Regulation of phosphatidylcholine synthesis in maturing type II cells: increased mRNA stability of CTP:phosphocholine cytidyltransferase

Martina HOGAN, Maciej KULISZEWSKI, Wensu LEE and Martin POST*

The Medical Research Council Group in Lung Development and the Neonatal Research Division, Department of Paediatrics, Hospital for Sick Children Research Institute, University of Toronto, Toronto, Ontario M5G1X8, Canada

Pulmonary surfactant phosphatidylcholine synthesis increases in fetal lung type II cells with advancing gestation. This increase is accompanied by an increase in gene and protein expression of CTP:phosphocholine cytidyltransferase (CT; EC 2.7.7.15), which catalyses a regulatory step in *de novo* phosphatidylcholine synthesis by fetal type II cells. In the present study we investigated the role of transcriptional and post-transcriptional mechanisms in the developmental induction of CT mRNA in maturing type II cells. We found that CT mRNA increased 2-fold from days 18 to 21 of fetal rat gestation (term 22 d). This increase in CT mRNA was not accompanied by a developmental increase in CT gene transcription. However, CT mRNA was more stable on day 21 ($t_{1/2}$ 48 h) compared with that on day 18 ($t_{1/2}$ 17 h). Gluco-

corticoids have been shown to enhance surfactant phosphatidylcholine synthesis in fetal type II cells. Therefore we also examined the effect of maternal glucocorticoid administration to pregnant rats at 19 d of gestation on CT mRNA expression in fetal type II cells isolated 24 h later. Glucocorticoid treatment did not increase type II cell CT mRNA. As reported previously, however, glucocorticoids increased CT activity in the microsomal membrane fraction of fetal type II cells, whereas no differences in cytosolic CT activity were observed. We conclude that the developmental increase in CT mRNA in fetal type II cells is due to a decreased breakdown of the CT transcript and that glucocorticoids regulate fetal type II cell CT activity at a post-translational level.

INTRODUCTION

Adequate amounts of pulmonary surfactant are essential for proper lung functioning immediately after birth. Pulmonary surfactant is produced by alveolar type II cells and is composed of lipids and proteins. Phospholipids are quantitatively the most important constituents of surfactant. Phosphatidylcholine (PC) makes up 70–85% of the phospholipids. The synthesis of PC increases during late gestation. The major route for synthesis of PC is the CDP-choline pathway. The reaction catalysed by CTP:phosphocholine cytidyltransferase (CT) is the rate-limiting step in this pathway [1]. Recently we reported that increased PC formation during fetal type II cell development is associated with an increase in CT activity [2]. The increase of CT activity in fetal type II cells during late gestation is partly due to an increase in CT mRNA and protein expression [3]. The developmental regulation of type II cell CT mRNA is unknown. Endogenous glucocorticoids play a central role in fetal lung maturation. Studies *in vivo* and *in vitro* have shown that glucocorticoids stimulate CT activity and PC synthesis [4]. Although glucocorticoids do not directly affect CT mRNA expression of fetal type II cells, recent studies suggest that they increase fetal type II cell CT mRNA [5] and CT activity [6] via a mesenchymal–epithelial cell interaction.

To further unravel the developmental regulation of CT mRNA expression in fetal type II cells, we first investigated whether the developmental increase in CT mRNA is due to an increased rate of transcription and/or increased mRNA stability. We then examined whether CT gene expression is increased in type II cells of fetuses after maternal glucocorticoid administration and, if so,

whether this is brought about at the level of transcription or mRNA stability.

MATERIALS AND METHODS

Cell culture

Female (200–250 g) and male (250–300 g) Wistar rats were purchased from Charles River (St. Constant, Quebec, Canada) and bred in our animal facilities. Rats were killed by diethyl ether inhalation on days 18 and 21 of gestation (term 22 d). The fetuses were removed aseptically from the mothers, the fetal lungs dissected out in cold Hank's balanced salt solution without calcium or magnesium [HBSS (–)] and cleared of major airways and vessels. The lungs were washed twice in HBSS (–), minced and suspended in HBSS (–). Fetal type II cells were isolated from the fetal lungs as previously described by Caniggia et al. [7]. All experiments were performed 24 h after the start of isolation. The viability and purity of the epithelial cultures were not affected by gestational age and were comparable to previously published data [3,7].

