0031-3998/86/2005-0473$02.00/0
PEDIATRIC RESEARCH

Vol. 20, No. 5, 1986
Printed in U.S.A.

Influence of Epidermal Growth Factor on Fetal Rat Lung Development *in Vitro*

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ABSTRACT. Epidermal growth factor (EGF) has been shown to enhance cell multiplication or differentiation in a number of developing tissues. We have examined the effects of this growth factor on the biochemical development of explants of fetal rat lung, cultured in serum-free medium for 48 h. EGF enhanced the rate of choline incorporation into phosphatidylcholine and disaturated phosphatidylcholine in a dose dependent fashion. Half maximal stimulation occurred at a concentration of 1.0 nM, similar to the Kd for EGF binding to rat lung cell membranes. There was also significant stimulation of acetate incorporation into all phospholipids, particularly phosphatidylglycerol (539%), and increased distribution of radioactivity from acetate in this phospholipid fraction. Exposure to EGF stimulated PC synthesis in 18- and 19-day explants (term is 22 days) whereas maximal enhancement of DNA synthesis occurred after this time. This sequence differs from that observed during early embryonic development when EGF initially enhances cell multiplication. An additive interaction with regard to enhancement of PC synthesis was observed with EGF and thyroid hormone, but not EGF and dexamethasone. EGF had no effect on the activity of the enzymes of the choline incorporation pathway of phosphatidylcholine synthesis or on the activity of enzymes involved with acidic phospholipid synthesis. Fetal lung EGF content and EGF binding capacity were not increased by glucocorticoid treatment and similarly glucocorticoid binding capacity was not increased by EGF. These data indicate that EGF enhances fetal rat lung phospholipid synthesis in a dose-dependent manner and suggest that this is a direct effect on the lung tissue mediated by specific receptors. (*Pediatr Res* 20: 473–477, 1986)

Abbreviations

EGF, epidermal growth factor
PC, phosphatidylcholine
DSPC, disaturated PC
T_3, triiodothyronine
RIA, radioimmunoassay

EGF is a polypeptide with a molecular weight of 6045 which was originally isolated from the mouse submaxillary gland. This growth factor stimulates epidermal growth, eyelid opening, and incisor eruption in neonatal animals. It has effects on many organs such as the stimulation of DNA, RNA, and protein synthesis and the acceleration of cell multiplication and differentiation (1).

EGF also enhances lung maturation *in vivo*. Injection of EGF into fetal lambs (2) or rabbits (3) results in accelerated morphologic maturation and evidence of increased lung compliance. EGF also enhances DNA synthesis in the tracheal and bronchial tree of chick embryo lung cultures; it was noted that the induction of lung budding from the trachea was similar to the effect produced by mesenchymal tissue (4). Previous studies of the effects of EGF on phospholipid synthesis *in vitro* have however yielded negative results. A preliminary report suggested that EGF alone had no effect on PC synthesis in organ cultures of fetal rat lungs. However, there was enhanced incorporation of choline into DSPC when EGF was combined with retinoic acid (5). Similarly a study with adult type II cells found that EGF had no effect on the rate of choline incorporation into PC (6). Both these studies used tissues that were grown in the presence of fetal calf serum, which contains EGF, and this could possibly have confounded the results.

During development of the mouse EGF binding capacity increases, but the affinity of the receptors for EGF decreases (7). It has been speculated that EGF may initially stimulate proliferation of embryonic cells and then enhance differentiation as the tissues mature. In this study we have examined the direct effects of EGF on the biochemical development of fetal rat lung in serum free organ culture, the relationship between the effects of EGF on lung cell multiplication and differentiation and the interactions among EGF, thyroid hormone, and corticosteroids.

