CORTISOL RECEPTORS IN RABBIT FETAL LUNG*

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SUMMARY. Rabbit fetal lung nuclei contain macromolecules which have the properties of physiological receptors for cortisol by the criteria of specificity of binding and saturation of binding sites at low concentrations of the hormone The number of nuclear receptor sites is relatively low at 20 days of gestation, reaches a maximum at about 28-30 days of gestation and drops slightly after birth. These results correlate well with previously reported changes in pulmonary epithelial cell maturation and surfactant concentrations in rabbit fetal lung extracts. Preliminary evidence for the presence of cytoplasmic cortisol-binding components in fetal lung cells is also presented.

INTRODUCTION. Numerous theoretical and experimental studies have established that the alveoli of mammalian lung are lined with a highly surface-active substance, designated pulmonary surfactant, which presumably reduces alveolar surface forces at low lung volumes during respiration, thereby preventing alveolar collapse (1-3). Present evidence indicates that this material is a lipoprotein with a high content of dipalmitoyl lecithin (4,5).

The fetal lung undergoes marked changes towards the end of gestation to permit its function as an organ of gas exchange. Maturation of the fetal lung involves both anatomical and biochemical characteristics associated with the appearance of pulmonary surfactant (5,6). The possibility that fetal steroids may influence pulmonary epithelial cell maturation was suggested by Buckingham et al (7), and supported by the observations of Liggins (8). Preliminary evidence that surfactant was present earlier than expected after cortisol infusion of fetal lambs was reported by de Lemos et al (9). These observations were subsequently confirmed in fetal rabbits by Kotas and Avery (10), who found that lung

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maturity, as defined by accelerated morphologic development and increased pulmonary surfactant, was increased by glucocorticoid treatment.

The mechanism by which cortisol accelerates lung maturation is not known. Since the interaction of steroid hormones with specific receptor molecules in their target tissues seems to be an early requisite step in their mechanism of action (11), we have examined fetal rabbit lung for the presence of cortisol receptors.

METHODS. Pregnant white New Zealand rabbits were operated on at 20-30 days of gestation under pentobarbitol anesthesia. The gravid uterus was exposed and the fetuses were removed and immediately decapitated. The fetal lungs were excised, placed in ice-cold Eagle's Hela medium, and minced with scissors. The lung minces were blotted and 1 gram aliquots of the tissue were incubated in 10 ml Eagle's Hela tissue culture medium containing an appropriate concentration of ^{3}H-cortisol (45 Ci/mMole) at 37° in an atmosphere of 95% O_2-5% CO_2. Competition studies were performed by incubating the tissue with ^{3}H-cortisol and a ten-fold excess of non-labeled steroid.

Following incubation, the lung minces were washed several times with 0.32M sucrose, 0.01 M Tris, 0.003 M $MgCl_2$, pH 7.4, buffer (Buffer A) and a 5% homogenate was prepared in the same buffer using a glass homogenizer with a teflon pestle. The homogenate was filtered through four layers of cheesecloth, the concentration of sucrose was adjusted to 0.25 M and 15 ml of the diluted homogenate was layered on top of 5 ml Buffer A and centrifuged at 1100 x g for 10 mins. The pellet was then washed three times with 20 ml of 0.25 M sucrose, 0.01 M Tris, 0.001 M $MgCl_2$, pH 7.4, buffer (Buffer B). Further purification of the nuclei was accomplished by homogenization of the crude nuclear pellet in 10 ml of 2.4 M sucrose, 0.01 M Tris, 0.001 $MgCl_2$, pH 7.4, buffer, followed by centrifugation at 100,000 x g for 1 hour. The purified nuclear pellet was homogenized in 3 ml of Buffer B and two 0.1-0.2 ml aliquots of the homogenate were taken for DNA determinations by the diphenylamine method (12). Separate 0.2 ml aliquots (in triplicate) of the purified nuclear pellet homogenate were mixed with 3 ml ethanol and, following

centrifugation, the supernatant was mixed with 10 ml of scintillation fluid and counted. In other experiments, the purified nuclear pellet was homogenized in 3 ml of 0.01 M Tris, 0.0015 M EDTA, 0.6 M KCl, pH 8.5, buffer (Buffer C), the homogenate was allowed to stand at 4° for 30 mins and then centrifuged at 70,000 x g for 15 mins. Aliquots of the supernatant (nuclear extract) were fil tered through small Sephadex G-50 columns at 4° to separate the bound from the free steroid.

