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# EFFECTS OF CORTISOL AND THYROXINE ON PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLGLYCEROL SYNTHESIS BY ADULT RAT LUNG ALVEOLAR TYPE II CELLS IN PRIMARY CULTURE

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## Summary

1. The effect of cortisol and thyroxine on the formation of phosphatidylcholines and phosphatidylglycerols was studied in adult rat lung type II cells in primary culture.

2. Addition of cortisol enhanced the incorporation of [*Me*-$^{14}$C]choline, [1-$^{14}$C]acetate, [1-$^{14}$C]palmitate, D-[U-$^{14}$C]glucose, and [1(3)-$^{3}$H]glycerol into total and disaturated phosphatidylcholines.

3. Cortisol also stimulated the formation of phosphatidylglycerols from labelled acetate, palmitate, glucose, and glycerol, but did not affect the formation of phosphatidylethanolamines.

4. Thyroxine alone did not significantly affect the formation of total and disaturated phosphatidylcholines nor that of phosphatidylglycerols or phosphatidylethanolamines.

5. Exposure of the cells to a combination of cortisol and thyroxine caused increases in the rates of synthesis of total and disaturated phosphatidylcholines from labelled choline, palmitate, and glycerol and in that of phosphatidylglycerols from labelled glycerol. These increases were about the same as those brought about by cortisol alone. In contrast to cortisol alone, the combination of cortisol and thyroxine did not significantly affect the entry of labelled acetate and glucose into phosphatidylcholines and phosphatidylglycerols.

6. The present results suggest that direct effects of glucocorticosteroids on the alveolar type II cell may play a role in the regulation of the synthesis of surfactant lipids in the adult lung.

## Introduction

During the later part of gestation, the lungs of the fetus mature and begin to produce pulmonary surfactant. This surface-active material lines the alveoli and prevents alveolar collapse during expiration by reducing alveolar surface tension (for reviews see Refs. 1—5). Dipalmitoylphosphatidylcholine is undoubtedly the principal constituent of pulmonary surfactant [6]. Phosphatidylglycerol, however, appears to be an essential minor component of surfactant [7].

Ample experimental evidence is available indicating that circulating glucocorticosteroids are involved in the regulation of fetal lung maturation [8—10]. Several reports [11—13] suggest that thyroid hormones exert similar stimulatory effects on lung development in fetal animals. The detailed mechanisms via which these hormones accelerate the synthesis of surfactant have, however, not yet been elucidated [14].

Although in the studies of the hormonal effects on lung lipid metabolism the adult lung has been relatively ignored, there is experimental evidence that glucocorticosteroids [15,16] and thyroid hormones [17] also affect lung morphology and lipid metabolism in the adult animal.

It is generally accepted that the alveolar type II cells are the most important, if not the only, producers of surfactant lipids [1,3,5]. In type II cells corticosteroid [18] and thyroid hormone [19] receptors have been demonstrated indicating that type II cells may be influenced by a direct action of these hormones. Studies with isolated type II cells have shown that glucocorticosteroids stimulate the synthesis of phosphatidylcholines from radioactive precursors [20—22]. However, the latter type of study has thus far only been carried out with cell line A549, derived from a human lung carcinoma [20] and with cells isolated from normal lung tissue by cloning [21,22]. Tumor cells are known often to differ metabolically from the normal cells from which they are derived. In addition, the A549 cells have been reported to differ from normal type II cells in morphology and content of disaturated phosphatidylcholines [23]. It is therefore uncertain whether results obtained with this cell line can be directly extrapolated to normal type II cells. A similar uncertainty applies to data from cells obtained by cloning, as the development of a cell line usually involves a transformation [24]. A second problem encountered in the use of cloned cells is the uncertainty about the cellular origin of the cell line (for discussion see Ref. 25).

Because of these uncertainties studies were undertaken to investigate whether cortisol has the same stimulatory effect on the formation of phosphatidylcholines in fresh type II cells from normal adult rat lung. As phosphatidylglycerols may also be essential for an optimal functioning of pulmonary surfactant [7], the synthesis of this phospholipid class was also included in these studies. Moreover, in view of the indications that thyroid hormones may influence lung lipid metabolism [11—13,17,19] the effect of thyroxine on the formation of phosphatidylcholines and phosphatidylglycerols in type II cells was also investigated.

