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GLUCOCORTICOIDS INCREASE PULMONARY EPIDERMAL GROWTH FACTOR RECEPTORS IN FEMALE AND MALE FETAL RABBIT

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Experimental evidence in animals and humans suggest that glucocorticoids enhance fetal pulmonary maturation. Mechanisms of glucocorticoid effects remain unclear; but apparently include up regulation of fetal pulmonary insulin and β-adrenergic receptors. A role of Epidermal Growth Factor (EGF) in fetal lung maturation through plasma membrane bound receptors has been recently proposed. Betamethasone, 0.085 mg/kg, was administered on 25th and 26th day of gestation to the rabbit doe. Fetal pulmonary EGF receptor characteristics in male or female fetuses were studied on the 27th day of pregnancy. The percent specific binding of 125-I-EGF to lung plasma membranes (LPM) and the number of receptor sites per mg of LPM protein or DNA content were significnatly higher in the glucocorticoid treated female as well as male fetuses when compared to the control pups, with no difference in the Kd. Presence of high affinity receptors for EGF and their up regulation by glucocorticoids support the hypothesis that EGF plays an important role in fetal lung maturation and that some of the beneficial effects of glucocorticoids in decreasing the incidence of HMD may be mediated through its interaction with EGF.

Hyaline Membrane Disease (HMD), secondary to pulmonary surfactant deficiency, remains the major cause of neonatal mortality and morbidity. Experimental evidence in animals and clinical trials in humans suggest that fetal pulmonary maturation can be enhanced by the administration of glucocorticoids to the mother or the fetus (1-7). The mechanisms of glucocorticoid effects in fetal lung development are incompletely understood; but apparently include increased glycogenolysis (8,9), stimulation of enzymes necessary for surfactant synthesis (9,10) and up regulation of fetal pulmonary insulin and β-adrenergic receptors (11-13).

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ABBREVIATIONS: HMD, Hyaline Membrane Disease; EGF, Epidermal Growth Factor; BSA, Bovine Serum Albumin; LPM, Lung Plasma Membrane; TCA, Trichloroacetic acid

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Effects of Epidermal Growth Factor (EGF) in morphologic and functional fetal pulmonary maturation are well established (14-17). The biological effects of EGF in the fetal lung are mediated via plasma membrane bound receptors (18-19). The degree of biological response to EGF can be influenced by the concentration and affinity of EGF receptors as well as post receptor events (20). In this study, we investigated if administration of synthetic glucocorticoid to the rabbit doe would influence the concentration and/or affinity of fetal pulmonary EGF receptors as has been demonstrated for insulin and β-adrenergic receptors (11-13).

MATERIALS AND METHODS

Pregnant New Zealand white rabbits of known gestation were purchased from a local supplier and housed individually. On 25th and 26th day of gestation (0 - breeding day, term ~ 31 days) the study group does received 0.085 mg/kg of betamethasone (Schering Corp., N.J.) intramuscularly while the control animals received saline only. The concentration of betamethasone is comparable to the dose used for prevention of HMD in human (5). When administered in the pregnant rabbit doe it increases fetal pulmonary β-adrenergic and insulin receptors (11,12) and inhibits endogenous corticosteroid production in the mother as well as the fetus (11). Each doe was sacrificed by I.V. pentobarbital on the 27th day of gestation. The fetuses were removed after hysterotomy, weighed and killed by cervical dislocation (11,18).

Isolation of Lung Plasma Membranes:

Lung Plasma Membranes (LPM) were isolated by differential centrifugation as previously described (18,21) with minor modifications. The significant amount of blood present in the fetal lung at the time of sacrifice interferes with accurate weight of the lung, protein and 5'-nucleotidase assay in the lung homogenate and the LPM. Therefore,in all experiments we ventilated the fetal lung via a catheter in the trachea and perfused heparinized saline (2 U-heparin/ml) through the pulmonary artery as described before (22). Most of the blood was removed within 1-2 minutes. Since there are differences in the developmental pattern of fetal lung maturation (23) and pulmonary responsiveness to glucocorticoid in the male and female fetuses (7), male or female lungs within the same litter were pooled and processed separately. Method of sex determination in the rabbit pup has been described (18). Lungs were trimmed of bronchial and connective tissue, cut into small pieces and homogenized in ice cold 0.3M sucrose 50mM Tris Hcl buffer (pH 7.45) in a Dounce homogenizer (w/v 1:10) at 4°C. The homogenate was centrifuged at 900xg for ten minutes at 4°C. The supernatant was adjusted to 0.1M NaCl and 0.2mM $MgSO_4$ and centrifuged at 3000xg for 20 minutes. The supernatant was further centrifuged at 140,000xg for 60 minutes at 4°C and the pellet was washed with 30ml of 50mM Tris Hcl, pH 7.45 buffer to remove the sucrose. LPM were harvested by centrifugation at 37,500 xg for 30 minutes and the pellet was resuspended in 50mM Tris Hcl buffer to yield 1-2 mg/ml of LPM protein. Using BSA as standards, protein concentration in the lung homogenate and LPM were quantitated by Lowry's method (24).

