of particular interest in light of the use of glucocor-

ticosteroid treatments antenatally to accelerate fetal lung maturation (3) and postnatally in preterm infants to prevent chronic lung disease (29). Routine use of systemic DEX in premature infants is not recommended because of concerns about negative side effects including abnormal neurologic development and reduced somatic growth, yet such use continues (30). Assuming that REDD1 induction and ensuing down-regulation of mTOR-dependent protein synthetic pathways would hold negative consequences for most tissues in light of the rapid growth and development occurring in the neonatal period, one could speculate that lung epithelia may escape negative effects of DEX treatment by this adaptation whereas other tissues do not. Clinical studies attempting to use inhaled corticosteroids in neonates at risk of chronic lung disease have so far been disappointing, but it is unclear whether this is due to the lung disease itself or the unique physiologic, anatomic, and technical issues involved in delivering inhaled drugs to the very low birth weight, ventilated infant (31). In light of the potentially specific effects of DEX on REDD1 in lung epithelial tissues, it appears worthwhile to continue to pursue attempts to optimize drug delivery via the inhaled route.

In summary, regulation of the cell signaling pathways mediated by REDD1 in response to both hypoxia and GC shows considerable evidence for cell-specific effects. Primary cultures of rat FDLE and fetal lung fibroblasts show no effect of changes between 21% and 3% O₂ on REDD1, and respond to DEX in opposite ways. These responses may be because of the adaptation of these cells to the fetal environment and an understanding of such pathways is potentially important in view of the use of antenatal glucocorticoids and controlled O₂ ventilation in premature infants.

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