Modulation of surfactant phosphatidylcholine by exogenous magnesium, hormones, fatty acids and insulin in juvenile rat lung organ cultures

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Zusammenfassung

In dieser Studie wurde die zeitliche Wirkung von exogen-zugeführten Hormonen, Magnesium und Fettsäuren auf die Biosynthese von oberflächenaktiven Lipiden, Eiweißen und DNS in Lungengewebskulturen junger Ratten untersucht.
Die zellulären Protein- und Phosphatidylcholinkonzentrationen stiegen an, während die DNS-Konzentration relativ stabil blieb. Zusätzlich erhöhten sich die zellulären Eiweiß-, DNS- und Phosphatidylcholinkonzentrationen dosisabhängig mit steigender Magnesiumkonzentration im Nährmedium wie folgt: freies Mg < niedriger Mg-Spiegel < hohe Mg-Konzentration. Ein Magnesiummangel kann somit *in-vitro* die gesamten zellulären Protein-, DNS- und Phosphatidylcholinkonzentrationen unterdrücken.
Die Applikation von Dexamethason und Trijodthyronin steigerte die Biosynthese von Phosphatidylcholin, DNS und Protein. Eine weitere Inkubation mit Palmitat oder Oleat zusammen mit Dexamethason und Trijodthyronin verstärkte die zellulären Protein-, DNS- und Phosphatidylcholinspiegel. Die Gabe von Insulin zu den Zellkulturen antagonisierte die Wirkung der Hormone und Fettsäuren auf die Phosphatidylcholin- und Eiweißsynthese.
Die Ergebnisse deuten darauf hin, dass die Magnesiumionenkonzentration die Synthese von oberflächenaktiven Lipiden, Eiweißen und DNS regulieren kann. Zusätzlich dazu steigern Dexamethason, Trijodthyronin und Fettsäuren die Produktion dieser Substanzen, wahrend Insulin diese Modulation verhindert.

Summary

This study examined the effect of time in culture or magnesium concentration and exogenous hormone and fatty acid supplementation on protein, DNA and surfactant lipid concentration in rat lung in organ culture.
Cellular protein and phosphatidylcholine (PC) concentrations increased with time in culture, whilst DNA concentration remained relatively stable. In addition, total cellular protein, DNA and PC concentrations increased in a dose dependent manner with increasing media magnesium concentration in the order: free magnesium < normal magnesium < high magnesium media. Thus, magnesium restriction or undernutrition suppress total cellular protein, DNA and PC concentrations *in-vitro*.
Dexamethasone and triiodothyronine administration enhanced protein, DNA and phosphatidylcholine concentration. Further incubations with either palmitate or oleate in combination with dexamethasone and triiodothyronine enhanced cellular protein, DNA and PC content. The addition of insulin to cultures however, abolished the hormonal and fatty acid effects on phosphatidylcholine and protein concentration.
These results indicate that magnesium ion concentration may regulate DNA, protein and surfactant lipid synthesis. In addition, dexamethasone, triiodothyronine and fatty acids can enhance DNA, protein and surfactant lipid concentration whilst insulin abolishes this modulation effect.

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Introduction

Respiratory Distress Syndrome (RDS), a primary cause of neonatal death results from insufficient production of surfactant by type II alveolar epithelial cells [1]. Glucocorticoids are known to stimulate surfactant lipid synthesis and surfactant protein expression. In addition, fatty acids are required for the biosynthesis of phospholipids [2] and are transported to lung cells via fatty acid binding proteins [3].
Magnesium plays a critical role in regulating lung function [4] and structure [5] and is essential for activating nearly all enzymatic systems. Maternal nutritional magnesium depletion and thus inadequate magnesium levels may contribute to neonatal respiratory disorders [6, 7]. Dietary magnesium supplementation in human pregnancy reduces the incidence of preterm delivery [8]. Hypomagnesaemia is common in diabetes [9] and infants of diabetic mothers are frequently hyperinsulinaemic with delayed fetal lung maturation [10]. However, the precise role of magnesium in the aetiology of RDS is unknown.
This study investigates the effect of time in culture or magnesium concentration on total cellular DNA, protein and surfactant lipid concentration in rat lung organ cultures. In addition, the modulation of surfactant phosphatidylcholine by exogenous hormone and fatty acid supplementation was studied and effects of insulin on these processes observed.

Methods and materials

Preparation of lung organ cultures

Lung tissue was aseptically removed from young juvenile male Wistar rats (9–10 weeks old) after CO_2 anaesthesia, washed free of blood cells with Phosphate Buffered Saline (PBS, pH 7.4) at 37 °C, pooled and then finely diced into 1 mm^3 pieces. The pieces were then mixed to give a representative sample. Organ cultures were initiated by inoculating 2×10^6 cells onto sterile collagen sponges (Colgen) ($1 \times 1 \times 0.7$ cm) in a 60 mm-diameter tissue culture dish [11, 12]. Cultures were

initiated only if cell viability in preparations was greater than 95 % using the Trypan Blue dye exclusion procedure.