5'-Nucleotidase Assay:

5'-Nucleotidase activity in the lung homogenate and LPM was determined by the method of Arkesteijn (25).

DNA Assay:

Lung homogenate DNA content was measured by Zamenof's modification (26) of Burton's technique (27).

125 I-EGF Binding Assay:

Purified mouse EGF (Collaborative Research, MA M.W. ~ 6045) was radioiodinated using carrier free 125 NaI (N.E.N., MA) by a modification of Greenwood and Hunter's method (28). Radioiodinated EGF and free NaI were separated on a Sephadex-G-25 column saturated with 0.1% BSA (29). 125-I-EGF with > 90% TCA precipitability was utilized for the binding assays (29). In preliminary experiments, optimal 125 I-EGF binding to LPM was noted at 37°C, pH 7.45 with an incubation time of 60 minutes and 300 µg/tube of BSA. Percent specific binding of 125-I-EGF to LPM was linear from 50 to 200 µg/tube of LPM protein. Each point in the displacement curve (Fig. 1) was assayed in triplicate using 125-I-EGF (95000 to 100000 cpm/tube) in presence of unlabelled EGF (Collaborative Research) at $5x10^{-10}$ to $5x10^{-8}$M concentrations in a final incubation volume of 0.3ml of 50mM Tris Hcl, pH 7.45, containing 100 to 200 µg of LPM protein and 300 µg of BSA in 1.5ml polypropylene tubes (Kew Scientific, OH). The binding reaction was terminated by adding 0.8ml of ice cold Tris Hcl buffer and by centrifugation at 12,500xg for five minutes. The supernatant was aspirated and the amount of radioactivity in the membrane pellet was counted in Packard gamma radiation counter for two minutes. Nonspecific binding was measured as the residual radioactivity in the presence of excess ($1x10^{-7}$M) unlabelled EGF and was subtracted from all points of the dose response curve to determine percent specific binding. The number of EGF binding sites and Kd were calculated by Scatchard analysis (30). Statistical significance was determined by Student's "t" test.

RESULTS

All data are expressed as means ± S.E.M. Number of fetuses per litter ranged from 2 to 10. Table I summarizes the results of this investigation. The fetal weight was significantly less in steroid treated animals. Lung homogenate protein and DNA content/gm of lung, percent recovery as LPM

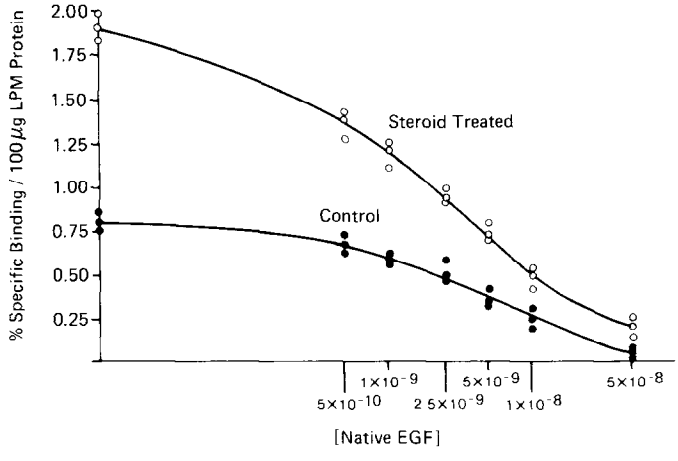

FIGURE 1: Displacement of 125-I-EGF binding to LPM in presence of various concentrations of unlabelled EGF in control and steroid treated fetus.

TABLE I: Fetal weight, lung homogenate and plasma membrane protein DNA and 5'-nucleotidase activity, number of 125 I-EGF binding sites and their Kd in male and female control and steroid treated fetuses.

	CONTROL MALE	CONTROL FEMALE	STEROID MALE	STEROID FEMALE	STATISTICAL SIGNIFICANCE
Fetal Weight (gms)	28.4 ± 1.7 (n=9)		21.7 ± 1.1 (n=7)		$p < 0.001$
Lung Homogenate Protein mg/gm of Lung	36.8 ± 2.8 (n=7)	39.8 ± 2.8 (n=5)	38.9 ± 1.5 (n=5)	35.8 ± 4.1 (n=5)	N.S.
% Recovery as LPM Protein	3.59 ± 0.21 (n=7)	3.7 ± 0.52 (n=5)	3.48 ± 0.26 (n=5)	3.11 ± 0.23 (n=5)	N.S.
DNA (mg) Content/gm of Lung	1.3 ± 0.1 (n=7)	1.53 ± 0.13 (n=6)	1.38 ± 0.14 (n=6)	1.47 ± 0.17 (n=6)	N.S.
5'-Nucleotidase Activity (units/mg of protein/min)					
(a) Homogenate	0.0303 ± 0.0039 (n=4)	0.026 ± 0.0005 (n=4)	0.0218 ± 0.00035 (n=3)	0.0198 ± 0.0009 (n=4)	N.S.
(b) LPM	0.1026 ± 0.0144	0.0925 ± 0.01646	0.0703 ± 0.0194	0.0936 ± 0.0048	N.S.
Fold Purification	3.49 ± 0.59	4.04 ± 0.544	3.53 ± 0.53	4.52 ± 0.448	N.S.
% Specific Binding/ 100 µgm LPM Protein	0.97 ± 0.14 (n=6)	0.99 ± 0.2 (n=4)	2.98 ± 0.49 (n=4)	2.23 ± 0.41 (n=5)	1 vs. 3 $p < 0.01$ 2 vs. 4 $p < 0.05$
Number of binding sites/ mg of LPM Protein X 10^{-10}	6.1 ± 1.5 (n=5)	7.5 ± 1.9 (n=3)	21.6 ± 3.7 (n=3)	21.2 ± 3.5 (n=4)	1 vs. 3 $p < 0.01$ 2 vs. 4 $p < 0.02$
Number of binding sites/ mg of DNA X 10^{-10}	7.1 ± 1.5 (n=5)	7.8 ± 0.4 (n=4)	19.8 ± 1.8 (n=3)	25.5 ± 2.5 (n=4)	1 vs. 3 $p < 0.001$ 2 vs. 4 $p < 0.001$
Kd X 10^{9}	3.7 ± 0.5 (n=5)	3.9 ± 0.6 (n=3)	5.1 ± 1.1 (n=3)	5.6 ± 1.0 (n=4)	N.S.

