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Glucocorticoid–thyroid synergism in lung maturation: A mechanism involving epithelial–mesenchymal interaction

(fibroblast-pneumonocyte factor/surfactant/alveolar type II cell/pulmonary fibroblast)

BARRY T. SMITH AND KARIM SABRY

Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT **We studied the effects of cortisol and triiodothyronine (T3) on 20-day fetal rat lung cell cultures. Cortisol enhanced the production of surfactant-associated saturated phosphatidylcholine while T3 did not. However, T3 potentiated the cortisol effect. We observed that T3 enhanced the response of cultures enriched with alveolar type II cells to fibroblast-pneumonocyte factor (FPF). Intracellular cAMP was increased by exposure of these cultures to FPF, and T3 potentiated this increase. Unlike cortisol, T3 had no effect on production of FPF by fetal lung fibroblasts, as determined by bioassay of fractions of fibroblast-conditioned medium partially purified by column chromatography. The time course of cortisol action on mixed (fibroblast/epithelial) cultures was in keeping with the proposed mechanism: glucocorticoid induction of FPF in fibroblasts, followed by FPF induction of cAMP in epithelia and, finally, by enhanced production of saturated phosphatidylcholine. Thus, glucocorticoid acting on mesenchyme and thyroid hormone acting on epithelium have a synergistic effect on expression of differentiated epithelial function.**

In late fetal life, maturation of the mammalian lung is heralded by the ability of alveolar epithelial type II cells to synthesize and secrete the pulmonary surfactant, which is necessary for successful postnatal gas exchange (1). Extensive evidence suggests that both glucocorticoids and thyroid hormones regulate surfactant synthesis in late fetal life. Glucocorticoids enhance surfactant-associated phospholipid synthesis by fetal lung in organ culture (2–4) or in mixed monolayer culture (5, 6), but their effect on clonally derived alveolar type II cells is very much attenuated (7). It is now evident that a major glucocorticoid effect on the alveolar epithelium is indirect: glucocorticoid (cortisol) induces production by fetal lung fibroblasts of fibroblast-pneumonocyte factor (FPF), which, in turn, increases phospholipid synthesis by alveolar epithelial type II cells *in vitro* (7) and *in vivo* (8). We here report that triiodothyronine (T3) acts directly on alveolar type II cells to enhance responsiveness to FPF.

MATERIALS AND METHODS

Cell Cultures. All cultures were prepared from 20-day fetal rat lungs. Mixed monolayer cultures were prepared as described (5), modified as follows: mixed cell suspensions were prepared by incubating finely minced lung in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution containing 0.05% trypsin (Worthington), DNase (10 μg/ml; Sigma), and 1% chicken serum (GIBCO) (9). They were plated in 24-well plates (10) at high cell density (4×10^6 cells per cm^2) and maintained in 2 ml of minimal essential medium/10% newborn calf serum for 3 days with daily medium changes.

Cultures enriched with respect to alveolar type II cells were prepared as described above, except that 2 hr after initial plating, at which time a majority of the fibroblasts had attached to the flask (11), the supernatant media were aspirated with unattached cells. The cells were pelleted at $50 \times g$ and the pellet was incubated at 37°C for 1 hr (9). The cells were then resuspended, plated, and maintained as described above. Some cultures prepared in this way were grown in the standard medium (minimal essential medium/10% newborn calf serum) with added T3 (10 nM).

Fetal lung fibroblast cell cultures were grown as described (11). After removal of the 2-hr supernates for preparation of enriched alveolar type II cell cultures as described above, the adherent cells (fibroblasts) were grown to confluence in minimal essential medium/10% newborn calf serum. They were then exposed to serum-free minimal essential medium with or without cortisol or T3 for 24 hr. The media were then collected, filtered, and used as the source of FPF.

Study of Cellular Phospholipid Synthesis. As an index of cellular maturation, we studied the ability of either mixed monolayer cultures or cultures enriched with respect to alveolar type II cells to incorporate labeled precursor into saturated phosphatidylcholine (PtdCho), the major functional component of the pulmonary surfactant. After 3 days growth under standard conditions, the media were aspirated and replaced with 2.0 ml of serum-free minimal essential medium containing the agent to be studied and 2 μCi of [^{3}H]choline (New England Nuclear; specific activity, 80 Ci/mmol; 1 Ci = 37 GBq). After 24 hr of incubation, the cells were recovered and studied for cell number and [^{3}H]choline incorporation into saturated PtdCho as described (10).

