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GLUCOCORTICOIDS AND FETAL LUNG DEVELOPMENT

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SUMMARY

Acceleration of fetal lung development by administration of glucocorticoid hormones has been demonstrated in a number of mammalian species. Dexamethasone 10^{-5} M significantly depressed protein content per explant in the presence and absence of insulin. Insulin did not alter the protein content but significantly increased radioactive choline incorporation. However, dexamethasone provided no added stimulation above that observed with insulin alone. The incorporation of radioactive choline and specific activities in isolated phosphatidylcholine were almost identical in control and dexamethasone (10^{-7} M) treated explants. The data presented demonstrate a modest but significant increase in choline incorporation using a concentration of dexamethasone of 10^{-9} M. At higher concentrations of dexamethasone, the incorporation was significantly depressed using an 8 day exposure time. Although choline incorporation increases as a function of gestational age with a burst in rate on the last day of fetal life, dexamethasone suppresses this activity at all developmental ages past 19.5 days. The only conclusion that appears valid at this time is the rat fetal lung system reported here differs in an unknown way when compared to the rabbit monolayer system, the human organ culture, and the *in vivo* situation.

INTRODUCTION

Acceleration of fetal lung development by administration of glucocorticoid hormones has been demonstrated in a number of mammalian species using physiological, morphological and biochemical parameters. The effect was first noted by Liggins following *in utero* infusion of cortisol or dexamethasone in the fetal lamb [1]. deLemos confirmed that glucocorticoid hormones increased the physiological maturity of the fetal lamb lung assessed by the surface activity of lung extracts and compliance (pressure–volume curves) [2]. A definitive study of Kotas and Avery demonstrated increased pulmonary compliance and surface activity following *in utero* administration of 9-fluoroprednisolone to fetal rabbits [3]. Morphological studies of rabbit lung following cortisol administration have shown acceleration of epithelial development and differentiation [4, 5]. Studies with the rat indicate a similar response to glucocorticoid hormones [6]. Clinical studies of the incidence of respiratory distress syndrome following therapeutic use of beta-methasone prior to premature delivery suggest that the hormone effects occur in human fetal lung [7–10].

Farrell[11] has shown that administration of 9-fluoroprednisolone to fetal rabbits resulted in increased activity of the CDP-choline diglyceride transferase in lung but had no effect on the activity of other enzymes in the methylation and CDP choline pathways of phosphatidylcholine synthesis. These data suggest that one level of control may be enzyme synthesis and specifically at the level of translation of mRNA since the increase in enzyme activity following hormone administration was blocked by prior treatment of the fetuses with cycloheximide but not by treatment with Actinomycin D.

A consideration of the optimal experimental system for evaluation of factors which directly affect biochemical control in lung during development has led to the development of an *in vitro* organ culture system for rat lung from the 15.5 day gestational age fetus [12].

The principal advantages of this system are the ease and precise control of hormone concentration, oxygen tension, pH and other environmental conditions and the capability to distinguish between direct effects of hormones on the lung and effects secondary to interaction of hormones with other target organs.

Experiments by Farrell and co-workers have shown that the incorporation of [^{14}C]-methyl-choline into the organic phase following chloroform:methanol (2:1 v/v) extraction of tissue homogenates is a valid measurement of the incorporation of choline into phosphatidylcholine, the major chemical constituent of surfactant [11, 13]. Using this criterion for surfactant synthesis, this study reports the inability of glucocorticoids to stimulate choline incorporation into lipids using the isolated fetal rat lung explant.

EXPERIMENTAL

Animals. Timed-bred Sprague–Dawley rats are obtained from Charles River Laboratories, Wilmington, MA.