## Materials and Methods

*Animals.* Female Wistar rats, 180—200 g, were obtained from the Central Institute for Breeding of Laboratory Animals in Zeist, The Netherlands. The rats had free access to standard laboratory chow and water.

*Isolation of type II cells.* Alveolar type II cells were isolated from rat lung by trypsinization and density gradient centrifugation [26,27]. The cells obtained in this way were further purified by differential adherence in primary monolayer culture [23,27]. The culture medium consisted of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 50 μg/ml gentamycin. The medium was gassed with 10% $CO_2$/90% air. During the first 3 h of culture at 37°C most macrophages adhere. The non-adherent cells were divided equally between 20 to 40 35-mm tissue culture dishes. The volume of the medium per dish was adjusted to 1.5 ml. Culturing was continued for 20 h, during which period most type II cells adhere but most contaminating cells do not. At the end of this period the medium and non-adherent cells were removed by aspiration. The adherent cells were washed with 1.5 ml fresh medium.

*Exposure of the purified type II cells to hormones.* The type II cells purified as described above were cultured for an additional 24 h period with either control medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 50 I.U./ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml amphothericin B) or the same medium with $10^{-5}$ M cortisol, $5 \cdot 10^{-7}$ M thyroxine or $10^{-5}$ M cortisol plus $5 \cdot 10^{-7}$ M thyroxine.

*Incubation of the cells with radioactive substrates.* At the end of the 24 h period of exposure to hormones the culture medium was removed by aspiration. The cells in the dishes were rinsed with a serum-free incubation medium containing 125 mM NaCl, 5 mM KCl, 2.5 mM $Na_2HPO_4$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 17 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 50 I.U./ml penicillin and 50 μg/ml streptomycin (pH 7.4). They were subsequently incubated in a reciprocating waterbath (25 cycles/min) for 5 h at 37°C in this medium (1.5 ml per dish) supplemented with labelled substrates to estimate the rate of lipid synthesis. The rate of precursor incorporation into lipids was linear for at least 6 h of incubation. The medium in each dish also contained the hormone(s), to which the cells in that particular dish had already been exposed, at the same concentration as before. As substrates the cells received either 0.025 mM [*Me*-$^{14}$C]choline (spec. act. $4.9 \cdot 10^4$ dpm/nmol) in the presence of 5.6 mM glucose and 0.2 mM palmitate, 1 mM [1-$^{14}$C]acetate (spec. act. $1.4 \cdot 10^4$ dpm/nmol) or 0.2 mM [1-$^{14}$C]palmitate (spec. act. $1.3 \cdot 10^4$ dpm/nmol) in the presence of 5.6 mM glucose and 0.05 mM choline, 5.6 mM D-[U-$^{14}$C]glucose (spec. act. $9.7 \cdot 10^3$ dpm/nmol) in the presence of 0.05 mM choline, or 0.1 mM [1(3)-$^3$H]glycerol (spec. act. $1.8 \cdot 10^5$ dpm/nmol) in the presence of 0.2 mM palmitate and 0.05 mM choline. Palmitate was complexed to bovine serum albumin in a molar ratio 5.3 : 1. During this incubation the culture dishes were each held in a 50 ml glass beaker which was covered with parafilm. The incubations were terminated by scraping the cells from the culture dishes with a rubber policeman and transferring cells plus medium to a tube containing 16 ml $CHCl_3/CH_3OH$ (1 : 1, v/v). The culture dishes were

rinsed with another ml of fresh medium, which was also transferred to the $CHCl_3/CH_3OH$ mixtures.

*Evaluation of the type II cell preparations.* The percentage type II cells in the dishes was assessed after the period of exposure to hormones by the modified Papanicolaou stain described by Kikkawa and Yoneda [28]. After staining the cells, the number of cells on the dishes was counted with the aid of an occular micrometer. In each experiment the dishes contained at least $0.15 \cdot 10^6$ cells. The adherent cells were 92 ± 1% (average ± S.D.; $N = 24$) type II cells. The viability was 91 ± 3% as judged from trypan blue exclusion.