METHODS

Tissue culture. All experiments were performed with explants of 16- through 22-day Sprague Dawley fetal rat lung cultured for 48 h. The organ culture technique has been described previously (8). One-mm^3 explants of fetal lung were placed on either side of a 60-mm tissue culture dish containing 2 ml Waymouth's MB 752/1 medium with 100 U penicillin and 100 μg streptomycin per ml but no serum. The dishes were rocked from side to side on a rocking platform so that at any one time half the explants were exposed to the culture medium and the other half to the atmosphere of 95% oxygen, 5% CO_2.

Phospholipid synthesis. Explants were grown in the absence (controls) or presence of EGF, dexamethasone, T_3, or combinations of these hormones for 48 h. The culture medium was then aspirated from those cultures that were used for isotope incorporation studies and replaced with 2 ml fresh medium containing 4 μCi/ml [methyl-^{3}H] choline chloride, 2.23 Ci/mol, or 6.7 μCi/ml [^{3}H]Na acetate, 6.4 Ci/mol. Incubation was continued for a further 4 h. The explants were then washed with ice cold 0.9%

Received October 21, 1985; accepted January 10, 1986.
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Supported by NIH Grants, HL 19752, HD 10192, and HL 30119.

NaCl, lipids were extracted, phospholipids were isolated by thin-layer chromatography and the radioactivity in the various phospholipid fractions was determined by methods described previously (8). Protein content was measured by the method of Lowry *et al.* (9) and DNA content by mithramycin binding as described by Hill and Whatley (10), using bovine serum albumin and calf thymus DNA as standards, respectively. Protein and DNA synthesis were evaluated by determining the rate of leucine or thymidine incorporation into precipitated macromolecules (11).

For enzyme assays the explants were homogenized with a Potter-Elvehjem tissue grinder in 0.5–1.5 ml 0.33 M sucrose, 10 mM Tris-HCl, 1 mM EDTA (pH 7.4). The protein concentration of the homogenate was 1.5–3.0 mg/ml. Aliquots of the homogenate were retained for enzyme assay and after centrifugation at 800 × *g* for 15 min and 12,500 × *g* for 10 min the supernate was fractionated into microsomal and cytosolic fractions by centrifugation at 105,000 × *g* for 1 h. Choline kinase (2.7.1.32) and cholinephosphotransferase (EC 2.7.8.2) were assayed in the homogenate and cholinephosphate cytidylyltransferase (EC 2.7.7.15) in the cytosolic fraction as described previously (12). In some experiments cholinephosphate cytidylyltransferase was also assayed in the cell-free homogenate and in the microsomal fraction. This enzyme was assayed both with and without addition of 1.1 mM phosphatidylglycerol to the reaction mixture (12, 13). Enzymes of acidic phospholipid synthesis, phosphatidate cytidylyltransferase (EC 2.7.7.41), CDPdiacylglycerol-inositol 3-phosphatidyltransferase (EC 2.7.8.11), and glycerophosphate phosphatidyltransferase (EC 2.7.8.5) combined with phosphatidylglycerophosphatase (EC 3.1.3.27) were also assayed in the homogenate (13).

Glucocorticoid binding. Binding of dexamethasone was assayed using intact cell techniques as described previously (14). Culture medium was removed from the explants and fresh Waymouth's medium containing 35 nM [^{3}H]dexamethasone with or without a 500-fold excess of nonlabeled dexamethasone was added. Incubation was continued for 3 h at 37° C. An aliquot of the culture medium was counted to determine the free steroid concentration. At the end of the incubation period, the tissue was washed with ice cold saline and homogenized in cold buffer. A nuclear and cytoplasmic fraction were prepared by centrifugation as described previously (14). The cytoplasmic fraction was applied to a Sephadex G25 column and the void volume collected and retained, in order to remove free dexamethasone. The nuclear fraction was washed twice and radioactivity in both fractions was determined by liquid scintillation counting. Nonspecific binding, determined in the presence of the 500-fold excess of dexamethasone, was subtracted from total binding to give specific binding.

