CORTICOSTEROID INDUCTION OF PHOSPHATIDIC ACID PHOSPHATASE IN FETAL RABBIT LUNG

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SUMMARY: Prenatal betamethasone therapy is known to reduce the incidence of respiratory distress syndrome in premature infants. We report that administration of an equivalent dose of this corticosteroid to pregnant rabbits increases phosphatidic acid phosphatase (PAPase) activity and the rate of choline incorporation into lecithin in fetal lung. Under these conditions there was no induction of choline phosphotransferase or glycerolphosphate phosphatidyltransferase. These results suggest that PAPase may be a key regulatory enzyme in surfactant synthesis during both normal and glucocorticoid-accelerated lung maturation.

INTRODUCTION: Respiratory distress syndrome (RDS) of premature newborn infants is associated with reduced levels of pulmonary surfactant, a material which contains surface active phosphatidylcholine (lecithin) as well as phosphatidylglycerol, other lipids, and specific proteins. As first reported by Liggins and Howie in 1972, the incidence of RDS is substantially reduced by administration of betamethasone, a synthetic corticosteroid, to women in premature labor (1,2). Lung development and appearance of alveolar surfactant is also accelerated in fetal animals exposed to corticosteroid (3). In the fetal rabbit, the rate of choline incorporation into lecithin is increased by administration of glucocorticoid to the fetus (4-6), mother (7), or cultured lung cells (8).

This hormonal effect presumably involves enzyme induction mediated by glucocorticoid receptors which are present in fetal lung (9-11), however, the identification of inducible enzymes has been uncertain. In 1973, Farrell and Zachman (4,5) reported that injection of rabbit fetuses with synthetic corticosteroid increased the activity of choline phosphotransferase (EC 2.7.8.2), the final enzyme for phosphatidylcholine synthesis in lung, but

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had no effect on cholinephosphate cytidyltransferase (EC 2.7.7.15), the preceding enzyme in the pathway. Recently, Rooney and associates (12, 13) found some increase in the level of cholinephosphate cytidyltransferase, but not of choline phosphotransferase, two days after fetal administration of cortisol; three days after treatment there was increased activity of glycerolphosphate phosphatidyltransferase (EC 2.7.8.5) an enzyme involved in the synthesis of phosphatidylglycerol. A regulatory role for any of these enzymes during normal development of the fetal rabbit lung is unlikely since it is reported that the activity of each declines during late gestation (14-16).

By contrast, Schultz et al. (15) recently reported a three-fold increase in the activity of phosphatidic acid phosphatase (PAPase, EC 3.1.3.4) during fetal lung development in the rabbit. This enzyme catalyzes the hydrolysis of phosphatidic acid to 1,2-diacyl-sn-glycerol (diglyceride) which is utilized as substrate by choline phosphotransferase. Microsomal PAPase and endogenous plasma corticoids (17) begin to increase about day 26 of gestation, preceding the accumulation of lamellar bodies in type II alveolar epithelial cells (18) and the increase in alveolar surfactant (14,16). We felt that the level of PAPase might be a regulatory factor for surfactant synthesis during normal lung development, and, as such, might be induced by glucocorticoids.

In this study we found that maternal betamethasone treatment stimulates PAPase activity and the incorporation of choline into lecithin. A preliminary report of some of this work has been published (19).

MATERIALS AND METHODS: We injected pregnant New Zealand white rabbits with 0.25 mg/kg Celestone Soluspan[1] or saline (controls) intramuscularly 12, 24 or 48 hr (one dose) or 24 and 48 hr (two doses) prior to sacrifice. In order to keep the duration of gestation constant, all rabbits were killed on day 26 of gestation. The does were anesthetized with intravenous pentobarbital, maternal blood was collected by cardiopuncture, fetal blood was obtained after decapitation, and the lung and liver were removed. Plasma glucocorticoid activity (cortisol plus betamethasone) was determined by radioreceptor assay (20). For measurement of choline incorporation, about 150 mg of lung were minced with a Brinkmann Tissue Chopper and added to a flask with 1 ml of Krebs-Ringer

[1] Celestone Soluspan (Schering Corp.) contains equal amounts of betamethasone acetate and betamethasone phosphate.

