Induction of thioredoxin and thioredoxin reductase gene expression in lungs of newborn primates by oxygen

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Das, Kumuda C., Xiao-Ling Guo, and Carl W. White. Induction of thioredoxin and thioredoxin reductase gene expression in lungs of newborn primates by oxygen. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L530–L539, 1999.—Thioredoxin (TRX) is a potent protein disulfide oxidoreductase important in antioxidant defense and regulation of cell growth and signal transduction processes, among them the production of nitric oxide. We report that lung TRX and its reductase, TR, are specifically upregulated at birth by O2. Throughout the third trimester, mRNAs for TRX and TR were expressed constitutively at low levels in fetal baboon lungs. However, after premature birth (125 or 140 of 185 days gestation), lung TRX and TR mRNAs increased rapidly with the onset of O2 or air breathing. Lung TRX mRNA also increased in lungs of term newborns with air breathing. Premature animals (140 days) breathing 100% O2 developed chronic lung disease within 7–14 days. These animals had greater TRX and TR mRNAs after 1, 6, or 10 days of life than fetal control animals. In 140-day animals given lesser O2 concentrations (as needed) who do not develop chronic lung disease, lung TRX and TR mRNAs were also increased on days 1 and 6 but not significantly on day 10. In fetal distal lung explant culture, mRNAs for TRX and TR were elevated within 4 h in 95% O2 relative to 1% O2, and the response was similar at various gestations. In contrast, TRX protein did not increase in lung explants from premature animals (125 or 140 days) but did in those from near-term (175-day) fetal baboons after exposure to hyperoxia. However, lung TRX protein and activity, as well as TR activity, eventually did increase in vivo in response to hyperoxia (6 days). Increases in TRX and TR mRNAs in response to 95% O2 also were observed in adult baboon lung explants. When TRX redox status was determined, increased O2 tension shifted TRX to its oxidized form. Treatment of lung explants with actinomycin D inhibited TRX and TR mRNA increases in 95% O2, indicating transcriptional regulation by O2. The acute increase in gene expression for both TRX and TR in response to O2 suggests an important role for these proteins during the transition from relatively anaerobic fetal life to O2 breathing at birth.

The biochemical adaptation of the lung to air or oxygen breathing at birth is incompletely understood. The sudden exposure of the air-blood interface of the lung to increased oxygen tension must pose an acute oxidative stress compared with the relatively anaerobic fetal environment. Indeed, the pulmonary epithelium usually is exposed to the highest oxygen tension present in the organism. In several mammalian species, there is a gradual increase in the lung activities of multiple antioxidant enzymes during the final 15–30% of gestation (17, 46). In the premature newborn, it has been suggested that failure to elevate the activities of these antioxidant enzymes can increase the lung damage caused by hyperoxia during treatment of respiratory distress syndrome (43).

Thioredoxin (TRX) is a potent protein disulfide oxidoreductase with importance in antioxidant defense (23, 30, 32, 37), regulation of cellular proliferation (5, 19), and regulation of gene expression through transcription factor activation (22, 31). This low-molecular-mass protein (12 kDa) has a highly conserved active site, Trp-Cys-Gly-Pro-Cys, present in plants, bacteria, and mammalian cells (23). TRX has been shown to have both direct and indirect antioxidant effects (6, 7, 14, 23, 24, 32). More recently, TRX has been shown to exert redox control over transcription factors in mammalian cells by dithiol-disulfide interchange to modulate their binding to DNA (22). In addition, TRX can reduce critical disulfides in numerous important proteins including glyceraldehyde-3-phosphate dehydrogenase (50) and endothelial nitric oxide synthase (eNOS) (35, 36), allowing activation of these enzymes. TRX, along with protein disulfide isomerase and related proteins, also plays an important role in the correct folding of newly synthesized proteins (10, 50). Although TRX expression has been detected in proliferating lung epithelium and other tissues in early human fetal life (9–23 wk) (18), little is known about its expression in the lung during the final third of gestation or the perinatal period. Because of the multiple functions of TRX in antioxidant defense and specifically the critical role of eNOS activity in producing NO during the fetal-to-neonatal transition, upregulation of the TRX system in the lung at birth could be an important biochemical event.