EGF binding. Pregnant rats were injected with 1 mg/kg betamethasone (Celestone) or saline (controls) on days 18 and 19 and the fetuses were removed on day 20. The lungs were excised, cleaned, and homogenized with a Potter-Elvehjem grinder in 0.3 M sucrose, 25 mM Tris pH 7.6, 10 mM $MgCl_2$. All procedures were performed at 0–4° C. The homogenate was centrifuged at 800 × *g* for 10 min. The supernatant was then centrifuged at 20,000 × *g* for 20 min. The pellet was retained and washed once in 25 mM Tris, 10 mm $MgCl_2$ buffer, and resuspended in the same buffer. EGF binding was determined by modification of the method of Adamson *et al.* (15).

The membranes (300 μg protein) were incubated with ^{125}I-EGF (0.06–0.08 ng EGF, 30,000–40,000 dpm) in a binding buffer (0.25 M Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 0.1% bovine serum albumin for 90 min at room temperature with continuous shaking. Total assay volume was 0.25 ml. Nonspecific binding was determined by measuring binding after addition of excess (1000-fold) unlabeled EGF. Unbound ^{125}I-EGF was separated from bound EGF by centrifugation. Dose-binding studies were also performed and Scatchard plots derived.

EGF levels. This portion of the study was performed with fetal mice, as a sensitive radioimmunoassay to mouse EGF had been developed previously. Pregnant mice were injected with 0.3 mg betamethasone or saline (controls) on days 15 and 16 of gestation and the fetuses examined on day 17. This time and dose had been shown to be optimal in a previous study of glucocorticoid effects on lung maturation (16). After removal the lungs were homogenized in 0.1 M phosphate buffer, pH 7.4, and centrifuged at 800 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 1 h and the pellet discarded. Ten μg/ml soybean trypsin inhibitor was added to prevent EGF degradation.

EGF levels in tissue supernatants were measured by RIA as described previously (17). Supernatants were also examined by radioreceptor assay as described by Nexo *et al.* (18) using membranes prepared from fresh human term placenta. Assay sensitivity was defined as the amount of EGF that caused 10% displacement of radiolabeled EGF from antibody or receptor. Maximum sample size in both assays was 200 μl. Recovery studies were performed by adding 1 ng standard EGF to 100 μl lung supernatant from either control or steroid-treated fetuses. The percent recovery was calculated from triplicate specimens.

Statistical analysis. Values reported are the mean ± SE of the number of experiments performed. In all tissue culture experiments a common pool of lung explants was prepared from approximately four litters. This pool of explants was then used for the various treatment groups. Each experiment was performed with triplicate flasks. Statistical analysis was by paired *t* test in those cases where one control and one treatment were being compared. In situations where multiple treatment groups were compared statistical analysis was by analysis of variance with the Student Newman Keuls test (19).

Materials. Radiochemicals were purchased from Amersham, Arlington Heights, IL and New England Nuclear, Boston, MA. EGF was obtained from Collaborative Research, Boston, MA and tissue culture medium from Grand Island Biological Co, Grand Island, NY.

RESULTS

EGF enhanced the rate of choline incorporation into PC and DSPC in the explants. As is shown in Figure 1 this was a dose-dependent phenomenon with a plateau occurring at about 10 nM (1 nM = 6.1 ng/ml). A concentration of 10 nM EGF was used in all further experiments. The concentration of EGF which produced half maximal stimulation was 1.03 nM (Table 1). This correlated closely with the concentration of EGF which resulted in 50% occupancy of the EGF receptor (Kd) in membranes