Organ culture. The technique has been published in detail [12]. Fetuses are harvested under sterile conditions at 15.5 days gestation following maternal

laparotomy using ether anesthesia. Dissection is performed in ice-cold buffered media using a dissecting microscope. Lungs are cultured as the gas–liquid interface in a humidified atmosphere of 95% air and 5% CO_2 at 37 degrees. Stainless steel wire grids (about 0.5 mm-square mesh, 5 × 10 mm area) covered with Millipore filter paper (GS 0.22 micron) are used to support the organs. The cultures (two lungs per dish) are placed in 35 × 10 mm plastic tissue culture dishes (Falcon Plastics, Oxnard, California) containing two mls Medium MD 705/1 with glutamine (Waymouth) from Grand Island Biological Company, Grand Island, New York, 14072. The medium is supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Company) and 100 units per ml Penicillin-G. The cultures are harvested by removing tissue from the filter with a cataract knife and washed with ice-cold 0.9% NaCl prior to homogenization.

Choline incorporation. Five μCi (methyl-^{3}H) choline chloride (S.A. 4.2 Ci/mmol, New England Nuclear, Boston, Mass., U.S.A.) was added to each 2 ml culture dish and the incubation continued for 20 hours. The radioactive choline chloride was added in ethanol solution (10 μl) after demonstrating that this amount of ethanol did not alter the incorporation rate.

The tissue was harvested as described above and homogenized in 0.2 mls ice-water using 1.5 ml glass homogenizers with a teflon pestle, or with a Model W140 cell disruptor from Heat Systems-Ultrasonics, Inc., Plainview, Long Island, NY, U.S.A., using a microtip with an output setting of 7. A two-minute disruption period was used in an ice water bath. Chloroform:methanol (2:1 v/v) was added to give a 20:1 ratio of chloroform:methanol to water. The samples were mixed, centrifuged and the organic phase removed and back-washed with an equal volume of distilled water:methanol (1:1 v/v). Using this procedure less than 0.5% of the unincorporated choline is carried over into the organic phase.

The organic phase was evaporated to dryness in scintillation vials. 10 mls toluene containing 6 gm/l. 2,5-diphenyl-1,3-oxazole and 0.1 mg/liter 1,4-bis(2,4-methyl-5-phenyl-1,3-oxazoyl) benzene was added and radioactivity was quantitiated using a Beckman LS-350 scintillation spectrometer. The specific activity of the choline in the tissue culture medium was determined by counting 10 μl aliquots of the medium in 10 mls Aquasol (New England Nuclear) and using the manufacturers stated or the known added choline concentration. The counting efficiency for tritium determined by internal standardization with [^{3}H]-toluene was 48%. The picomoles of choline incorporated per 20 h incubation was then calculated by dividing the c.p.m. in the lipid extract by the experimentally determined specific activity of the incubation medium.

Thin layer chromatography of four samples using silica gel G and a solvent system of chloroform:methanol:water (65:25:4, by vol.) showed that the radioactivity was incorporated into two spots corresponding to phosphatidylcholine and sphingomyelin standards (Sigma Chemical Co.). The radioactivity in the sphingomyelin spot was less than 5% of that incorporated into phosphatidylcholine. Therefore, choline incorporated into the phospholipid fraction in this system is primarily in phosphatidylcholine as reported by Farrell for lung slices [11].

Hormone exposure. Dexamethasone phosphate was obtained from Napp Chemicals, Inc., 199 Main St., Lodi, NJ or Merck Sharp and Dohme, Rahway, NJ. Tri-iodothyronine and insulin were obtained from Sigma Chemical Co., St. Louis, MO. Dexamethasone phosphate was dissolved in phosphate buffered saline (PBS) or tissue culture medium. Small amounts of dilute HCl and NaOH were used to solubilize the insulin and tri-iodothyronine, respectively; and these were diluted with the PBS or tissue culture medium. Identical aliquots of each vehicle were added to the controls.

RESULTS

The effect of 10^{-7} M dexamethasone on explant content of lipid phosphorous, DNA and protein content and choline incorporation was studied following 6 days in culture. The data in Table 1 demonstrate a significant depression under these conditions of the explant tissue constituents with no alteration of radioactive choline incorporation into the phospholipid fraction.