bicarbonate solution (pH 7.4) containing 3% bovine serum albumin; following 10 minutes of equilibration, 0.5 μCi of [3H]choline chloride[2] (2.5 mCi/mmol) were added and the tissue was incubated with shaking for one hour at 37°C under 95% O_2: 5% CO_2. After washing with cold 0.9% NaCl, the lipids were extracted with chloroform: methanol (2:1), washed by the method of Folch et al. (21), and the radioactivity measured. Thin layer chromatography indicated that more than 95% of the [3H] choline incorporated into lipids was found in lecithin. For the enzyme assays, frozen tissues were homogenized in five volumes of 0.01 M Tris-HCl, 0.25 M sucrose, 1 mM EDTA at pH 7.4; microsomes were prepared by centrifuging homogenate for 20 minutes at 15,000 x g and then centrifuging the supernatant for one hr at 100,000 x g. Preliminary studies demonstrated that initial enzyme velocities were proportional to protein concentration in each case and that product formation was linear with time for at least 10, 15 and 60 minutes for choline phosphotransferase, glycerolphosphate phosphatidyltransferase and PAPase, respectively. The reaction system for PAPase was a modification of the method of Coleman and Hubscher (22) and contained 0.5 mg phosphatidic acid (prepared from egg lecithin, Sigma), 0.025-0.1 mg of protein, and 50 mM sodium maleate buffer (pH 6.5) in a total volume of 0.25 ml; free phosphate was measured by the method of Baginski and Zak (23). We assayed the other enzymes using slight modifications of the methods of Rooney et al. (12). For choline phosphotransferase the reaction mixture contained 50 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, 5 mM glutathione, 0.1 mM [^{14}C]CDP-choline (1 mCi/mmol), lung homogenate (0.3-0.6 mg protein), and 0.88 mM diolein prepared in 0.006% Triton X-100. Aliquots were removed after various times at 37°C and processed on filter paper following the technique of Goldfine (24). The reaction system for glycerolphosphate phosphatidyltransferase contained 70 mM Tris-HCl (pH 7.4), 0.01% Triton X-100, 0.15 mM CDP-dipalmitin, 0.2 mM [^{14}C]glycerol-3-phosphate (2.5 mCi/mmol) and lung homogenate (0.2-0.5 mg protein). Aliquots were processed as for choline phosphotransferase. Protein was assayed by the method of Lowry et al. (25).

In some experiments, we assayed the left lung for DNA (26), total lipid (27), and disaturated phosphatidylcholine (27).

RESULTS: The results for plasma glucocorticoids, choline incorporation, and enzyme activities are shown in the Table. Plasma glucocorticoid activity was maximal at one and two hr after injection of betamethasone with mean ± SE levels of 152 ± 16 and 36.2 ± 1.5 μg cortisol equivalents/100 ml for 3 mothers and 5 fetuses, respectively. Values returned to control levels between 24 and 48 hr. For comparison, maximal glucocorticoid levels in women and their newborn infants one hr after prenatal betamethasone treatment are about 100 and 24 μg/100 ml, respectively, and betamethasone disappears from both maternal and cord blood within two days (28).

The rate of choline incorporation by fetal lung shows a significant increase of 27% by 12 hr and 53% by 24 hr. After 48 hr, if only one injection is given, there is a trend

[2] All radioactive compounds were obtained from New England Nuclear.

TABLE: EFFECT OF BETAMETHASONE ADMINISTRATION ON PLASMA GLUCOCORTICOIDS, CHOLINE INCORPORATION, AND ENZYME ACTIVITIES

Determination (n)+	Control‡	Time of Maternal Injections 12 hr	24 hr	48 hr	24 + 48 hr
Plasma Glucocorticoids (μg cortisol equivalents/100 ml)					
Maternal (3-7)	4.2±0.7	25.8±3.8*	5.0±0.3	3.6±2.3	8.9±2.9*
Fetal (3-10)	1.3±0.1	4.1±0.4*	3.2±0.5*	2.7±1.2	5.9±0.3*
Choline Incorporation (10-25) (pmol x mg wet lung^{-1} x hr^{-1})	32.5±1.1	41.3±2.6*	49.7±2.3*	40.7±4.2*	53.3±2.1*
Phosphatidic Acid Phosphatase (nmol x mg protein^{-1} x min^{-1})					
Lung Homogenate (8-19)	6.6±0.5	7.4±0.3*	9.5±0.5*	10.8±0.7*	12.8±0.6*
Lung Microsomes (8-14)	10.5±0.4	12.9±0.7*	14.6±0.6*	14.1±1.0*	19.0±0.8*
Liver Homogenate (8)	7.2±0.6	---	5.6±0.2*	5.9±0.5*	5.0±0.2*
Choline Phosphotransferase (8-16) (pmol x mg protein^{-1} x min^{-1})	466±38	---	380±29	372±47	493±22
Glycerolphosphate Phosphatidyltransferase (8-16) (pmol x mg protein^{-1} x min^{-1})	77.8±6.1	82.0±4.9	60.3±4.7	67.9±8.2	78.0±8.7

All values are given as mean ± SE.

* $p < 0.05$ compared with control by unpaired t test.