TRX reductase (TR; EC 1.6.4.5) is member of the pyridine nucleotide-disulfide oxidoreductase family that includes glutathione reductase, lipooamide dehydrogenase, mercuric reductase, and NADH peroxidase (47). Members of the family are homodimeric proteins, each subunit of which has a redox active disulfide and a tightly bound FAD group that mediates the transfer of reducing equivalents from NAPDH to its own disulfide bond and then to the disulfide bond of the substrate (47). Physiological substrates for TR include TRX and protein disulfide isomerase (29). TR has diverse functions in the cell. Among these, it provides reducing equivalents through TRX for ribonucleotide reductase, the first unique step in DNA synthesis.

Here we report that the expression of both TRX and TR is promptly upregulated in the newborn lung at

THE BIOCHEMICAL ADAPTATION OF THE LUNG TO AIR OR OXYGEN BREATHING AT BIRTH IS INCOMpletely UNDERstood. THE SUDDEN EXPOSURE OF THE AIR-BLOOD INTERFACE OF THE LUNG TO INCREASED OXYGEN TENSION MUST POSE AN ACUTE OXIDATIVE STRESS COMPARED WITH THE RELATIVELY ANAEROBIC FETAL ENVIRONMENT. INDEED, THE PULMONARY EPITHELIUM USUALLY IS EXPOSED TO THE HIGHEST OXYGEN TENSION PRESENT IN THE ORGANISM. IN SEVERAL MAMMALIAN SPECIES, THERE IS A GRADUAL INCREASE IN THE LUNG ACTIVITIES OF MULTIPLE ANTIOXIDANT ENZYMES DURING THE FINAL 15–30% OF GESTATION (17, 46). IN THE PREMATURE NEWBORN, IT HAS BEEN SUGGESTED THAT FAILURE TO ELEVATE THE ACTIVITIES OF THESE ANTIOXIDANT ENZYMES CAN INCREASE THE LUNG DAMAGE CAUSED BY HYPEROXIA DURING TREATMENT OF RESPIRATORY DISTRESS SYNDROME (43).

THIOREDOXIN (TRX) IS A POTENT PROTEIN DISULFIDE OXIDOREDUCTASE WITH IMPORTANCE IN ANTIOXIDANT DEFENSE (23, 30, 32, 37), REGULATION OF CELLULAR PROLIFERATION (5, 19), AND REGULATION OF GENE EXPRESSION THROUGH TRANSCRIPTION FACTOR ACTIVATION (22, 31). THIS LOW-MOLECULAR-MASS PROTEIN (12 kDa) HAS A HIGHLY CONSERVED ACTIVE SITE, TRP-CYS-GLY-PRO-CYS, PRESENT IN PLANTS, BACTERIA, AND MAMMALIAN CELLS (23). TRX HAS BEEN SHOWN TO HAVE BOTH DIRECT AND INDIRECT ANTIOXIDANT EFFECTS (6, 7, 14, 23, 24, 32). MORE RECENTLY, TRX HAS BEEN SHOWN TO EXERT REDOX CONTROL OVER TRANSCRIPTION FACTORS IN MAMMALIAN CELLS BY DIITHIOL-DISULFIDE INTERCHANGE TO MODULATE THEIR BINDING TO DNA (22). IN ADDITION, TRX CAN REDUCE CRITICAL DISULFIDES IN NUMEROUS IMPORTANT PROTEINS INCLUDING GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (50) AND ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) (35, 36), ALLOWING ACTIVATION OF THESE ENZYMES. TRX, ALONG WITH PROTEIN DIISULFIDE ISOMERASE AND RELATED PROTEINS, ALSO PLAYS AN IMPORTANT ROLE IN THE CORRECT FOLDING OF NEWLY SYNTHESIZED PROTEINS (10, 50). ALTHOUGH TRX EXPRESSION HAS BEEN DETECTED IN PROLIFERATING LUNG EPITHELIUM AND OTHER TISSUES IN EARLY HUMAN FETAL LIFE (9–23 wk) (18), LITTLE IS KNOWN ABOUT ITS EXPRESSION IN THE LUNG DURING THE FINAL THIRD OF GESTATION OR THE PERINATAL PERIOD. BECAUSE OF THE MULTIPLE FUNCTIONS OF TRX IN ANTIOXIDANT DEFENSE AND SPECIFICALLY THE CRITICAL ROLE OF eNOS ACTIVITY IN PRODUCING NO DURING THE FETAL-TO-NEONATAL TRANSITION, UPREGULATION OF THE TRX SYSTEM IN THE LUNG AT BIRTH COULD BE AN IMPORTANT BIOCHEMICAL EVENT.

