Repetitive prenatal glucocorticoid therapy reduces oxidative stress in the lungs of preterm lambs

FRANS J. WALTHER,1 ALAN H. JOBE,2 AND MACHIKO IKEGAMI2
1Department of Pediatrics, Charles R. Drew University of Medicine and Science 90059, and Perinatal Research Laboratories, Harbor-UCLA Research and Education Institute, University of California Los Angeles School of Medicine, Los Angeles, California 90502; and 2Division of Pulmonary Biology, Children’s Hospital Medical Center, Cincinnati, Ohio 45229

Walther, Frans J., Alan H. Jobe, and Machiko Ikegami. Repetitive prenatal glucocorticoid therapy reduces oxidative stress in the lungs of preterm lambs. J. Appl. Physiol. 85(1): 273–278, 1998.—Repetitive courses of maternal prenatal glucocorticoids are often used in high-risk pregnancies with threatening preterm labor to induce lung maturation, but the effects on the cellular oxidant-antioxidant balance in the fetal lung have not been evaluated. We investigated the effect of repetitive treatment with glucocorticoids, beginning early in gestation, on oxidative stress in the preterm ovine lung. Pregnant ewes were randomized to receive one, two, three, or four doses of 0.5 mg/kg betamethasone or saline placebo at 7-day intervals on 104, 111, 118, and 124 days gestation (n = 11 for each group). All lambs were delivered preterm at 125 days gestation, and lung tissue was assayed for antioxidant enzymes, lipid hydroperoxides, and carbonyl proteins. Lung manganese superoxide dismutase, catalase, and glutathione peroxidase activity increased after 1 dose of betamethasone given at 104 days gestation, whereas copper-zinc superoxide dismutase activity increased after 2 doses given at 104 and 111 days gestation. The activity of all four antioxidant enzymes further increased with additional doses and was maximal after four doses of betamethasone. Lung lipid hydroperoxide levels and carbonyl protein content decreased stepwise after each dose of betamethasone and were lowest after four doses. Repetitive prenatal glucocorticoid therapy increases antioxidant enzyme activity and reduces oxidative stress in the lungs of preterm lambs, and these effects begin early in gestation and persist for 2–3 wk.

METHODS

Animal protocol. These experiments were performed in Western Australia with timed pregnant merino ewes with singleton pregnancies, confirmed by ultrasound at 60 days gestation, that were bred by the Western Australian Department of Agriculture. At 101 days gestation, the ewes were treated with an intramuscular injection of 150 mg medroxyprogesterone (Depo-Provera, Upjohn, Kalamazoo, MI) to minimize the occurrence of preterm labor and abortion induced by glucocorticosteroids in sheep (16). Three days later, at 104 days gestation, the ewes were randomized to receive an intramuscular injection of either normal saline or 0.5 mg/kg betamethasone (Celestone Chronicosde, Schering, New South Wales, Australia) and subsequent injections of saline or betamethasone at 7-day intervals. There were four treatment groups of 11 animals, each of which received betamethasone on 104 days gestation only (1 dose); on 104 and 111 days gestation (2 doses); on 104, 111, and 118 days gestation (3 doses); or on 104, 111, 118, and 124 days gestation (4 doses). Saline was injected on those days that betamethasone was not administered. A control group of animals received four
weekly injections of saline. The animals remained undisturbed except for the injections, and all fetuses were delivered by cesarean section at 125 days gestation. Delivery and postnatal assessment of the preterm lambs and the effects of prenatal betamethasone therapy on postnatal lung function, birth weight, and plasma cortisol, thyroid hormones, and catecholamine levels have previously been described in detail (13). In brief, the preterm lambs were sedated and mechanically ventilated with 100% oxygen to achieve mean arterial PCO2 values of ~50 Torr. After 40 min of ventilation, the lambs were killed with pentobarbital sodium, the chest was opened, and a pressure-volume curve was performed (13). Pieces of the right lower lobe of each lung were frozen for assay of antioxidant enzymes.

Pieces of ~300 mg of lung tissue were homogenized in 3 ml of 5 mM potassium phosphate buffer (pH 7.8) containing 1% Triton X-100 for 90 s at high speed with a Brinkmann polytron (Brinkmann Instruments, Westbury, NY). These lung homogenates were centrifuged at 15,000 g for 10 min at 4°C, and the supernatant was used for antioxidant enzyme assays, DNA, protein, and protein carbonyl content. For lipid hydroperoxide assays, pieces of ~300 mg of lung tissue were extracted with a modified Bligh and Dyer method by using dichloromethane and methanol (containing 50 µg/ml butylated hydroxytoluene to inhibit formation of additional lipid peroxides) as solvents (25).