Endogenous total cellular PtdCho was determined by TLC as described by Torday *et al.* (12) on both mixed cultures and enriched type II cell cultures. For saturated PtdCho, aliquots of the lipid extracts were treated with osmium tetroxide prior to chromatography (12). Results are expressed as percent of total PtdCho represented by saturated PtdCho.

Preparation of Fibroblast-Pneumonocyte Factor. For study of T3 interaction with FPF, a partially purified preparation (herein designated FPF-II) was used. FPF-II was prepared from conditioned serum-free medium from cortisol (1 μM)-treated 20-day rat fetal lung fibroblasts grown as described above (11). Two liters of this medium, containing 76 mg of protein, was partially desalted and concentrated in a stirred cell system across a membrane with molecular mass cutoff of 1,000 daltons (Amicon). The concentrate was lyophilized, suspended in 1 M acetic acid, and chromatographed on a Bio-Gel P60 column as described (8). Column fractions 20–41 (approximate molecular mass, 12,000–4,000 daltons) were pooled, lyophilized, and suspend-

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Abbreviations: T3, triiodothyronine; PtdCho, phosphatidylcholine; FPF, fibroblast-pneumonocyte factor.

ed in 1.0 M HCl followed by neutralization with an equal volume of 1.0 M NaOH (8) to a final concentration of 1 mg/ml. The total yield was 456 μg of protein. On $NaDodSO_4$/polyacrylamide gel electrophoresis (10), major bands were seen at 12,000, 10,000, 8,000, and 5,000 daltons.

Determination of Intracellular cAMP Levels. Cultures enriched with respect to alveolar type II cells were grown for 3 days in the presence or absence of 10 nM T3 and then incubated for 24 hr in serum-free medium with or without added FPF-II (10–1,000 ng/ml). The media were then removed and the cells were frozen at −70°C. The frozen cells were scraped from the plastic surface with a rubber policeman and homogenized in 1.0 ml of 6% trichloroacetic acid. [^{3}H]cAMP was added at this stage to correct for recovery. The homogenates were spun at 2,500 × g for 15 min and the supernatants were extracted with four 5-ml portions of water-saturated ether. The resulting aqueous phase was lyophilized, suspended in 0.05 M sodium acetate (pH 6.2) and assayed for cAMP using an ^{125}I-labeled cAMP assay kit (New England Nuclear). The samples and standards were acetylated prior to assay, giving a working range of 0.01–0.5 pmol.

Regulation of FPF Production by Fetal Lung Fibroblasts. Fetal lung fibroblasts prepared as described above were grown to confluence and then incubated for 24 hr in the presence of serum-free minimal essential medium alone or containing 1 μM cortisol, 10 nM T3, or both. Twenty-five milliliters of each conditioned medium was lyophilized, suspended in 5 ml of 1.0 M acetic acid, and chromatographed on a column (2.5 × 60 cm) packed with Bio-Gel P60 (10). The column was eluted with 1.0 M acetic acid at a flow rate of 10 ml/hr. Two-milliliter fractions were collected. The fractions were lyophilized, suspended in 0.5 ml of 1.0 M HCl, and neutralized with equal volumes of 1.0 M NaOH, and 0.1-ml aliquots were tested for FPF activity with enriched alveolar type II cell cultures as described above. Because of the large number of fractions to be assayed, each of the four sets of column fractions was assayed in a single assay, along with four control cultures that had been incubated in serum-free minimal essential medium alone. In the two column runs (medium conditioned in the presence of cortisol and medium conditioned in the presence of T3/cortisol) in which FPF activity was detected, this activity eluted in fractions 36–43. Thus, for each column run, the assay results for fractions 36–43 were compared with the four control wells run in each assay (see Table 2).

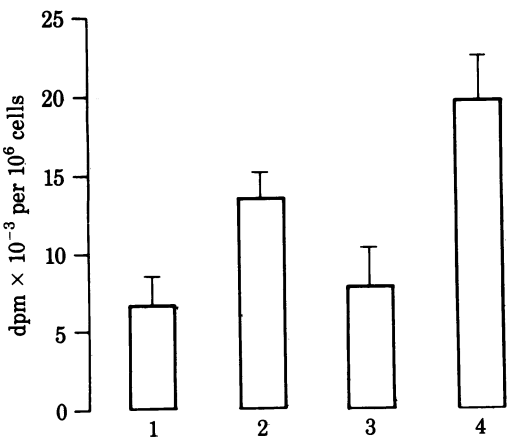

FIG. 1. Effects of cortisol (bar 2), T3 (bar 3), and the two together (bar 4) on [^{3}H]choline incorporation into saturated PtdCho by mixed monolayer cultures of 20-day fetal rat lung. Bar 1: Control. Results represent mean ± SD for six replicate cultures for each group.