Culture conditions

Organ cultures were serum free and contained 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin, 0.25 µg ml^{-1} Fungizone, and 5 U ml^{-1} Nystatin. Cultures were maintained in 5 ml RPMI 1640 media in a humidified chamber at 37 °C in 95 % air and 5 % CO_2 for four days. At the end of the incubation period, the culture media was decanted, cultures were washed three times with PBS at 37 °C and the cells removed from the collagen sponge matrix. Cells were then sonicated in 1 ml PBS using an Ultra Sonicator (Microson Heat Systems) to disrupt them and stored at –20 °C until utilised for total phosphatidylcholine, DNA and protein analysis.

Experimental methods

i. Effect of time in culture

Cultures were initiated as described above and maintained in normal magnesium RPMI 1640 culture media (containing 0.4 mmol l^{-1} total magnesium) for four days. A number of cultures were terminated each day for the analysis of cellular protein, DNA and total phosphatidylcholine concentrations.

ii. Effect of magnesium concentration in the media

Cultures were maintained for four days in either 5 ml magnesium free (without magnesium), normal magnesium (containing 0.4 mmol l^{-1} total magnesium) or high magnesium (containing 4.4 mmol l^{-1} total magnesium) RPMI 1640 culture media. High magnesium media was prepared by supplementing normal magnesium media with $MgSO_4.7H_2O$ to obtain the appropriate concentration. At the end of the culture period, cellular protein, DNA and total phosphatidylcholine concentrations were determined.

iii. Effect of hormones and fatty acids

Dexamethasone and triiodothyronine were added to cultures for the entire culture period (4 days) at concentrations of 10^{-6} and 10^{-7} mol l^{-1} respectively. Concentrations employed were comparable to those used in past experiments [12]. Cultures were maintained in normal magnesium RPMI 1640 culture media. Parallel cultures were supplemented for the entire culture period with exogenous fatty acids. The sodium salts of palmitate and oleate were complexed to bovine serum albumin (essentially fatty acid free) to give final concentrations of 200 µmol l^{-1} fatty acid and 3 % (w/v) albumin as reported previously [12]. Control cultures received no additions. At the end of the culture period, cellular protein, DNA and total phosphatidylcholine concentrations were determined.

iv. Effect of Insulin

Cultures maintained in normal magnesium media were supplemented with dexamethasone and triiodothyronine and additionally received either palmitate or oleate or a combination of both fatty acid preparations for four days. The effect of insulin (0.5 U ml^{-1}) supplementation on the hormonal and fatty acid action was observed and cellular protein, DNA and total phosphatidylcholine concentrations was analysed at the end of the culture period.

Experimental analysis

On completion of experiments, total protein and DNA concentrations were determined on cell homogenates by the methods of *Lowry* [13] and *Sterzel* [14] respectively. Total phosphatidylcholine concentration was measured using an enzymatic phospholipase D method [15].

Chemicals, culture media and test agents for supplementation were obtained from Sigma Chemical Co. (Poole, Dorset, UK), BDH (Liverpool, UK) or Gibco BRL (Paisley, Scotland).

Statistical analysis

The results of the effect of exogenous supplements on protein, DNA and phosphatidylcholine concentrations are representatives of six experimental cultures and each point represents the mean of triplicate analysis. Results are expressed as means ± S.D. Statistical analysis was performed using an ANOVA test and the Bonferroni post hoc test was used to locate the source of significant variance. Differences were taken as statistically significant when $P < 0.05$.

Results

Using rat lung in organ culture we investigated the influence of magnesium, dexamethasone, triiodothyronine, fatty acids and insulin on total cellular protein, DNA and phosphati-

Tab. 1: Effect of time in culture on phosphatidylcholine, protein and DNA concentration by 2×10^6 rat lung cells in organ culture.

Time in culture (days)	Phosphatidylcholine (PC) concentration mg PC / 2×10^6 cells	Protein concentration mg Protein / 2×10^6 cells	DNA concentration mg DNA / 2×10^6 cells
1 (n = 6)	0.109 ± 0.007	0.139 ± 0.006	0.015 ± 0.003
2 (n = 6)	0.118 ± 0.006	0.271 ± 0.068 *	0.016 ± 0.001
3 (n = 6)	0.136 ± 0.009 *, **	0.358 ± 0.058 *	0.017 ± 0.003
4 (n = 6)	0.179 ± 0.001*, **, ***	0.393 ± 0.068 *	0.017 ± 0.004

Results are means ± S.D for six experimental cultures.
* Compared with day 1, $P < 0.05$. ** Compared with day 2, $P < 0.05$. *** Compared with day 3, $P < 0.05$.