TRX REDUCTASE (TR; EC 1.6.4.5) IS MEMBER OF THE PYRIDINE NUCLEOTIDE-DISULFIDE OXIDOREDUCTASE FAMILY THAT INCLUDES GLUTATHIONE REDUCTASE, LIPOOAMIDE DEHYDROGENASE, MERCURIC REDUCTASE, AND NADH PEROXIDASE (47). MEMBERS OF THE FAMILY ARE HOMODIMERIC PROTEINS, EACH SUBUNIT OF WHICH HAS A REDOX ACTIVE DISULFIDE AND A TIGHTLY BOUND FAD GROUP THAT MEDIATES THE TRANSFER OF REDUCING EQUIVALENTS FROM NAPDH TO ITS OWN DISULFIDE BOND AND THEN TO THE DISULFIDE BOND OF THE SUBSTRATE (47). PHYSIOLOGICAL SUBSTRATES FOR TR INCLUDE TRX AND PROTEIN DIISULFIDE ISOMERASE (29). TR HAS DIVERSE FUNCTIONS IN THE CELL. AMONG THESE, IT PROVIDES REDUCING EQUIVALENTS THROUGH TRX FOR RIBONUCLEOTIDE REDUCTASE, THE FIRST UNIQUE STEP IN DNA SYNTHESIS.

Here we report that the expression of both TRX and TR is promptly upregulated in the newborn lung at
birth in response to hyperoxia. Increases in both mRNAs, TRX protein, and both activities could be detected. Studies in fetal lung cultures demonstrated that ambient oxygen tension rather than some other factor related to labor, delivery, or the postnatal environment was crucial in regulating TRX and TR gene expression.

METHODS

Materials

Goat anti-human (h) TRX polyclonal antibody, hTRX, and bovine TR were purchased from American Diagnostica (Greenwich, CT). Digoxigenin (Dig)-3′-o-succinyl-e-aminoacproacid-N-hydroxysuccinimide ester, dimethyl sulfoxide, 2.2′-azino-di(3-ethylbenzthiazoline-6-sulfonate) (ABTS), and anti-Dig peroxidase were purchased from Boehringer Mannheim. Maxi-Sorp 8 strip modules were purchased from Nunc (Roskilde, Denmark). Normal goat serum and Tween 20 were obtained from Pierce Chemical (Rockford, IL). Actinomycin D was purchased from Calbiochem. All other chemicals were of the purest available grade. Buffers and solutions were prepared in Milli-Q water.

Animal Studies

All animal care procedures were performed according to the National Research Council’s Guide for the Care and Use of Laboratory Animals. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Southwest Foundation for Biomedical Research (San Antonio, TX). Fetal baboons of varying gestational ages (±2 days) were delivered by hysterotomy. Gestational ages were determined by timed matings as previously described, with confirmation by ultrasound at intervals during pregnancy. Fetal (gestational control) animals were killed at delivery before the first breath, and the lungs were processed immediately for biochemical assay. Fetal peripheral lung specimens were cut and frozen immediately in liquid nitrogen. For fetal lung explant culture (see Fetal Lung Culture), specimens (1–3 g) were cut, placed in sterile Waymouth medium (50 ml), and shipped overnight (at 4°C) to Denver, CO. In a few cases, lung specimens, obtained as above, were taken from newborn baboons after a natural delivery at term gestation (185 days).