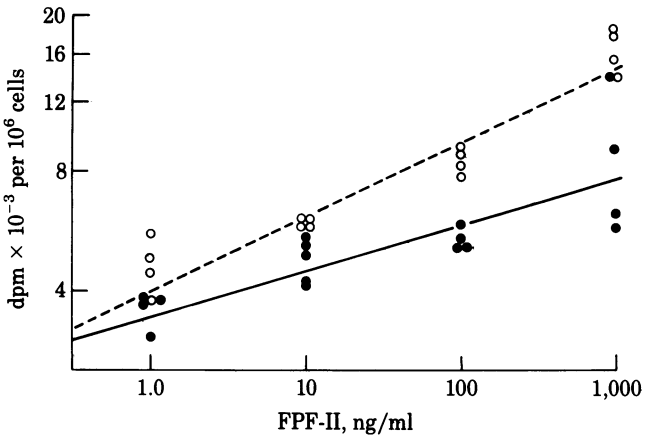

FIG. 2. Effect of partially purified fibroblast-pneumonocyte factor (FPF-II) on [^{3}H]choline incorporation into saturated PtdCho by fetal rat lung cell cultures enriched with respect to alveolar type II cells. Cells incubated with FPF-II showed a significantly greater response if they had been grown in T3-containing medium (○, data; ---, regression line) as compared with control cells (●, data; ——, regression line).

Time Course of Cortisol Effect on cAMP and Phospholipid Synthesis by Mixed Monolayer Cultures. Replicate mixed monolayer cultures were grown to confluence and then incubated in serum-free minimal essential medium containing [^{3}H]choline (as above) with or without added cortisol (1 μM). At 1, 6, 12, 18, and 24 hr of incubation, four replicate cultures from each group were assayed for [^{3}H]choline incorporation into saturated PtdCho and an additional four replicates from each group were assayed for cAMP.

Statistical. Means were compared by using Student's *t* test or, where more than two variables were compared, by analysis of variance using the least significant difference technique.

RESULTS

After 3 days of growth, cultures enriched with respect to alveolar type II cells contained (by electron microscopic morphometry) 50.5 ± 1.0% alveolar type II cells, as compared with 28.6 ± 3.8% for mixed monolayer cultures (mean ± SD, n = 4 for each group). Similarly, in enriched cultures, saturated PtdCho represented 60.9 ± 7.0% of total PtdCho, as compared with 29.8 ± 8.6% in mixed monolayer cultures.

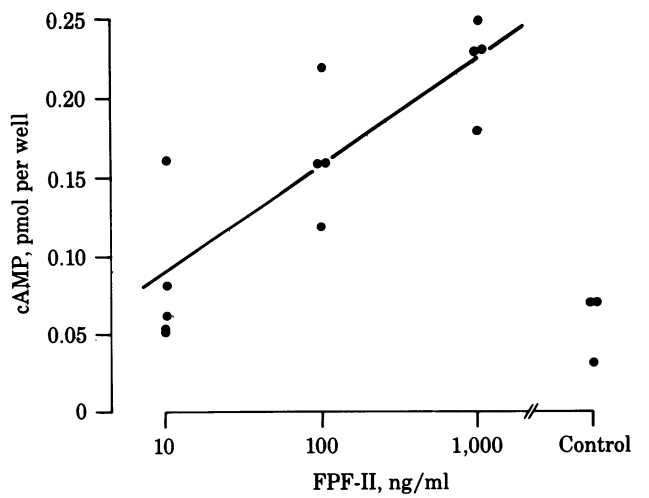

FIG. 3. Effect of FPF on intracellular cAMP content of 20-day fetal rat lung cell cultures enriched with respect to alveolar type II cells. Incubation of cells with FPF-II gave a dose-responsive increase in intracellular cAMP content. (r = 0.82, P < 0.001.)

Table 1. Effects of FPF and T3 on cAMP content of alveolar type II cell-enriched fetal rat lung cell cultures

Addition	cAMP, pmol per culture	P
None (control)	0.11 ± 0.04	
FPF (1 μg/ml)	0.18 ± 0.05	0.09 vs control
T3 (10 nM)	0.10 ± 0.04	
FPF (1 μg/ml)/T3 (10 nM)	0.31 ± 0.12	0.001 vs control; 0.01 vs FPF

Data represent mean ± SD for six cultures per variable in a single representative experiment.