Tab. 2: Effect of media magnesium concentration on phosphatidylcholine, protein and DNA concentration by 2×10^6 rat lung cells in organ culture.

Magnesium concentration	Phosphatidylcholine (PC) concentration mg PC / 2X10^6 cells	Protein concentration mg Protein / 2×10^6 cells	DNA concentration mg DNA / 2×10^6 cells
Mg free	0.035 ± 0.012	0.136 ± 0.03	0.003 ± 0.003
Normal Mg	0.073 ± 0.033 *	0.321 ± 0.022 *	0.012 ± 0.001 *
High Mg	0.147 ± 0.015 *, **	0.398 ± 0.025 *, **	0.029 ± 0.001 *, **

Results are means ± S.D for six experimental cultures.
* Compared with culture in magnesium free media, $P < 0.05$. ** Compared with culture in normal magnesium media, $P < 0.05$.

Tab. 3: Effect of hormone supplementation on phosphatidylcholine, protein and DNA concentration by 2×10^6 rat lung cells in organ culture.

Exogenous supplementation	Phosphatidylcholine (PC) concentration mg PC / 2×10^6 cells	Protein concentration mg Protein / 2×10^6 cells	DNA concentration mg DNA / 2×10^6 cells
Control (No addition)	0.064 ± 0.019	0.159 ± 0.035	0.013 ± 0.001
Dexamethasone (Dex)	0.110 ± 0.031 *	0.216 ± 0.029	0.048 ± 0.008 *
Triiodothyronine (T_3)	0.100 ± 0.009	0.197 ± 0.139	0.036 ± 0.016 *
Dexamethasone / Triiodothyronine	0.165 ± 0.027 *, **	0.371 ± 0.082 *, **	0.053 ± 0.014 *

Results are means ± S.D for six experimental cultures.
* Compared with control culture, $P < 0.05$. ** Compared with either Dex or T_3 supplementation.

dylcholine concentrations after four days in culture.

i. Effect of time in culture

Cellular protein and phosphatidylcholine (PC) concentrations increased with time in culture, whilst DNA concentration (Tab. 1) remained relatively stable. Cellular protein concentrations at two, three and four days in culture were significantly increased over that observed at day one ($P < 0.05$). PC concentrations after three and four days in culture were significantly greater than that at day one ($P < 0.05$ respectively).

ii. Effect of magnesium concentration in the media

Total cellular protein, DNA and phosphatidylcholine (PC) (Tab. 2) concentrations increased in a dose dependent manner with increasing media magnesium concentration in the order: free magnesium < normal magnesium < high magnesium media. As such, protein, DNA and PC concentrations were significantly increased in cells cultured in normal and

Tab. 4: Effect of fatty acid supplementation and exogenous insulin on phosphatidylcholine, protein and DNA concentration by 2×10^6 rat lung cells in organ culture.

Exogenous supplementation	Phosphatidylcholine (PC) concentration mg PC / 2×10^6 cells	Protein concentration mg Protein / 2×10^6 cells	DNA concentration mg DNA / 2×10^6 cells
Control	0.081 ± 0.010	0.266 ± 0.063	0.014 ± 0.003
Hormones	0.111 ± 0.003	0.339 ± 0.033	0.023 ± 0.005
Hormones / Palmitate	0.114 ± 0.006 *	0.423 ± 0.009 *	0.030 ± 0.009 *
Hormones / Oleate	0.109 ± 0.008 *	0.447 ± 0.038 *	0.031 ± 0.014 *
Hormones / Palmitate / Oleate	0.201 ± 0.013 *, **, ***, ****	0.560 ± 0.096 *, **, ***, ****	0.047 ± 0.009 *, **, ***
Hormones / Insulin	0.071 ± 0.006 **	0.211 ± 0.013 * **	0.023 ± 0.008
Hormones / Palmitate / Insulin	0.070 ± 0.24 ***	0.214 ± 0.013 ***	0.040 ± 0.010
Hormones / Oleate / Insulin	0.054 ± 0.015 ****	0.220 ± 0.031 ****	0.039 ± 0.005

Cultures were maintained in normal magnesium media and received hormones (dexamethasone, Dex and triiodothyronine, T_3) and fatty acids (either palmitate or oleate, or palmitate and oleate).

Results are means ± S.D for six experimental cultures.