*Lipid analyses.* Lipids were extracted from the $CHCl_3/CH_3OH$ mixture by the method of Sundler et al. [29] after addition of lung lipid extract (0.2 μmol phosphorus). Phosphatidylcholines were isolated from lipid extracts of cells incubated with labelled choline by thin-layer chromatography on silicagel H plates with $CHCl_3/CH_3OH$/acetic acid/$H_2O$ (50 : 30 : 8 : 4, v/v) as eluent [30]. In experiments with the other precursors phosphatidylcholines, phosphatidylglycerols and phosphatidylethanolamines were separated by two-dimensional thin-layer chromatography on boric acid-impregnated silicagel G plates with $CHCl_3/CH_3OH/H_2O$/conc. $NH_3$ (70 : 37 : 4 : 6, v/v) as developing solvent in the first direction and with $CHCl_3/CH_3OH/H_2O$ (65 : 35 : 5, v/v) as eluent in the second direction [31]. The lipids were visualized with iodine vapour. Phosphatidylglycerols and phosphatidylethanolamines were transferred directly into scintillation vials while phosphatidylcholines were extracted from the silicagel according to Bligh and Dyer [32]. Disaturated phosphatidylcholines were isolated from total phosphatidylcholines by the method of Mason et al. [33]. Radioactivity was measured in a liquid scintillation mixture described by Pande [34]. Counting efficiency was determined by the channels ratio method.

*Materials.* Trypsin (twice crystallized) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. and all radioactive chemicals from the Radiochemical Centre, Amersham, U.K.

## Results

*Incoporation of various precursors into phospholipids*

Table I shows the incorporation of several phospholipid precursors into adult rat type II cells during a 5 h incubation after 47 h of culture in the absence of added hormones. The rates of incorporation of the precursors into phosphatidylcholines and the high degree of saturation of the synthesized phosphatidylcholines are about the same as those reported previously for type II cells in primary culture for 20 h [35].

It can be seen that the cells also have the capability to synthesize phosphatidylglycerols. The percentages of phosphatidylglycerols relative to total lipids synthesized from labelled acetate or glycerol are of the same order as those reported by Mason and Williams [23] for cultured adult rat type II cells, while the percentages obtained with [1-$^{14}$C]palmitate and [U-$^{14}$C]glucose are of the same order as those reported by Smith and Kikkawa [36] for adult rabbit type II cells.

As eight molecules of acetate are necessary for the formation of one palmitate molecule, the rate of incorporation into phosphatidylglycerols and

TABLE I

INCORPORATION OF VARIOUS PRECURSORS INTO TOTAL AND DISATURATED PHOSPHATIDYLCHOLINES, PHOSPHATIDYLGLYCEROLS AND PHOSPHATIDYLETHANOLAMINES BY ADULT RAT LUNG TYPE II CELLS IN THE ABSENCE OF HORMONES

After culturing for 47 h, the isolated type II cells were incubated with the various precursors. The data are the averages ± S.E. of at least eight experiments (see Table II). PC, phosphatidylcholines; DSPC, disaturated phosphatidylcholines; PG, phosphatidylglycerols; PE, phosphatidylethanolamines.

| Labelled substrate | Incorporation into PC (nmol/h per $10^6$ cells) | PC as % of total lipids (label incorporated) | DSPC as % of total PC (label incorporated) | Incorporation into PG (nmol/h per $10^6$ cells) | PG as % of total lipids (label incorporated) | Incorporation into PE (nmol/h per $10^6$ cells) | PE as % of total lipids (label incorporated) |
|---|---|---|---|---|---|---|---|
| [*Me*-$^{14}$C]Choline | 1.36 ± 0.09 | 76 ± 1 | 90 ± 3 | — | — | — | — |
| [1-$^{14}$C]Acetate | 2.22 ± 0.33 | 44 ± 1 | 66 ± 4 | 0.21 ± 0.02 | 4 ± 1 | 0.16 ± 0.02 | 3 ± 1 |
| [1-$^{14}$C]Palmitate | 6.72 ± 0.81 | 62 ± 2 | 88 ± 3 | 0.86 ± 0.08 | 8 ± 1 | 0.47 ± 0.06 | 4 ± 1 |
| D-[U-$^{14}$C]Glucose | 0.61 ± 0.02 | 50 ± 2 | 52 ± 3 | 0.11 ± 0.01 | 9 ± 1 | 0.05 ± 0.01 | 4 ± 1 |
| [1(3)-$^{3}$H]Glycerol | 0.23 ± 0.03 | 42 ± 2 | 72 ± 2 | 0.07 ± 0.01 | 12 ± 1 | 0.02 ± 0.01 | 4 ± 1 |