The binding of cortisol to cytoplasmic components of fetal lung and liver was also studied. The tissues were homogenized in 0.01 M Tris, 0.0015 M EDTA, pH 7.4, buffer (Buffer D) and soluble fractions (cytosol) were prepared by centrifuging the homogenates at 224,000 x g for 1 hour. The cytosol fractions were incubated for 2 hours at 4° with 10^{-8}M of ^{3}H-cortisol and fractionated by gel filtration on Sephadex G-200 columns. The methods of gel filtration have been previously described (13).

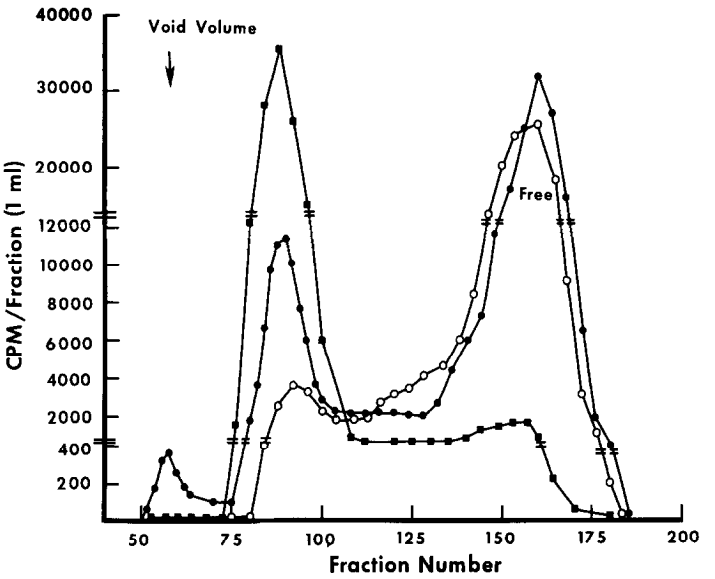

Fig. 1. Gel filtration on Sephadex G-200 of rabbit fetal lung and liver cytosols and of serum pre-incubated with 1 x 10^{-8}M ^{3}H-cortisol for 2 hours at 4°. One-gram aliquots of tissues from rabbit fetuses at day 28 of gestation were homogenized with 3 ml of Buffer D. Following centrifugation of the homogenate at 224,000 x g for 1 hr, 2-ml aliquots of the cytosol fractions were applied on the column. Elution was carried out at 4° at a flow rate of 6 ml per hr with Buffer D. One-ml fractions were collected. Column, 2.5 x 30 cm; bed volume, 153 ml. ●—●, lung cytosol; ○—○, liver cytosol; ■—■, fetal serum (10-fold dilution with Buffer D).

RESULTS AND DISCUSSION. Figure 1 shows that cytosols from rabbit fetal liver and lung contain a cortisol-binding macromolecule with an elution pattern similar to that of serum cortisol-binding globulin (CBG) when examined by filtration on Sephadex G-200. It is likely that liver and lung cytosols contain blood contaminants and that the cortisol-binding component shown in Fig. 1. is due to contamination by serum CBG present in the tissue fractions. However, since a far greater amount of cortisol is bound to the CBG-like component in cytosol compared to liver cytosol (Fig.1), the lung cytosol at least appears to contain a cellular cortisol-binding molecule in addition to possible contamination by serum CBG. Further fractionation of the lung and liver cytosols by chromatography on DEAE-Cellulose columns is in progress. In addition to the major CBG-like component, lung cytosol contains a minor cortisol-binding component (eluted with the void volume) which is not present in liver cytosol or in serum (Fig.1). These results indicate that the rabbit fetal lung cells contain one or more cytoplasmic cortisol-binding macromolecules. The specificity of these components for cortisol-binding has not yet been studied.

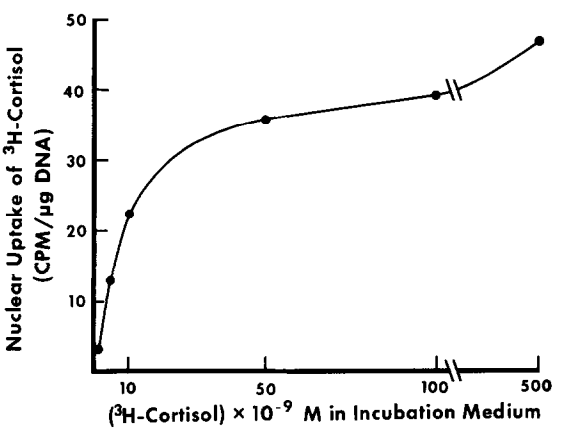

Fig. 2. Kinetics of ^{3}H-cortisol uptake by rabbit fetal lung nuclei. One-gram aliquots of lung minces (from fetuses at day 28 of gestation) were incubated with increasing amounts of ^{3}H-cortisol in 10 ml of Eagle's Hela tissue culture medium at 37° for 2 hrs. Purified nuclei were prepared as described in METHODS. The purified nuclear pellets were homogenized in Buffer B and aliquots of the homogenates were extracted with 3 ml of ethanol and counted. Separate aliquots of the purified nuclear pellet homogenates were taken for DNA determinations.