The combined effect of triiodo-thyronine (2.5×10^{-5} M) and dexamethasone (1×10^{-5} M) on

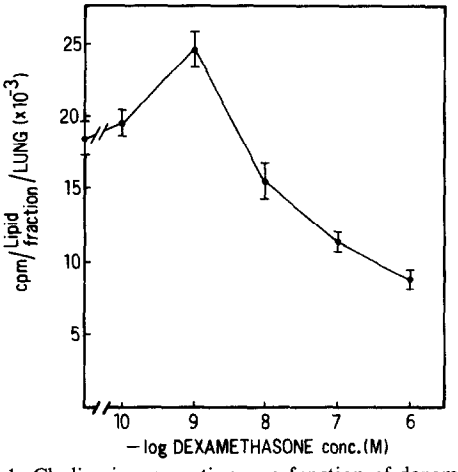

Fig. 1. Choline incorporation as a function of dexamethasone concentration in the culture medium. Fetal lung was cultured for eight days in medium containing varying dexamethasone concentrations. Twenty hours before harvest of the cultures 5 microcuries (methyl-^{3}H) choline chloride was added to the 2 mls in each culture dish. Incorporation of isotope has been shown to be essentially linear during a 20 h incorporation period. The cultures were harvested and radioactivity in the phospholipids quantitated as described in methods. The c.p.m. incorporated into each lipid fraction during the 20 h incubation period is represented on the ordinate. Each point on the graph represents the mean ± S.E.M. for eight separate cultures.

Table 1. Growth and lipid synthesis in lung cultures incubated in the presence of 10^{-7} M dexamethasone phosphate for six days compared to control cultures

	nmol Lipid phosphorus	μg DNA	μg Protein	c.p.m. [^{3}H]methyl choline incorporation into phospholipid
Control	359 ± 12.5	317.3 ± 10.09	1,957.5 ± 68.6	66,837 ± 2,194
Dexamethasone	289 ± 23.2	161.3 ± 10.77	1,263.8 ± 71.40	65.466 ± 4.741
	$P < 0.025$	$P < 0.001$	$P < 0.001$	N.S.

Each sample consists of ten lung cultures from 15.5 day gestational age fetal rat lung cultured six days. The culture medium was Waymouth MD 705/1 supplemented with 10% adult rat serum and 100 units/ml penicillin G. [^{3}H]methyl choline chloride (2.5 μCi/ml) was added 20 h prior to harvest.

choline incorporation was measured following 4 and 7 days exposure to T_3 with two groups being exposed to dexamethasone the last 2 days (Table 2). This maneuver did not increase radioactive choline incorporation based on the protein content of the explant.

The combined effect of long term exposure (8 days) to insulin (5 μg/ml) and dexamethasone (10^{-5} M) on choline incorporation was then measured in the explant system. The data are presented in Table 3. Dexamethasone in the concentration used significantly depressed protein content per explant in the presence and absence of insulin ($P < 0.001$). Insulin did not alter the protein content but significantly increased radioactive choline incorporation ($P < 0.02$). However, dexamethasone provided no added stimulation above that observed with insulin alone.

The specific activity of the [^{3}H]-choline relative to phosphatidylcholine phosphorus was determined after explant exposure to a lower concentration (10^{-7}) dexamethasone for 6 days by isolating the end product using thin layer silica gel chromatography. The incorporation and specific activities were almost identical in control and dexamethasone treated explants (Table 4).

Because 10^{-5} to 10^{-7} M dexamethasone depressed choline incorporation, we exposed explants to 0–10^{-6} M dexamethasone concentrations for 8 days. The data presented in Fig. 1 demonstrate a modest but significant ($P < 0.01$) increase in choline incorporation using a concentration of 10^{-9} M. At a concentration of dexamethasone greater than 10^{-7} M, the incorporation was significantly depressed using this exposure time.