Antioxidant enzyme activities and protein and DNA content. Antioxidant enzymes measured included MnSOD, CuZnSOD, catalase, and glutathione peroxidase activity. Total superoxide dismutase (SOD) activity was assayed by inhibition of the reduction of cytochrome c in the xanthine oxidase reaction (18) by using a modification described by Forman and Fisher (6). MnSOD was measured by repeating the assay after the addition of 1 mM potassium cyanide, which completely blocks the activity of CuZnSOD. CuZnSOD was determined by subtracting MnSOD from total SOD activity. One unit of SOD enzyme activity is defined as the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50%. Catalase activity was measured by the rate of reduction of H2O2 substrate, followed spectrophotometrically at 240 nm (11). One unit is defined as the amount of enzyme required to oxidize 1 nmol H2O2 substrate, and was calculated using an extinction coefficient of 0.046 mmol/cm2 at 240 nm. Glutathione peroxidase activity was assayed spectrophotometrically at 340 nm by the rate of oxidation of NADPH (21). The assay mixture for measurement of this cystolic enzyme includes cumene hydroperoxide as primary substrate, with sodium azide added to inhibit contributing activity from catalase enzyme. The amount of protein in the homogenates was determined by a modification of the Lowry method (17) by using bovine serum albumin as a standard. DNA was measured by using the diphenylamine reaction and calf thymus DNA as a standard (3).

Carbonyl protein content. Protein oxidation was determined by quantitating the carbonyl protein content of the lung homogenates. Carbonyl proteins were measured by reaction with 2,4-dinitrophenylhydrazine (DNPH) (22). After removal of nucleic acids with streptomycin sulfate, the proteins in the homogenates were reacted with 10 mM DNPH in 2.5 M HCl or 2.5 M HCl only and were precipitated with 20% TCA. The pellets were washed with 10% TCA and ethanol-ethyl acetate (1:1 vol/vol) to remove the free DNPH and lipid contaminants and were solubilized in guanidine hydrochloride. The difference in absorption between the DNPH-treated samples vs. their HCl control samples was determined spectrophotometrically at 370 nm. Total protein in the HCl-treated samples was determined spectrophotometrically at 280 nm by using bovine serum albumin dissolved in guanidine hydrochloride as a standard. The results were expressed as nanomoles of carbonyl per milligram protein by using a molecular absorption coefficient of 22,000 for the DNPH derivatives.

Lipid hydroperoxides. Lipid hydroperoxide levels were measured by a specific lipid hydroperoxide assay (K-Assay LPO-CC kit, Kamiya Biomedical, Seattle, WA) (24) in lung tissue samples extracted with dichloromethane and methanol. This assay uses the observation (20) that hemoglobin catalyzes the reaction of lipid hydroperoxides with the methylene blue derivative MCDP (10-N-methylcarbamoyl-3,7-dimethylaminio-10-H-phenothiazine), forming an equimolar concentration of methylene blue with maximum absorbance at 675 nm. Cumene hydroperoxide was used as an external standard in this assay.

The linear range of the Kamiya LPO-CC assay is between 2 and 300 nmoles/ml for quantitative determination, specificity is within ±10%, and absorbance varies <3% for 10 repeated assays. The assay specifically and directly measures lipid peroxides. The length of the hydrocarbon portion of the peroxides does not correlate with its reactivity with MCDP, but the structure appears to be more important. Each lipid alcohol has a different potential for oxidizing MCDP. Alcohols derived from R-OOH type peroxides (such as cumene hydroperoxide) all have relatively the same oxidation potential. Alcohols from R-OO-R type peroxides (such as benzoyl peroxide) have a lower oxidation potential. Because the assay measures color production within a set time, the latter peroxides will not be measured to the same degree since they oxidize little MCDP. Hydrogen peroxide, benzoinperoxides, and peracetic acid oxidize MCDP very slowly and will not be detected in the assay.

Statistics. All data are reported as means ± SD. Lung antioxidant enzyme activity is expressed as units per milligram DNA, protein oxidation as nanomoles of carbonyl per milligram protein, and lipid hydroperoxide levels as nanomoles per milligram DNA. Group means were compared by ANOVA, followed by post hoc analysis by using the Student-Newman-Keuls test when significant differences were detected. A P value < 0.05 was considered to be significant.