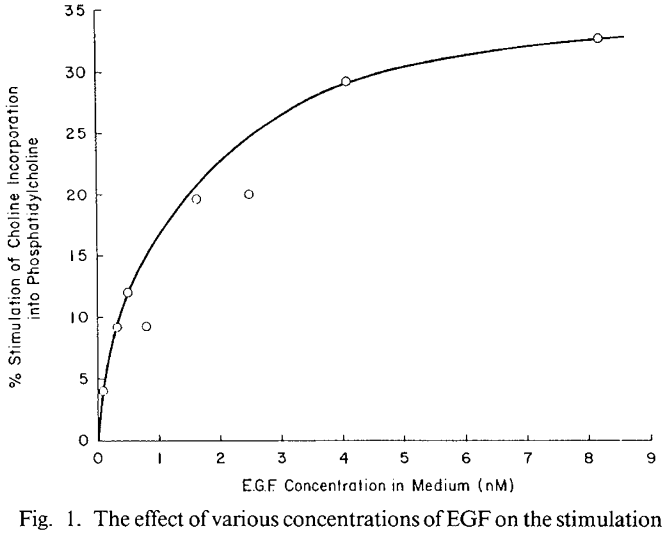

Fig. 1. The effect of various concentrations of EGF on the stimulation of choline incorporation into PC in explants of 18-day fetal rat lung cultured for 48 h. Data points represent the mean of two to four experiments.

derived from fetal rat lung. The Scatchard plot derived from 18-day fetal lung membranes revealed a single class of EGF receptors.

EGF also enhanced the incorporation of acetate into a number of phospholipids. As is shown in Table 2 there was 126% stimulation of acetate incorporation into total phospholipids. The stimulation of incorporation into PC and DSPC was similar to that into total phospholipid, whereas there was a very marked 539% stimulation of incorporation into phosphatidylglycerol. When the data were analyzed as the distribution of radioactivity from acetate in the various phospholipid fractions (Table 3) there was a significant increase in the percentage of radioactivity in phosphatidylglycerol, but not in PC or DSPC. This suggests that EGF may have a specific effect on the synthesis of this surfactant-associated phospholipid.

The effect of EGF was dependent on gestational age. As is shown in Figure 2, EGF enhanced the incorporation of choline into PC in explants derived from 16- to 21-day rats. The stimulation was significant in the day 18 and 19 explants. By contrast, the most marked enhancement of incorporation of thymidine into DNA occurred later. Although some stimulation was seen from 16–18 days the major effect occurred at 20–22 days, so that the pattern was almost the reciprocal of that observed with choline incorporation into PC. Enhancement of thymidine incorporation was significant at all four gestational ages studied. EGF had a smaller effect on leucine incorporation into protein (data not shown). Stimulation ranged from 10% on day 16 to about 25% on days 18, 20, and 22. The only statistically significant difference was found on day 20 at which time 25% stimulation above control values was observed.

In order to explore possible interactions between EGF, T_3, and dexamethasone explants of 18-day lung were cultured for 48 h in the presence of concentrations of these hormones that had been shown to be optimal in previous studies (Table 4). Whereas there was enhancement of choline incorporation into PC and DSPC after exposure to all of these agents, EGF in combination with T_3 resulted in stimulation that was about equal to the arithmetic sum of the individual effects of these two hormones, but EGF did not enhance the effect of dexamethasone. Thus there was an additive interaction between EGF and T_3, but not between EGF and dexamethasone.

One explanation for the observation that EGF did not have an additive interaction with dexamethasone could be that both these agents act at similar metabolic sites. The interactions between these hormones were explored at a number of levels. EGF did not enhance either cytoplasmic or nuclear binding of dexamethasone in explants of 18-day fetal lung exposed to the hormone for 48 h and betamethasone treatment of fetal rats *in vivo* on days 18 and 19 did not result in a significant increase in EGF binding in the lungs of 20-day gestation fetuses. (Control: 4.18 ± 0.42% of total EGF in assay bound per 300 μg protein cell membrane; betamethasone: 4.69 ± 0.34%; $n = 10$.) Whereas dexamethasone significantly enhanced the activity of choline-phosphate cytidylyltransferase in the cytosolic fraction (assayed without added phosphatidylglycerol) as reported previously (13) EGF did not (Table 5). EGF also had no effect on the activity of

Table 1. *Comparison of dissociation constant (Kd) for specific EGF binding and half maximal concentration for enhancement of choline incorporation into PC (mean ± SE)*

	EGF concentration (nM)	*n*
Kd	0.96 (0.80, 1.12)	2
Half-maximal concentration*	1.03 ± 0.09	3

* Explants of 18 day fetal rat lung were cultured for 48 h in the presence of varying concentrations of EGF.