The kinetics of the nuclear uptake of ^{3}H-cortisol following incubations of rabbit fetal lung minces at 37° is shown in Fig. 2. Saturation of the nuclear binding sites is reached when the ^{3}H-cortisol concentration in the incubation medium is 5 x 10^{-8} to 1 x 10^{-7}M. This concentration is within the physiological range of cortisol in blood. At higher cortisol concentrations, further nuclear uptake of ^{3}H-cortisol (probably non-specific) is observed (Fig.2). As shown in Table 1, the nuclear uptake of ^{3}H-cortisol in fetal lung is specific since only steroids with glucocorticoid activity compete for the binding sites. In addition, the ability of different glucocorticoids to compete with ^{3}H-cortisol uptake by

Table 1. EFFECT OF NON-LABELED STEROIDS ON THE NUCLEAR UPTAKE OF ^{3}H-CORTISOL IN RABBIT FETAL LUNG*

Non-labeled steroid added in incubation medium	Nuclear uptake of ^{3}H-cortisol (cpm/ µg DNA)	% Competition
-	43.5	-
Cortisol	6.8	84
9α-Fluorocortisol	4.1	91
Corticosterone	19.6	55
Estradiol-17β	39.6	9
Progesterone	44.5	0
Testosterone	45.8	0

* One-gram aliquots of rabbit lung minces (from fetuses at day 28 of gestation) were incubated in 10 ml of Eagle's Hela medium containing 1 x 10^{-7}M ^{3}H-cortisol with or without a ten-fold excess of non-labeled steroid for 2 hours at 37°. Nuclear uptake of ^{3}H-cortisol was measured as described in Fig. 2.

lung nuclei correlates well with their biological activity. About 70-90% of the tritiated steroid could be extracted from lung nuclei with Buffer C. In the nuclear extract 50-60% of the labeled steroid is bound to macromolecules (Fig.3). Preliminary evidence indicates that the labeled steroid extracted from the nuclei is cortisol. Fig. 3 also shows that the amount of bound steroid in nuclear ex-

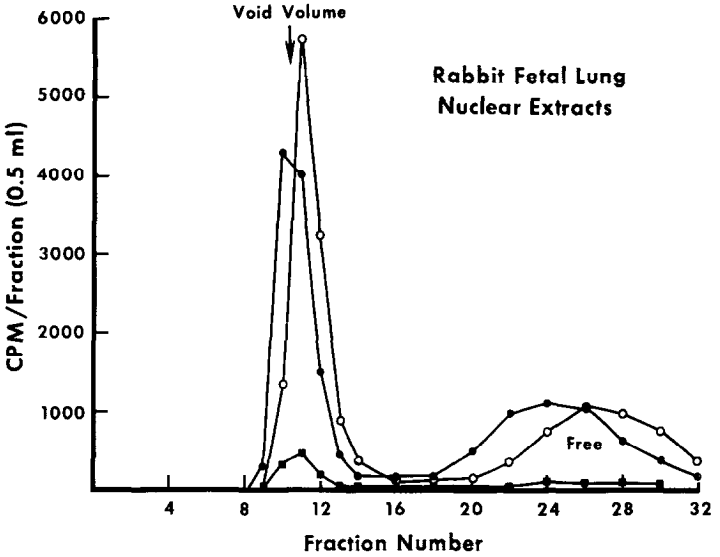

Fig. 3. Gel filtration on Sephadex G-50 columns of rabbit fetal lung nuclear extracts prepared after incubation of lung minces (from fetuses at day 28 of gestation) with: ●—●, 1 x 10^{-7}M ^{3}H-cortisol; ○—○ , 1 x 10^{-7} ^{3}H-cortisol + 1 x 10^{-6}M estradiol-17β; ■—■ , 1 x 10^{-8}M ^{3}H-cortisol + 1 x 10^{-6}M cortisol. Techniques of tissue incubation and preparation of nuclear extracts with Buffer C are described in METHODS.

tracts is greatly decreased when lung tissue is incubated in the presence of a ten-fold excess of non-labeled cortisol but a ten-fold excess of estradiol-17β has no effect. A comparison of the nuclear uptake of cortisol in lung tissues of rabbit fetuses from day 20 to day 30 of gestation and of newborn rabbits is shown in Fig. 4. In these experiments lung tissues were incubated with 1 x 10^{-7}M ^{3}H-cortisol for 2 hours at 37° as these conditions were found to result in saturatio of the specific nuclear sites and minimal non-specific uptake. The amount of ^{3}H-cortisol taken up by nuclei, expressed as cpm/μg DNA, increases from day 20 of gestation (the earliest time studied) reaching a maximum at about 28-30 days of gestation. Somewhat lower nuclear uptake of cortisol was observed in lungs of newborn rabbits.