Maternal treatment of fetal lung with glucocorticoids

On day 19 of gestation, pregnant rats were injected intraperitoneally with dexamethasone phosphate [0.2 or 1 mg/kg] (SABEX, Boucherville, Quebec, Canada). Controls were injected with vehicle alone. Approx. 24 h after the injections, the fetuses were delivered by hysterotomy, killed, and their lungs isolated. The freshly excised lungs were used for the preparation of fetal lung type II cells as described previously [7].

Abbreviations used: CT, CTP:phosphocholine cytidyltransferase; HBSS(–), Hank's balanced salt solution without magnesium; PC, phosphatidylcholine; RT-PCR, reverse transcriptase-PCR; SP-B, SP-C, surfactant proteins B and C.

* To whom correspondence should be addressed.

Northern analysis

Total RNA was isolated from fetal type II cells essentially as described by Chirgwin et al. [8]. Total RNA was fractionated on 1% (w:v) agarose gels containing 6% (v:v) formaldehyde, transferred to a nylon membrane and fixed by UV cross-linking. Hybridizations with rat [32 P]CT cDNA, complementary to nucleotides 1–706 of rat lung CT, and washings were performed by the method of Hogan et al. [3]. The films were quantified by an Ultrosan XL laser densitometer (LKB, Bromma, Sweden).

Nuclear run-on transcription assays

Nuclear run-on transcription reactions were performed with isolated nuclei and [α - 32 P]UTP as described previously [9,10]. Briefly, nuclei of day 18 and 21 fetal type II cells were incubated with [α - 32 P]UTP (800 Ci/mmol) for 30 min at 30 °C. Labelled RNA was isolated by lysis in 4 M guanidinium thiocyanate followed by centrifugation on a 5.7 M caesium chloride cushion. Hybridizations were performed with equal numbers of labelled RNA counts (approx. 3×10^6 c.p.m.) on replicate filters prepared by slot-blotting 10 μ g of linearized cDNA probes for CT, β -actin, glyceraldehyde 3-phosphate dehydrogenase and 18 S rRNA. The filters were hybridized in plastic vials at 55 °C for 48 h, then washed. Washed membranes were exposed to X-ray film with intensifying screens and signals were quantified by scanning laser densitometry. After autoradiography, membranes were counted in a liquid-scintillation counter to determine the amount of bound radioactivity. RNA degradation was assessed by spotting an aliquot of labelled RNA on DE81 filters and washing extensively with 0.5 M Na_2HPO_4 to remove free nucleotides [11]. No degradation of RNA during the hybridization was observed. Transcription rates quantified by either autoradiography or radioactivity were similar. Signals were corrected for background (linearized PCR DNA vector).

RNA stability

To measure the degradation rates of CT mRNA, actinomycin D was added to day 18 and day 21 fetal type II cell cultures at a concentration of 5 μ g/ml, a concentration that we previously found to inhibit RNA synthesis completely [9,10]. Total RNA from four 75 cm² tissue culture flasks for each time point was isolated and processed for Northern blot analyses at 0, 3, 6, 12 and 24 h after the addition of actinomycin D. Blots were probed for the CT gene, then reprobed for β -actin to correct for any error in the amount of RNA loaded and also to rule out any undesirable effects of actinomycin D on cell toxicity. The half-life ($t_{1/2}$) of RNA was calculated from the equation $t_{1/2} = \ln 2/k$ (degradation rate constant $k = -2.303 \times \text{slope}$) [12].

Cytidyltransferase activity assay

CT activity was assayed by measuring the rate of incorporation of [$\text{methyl-}^{14}\text{C}$]phosphocholine (New England Nuclear, Mississauga, Ontario, Canada) into CDPcholine. Cell fractionations and CT assays were performed by the method of Zimmermann et al. [2].

Statistical analysis

Data are presented as means $\pm$ S.E.M. Statistical differences between groups were analysed by unpaired *t*-test. Statistical significance was accepted at the $P < 0.05$ level (two-tailed).