RESULTS

Total lung SOD activity (Fig. 1) was 70 ± 14 U/mg DNA in the saline controls and increased to 75 ± 16 U/mg DNA after one dose, to 88 ± 16 U/mg DNA after two doses (P = 0.01 vs. controls), to 101 ± 14 U/mg DNA after three doses (P < 0.001 vs. controls), and to 118 ± 21 U/mg DNA after four doses of betamethasone (P < 0.001 vs. controls). Like total SOD activity, both MnSOD and CuZnSOD activity (Fig. 1) increased stepwise with each dose of betamethasone and exceeded the activity of controls after, respectively, one to four (P < 0.01) and two to four doses of betamethasone (P < 0.05). The ratio of MnSOD to total SOD activity increased from 0.176 ± 0.022 in controls to 0.254 ± 0.021 after four doses of betamethasone (P < 0.001), indicating a relatively larger increase in MnSOD than in CuZnSOD activity with increasing doses of betamethasone. Total SOD and CuZnSOD activity was similar after two to three doses but further increased after a fourth dose of betamethasone (P = 0.04), secondary to a significant increase in MnSOD activity (P = 0.03).

Like SOD activity, both lung catalase and glutathione peroxidase activity increased with increasing doses
of betamethasone (Fig. 2). After one to four doses of betamethasone, lung catalase and lung glutathione peroxidase activity were significantly higher than in controls ($P < 0.005$ and $P < 0.05$, respectively). A fourth dose of betamethasone increased glutathione peroxidase ($P < 0.02$) but not catalase activity (Fig. 2).

Lung lipid hydroperoxide levels (Fig. 3) decreased with increasing doses of betamethasone from $25 \pm 3$ nmol/mg DNA in the controls to $15 \pm 2$ nmol/mg DNA after four doses of betamethasone ($P < 0.001$ for 1–4 doses vs. controls). Although lipid hydroperoxide levels were lowest after four doses of betamethasone, the differences between the groups treated with three or four doses were not significant.

Lung carbonyl protein content (Fig. 4) decreased with repetitive doses of betamethasone from $10.5 \pm 1.9$ nmol/mg protein in the controls to $4.0 \pm 0.9$ nmol/mg protein after four doses of betamethasone ($P < 0.001$ for 1–4 doses vs. controls). The differences in carbonyl protein between groups treated with three and four doses of betamethasone were significant ($P = 0.01$).

**DISCUSSION**

These experiments evaluated the persistence and efficacy of the antioxidant response to repetitive prenatal doses of glucocorticoids at 7-day intervals between 104 and 124 days gestation in the lungs of preterm lambs delivered at 125 days gestation. Lung antioxidant enzyme activity increased and lipid hydroperoxide levels and carbonyl protein content decreased with increasing doses of prenatal glucocorticoids, indicating a protective effect against oxidative stress. Prenatal glucocorticoid therapy was initiated early in gestation, and one maternal dose of betamethasone administered 21 days before preterm delivery had a persistent effect on lung antioxidant enzyme activity, lipid peroxidation, and protein oxidation. The antioxidant responses to two and three doses of glucocorticoids, administered 14 and 7 days before preterm delivery, were similar to and larger than the response to one dose of glucocorticoids, administered 21 days before delivery. The response to a fourth dose of prenatal glucocorticoids, administered 24 h before preterm delivery, was less consistent, because only MnSOD and glutathione peroxidase activity and carbonyl protein content changed significantly after the fourth dose. These data indicate that the effect of...
Antenatal glucocorticoids on lung antioxidant enzymes, lipid peroxidation, and protein oxidation persists for 14–21 days. We recently observed that the positive effect of a single fetal dose of betamethasone on lung antioxidant enzyme activity occurred within 24 h after exposure, persisted for a period of 7 days without a major change in the magnitude of the response, and led to a reduction in lipid hydroperoxide formation (29). The relatively fast and persistent antioxidant response to prenatal glucocorticoids in the fetal lamb lung not only underscores the efficacy of early and repetitive prenatal glucocorticoid administration but also suggests that repetitive doses at intervals longer than 7 days may be as effective to prepare the lung for postnatal oxidant stress.

Although the effects of prenatal glucocorticoid administration on fetal lung function and surfactant metabolism have been extensively researched, limited data are available on the effects on the antioxidant capacity of the fetal lung (7, 8, 15, 29, 30). The lung surfactant and antioxidant enzyme systems have similar development profiles: both mature in late gestation (9, 31) and accelerate their maturation in response to prenatal glucocorticoid therapy (7, 8, 15, 29, 30). This study is unique not only for its repetitive glucocorticoid dosing regimen beginning early in gestation but also because it describes the effects of prenatal glucocorticoid therapy on the activity of both cellular types of SOD instead of total SOD only. Glucocorticoids increased the activity of both types of cellular SOD but stimulated mitochondrial MnSOD activity to a greater extent than cytosolic CuZnSOD, providing pivotal resistance against oxidative stress in the mitochondria.