The previous experiments were all performed using lungs from 15.5 days gestation fetuses for the explant and growing them in a tissue culture medium for 4–8 days *in vitro*. In the experiment in Fig. 2, lungs harvested daily at gestational ages between 15.5 days and

Table 2. Long-term exposure to triiodo-thyronine with short-term exposure to 10^{-5} M dexamethasone

	pmol choline incorporated per μg protein per 20 h	
Four days incubation		
Control	52.28 ± 2.56	$n = 10$
+ T_3 (25 micromolar)	53.11 ± 3.35	$n = 5$
+ T_3 + (2 days with 10^{-5} M dexamethasone)	53.97 ± 5.68	$n = 4$
Seven days incubation		
Control	74.57 ± 4.34	$n = 5$
	59.85	
+ T_3 (25 micromolar)	53.66	$n = 2$
	68.99	
T_3 + (2 days with 10^{-5} M dexamethasone)	73.88	$n = 2$

Table 3. Effects of dexamethasone and insulin on choline incorporation into phospholipid

	pmol incorporation per μg protein per 20 h	μg protein per culture
Control	70.12 ± 2.75	115.71 ± 3.41
Dexamethasone (1×10^{-5} M)	75.09 ± 3.99	69.08 ± 3.81
Insulin (5 μg/ml)	83.14 ± 5.04	113.71 ± 4.41
Insulin + dexamethasone	84.53 ± 4.22	74.32 ± 4.46

Fetal lung (15.5 days gestational age) cultured for eight days in Waymouth's MD 705/1, 10% fetal calf serum, 100 units/ml Penicillin G. [^{3}H]-methyl choline chloride (2.5 μCi per ml) added 20 h prior to harvest.

Table 4. Specific activity of isolated phosphatidylcholine from lung cultures incubated six days with 1×10^{-7} M dexamethasone compared to control cultures

	c.p.m. [^{3}H]-methyl choline per μg P_i	Total P_i isolated in phosphatidylcholine fraction (μg)
Control		
$n = 4$	7,470 ± 430	2.53 ± 0.15
Dexamethasone		
$n = 5$	7,662 ± 648	2.44 ± 0.01

birth (22.5 days) were exposed to 10^{-7} M dexamethasone for 3 days prior to measuring choline incorporation. Although choline incorporation increases as a function of gestational age with a burst in rate on the last day of fetal life, dexamethasone suppresses this activity at all developmental ages past 19.5 days.

DISCUSSION

The rationale for these experiments was based on the observation that hormones produce effects on fetal lung when administered *in vivo* and the glucocorticoid effect appears to result from a direct interaction of the hormone with lung and should be demonstrable in a suitable *in vitro* system [14]. The presence of specific glucocorticoid hormone receptors in the nucleus and cytoplasm of fetal lung cells [15–18] has been repeatedly documented. The interaction has been shown to be similar to that observed in other target organs in terms of hormone receptor quantity and specificity [17, 18].

Farrell and Avery have recently reviewed the glucocorticoid effect on fetal lung surfactant induction in relation to human hyaline membrane disease and have concluded the experimental and clinical evidence justifies the use of these hormones in the human [14]. The reasons for the failure of dexamethasone to increase the rate of choline incorporation into phospholipids in this organ explant system are not apparent to the authors. One possibility is that enhancement of phosphatidylcholine synthesis by hormones is obscured under the optimal conditions used in this system. The rates of choline incorporation observed here are considerably greater than those reported by Ekelund *et al.* in a human organ culture system [19]. This is likely due to the choline concentration in the Parker 199 medium used by these investigators, 3.6×10^{-6} M compared to 1.79×10^{-3} M in the Waymouth's MD 705/1 used in our studies.

Torday *et al.* have reported glucocorticoids stimulate choline incorporation into phospholipids in monolayers of rabbit fetal lung cells [20]. Ekelund *et al.* using pieces of human lung from second trimester fetuses in organ culture report cortisol acetate (2.5×10^{-6} M) induction of lamellar bodies in type II pneumocytes and increased choline incorporation