* Compared with control culture, $P < 0.05$. ** Compared with Dex and T_3 supplementation, $P < 0.05$. *** Compared with hormones and palmitate, $P < 0.05$. **** Compared with hormones and oleate, $P < 0.05$.

high magnesium media compared to that in the absence of magnesium ($P < 0.05$ respectively).

iii. Effect of hormones and fatty acids

a) Effect of hormones

Dexamethasone and triiodothyronine administration enhanced protein, DNA and PC concentration in rat lung organ cultures. Dexamethasone but not triiodothyronine significantly increased PC concentrations above control values ($P < 0.05$) (Tab. 3). However, simultaneous administration of dexamethasone and triiodothyronine produced a synergistic increase in protein and PC concentrations (Tab. 3) above control values and values obtained on administration of these hormones separately ($P < 0.05$ respectively). Similarly, DNA concentration of cultures was increased above control values by dexamethasone and triiodothyronine supplementation either singly or in combination ($P < 0.05$ respectively).

b) Effect of fatty acids

Incubation with 200 μmol l^{-1} palmitate-albumin or oleate-albumin in addition to hormones dexamethasone and triiodothyronine caused a significant stimulation of cellular phosphatidylcholine (PC), protein and DNA content (Tab. 4), ($P < 0.05$ respectively) compared to controls. Furthermore, the incubation with both palmitatealbumin and oleate-albumin in addition to hormones stimulated PC, protein and DNA concentration above control values ($P < 0.05$) and above values for cells supplemented with hormones ($P < 0.05$), or with hormones plus palmitate-albumin ($P < 0.05$), or oleate-albumin ($P < 0.05$).

iv) Effect of insulin

The addition of insulin to cultures abolished the hormonal and fatty acid effects on phosphatidylcholine, and protein concentration (Tab. 4) ($P < 0.05$ respectively). Conversely, whilst the addition of insulin to cultures abolished the hormonal effect on DNA concentration (Tab. 4), the fatty acid effect was preserved.

Discussion

Dexamethasone, triiodothyronine and fatty acids were previously shown to stimulate the synthesis and accumulation of fetal human lung phosphatidylcholine (PC) and preferentially increase dipalmitoylphosphatidylcholine (DPPC) *in-vitro* [11, 12]. Our findings illustrate an increase in total cellular protein and PC concentrations with time in culture (Tab. 1), and on exogenous supplementation with dexamethasone and triiodothyronine and clearly support these and other previous observations [16, 17]. We also describe a synergistic increase in protein, DNA and PC concentrations on simultaneous administration of dexamethasone and triiodothyronine (Tab. 3).

Mechanisms of phosphatidylcholine acyl remodelling by the human fetal lung *in-vitro* have been defined [12]. These results suggested that principally their incubation conditions and not their state of apparent maturation [11] determine the synthesis pattern of PC species by lung cells. This conclusion was supported by our observations that incubations with either palmitate or oleate or a combination of both with dexamethasone and triiodothyronine enhanced total cellular PC, protein and DNA concentration (Tab. 4), consistent with these and other reports [18, 19]. In addition, our results suggest that insulin inhibits the hormonal and fatty acid effects on PC and protein concentration and inhibits the hormonal effect on DNA concentration (Tab. 4), in accordance with previous findings [20, 21, 22]. However, this antagonistic effect of insulin requires further study to ascertain whether phosphatidylcholine and surfactant specific protein syntheses are regulated independently and to determine the role of insulin in respiratory distress syndrome in metabolic conditions such as poorly controlled diabetic pregnancies.

Magnesium ions play an important role in protein, DNA and lipid synthesis. Magnesium regulates metabolism, growth and protein synthesis and low magnesium concentrations can lead to growth failure [23, 24]. This conclusion was supported by our observation that total cellular protein, DNA and PC concentrations were lowest in cells cultured in a magnesium free media and were increased with increasing magnesium concentration in the media (Tab. 2). In otherwords, these results suggest that culture media magnesium restriction or under-nutrition as indicated by culture in magnesium free media suppressed synthesis of all these parameters. However, because our model does not discriminate between individual phosphatidylcholine species or between total cellular protein and the surfactant specific proteins, these factors will need to be explored in detail to determine the precise role of magnesium in surfactant lipid and protein synthesis, storage and secretion. Our findings are consistent with the suggestion that magnesium deficiency may be implicated as an aetiological factor in Respiratory Distress Syndrome (RDS) as reported previously [25].

The synthesis of PC species and the surfactant specific proteins by alveolar type II cells in response to a variety of hormones and alterations in culture conditions is also worthy of further study to reveal specific mechanisms important in surfactant dysfunction and the aetiology of RDS. Although the relationship between glucocorticoids and lung development is well established, the relative importance and contribution of specific substrates to the fetal lung surfactant system remains to be more clearly defined.

Acknowledgements

We wish to thank the University of Wolverhampton for financial support.

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