The effects, in serum-free media, of 1 μM cortisol and 10 nM T3, alone and in combination, on the incorporation of [^{3}H]choline into saturated PtdCho by mixed cultures of fetal rat lung cells are summarized in Fig 1. As shown, cortisol alone increased activity over control values: cortisol treated, 13,516 ± 1,700 cpm/10^6 cells; control, 6,658 ± 1,861 (mean ± SD, $P < 0.001$). In contrast, T3 alone has no apparent effect (7,930 ± 2,431; P, not significant), but significantly increases the cortisol effect when the two are incubated together (19,611 ± 2,673, $P < 0.001$ as compared with cortisol alone).

This apparent synergistic effect of T3 and cortisol was further evaluated by studying the effect of prior treatment with T3 on the response of fetal rat lung cultures enriched with respect to alveolar type II cells to a partially purified preparation of FPF, here designated FPF-II. As shown in Fig. 2, cells previously treated with 10 nM T3 had an enhanced responsiveness to FPF over the dose range 1–1,000 ng/ml. In the presence of T3, the slope of the dose–response curve was significantly increased ($t = 5.95$, $P < 0.001$). These data can also be expressed by the statement that the *relative potency* (13) of FPF in the presence of T3 is 2.014. The lack of an effect of T3 alone is further emphasized by the observation that the y intercepts are not significantly different ($t = 0.91$, P, not significant).

We thus hypothesized that T3 acts directly on the alveolar type II cell to enhance its responsiveness to FPF. To test this hypothesis, we examined the intracellular levels of cAMP in cells exposed to FPF in the presence and absence of T3. As shown in Fig. 3, incubation with FPF-II gave a dose-responsive increase in intracellular cAMP levels in fetal rat lung cell cultures enriched with respect to alveolar type II cells ($r = 0.82$, $P < 0.001$). We also found (Table 1) that prior treatment with T3 did not itself increase intracellular cAMP levels but, in cells previously treated with T3, the intracellular cAMP response to FPF (1,000 ng/ml) was enhanced (0.31 ± 0.12 vs 0.18 ± 0.05 pmol per culture, $P = 0.01$).

The effects of cortisol and T3 on FPF production by fetal lung fibroblast cultures are summarized in Table 2. Column fractions 36–43 of media conditioned by lung fibroblasts exposed to control medium alone or medium containing cortisol, T3, or both were incubated with enriched alveolar type II cell cultures and their ability to incorporate [^{3}H]choline into saturated PtdCho was compared with that of intra-assay control cultures. In the presence of fractions from serum-free medium alone or medium containing 10 nM T3, no stimulation was seen. In contrast, media conditioned by fetal lung fibroblasts exposed to 1 μM cortisol or to cortisol/T3 showed significant stimulatory activity (214% and 170% of control, respectively, $P < 0.0001$). The degree of stimulation by cortisol alone and by cortisol/T3 was not significantly different.

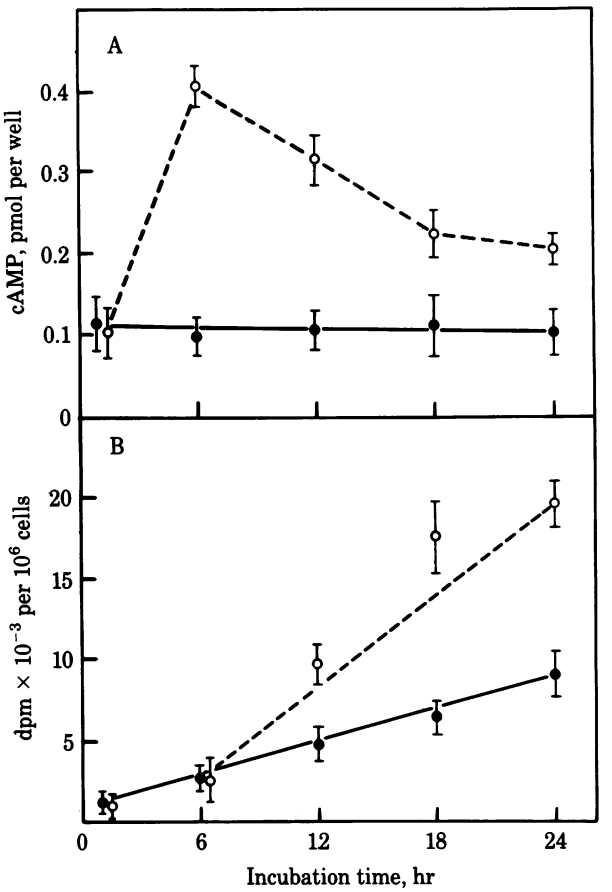

FIG. 4. Time course of effects of cortisol on mixed monolayer cultures of 20-day fetal rat lung. Data represent mean ± SD for four cultures per time point in a single experiment. (*A*) Effect on intracellular cAMP. (*B*) Effect on [^{3}H]choline incorporation into saturated PtdCho. ○, Cortisol treated; ●, control.