\+ Number of animals examined at each time (maximum of four fetuses per litter).

‡ Control values for all times of saline exposure were similar and were combined.

toward a lower rate, however, this value is not significantly different from the 24 hr value; with two doses, the rate of incorporation is 64% greater than the control. Later in gestation (28-29 days), the rate of choline incorporation in untreated animals was higher (46.5 ± 2.8 pmol x mg lung^{-1} x hr^{-1}, n=24) and there was no effect of 48 hr treatment with two doses of betamethasone (46.5 ± 3.5, n=10).

PAPase activity is significantly increased in both lung homogenate and lung microsomes by 12 hr, and it nearly doubles after two doses of steroid. With only one injection PAPase activity appears to plateau between 24 and 48 hr. In the liver, PAPase activity decreases slightly (18-30%, $p < 0.05$) after betamethasone treatment.

There was no significant effect of treatment on the specific activity of choline phosphotransferase and glycerolphosphate phosphatidyltransferase in lung homogenate.

We investigated the effect of administering one-tenth of the usual dose of betamethasone (0.025 mg/kg every 12 hr for four injections). PAPase activity in lung homogenate was 7.2 ± 0.2 nmol x mg protein^{-1} x min^{-1} and choline incorporation by lung was 34.3 ± 1.3 pmol x mg lung^{-1} x hr^{-1} in 24 animals. These values are not significantly different from control levels. After an intermediate dose of steroid (0.1 mg/kg every eight hr for 3 or 6 injections), the activity of PAPase in lung homogenate was similar to the levels obtained at the usual dose.

Steroid treatment for 12 or 24 hr did not significantly affect fetal body or lung weight; at 48 hr after two injections mean ± SE body (16.0 ± 0.7 g) and lung (0.441 ± 0.03 g) weight in 33 fetuses were decreased compared with 37 control fetuses (18.0 ± 0.5 g and 0.533 ± 0.02 g, respectively, $p < 0.05$).

In preliminary experiments we examined the DNA content and lipid concentration in four fetuses from a control litter and in four fetuses from each of three rabbits pretreated with betamethasone. There was no apparent effect of one dose of steroid for 12, 24 or 48 hr compared with mean ± SE control levels which were 8.45 ± 0.36 mg DNA/g lung wet weight, 2.29 ± 0.054 mg total tissue lipid/mg DNA, and

9.47 ± 0.094 μg P of disaturated lecithin/mg DNA.

DISCUSSION: In this study we administered corticosteroid to the doe, avoiding the apparent maturational effects caused by surgery and saline injection of the fetus (6,13). With two doses of betamethasone there was some growth retardation, probably secondary to premature placental aging (29). However, a relatively low dose of betamethasone, or of cortisol (7), avoids the severe growth retardation and fetal death associated with higher doses of maternal cortisol (30). We chose a dose of betamethasone equivalent to that which is used for prevention of RDS in the human fetus (1), and similar plasma levels and clearance rates of steroid were found. In this regard treatment of the pregnant rabbit with betamethasone appears to be an appropriate model for investigating the mechanism of glucocorticoid effects on lung development in the human fetus.

Our results indicate that maternal betamethasone treatment stimulates both PAPase activity and choline incorporation in fetal rabbit lung. There is a close association between these two events in regard to the dose of steroid required, the time course, and the extent of the increase. At 48 hr activity in both systems was greater after two doses rather than one dose of steroid, indicating that induction requires continued elevation of plasma glucocorticoid levels. The extent of the increase in both PAPase and choline incorporation during normal development is similar to that which occurs precociously after two days of betamethasone treatment. Thus, the level of PAPase, by controlling the supply of diglycerides, may be a key regulatory factor for the rate of surfactant synthesis during both normal and glucocorticoid-accelerated lung maturation.

One dose of maternal steroid apparently has little effect on tissue lipid concentrations, consistent with results obtained after direct treatment of the fetus (6,13). Further experiments, in more mature fetuses, are needed to examine the effect of betamethasone on morphology and on the phospholipid content of lung wash (13). Also, we found no increase in in two other lung enzymes which have been reported to be induced after steroid treatment

of the fetus. It is possible that the increase in choline phosphotransferase observed by Farrell and Zachman (4,5), using dipalmitin as substrate, resulted from increased levels of endogenous diglycerides which were secondary to enhanced PAPase activity. In a recent study Oldenborg and Van Golde (31) report that exogenous dipalmitin is not utilized as substrate for choline phosphotransferase in vitro. They suggest that assays performed with dipalmitin may measure changes in the content of endogenous diglycerides rather than alterations in the specific activity of choline phosphotransferase. In our study we used diolein which increased choline phosphotransferase activity three-fold over the basal level without added substrate.

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