TABLE II

EFFECTS OF CORTISOL AND THYROXINE ON THE INCORPORATION OF VARIOUS PRECURSORS INTO PHOSPHATIDYLCHOLINES, DISATURATED PHOSPHATIDYLCHOLINES, PHOSPHATIDYLGLYCEROLS AND PHOSPHATIDYLETHANOLAMINES IN ADULT RAT LUNG TYPE II CELLS

The ratio treated/control was calculated in each separate experiment. The data are averages ± S.E. of the indicated number of experiments. Statistical significance was determined by using Student's *t*-test. PC, phosphatidylcholines; DSPC, disaturated phosphatidylcholines; PG, phosphatidylglycerols; PE, phosphatidylethanolamines.

| Labelled substrate | Lipid synthesized | No. of experiments | Incorporation into individual phospholipids in control (nmol/h per $10^6$ cells) * | Ratio treated/control | | |
|---|---|---|---|---|---|---|
| | | | | Cortisol | Thyroxine | Cortisol plus thyroxine |
| [*Me*-$^{14}$C]Choline | PC | 16 | 1.36 ± 0.09 | 1.27 ± 0.05 ** | 1.13 ± 0.06 | 1.24 ± 0.06 ** |
| | DSPC | 16 | 1.21 ± 0.14 | 1.29 ± 0.06 ** | 1.17 ± 0.08 | 1.23 ± 0.05 ** |
| [1-$^{14}$C]Acetate | PC | 8 | 2.22 ± 0.33 | 1.27 ± 0.04 ** | 1.03 ± 0.04 | 1.08 ± 0.07 |
| | DSPC | 8 | 1.49 ± 0.26 | 1.27 ± 0.04 ** | 1.06 ± 0.06 | 1.05 ± 0.09 |
| | PG | 8 | 0.21 ± 0.02 | 1.33 ± 0.05 ** | 1.06 ± 0.05 | 1.04 ± 0.09 |
| | PE | 8 | 0.16 ± 0.02 | 0.87 ± 0.13 | 0.99 ± 0.09 | 1.05 ± 0.16 |
| [1-$^{14}$C]Palmitate | PC | 10 | 6.72 ± 0.81 | 1.16 ± 0.03 ** | 1.07 ± 0.04 | 1.28 ± 0.07 ** |
| | DSPC | 10 | 5.88 ± 0.76 | 1.22 ± 0.05 ** | 1.05 ± 0.04 | 1.28 ± 0.06 ** |
| | PG | 10 | 0.86 ± 0.08 | 1.35 ± 0.03 ** | 1.06 ± 0.05 | 1.09 ± 0.03 |
| | PE | 8 | 0.47 ± 0.06 | 1.13 ± 0.04 | 1.07 ± 0.03 | 1.20 ± 0.16 |
| D-[U-$^{14}$C]Glucose | PC | 10 | 0.61 ± 0.02 | 1.44 ± 0.10 ** | 1.04 ± 0.06 | 1.05 ± 0.07 |
| | DSPC | 10 | 0.30 ± 0.04 | 1.51 ± 0.12 ** | 1.03 ± 0.02 | 1.06 ± 0.04 |
| | PG | 10 | 0.11 ± 0.01 | 1.20 ± 0.04 ** | 1.06 ± 0.08 | 0.86 ± 0.10 |
| | PE | 8 | 0.05 ± 0.01 | 1.06 ± 0.03 | 1.09 ± 0.08 | 1.03 ± 0.08 |
| [1(3)-$^{3}$H]Glycerol | PC | 8 | 0.23 ± 0.03 | 1.23 ± 0.05 ** | 1.05 ± 0.07 | 1.22 ± 0.06 ** |
| | DSPC | 8 | 0.17 ± 0.03 | 1.37 ± 0.06 ** | 1.05 ± 0.02 | 1.32 ± 0.06 ** |
| | PG | 8 | 0.07 ± 0.01 | 1.53 ± 0.07 ** | 1.07 ± 0.09 | 1.37 ± 0.03 ** |
| | PE | 8 | 0.02 ± 0.01 | 1.06 ± 0.05 | 1.05 ± 0.05 | 1.24 ± 0.05 ** |