Time courses of the affects of 1 μM cortisol on cAMP level (*A*) and [^{3}H]choline incorporation into saturated PtdCho (*B*) by mixed monolayer cell cultures are shown in Fig. 4. The cAMP level was not different between control and cortisol-treated cultures after 1 hr of incubation but rose dramatically in cortisol-treated cultures after 6 hr, with a subsequent decline, although not to control levels, over the 24-hr observation period. In con-

Table 2. FPF activity produced by fetal lung fibroblasts in response to 1 μM cortisol, 10 nM T3, or both

	Control	Cortisol treated	T3 treated	Cortisol/T3 treated
Intra-assay control wells	5,107 ± 2,179	6,331 ± 2,885	3,732 ± 1,021	4,553 ± 1,855
Fractions 36–43	5,470 ± 2,041	13,564 ± 1,057*	3,312 ± 1,564	8,126 ± 1,551*
% control	107	214	89	178

Results (mean ± SD) are expressed as dpm of [^{3}H]choline incorporated into saturated PtdCho per 10^6 cells in cultures enriched with respect to alveolar type II cells.
*$P < 0.0001$.

trast, [^{3}H]choline incorporation into saturated PtdCho rose linearly, with cortisol-treated cultures exceeding the activity of control cultures at 12 hr and thereafter.

DISCUSSION

The lack of effect of T3 on mixed fetal rat lung monolayer cultures under serum-free conditions is in contrast to the effect of thyroxine on similar cultures in the presence of serum (which contains glucocorticoid) (6) and in organ culture systems (14), although Gross and Wilson (14) have recently presented evidence that glucocorticoid and T3 do have supra-additive effects on fetal lung organ cultures. Our observations suggest that T3 acts directly on the alveolar epithelium to enhance its responsiveness to FPF, which is produced in the mesenchyme under glucocorticoid regulation. This view is supported by observations of nuclear T3 receptors in cultured alveolar epithelial cells (15, 16) and of preferential uptake of T3 by the perinatal rat lung (17). Further evidence that, in contrast to that of cortisol, T3 action is directly on the epithelium is our observation that T3 has no effect on FPF production by the mesenchyme, either alone or in combination with cortisol. The observations that FPF increases intracellular cAMP and that this increase is greater in the presence of T3 suggest that FPF acts through a cell surface membrane receptor system that can be influenced by T3. It will be interesting to determine whether T3 can regulate FPF receptors, as has been suggested recently for fetal lung β-adrenergic receptors (18).

The time course of cortisol action on mixed monolayer lung cell cultures (Fig. 4) is in keeping with our model of glucocorticoid effect on surfactant synthesis. cAMP is not elevated after 1 hr of exposure to cortisol but is very significantly elevated after 6 hr, then declines somewhat (but remains above control levels) over the next 18 hr. The 1- to 6-hr lag time probably represents the time period necessary for glucocorticoid-induced synthesis of FPF by mesenchymal cells. Subsequent to the rise in intracellular (and presumably intraepithelial) cAMP, [^{3}H]choline incorporation into saturated PtdCho is enhanced.

Multihormonal control of T3–gene interactions is a general characteristic of thyroid hormone action (19), and the hormonal interrelationships can be complex and variable, even a single cell type (20, 21). In the present study, we have identified a mechanism of glucocorticoid–thyroid hormone interaction in which the hormones act on different germ cell layers: glucocorticoid, on mesenchyme to induce FPF production and thyroid hormone, on epithelium to increase the response to FPF. A similar synergism has recently been observed in chicken embryonic duodenal cells in an epithelial/mesenchymal bilayer cell culture system (22). These observations suggest that, in future, it may be possible to improve prenatal therapy for the prevention of hyaline membrane disease (lung immaturity) (23) by using combinations of hormones, particularly in those infants who appear to be resistant to glucocorticoid therapy alone (24). Indeed, such an approach has already been shown to be feasible in animals (25).

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