Table 3. *Effect of EGF on distribution of radioactivity from acetate into surfactant-associated phospholipids**

Phospholipid	Control (%)	EGF (%)	*p*
PC	70.4 ± 1.1†	71.7 ± 0.3	NS‡
Disaturated PC	39.7 ± 0.9	37.1 ± 1.8	NS
Phosphatidylglycerol	1.7 ± 0.2	4.6 ± 0.5	<0.005

* Culture conditions were as described in Table 2.
† Values represent the mean ± SE of four to five experiments.
‡ Statistical significance was evaluated by paired *t* test.

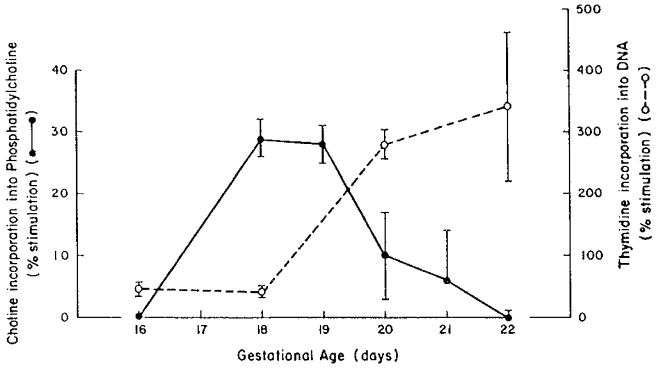

Fig. 2. The effects of exposure of explants of fetal rat lung at varying gestational ages to 10 nM EGF for 48 h. The stimulation of choline incorporation into PC (a differentiated function) and thymidine incorporation into DNA (a marker of cell multiplication) were determined. Data represent the mean ± SE of four to nine experiments.

Table 2. *Effects of EGF on acetate incorporation into phospholipid**

Phospholipid	Acetate incorporation (dpm/h/mg protein × 10-2) Control	EGF	EGF/control	*p*
PC	370 ± 43†	794 ± 103	2.14	<0.01‡
Disaturated PC	209 ± 25	404 ± 36	1.93	<0.001
Phosphatidylglycerol	8.4 ± 1.0	53.4 ± 9.8	6.39	<0.01
Phosphatidylethanolamine	62.8 ± 6.8	136 ± 13	2.16	<0.005
Phosphatidylinositol + phosphatidylserine	35.9 ± 3.9	63.9 ± 5.8	1.78	<0.001
Sphingomyelin	22.1 ± 2.6	37.6 ± 4.5	1.70	<0.01
Lysophosphosphatidylcholine	10.3 ± 2.5	18.4 ± 3.1	1.80	<0.05
Cardiolipin	5.1 ± 0.4	11.1 ± 1.2	2.17	<0.005
Total phospholipid	497 ± 53	1113 ± 114	2.26	<0.005

* Explants of 18-day fetal rat lung were cultured in the presence or absence (controls) of 10 nM EGF for 48 h.
† Values represent the mean ± SE of four to five experiments.
‡ Statistical significance was evaluated by paired *t* test.

Table 4. *Interactions between EGF, dexamethasone, and T_3**

	Choline incorporatoin into			
	PC	Stimulation	Disaturated PC	Stimulation
Control	694 ± 54		152 ± 13	
EGF	897 ± 29†,‡	29%	194 ± 14‡	28%
Dexamethasone	1042 ± 98†,‡	50%	266 ± 27†	75%
T_3	922 ± 69†,‡	33%	192 ± 20‡	26%
EGF + dexamethasone	1014 ± 94†,‡	46%	243 ± 28†	60%
EGF + T_3	1261 ± 120†	82%	244 ± 17†	61%

* Data are expressed as pmol/h/mg protein and represent the mean ± SE of four experiments. Statistical difference was evaluated by repeated measures analysis of variance with the Student Newman Keuls test. Explants of 18 day lung were cultured for 48 h. The concentration of hormones was: EGF, 10 nM; dexamethasone, 100 nM; T_3, 100 nM.