* Data taken from Table I.

** $P < 0.01$.

phosphatidylethanolamines of fatty acids synthesized de novo from labelled acetate was an order of magnitude slower than the rate of incorporation of exogenous palmitate (Table I). The same difference was observed in the synthesis of phosphatidylcholines (Table I and Ref. 35).

Maximally, 2.5% of the labelled glucose incorporated into lipids was recovered in triacylglycerols (not shown).

*Effects of cortisol and thyroxine on the synthesis of phosphatidylcholines, phosphatidylglycerols and phosphatidylethanolamines*

The number of type II cells per dish, the purity and viability (as judged from trypan blue exclusion) were not affected by the hormones to which the cells were exposed.

The effects of cortisol and thyroxine on the rate of incorporation of labelled precursors into total and disaturated phosphatidylcholines and into phosphatidylglycerols and phosphatidylethanolamines are shown in Table II. Exposure of the type II cells to cortisol had a significant stimulatory effect ($P < 0.01$) upon the incorporation of [*Me*-$^{14}$C]choline, [1-$^{14}$C]acetate, [1-$^{14}$C]palmitate, D-[U-$^{14}$C]glucose and [1(3)-$^{3}$H]glycerol into total and disaturated phosphatidylcholines. It also enhanced the incorporation of the last four precursors into phosphatidylglycerols, but did not affect the formation of phosphatidylethanolamines. The synthesis of neutral lipids was not influenced by cortisol (not shown). The percentage disaturation of phosphatidylcholines synthesized from the various precursors was not significantly influenced by cortisol. The formation of total and disaturated phosphatidylcholines and that of phosphatidylglycerols was not significantly influenced by thyroxine.

Exposure of the cells to a combination of cortisol and thyroxine caused increases in the rates of formation of phosphatidylcholines and disaturated phosphatidylcholines from labelled choline, palmitate and glycerol and in that of phosphatidylglycerols and phosphatidylethanolamines from labelled glycerol. The increases in the rates of synthesis of phosphatidylcholines and phosphatidylglycerols were about the same as those brought about by cortisol alone. In contrast to cortisol alone, the combination of cortisol and thyroxine did not significantly affect the entry of labelled acetate and glucose into phosphatidylcholines, disaturated phosphatidylcholines and phosphatidylglycerols.

It has been reported that in adult rat lung triiodothyronine is the principal thyroid hormone [37]. In addition, evidence is accumulating that thyroxine exerts its hormone effect by serving as a precursor for triiodothyronine [38]. Therefore, in some experiments (not shown) type II cells were exposed to triiodothyronine instead of thyroxine to investigate the effect of this thyroid hormone on the incorporation of [*Me*-$^{14}$C]choline into total and disaturated phosphatidylcholines. No significant effect of triiodothyronine on the formation of these lipids could be demonstrated.

## Discussion

In the present study it is demonstrated that adult rat lung type II cells in monolayer culture for 47 h have the capability to synthesize both phosphatidylcholines with a high degree of saturation and phosphatidylglycerols. This

capability is an important criterion for the viability of type II cells in view of their role as producers of surfactant. During the first two days, the cultures are stable and do not divide, as was shown by Mason et al. [27]. The DNA content of the cultures decreases rather than increases. It has been observed [27] that only after more extended periods (three days or longer) does the number of lamellar bodies per cell decrease. Therefore, it is very likely that the metabolic activities associated with the synthesis of surfactant, as reported in this paper, occur much more quickly than changes in the cultures.