RESULTS AND DISCUSSION

In previous experiments with fetal type II cells, we found by using reverse transcriptase-PCR (RT-PCR) that CT mRNA increased 2-fold with advancing gestation [3]. To investigate whether the increase in CT mRNA was accompanied by an increase in CT mRNA synthesis, we compared the rate of transcription of CT in type II cells derived from the late pseudoglandular stage (day 18) and early saccular stage (day 21) of rat lung development. In agreement with our previous finding [3], Northern hybridizations showed that the steady-state mRNA levels of CT increased approx. 2-fold between 18 and 21 d of gestation (Figure 1 and Table 1). Using a 5' end CT probe in the nuclear transcription run-on assay, we failed to detect significant differences in the transcriptional rate for CT in nuclei of 18 d and 21 d fetal type II cells (Figure 1 and Table 1). Because Margana and Boggaram [13] reported that transcription across the surfactant protein B gene is non-equimolar, we compared the

Figure 1 Developmental regulation of CT gene transcription and CT mRNA accumulation in maturing fetal rat type II cells

Autoradiogram (a) of nuclear run-on transcription assay of the CT gene in type II cells from 18 and 21 d of fetal gestation. Isolated nuclei from fetal type II cells were incubated with [32 P]UTP. Labelled RNA (3×10^6 c.p.m.) was hybridized to nylon membranes containing equimolar amounts of 3' end (nt 684–1510) and 5' end (nt 1–706) CT cDNA in PCR vector as probes [3]. Control PCR probes contained β -actin, glyceraldehyde 3-phosphate dehydrogenase and 18 S rRNA cDNA. PCR vector alone was used as a negative control. (b) Autoradiogram of RNA from day 18 and day 21 fetal type II cells after hybridization with 32 P-labelled 5' end CT cDNA.

Table 1 CT gene transcription and CT mRNA accumulation in fetal type II cells

Autoradiograms of Northern blots and transcription run-on assays were quantified by laser-scanning densitometry. In Northern blot analysis, CT mRNA signals were normalized to β -actin mRNA levels. Also CT transcription signals were normalized to β -actin signals. Data are means $\pm$ S.E.M. for three separate experiments. * $P < 0.05$.

Gestational age	Transcriptional activity (arbitrary units)		CT mRNA (arbitrary units)
	3' end CT	5' end CT	
Day 18	0.83 $\pm$ 0.26	0.67 $\pm$ 0.12	1.00 $\pm$ 0.10
Day 21	0.75 $\pm$ 0.10	0.52 $\pm$ 0.05	1.87 $\pm$ 0.26*

Figure 2 Degradation rates of mRNA for CT in maturing fetal rat type II cells

(A) Cells isolated from 18 and 21 d fetal rat lung were incubated in the presence of 5 μ g/ml actinomycin D. At the times indicated, total RNA was isolated, immobilized on nylon membranes and hybridized with 32 P-labelled CT and β -actin cDNA. (B) Autoradiograms were scanned by laser densitometer. Because the half-life of actin seemed not to change in maturing type II cells, CT mRNA signals were normalized to β -actin mRNA levels. Data from three separate experiments were analysed and the best fit was obtained by linear regression analysis. The half-life ($t_{1/2}$) of CT mRNA in fetal type II cells increased with advancing gestation [$t_{1/2}$ (18 d) = 17 h, compared with $t_{1/2}$ (21 d) = 48 h]. Symbols: $\circ$, 18 d; $\bullet$, 21 d.

transcriptional activity of both the 3' end and the 5' end of the CT gene. Independent of gestation, the transcription across the gene was equimolar for both regions (Figure 1 and Table 1). We have previously reported 1.5–2.5-fold changes in transcription for platelet-derived growth factor genes in fetal lung type II cells during development by using similar methods for nuclei isolation and transcription run-on assay [9]; it is therefore unlikely that the observed constant rate of CT transcription during development is due to insensitivity of the nuclear run-on assay. The results of the run-on assays suggest that the gestational increase in CT mRNA is not due to an increase in CT gene transcription. We then assessed the contribution of mRNA stability of CT in the overall steady-state levels of this gene. Again, the degradation rate of CT mRNA was measured in type II cells of 18 and 21 d of gestation. The half-lives of CT and β -actin mRNAs were determined by measuring their degradation at various time intervals after the addition of sufficient actinomycin D to inhibit mRNA synthesis. Actinomycin D had no effect on the turnover of β -actin message (Figure 2). Densitometric analysis of the CT

Figure 3 Effect of maternal administration of glucocorticoids on CT gene expression in fetal type II cells

