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

FRANS J. WALther, ALAN H. JOBE, and MACHIKO IKEgami

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 90022; 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. superoxide dismutase; catalase; glutathione peroxidase; lipid peroxidation; carbonyl proteins

METHODS

Prenatal glucocorticoid administration reduces the incidence and severity of the respiratory distress syndrome and decreases the incidence of patent ductus arteriosus, chronic lung injury (bronchopulmonary dysplasia), intracranial hemorrhage, necrotizing enterocolitis, and neonatal mortality (5, 33). On the basis of these findings, prenatal betamethasone therapy has been recommended for broad use in high-risk pregnancies with threatening preterm labor (19). The meta-analysis of Crowley (5) indicates that prenatal glucocorticoid therapy is optimally effective if the treatment-to-delivery interval is 24 h to 7 days (12). However, 20% of preterm deliveries occur within 24 h and 40–50% after a delay longer than 7 days (1, 12). As a result, obstetricians are frequently administering repetitive courses of maternal glucocorticoid therapy at 7-day intervals to achieve a maximal fetal response in pregnancies with early preterm labor (10, 32), although there are no randomized-controlled trial data to support this practice.

Oxygen-induced cellular damage is mediated by free radicals, such as superoxide, peroxides, and hydroxyl radicals. Free radical damage to immature lung tissue may be of bronchopulmonary dysplasia in preterm infants ventilated for respiratory distress syndrome (28). The primary defense against free radicals is provided by the intracellular antioxidant enzymes manganese superoxide dismutase (MnSOD), copper-zinc superoxide dismutase (CuZnSOD), catalase, and glutathione peroxidase. Lung antioxidant enzyme activity is low in preterm animals and increases rapidly, in parallel with phospholipid content, during the final 10–15% of gestation (9, 31). Prenatal glucocorticoids not only improve perinatal lung function but also accelerate the late gestational rise in fetal lung antioxidant enzyme activity (7, 8, 15, 29, 30). A single fetal dose of glucocorticoids injected between 24 h and 7 days before preterm delivery increases lung antioxidant enzyme activity and reduces lipid hydroperoxide formation during immediate postdelivery oxygen exposure in preterm lambs (29). The effects of repetitive courses of maternal prenatal glucocorticoids on the cellular oxidant-antioxidant balance in the fetal lung have not been evaluated. Therefore, we designed a multiple-retreatment protocol beginning early in gestation to characterize postnatal oxidative stress in the lungs of preterm lambs after up to four fetal exposures to betamethasone.

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 Chronodose, 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 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 catalyzing the reaction of lipid hydroperoxides with the methylenedimethyloxazoline (MDMPO) probe as described by using an extinction coefficient of 0.046 µmol/cm² at 240 nm. Glutathione peroxidase activity was assayed spectrophotometrically at 340 nm by the rate of oxidation of NADPH (21). The Glutathione peroxidase activity increased with increasing doses of betamethasone and exceeded the activity of CuZnSOD activity (Fig. 1) increased stepwise with increasing doses of betamethasone (Fig. 1) increased stepwise with increasing doses of betamethasone.

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

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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 betamethasone 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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REFERENCES