In the preceding section it was shown that in isolated adult rat type II cells in primary culture the glucocorticosteroid cortisol increases the rate of incorporation of all precursors tested into total and disaturated phosphatidylcholines and into phosphatidylglycerols. This is the first report on the stimulatory effect of a glucocorticosteroid on phosphatidylcholine synthesis in fresh type II cells in short-term culture. It is also the first report on the stimulatory effect of a glucocorticoid upon phosphatidylglycerol synthesis in isolated type II cells. Possmayer et al. [39] have reported that corticosteroids may exert a general stimulatory effect on the synthesis of pulmonary phospholipids. Their suggestion is not corroborated by the findings in the present study considering that cortisol stimulated the formation of the major surfactant lipids, phosphatidylcholines and phosphatidylglycerols, but not that of phosphatidylethanolamines. Under only one condition (glycerol as substrate and the combination of cortisol and thyroxine as effector) was the formation of phosphatidylethanolamines enhanced.

The data corroborate earlier studies with whole lung [16] that cortisol may not only accelerate the synthesis of surfactant lipids in fetal lung, but may also be involved in regulating this process in adult lung.

Although it has been reported [17] that thyroid hormones injected into adult rats caused an increase in the amount of surfactant obtained by alveolar wash and an enlargement of type II alveolar cells, no significant effect of thyroxine on the incorporation of radioactive precursors into phosphatidylcholines and phosphatidylglycerols by isolated adult rat type II cells was observed in the present studies. This is consistent with the observation of Mason et al. [40] that thyroxine treatment of adult rats did not alter the amount of phosphatidylcholines in the lungs. It should be emphasized, however, that the type II cells used in the present study were isolated from euthyroid animals. This may be the reason that no effect by thyroxine was observed.

Farrell et al. [41] have reported that in type II cells obtained from adult rat lung by cloning, the specific activity of cholinephosphotransferase, the enzyme catalyzing the terminal step of phosphatidylcholine synthesis, was increased 7-fold by the combined action of cortisol and thyroxine. Cortisol alone produced only a slight elevation in enzyme activity, while thyroxine alone caused a decrease in activity. Although the activity of cholinephosphotransferase in the type II cells in primary culture used in the present study may also have been higher in the presence of cortisol and thyroxine simultaneously than in the presence of cortisol alone, this is not apparent from the data concerning the incorporation of labelled precursors into phosphatidylcholines (Table II).

From the data on the precursor incorporations it is difficult to conclude which enzymes involved in the synthesis of phosphatidylcholines and phos-

phatidylglycerols are influenced by hormone treatment. The observations that cortisol alone stimulated the incorporation of both [1-$^{14}$C]acetate and [1-$^{14}$C]-palmitate into total and disaturated phosphatidylcholines, but that the combination of cortisol and thyroxine does not stimulate the incorporation of [1-$^{14}$C]acetate, might indicate that thyroxine has a inhibitory effect on a cortisol-stimulated fatty acid synthesis. However, direct measurement of the activity of the various enzymes involved in lipid metabolism after exposure of type II cells to the hormones will be necessary to pinpoint the hormonally regulated steps.

The studies reported here were concerned with the direct action of cortisol on isolated type II cells. Recent reports by Smith [22,42] demonstrated that an aligopeptide factor produced by fetal lung fibroblasts in response to cortisol, stimulated the formation of disaturated phosphatidylcholines in cloned type II cells. The fibroblast-pneumonocyte factor accelerated fetal lung maturation in vivo [42]. The suggestion was made that the glucocorticosteroid effect on lung maturation might be dependent upon intracellular interactions. The effect of cortisol on the synthesis of disaturated phosphatidylcholines mediated by the fibroblast-pneumonocyte factor was greater than and occurred at a lower cortisol concentration than the direct effect of cortisol [22]. It would be of interest to compare whether the direct cortisol stimulation and the oligopeptide-mediated stimulation are brought about by effects on the same enzymes in the type II cells.

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