Pregnant rats were injected with either dexamethasone or vehicle alone at 19 d of gestation, and the fetuses were delivered at 20 d. Fetal type II cells were isolated from the fetal lungs and total RNA was extracted from the cells. RNA (10 μ g) was analysed by Northern blotting with 32 P-labelled CT or β -actin cDNA. (a) Northern blot of fetal type cell RNA from control (C) or dexamethasone-treated (0.2 mg/kg) (D) pregnant rats. Data represent two separate experiments. (b) Northern blot of fetal type cell RNA from control (C) or dexamethasone-treated (1 mg/kg) (D) pregnant rats. Data are representative of four separate experiments. (c) Northern blot of whole fetal lung RNA from control (C) or dexamethasone-treated (1 mg/kg) (D) pregnant rats. Data represent three separate experiments. (d) Northern hybridization of whole fetal lung RNA from control (C) or dexamethasone-treated (1 mg/kg) (D) pregnant rats with 32 P]SP-C cDNA. Equal RNA loading and transfer was demonstrated by hybridizing the same blot with β -actin probe.

mRNA in the presence of actinomycin D was therefore normalized to the β -actin message after reprobing the blot with β -actin. The results of the mRNA stability assay are shown as decay curves in Figure 2. CT message was more rapidly degraded in type II cells from the late pseudoglandular stage ($t_{1/2}$ 17 h) than in type II cells from the early saccular stage ($t_{1/2}$ 48 h) of lung development. This increase in mRNA stability for CT in maturing type II cells was correlated with the observed increase in mRNA. Recently Fraslon-VanHulle et al. [14] reported that epithelial cells isolated from day 17 fetal rat lung and cultured in a serum-free medium on a basement membrane matrix showed a spontaneous increase in CT mRNA. A spontaneous increase has also been observed in fetal lung in explant culture [15]. Although it was concluded that the expression of CT mRNA is partly regulated developmentally at the pretranslational level, the molecular mechanism for this increase in CT mRNA was not investigated.

Recent studies have investigated the molecular mechanisms responsible for developmental increases in mRNA content of the surfactant proteins B (SP-B) and C (SP-C), which are essential for biophysical and physiological activity of surfactant [16,17]. The developmental accumulation of SP-B mRNA in fetal rabbit lung appears to be due to both enhanced transcription and mRNA stability [13,18]. Recently, Boggaram and Margana [19] reported that mRNA stabilization primarily accounts for the accumulation of SP-C mRNA during prenatal rabbit lung development. Thus it is possible that mRNA stabilization is a

Table 2 Effect of maternal glucocorticoid administration on CT mRNA expression in fetal lung and type II cells

Autoradiograms of Northern blots (1 mg/kg dexamethasone treatment) of Figure 3 were quantified by laser-scanning densitometry. In Northern blot analysis, CT mRNA signals were normalized to β -actin mRNA levels. Data are means $\pm$ S.E.M. for at least three separate experiments.

Treatment	CT mRNA (arbitrary units)	
	Whole lung	Type II cells
Control	1.00 $\pm$ 0.08	1.00 $\pm$ 0.09
Dexamethasone	1.15 $\pm$ 0.12	0.82 $\pm$ 0.08

Figure 4 Southern analysis of CT RT-PCR products of fetal lung mRNA after maternal administration of glucocorticoids

Pregnant rats were injected with either 0.2 mg/kg dexamethasone or vehicle alone at 19 d of gestation, and the fetuses were delivered at 20 d. Total RNA was extracted from the lungs, reverse transcribed and amplified by 20 cycles of PCR [3]. RT-PCR products were analysed by Southern blotting with 32 P-labelled CT cDNA. C, control; D, dexamethasone. Data represent three separate experiments.

common mechanism by which maturing type II cells co-ordinate the gene expression of CT, SP-B and SP-C. Such co-ordination between surfactant lipid and protein synthesis is essential for a properly functioning surfactant at term.