The results presented in this report demonstrate that rabbit fetal lung nuclei contain a macromolecule with a specific affinity for glucocorticoids and a limited number of binding sites which are saturated with physiological concentrations of cortisol. Preliminary evidence indicates that cortisol-binding com-

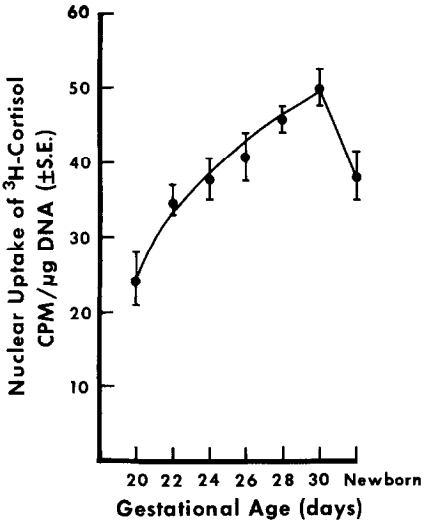

Fig. 4. Changes in uptake of ^{3}H-cortisol (per unit DNA) by lung nuclei of the developing rabbit fetus. Each point represents the mean of 5-6 determinations. For each determination, lungs from all fetuses of the same litter (5-10 fetuses) were pooled, the tissue was minced and a one-gram aliquot of the minces was incubated in 10 ml of Eagle's Hela medium containing 1 x 10^{-7}M ^{3}H-cortisol for 2 hrs at 37°. The nuclear uptake of ^{3}H-cortisol was then measured as described in Fig. 2.

ponents may also be present in the cytoplasm of fetal lung cells. Of particular interest is the finding that the nuclear uptake of cortisol in lung tissue increases during the last third of gestation reaching a maximum at about 28-30 days. This observation correlates well the concentration of surface-active phospholipids in lung extracts of rabbit fetuses (10,14,15). Pulmonary surfactant is detectable at day 24 of gestation and is markedly increased from day 24 to the end of gestation (10,14,15).

The physiological significance of these findings is difficult to evaluate at the present time. It has been proposed that the mechanism of enzyme induction in liver cells is mediated by cytoplasmic and nuclear cortisol receptors (11). It is known that a variety of enzymes (e.g. invertase, alkaline phosphatase, glutamine synthetase, tyrosine aminotransferase) can be induced in the immature animal by adrenal steroids. One could speculate that cortisol induces one or more of the enzymes necessary for surfactant synthesis and stimulates lung maturation.

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REFERENCES.

1. Avery, M.E. and Mead, J., Am. J. Dis. Child 97, 517, 1959.
2. Clements, J.A., Physiologist 5, 11, 1962.
3. Abrams, M.E., J. Appl. Physiol. 21, 718, 1966.
4. Pattle, R.E. and Thomas, L.C., Nature 189, 844, 1961.
5. Buckingham, S. and Avery, M.E., Nature 193, 688, 1962.
6. Humphreys, P.Q. and Strang, L.B., J. Physiol.(London) 192, 53, 1967.
7. Buckingham, S., McNary, W.F., Sommers, S.C. and Rothschild, J., Federation Proc. 27, 328, 1968.
8. Liggins, G.C., J. Endocrinol. 45, 515, 1969.
9. DeLemos, R.A., Shermeta, D.W., Knelson, J.H., Kotas, R.V. and Avery, M.E., Pediat. Res. 3, 505, 1969.
10. Kotas, R.V. and Avery, M.E., J. Appl. Physiol. 30, 358, 1971.
11. Raspé, G. (ed.) Schering Workshop on Steroid Hormone Receptors, Advances in the Biosciences 7, Pergamon Press, 1971.
12. Schneider, W.C., in S.P. Colowick and N.O. Kaplan (eds.) Methods in Enzymology, vol. 3, Academic Press, New York, 1957, p.680.
13. Giannopoulos, G. and Gorski, J., J. Biol. Chem. 246, 2530, 1971.
14. Kikkawa, Y., Motoyama, E.K. and Gluck, L., Am. J. Pathol. 52, 177, 1968.
15. Gluck, L., Sribney, M. and Kulovich, M.V., Pediat. Res. 1, 247, 1967.