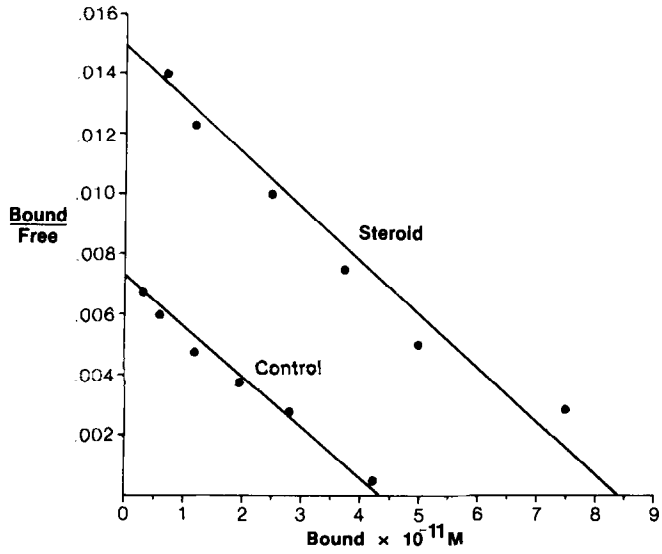

FIGURE 2: Representative Scatchard plot from control and steroid treated fetus.

protein, 5'-nucleotidase activity/mg of homogenate or LPM protein and the degree of enrichment as judged by 5'-nucleotidase activity in the LPM were similar in control and study group animals.

In all experiments, the Scatchard plots were linear, suggesting one order of binding sites (Fig. 2). The percent specific binding of I-125-EGF to LPM, number of binding sites per mg of LPM protein of DNA content and Kd were similar in male and female fetuses in the control group animals (Table I). The percent specific binding of I-125-EGF to LPM and the number of EGF binding sites/mg of LPM protein or DNA content in the steroid treated male or female fetuses was significantly higher when compared to the respective controls. The degree of up regulation i.e. increase above control, was similar in female as well as male fetuses in the study group animals. Although there was a trend towards increase in the Kd i.e. decrease in the receptor affinity, in steroid treated male as well as female fetuses, the changes were not significant ($p > 0.05$).

DISCUSSION

Administration of pharmacologic concentration of betamethasone to the rabbit doe at a critical period of rapid cellular proliferation

retards fetal development resulting in decreased body as well as lung weights (31). In this study, we administered ten times less betamethasone than the above mentioned study (31) and observed a significant reduction in the fetal body weight with no apparent decrease in the fetal lung protein or DNA content.

Betamethasone increased fetal pulmonary EGF receptors in the male as well as the female fetus in the present study. Whether an increase in the EGF receptors would imply an amplification of the biological effect of EGF in fetal pulmonary maturation remains unknown. However, the presence of high affinity receptors for EGF and their up regulation by betamethasone would further support the hypothesis that EGF plays an important role in fetal lung maturation and that some of the effects of betamethasone in decreasing the incidence of HMD may be mediated through an increase in pulmonary EGF receptors.

The subcellular mechanism of glucocorticoids effects in up regulating fetal pulmonary insulin, beta adrenergic as well as EGF receptors are unknown. Recently Maniscalo et al have demonstrated that cycloheximide, an inhibitor of protein synthesis, eliminates dexamethasone induced increase in fetal pulmonary β-adrenergic receptors (13). Whether up regulation of insulin or EGF receptors from glucocorticoid stimulation can be inhibited by cycloheximide requires further investigation.

Since LPM rather than isolated cells were used in this investigation, location of EGF receptors and their up regulation from glucocorticoid stimulation within different fetal pulmonary cells remains unknown. Since the presence of EGF receptors on adult rat Type II pneumocytes has been demonstrated (32) and EGF enhances fetal pulmonary surfactant synthesis (14-16), it is likely that fetal Type II cells possess EGF receptors.

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