Developmental regulation of genes at the post-transcriptional level (mRNA stability) has also been reported to play an important role during oocyte maturation and red cell development [20]. The mechanism of increased CT mRNA stability in type II cells with advancing gestation is not yet defined. Because glucocorticoids accelerate lung maturation and stimulate surfactant synthesis [4], we investigated whether glucocorticoids are responsible for this developmental increase in CT mRNA stability. Glucocorticoids have been shown to alter the mRNA stability of many genes [20]. The stability of SP-B mRNA is increased by glucocorticoids in rabbit [13,18] and human [21,22] fetal lung *in vitro*. Also, glucocorticoids increased the half-life of SP-C in rabbit fetal lung *in vitro* [19]. We found that maternal glucocorticoid administration at either 200 μ g/kg or 1 mg/kg did not increase CT mRNA expression in whole fetal rat lung or type II cells isolated from the fetuses when assessed by either Northern hybridization (Figure 3 and Table 2) or RT-PCR followed by Southern blotting (Figure 4). In contrast, administration of dexamethasone (1 mg/kg) to pregnant rats increased the expression of SP-C mRNA in whole fetal lung (Figure 3), consistent with previous findings [23,24]. In addition we have demonstrated an increase in transforming growth factor β_3 mRNA expression in fibroblasts isolated from lungs of fetuses of pregnant mothers treated with dexamethasone [25]. In agreement with our previous studies [6], we found that on glucocorticoid administration to pregnant mothers, CT activity increased in the microsomal membrane fraction of type II cells isolated from the lungs of the fetuses (Table 3). There was no change in the

Table 3 Effect of maternal administration of glucocorticoids on CT activity in fetal type II cells

Pregnant rats were treated as described for Figure 3. Fetal type II cells were isolated, homogenized and fractionated. Enzyme activity was measured in the different subcellular fractions. Values are means $\pm$ S.E.M. for four separate experiments, each carried out in triplicate. Abbreviation used: n.s., not significant.

Subcellular fraction	Activity (nmol/min per mg of protein)			P
	Control	Dexamethasone	Treated/control	
Cytosol	0.26 $\pm$ 0.02	0.30 $\pm$ 0.06	0.87	n.s.
Microsomal	0.69 $\pm$ 0.12	1.32 $\pm$ 0.24	1.91	< 0.05

cytosolic activity (Table 3). The observation that glucocorticoids did not increase the CT mRNA content in fetal type II cells argues against a role of glucocorticoids in stabilizing CT mRNA during development. Recently, Batenburg and Elfring [5] observed that exposure of isolated fetal type II cells to cortisol did not affect CT gene expression. Cells exposed to medium conditioned by fibroblasts in the presence of cortisol showed a small but significant increase in CT mRNA, suggesting a pre-translational regulation by glucocorticoids. In contrast, we have shown previously that treatment of fetal type II cells with cortisol-conditioned medium resulted in a rapid activation of the enzyme; we concluded that conditioned medium of cortisol-exposed fibroblasts regulates CT at a post-translational level [26]. In fetal rat lung *in vitro*, glucocorticoids increased the activity but not the amount of CT [27]. Unfortunately, only the cytosolic CT activity was measured. Our previous studies with fetal type II cells demonstrated that CT protein and activity increased in the microsomal fraction with advancing gestation, with no developmental change in the cytosolic fraction [2,3]. Our present observations are compatible with glucocorticoids' stimulating CT activity in fetal type II cells via an increased membrane association. The mechanism by which glucocorticoids induce CT binding to membranes remains to be investigated. Recently we found that cytosolic but not microsomal lipids are involved in the developmental activation of cytosolic CT in fetal type II cells at late gestation [28]. Furthermore CT-membrane interactions are promoted by cytosolic lipids such as fatty acids [29–32]. Glucocorticoids have been shown to stimulate fatty acid synthesis in fetal rat lung [33,34] and type II cells [5] *in vitro*. Inhibition of fatty acid synthesis abolished CT activation by glucocorticoids in fetal rat lung *in vitro* [35]. Thus it is likely that the glucocorticoid-induced microsomal CT activation in fetal type II cells is secondary to an increase in fatty acids.

In conclusion, we provide new insight into the regulation of surfactant PC synthesis in maturing type II cells. Our results show that the developmental increase in mRNA for cytidylyl-transferase, which catalyses a rate-limiting step in *de novo* PC synthesis in type II cells, is caused by mRNA stabilization. In contrast with SP-B and SP-C gene expression, glucocorticoids seem not to regulate CT mRNA stability in fetal type II cells. Identification of sequence motifs within the 3' untranslated region of CT mRNA that regulate RNA stability remain to be investigated. The 3' untranslated region of lung CT mRNA contains UA-rich regions [3], which have been implicated in determining RNA degradation [36]. Further investigation focusing on the putative 'turnover' proteins that interact with these 3' untranslated region elements may unravel the developmental control of CT mRNA stability in fetal type II cells [36]. Our data also suggest that the developmental increase in PC

synthesis in type II cells at late fetal gestation [2] is due to an increase in CT protein expression [3] and a glucocorticoid-induced activation of CT, most probably via fatty acids.

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