Oxidative stress, i.e., an increased exposure to oxidants and/or decreased antioxidant capacity, can lead to cell injury when free radicals attack cellular molecules such as lipids, proteins, and nucleic acids. Lipid peroxidation is a peroxyl radical-mediated process in which polyunsaturated fatty acids present in the cell membrane are transformed to lipid hydroperoxides. Oxidation of structural proteins or of enzymes by free radicals, e.g., alkoxy and peroxyl radicals, usually renders them dysfunctional because of modifications such as deamination, introduction of carbonyl groups (—C=O) into the side chains, and cleavage of peptide bonds. Quantitation of lipid peroxidation and carbonyl proteins provides a method to determine the extent of free radical-mediated tissue damage, but data on the levels of these macromolecules in the lung after prenatal glucocorticoid therapy have been limited to measurements of lipid hydroperoxide levels after a single fetal dose of betamethasone 24 h to 7 days before delivery at 128 days gestation (29). The progressive decrease in lipid hydroperoxide products and carbonyl proteins with increasing numbers of betamethasone doses suggests a reduction in the generation of free radicals in the preterm lamb lung secondary to increased lung antioxidant enzyme activity.

Prenatal glucocorticoid therapy accelerates normal events in the surfactant and antioxidant systems during the later stages of fetal lung development. A short-term exposure (24–48 h) has a rapid effect on lung structure and improves lung compliance, increases lung volume, and decreases capillary-alveolar protein leakage (14), whereas a more prolonged exposure results in a coordinated increase in the production and...
secretion of surfactant components (2, 13). Exposures as short as 24 h increase the activity of the antioxidant enzyme system (29), and the present data indicate that this effect persists for 2–3 wk. Although the delivery methods (fetal intramuscular injection vs. maternal treatment) and the gestational ages at delivery (128 vs. 125 days gestational age) are different, a quantitative comparison of the effect of a single fetal intramuscular dose of betamethasone at 124 days gestation (4 days before delivery at 128 days gestational age) in our previous study (31) with the effect of four maternal doses of betamethasone in this study shows only small, statistically insignificant differences in the lung antioxidant enzyme response. Multiple-dose treatment, however, leads to a larger reduction in lung lipid hydroperoxide products (average values decreased by 39 vs. 24%). The glucocorticoid-induced increase in antioxidant capacity can curtail free radical damage to the immature lung during ventilation and hyperoxia and reduce oxidative stress, an important cause of bronchopulmonary dysplasia in the ventilated preterm infant (28). In dual ways, prenatal glucocorticoid therapy improves lung function, reducing the need for ventilatory assistance (2), and augments antioxidant capacity, reducing oxidative stress in the fetal lung (29). These combined effects will ultimately decrease the incidence and severity of bronchopulmonary dysplasia and improve the outcome of preterm infants (5, 26, 33).

What is the physiological relevance of the lipid hydroperoxide and carbonyl protein levels relative to the ultimate development of oxygen-mediated lung injury? Immaturity is probably the most important factor explaining free radical-mediated lipid peroxidation and protein oxidation in the lungs of preterm infants (27, 28), and both lipid hydroperoxides and carbonyl proteins are related to the development of chronic lung disease. We presume that the magnitude of the antioxidant response to prenatal glucocorticoids affords some protection with prolonged oxygen exposure.

A possible limitation of this study is that the findings in preterm lambs may not be applicable across species. Preterm rats treated prenatally with dexamethasone manifest no increase in lung antioxidant enzyme activity (4) and develop pulmonary pathological abnormalities mimicking bronchopulmonary dysplasia and increased levels of lung hydroxyproline after prolonged exposure to hyperoxia. Furthermore, a recent preliminary study (23) detected higher rates of urinary malondialdehyde excretion in the first 3 days of life in a group of very-preterm infants who received prenatal beta-methasone treatment.

We conclude that, in fetal lambs delivered at 125 days gestation, repetitive prenatal glucocorticoid therapy increases lung antioxidant enzyme activity and persists over a period of 14–21 days. The increase in lung antioxidant enzyme activity is associated with reduced lipid hydroperoxide and carbonyl protein formation during immediate postdelivery oxygen exposure, indicating more efficient free radical scavenging in lung tissue after prenatal glucocorticoid therapy. These data suggest that fetal lung maturation of the antioxidant system resulting from prenatal glucocorticoid therapy far exceeds the now empirically used 1-wk time interval.

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Address for reprint requests: F. J. Walther, Harbor—UCLA REI, 1124 W. Carson St., RB-1, Torrance, CA 90502 (E-mail: fwalther@ucla.edu).

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