† Significantly different from control value.

‡ Significantly different from EGF + T_3 value.

Table 5. *Effects of EGF and dexamethasone on activity of enzymes of PC synthesis**

	Enzyme activity (pmol/min/mg protein)			
Enzyme	Control	EGF	Dexamethasone	EGF + dexamethasone
Cholinephosphate cytidylyltransferase	629 ± 71 (10)	689 ± 131 (5)	1031 ± 110 (10)†	868 ± 185 (5)‡
Choline kinase	1245 ± 147 (6)	1264 ± 119 (6)	1401 ± 112 (5)	1484 ± 165 (5)‡
Choline-phosphotransferase	1478 ± 185 (7)	1390 ± 167 (7)	1547 ± 118 (6)	1912 ± 137 (6)‡

* Data represent the mean ± SE of the number of experiments in parentheses. Statistical significance was assessed by analysis of variance with the Student Newman Keuls test. Culture conditions and hormone concentrations were as described in Table 4.

† Significantly different from control value.

‡ Not significantly different from dexamethasone value.

this enzyme in the homogenate or microsomal fractions when assayed with or without phosphatidylglycerol (data not shown). The effect of combining EGF and dexamethasone was not significantly different from that of dexamethasone alone. Neither EGF nor dexamethasone had a significant effect on the activities of the other enzymes in the choline incorporation pathway of PC synthesis, choline kinase, and cholinephosphotransferase. The effect of EGF on the activity of enzymes involved with the synthesis of the acidic phospholipids, phosphatidylglycerol, and phosphatidylinositol, was also examined. EGF did not increase the activities of phosphatidate cytidylyltransferase, glycerophosphate phosphatidyltransferase, and phosphatidylglycerophosphatase combined, or CDPdiacylglycerolinositol 3-phosphatidyltransferase.

The effect of *in vivo* glucocorticoid administration on EGF levels in lung tissue was studied in fetal mice. The level of EGF in fetal mouse lung tissue was essentially undetectable by both radioreceptor and RIA in control and betamethasone treated fetuses. The sensitivities of the RIA and radioreceptor were 10 and 85 pg/tube, respectively. Using the RIA, EGF values of 33 pg/ml (10 pg/mg protein) for controls and 55 pg/ml (19 pg/mg protein) for the steroid treatment group could be extrapolated from the lower end of the assay curve (between 0 and 10% displacement of label). However, no significance was assigned these extremely low values.

The mean (± SEM) recovery of added standard EGF to 100 μl of supernatant in the RIA was 112 ± 6 and 99 ± 2% for control and steroid treatment groups, respectively. These results would indicate the absence of significant degradative activity in the supernatant samples. In the radioreceptor, comparable recoveries of 92 ± 3 and 87 ± 3%, respectively, were obtained.

DISCUSSION

The data presented in this report indicate that EGF acts directly on fetal rat lung to enhance both biochemical maturation and growth. Our findings are in general agreement with a recent report that injection of EGF into fetal rats *in vivo* enhances the rate of choline incorporation into PC and decreases lung glycogen content (20). Although EGF enhances lung development *in vivo*, it could be acting indirectly, *e.g.* by influencing the levels of other hormones. In order to demonstrate that EGF acted directly on the lung it was necessary to examine its effects *in vitro*. In previous studies with type II cells from adult lung or with fetal lung explants, cultured in medium containing serum, enhancement of PC synthesis had not been observed after exposure to EGF (5, 6). We have shown that the enhancement of choline incorporation into DSPC is dose dependent and correlates with EGF binding. Although some caution must be exercised in comparing binding in isolated cell membranes to biochemical effects in lung explants, the fact that the half maximal concentration for enhancement of choline incorporation into PC and the Kd for EGF binding are very similar suggests that the EGF effect on PC synthesis is mediated by binding to specific EGF receptors.