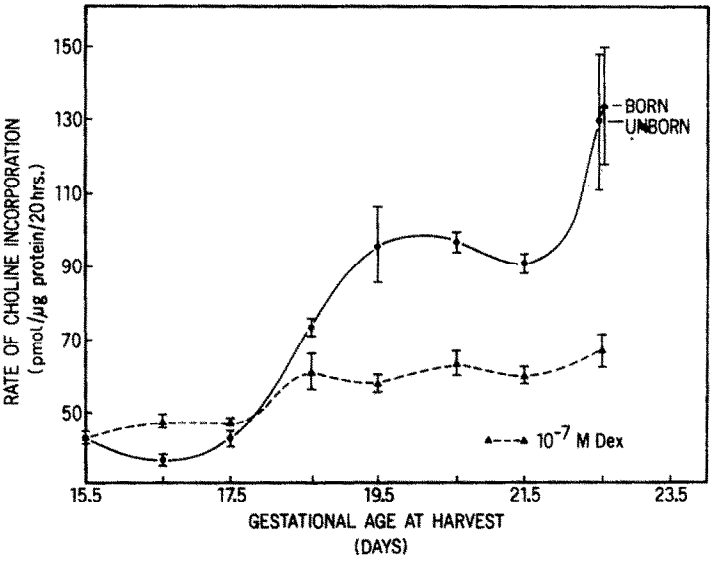

Fig. 2. The effect of gestational age at the time of culture on the rate of choline incorporation in fetal rat lung. Lung tissue was obtained at the gestational ages plotted on the abscissa. The tissue was cut into 1–2 mm^3 pieces for culture, except for 15.5 day lung which is small enough to culture whole. The culture period was three days and (methyl-^{3}H)-choline incorporation into phospholipid was determined as described in the legend to Fig. 1. Dexamethasone was present at 10^{-7} M concentration during the entire culture period for the curve represented by the dashed line. The points represent the mean ± S.E.M. for determinations on four cultures.

following 1–6 day explant exposure [21]. This group also reports a net increase in the explant content of lecithin and total phospholipid after 6 days exposure to the above cortisol concentration [19]. Another difference in their experiment and the one reported here is the serum and culture media. They used Parker 199:human serum 8:2 and we used Waymouths MD 705/1:fetal calf serum 9:1. Smith *et al.* have reported that insulin antagonizes the cortisol stimulation of choline incorporation in their rabbit fetal lung monolayer system [22]. The only conclusion that appears valid at this time is the rat fetal lung system reported here differs in an unknown way when compared to the rabbit monolayer system, the human organ culture, and the *in vivo* situation.

Multiple hormonal interactions during development have been studied most extensively in the investigation of the hormone regulation of the molecular processes during growth, differentiation and secretory function of the mammary gland [23–25]. In the mammary gland explant system, cell proliferation occurs as the result of stimulation by insulin [26, 27] or epithelial growth factors [28] and prolactin controls differentiation of the epithelium into secretory alveolar cells in the proper hormonal environment. Glucocorticoids apparently have a role in preparing mammary epithelial cells for prolactin stimulation, as cells grown in the absence of hydrocortisone must be exposed to the hormone 48 h before newly formed daughter cells can synthesize the milk proteins in response to prolactin [29]. It seems unlikely that multiple hormonal interactions are unique to mammary gland development and further study of the interactions involved in fetal lung development may eliminate the discrepancies.

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DISCUSSION

Solomon. When is the critical time in the 22 day gestation of the rat.

Hughes. For surfactant appearance, about 19 days.

Solomon. So if you give the corticosteroid to the rat fetus at 4 days you should see all the parameters that Dr. Farrell mentioned with the lung capacity and the increase in progestin.

Hughes. That's correct. These experiments are more difficult to do with the rat because the fetuses are so small but in the rabbit model this is certainly true.

Solomon. Haven't papers appeared on the rat. You have such a nice culture model but it doesn't do what the *in vivo* does.

Hughes. There are fewer published experiments with the rat, but available evidence indicates that the two animal systems (rat and rabbit) behave in a similar way. The culture tissue exhibits both metabolic and morphological changes typical of maturation. Because of the number of correlations observed in the *in vivo* and *in vitro* systems, the response to glucocorticoids *in vivo* may be mediated by cells outside the lung and not a direct effect on the Type II cell, *per se*.