EGF is unusual in that it enhances both cell multiplication and differentiation. These effects seem to occur at different times during gestation. Adamson and Meek (7) have suggested that EGF may initially stimulate cell proliferation in embryonic tissues and later enhance differentiation. We found that enhancement of choline incorporation into PC, a marker of differentiated function, was most pronounced with explants of 18- or 19-day lung whereas stimulation of thymidine incorporation into DNA occurred primarily after day 20. These data could be interpreted as implying that EGF enhances maturation of the type II cell earlier in gestation and cell multiplication later on and appear to conflict with the hypothesis of Adamson and Meek (7). This may relate to the fact that we were studying the effects of EGF in late gestation when the type II cells were approaching maturity. Different results might have been obtained had we studied early embryonic lung.

EGF specifically enhanced the synthesis of the surfactant-associated phospholipid, phosphatidylglycerol. The increased incorporation of acetate into all the phospholipids could represent

a true increase in phospholipid synthesis or could merely reflect changes in the intracellular pool of acetate. However, the fact that the percentage of phospholipid radioactivity in phosphatidylglycerol increased from 1.7 to 4.6% strongly suggests that there was a specific effect on this phospholipid.

EGF appears to mediate thyroid action in developing mouse skin. Administration of thyroid to newborn mice results in changes which are similar to those brought about by EGF and Hoath *et al.* (17) have shown that this is associated with increased EGF levels in the skin. Some of the effects of EGF on fetal lung phospholipid synthesis appear to be similar to those of dexamethasone but not to those of thyroid hormone. In previous studies we have shown that whereas dexamethasone treatment increases the distribution of radioactivity from acetate into phosphatidylglycerol and DSPC in fetal lung explants, thyroid does not (21). When saturating concentrations of EGF, dexamethasone, and T_3 were added to the explants alone or in combination there was an additive interaction between T_3 and EGF. This suggests that these two agents act at different biochemical sites. There was, however, no such additive interaction between EGF and dexamethasone. There are a number of possible explanations for this. If dexamethasone exposure resulted in increased tissue levels of EGF then one might not see further enhancement after adding EGF to the culture medium. Alternatively EGF and dexamethasone could be acting at similar metabolic sites, *e.g.* both agents could enhance the activity of a specific enzyme. If maximal induction was brought about by dexamethasone, addition of EGF would not result in further stimulation.

Possible interactions between EGF and glucocorticoids were therefore explored at a number of levels. Neither hormone increased the binding capacity of the other and there was no increase in tissue EGF levels after exposure to glucocorticoid. Glucocorticoids have been reported to increase EGF binding in human fibroblasts (22) and in fetal rabbit lung tissue (23). Our failure to observe a change in EGF binding capacity after glucocorticoid treatment in the rat may be due to species differences. In previous experiments we have shown that glucocorticoids increase the activity of cholinephosphate cytidylyltransferase, the rate limiting enzyme in the choline incorporation pathway of PC synthesis (13). This effect was confirmed in this study, but EGF did not influence the activity of this enzyme nor that of any of the other enzymes examined. Thus EGF and glucocorticoids are similar in that they both stimulate the incorporation of choline into PC and DSPC and preferentially enhance the incorporation of acetate into phosphatidylglycerol. They differ in that glucocorticoids selectively increase the incorporation of acetate into DSPC and the activity of cholinephosphate cytidylyltransferase, whereas EGF does not. An explanation for the absence of an additive interaction between EGF and dexamethasone awaits further study.

The data presented herein show that EGF enhances phospholipid synthesis by fetal rat lung in a dose-dependent fashion and suggest that it does so by a direct action on lung tissue which is mediated by specific EGF receptors.

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