In other studies pertinent to the effect of variable oxygen tension in vivo and to the development of bronchopulmonary dysplasia (BPD) after hyaline membrane disease, treatment groups were delivered at either 140 or 125 ± 2 days of gestation and immediately placed on positive-pressure ventilation. Animals of 140 days gestation were given either continuous 100% oxygen or an inspired oxygen tension as needed (pro re nata [PRN]) to maintain arterial PO2 at 40–50 Torr. Within 10 days, those 140-day animals given 100% oxygen develop lung histopathological lesions that closely resemble human BPD, whereas the PRN animals do not develop these lesions, allowing near-normal lung development (12, 13). More premature animals of 125 days gestation received immediate resuscitation with artificial surfactant, positive-pressure ventilation, and PRN oxygen. These animals also develop BPD despite receiving lesser (PRN) concentrations of oxygen (42). In either case, all animals received state-of-the-art care in a neonatal intensive care unit for up to 17 days. After treatment, the animals were killed by administration of intravenous pentobarbital sodium. The lungs were perfused via the pulmonary artery with phosphate-buffered saline (37°C), and distal lung tissue was dissected free from major airways and central structures and processed immediately as for fetal tissue.

Fetal Lung Culture

Distal fetal lung tissue (gestational age 125–175 days) was dissected into 1-mm3 pieces, with careful attention to removal of any attached airways. Explant pieces were placed at 4–5/well in 3.5-cm-diameter wells (6-well plates) that had been precoated with 2 ml of Waymouth A157 medium containing penicillin, streptomycin, and amphotericin B but lacking fetal bovine serum. This coating material also contained ultrapure agarose (0.5% wt/vol; Fisher) that had been dissolved by brief boiling (91°C in Denver) and was then allowed to harden on the plates by brief chilling. Plates were again warmed to room temperature before use. After they were placed on the plates, the explants were then overlayed with 1 ml of complete Waymouth A157 medium containing penicillin, streptomycin, and amphotericin B in addition to 10% fetal bovine serum. The explants were then placed in a 1% oxygen-5% carbon dioxide-94% nitrogen-containing certified gas mixture in a plastic modular exposure chamber (Billups-Rothenberg, La Jolla, CA) and allowed to equilibrate for 16 h (37°C). After this period of equilibration, the medium was changed, and the explants were exposed for an additional period of 24 h or more to otherwise identical conditions but with variable oxygen tension. After all exposures, the medium was aspirated and the explants were washed once with cold Hanks’ balanced salt solution. Thereafter, the explants were either homogenized in guanidinium isothiocyanate for mRNA analysis or frozen in liquid nitrogen until further biochemical analysis.

Isolation of Lung Total RNA and Northern Analysis

Lung tissue was homogenized in guanidinium isothiocyanate with a Virtishear homogenizer followed by centrifugation of the lysate in cesium chloride (147,000 g at 20–25°C) in a Beckman ultracentrifuge with a modified method of Sambrook et al. (40). Total RNA was precipitated spectrophotometricaly. Twenty micrograms of RNA were resolved by electrophoresis in 1% agarose-2.5 M formaldehyde gel in a buffer containing 20 mM MOPS and 1 mM EDTA (pH 7.4). RNA was transferred to nitrocellulose, and the blots were prehybridized for 2–12 h in 50% formamide, 0.75 M sodium chloride, 0.075 M sodium citrate (pH 7.0), 5× Denhardt’s solution, 50 µg/ml of salmon sperm DNA, and 0.1% SDS at 42°C. The blots were hybridized with cDNA for hTRX (a generous gift from Dr. E. E. Wollman, Hôpital Cochin, Paris) (48) and hTR (a generous gift from Dr. G. Powis, Arizona Cancer Center, Tucson, AZ) (20), labeled with [32P]UTP with a 5′-terminal DNA-labeling kit (GIBCO BRL). Autoradiographs were made by exposing blots to X-ray film (Kodak) at −70°C with intensifying screens. In some autoradiographs, densitometry was performed with Image 1.61 software (National Institutes of Health, Bethesda, MD). Some of the blots were exposed to phosphor screens (Molecular Dynamics), and densitometry was performed on phosphor screens with a Macintosh computer with Molecular Dynamics ImageQuant version 3.3 software.

Quantitation of TRX Protein by ELISA

Lung tissue stored in liquid nitrogen was homogenized in 50 mM Tris-HCl (pH 7.5) containing EDTA (1 mM), phenylmethylsulfonyl fluoride (1 mM), leupeptin (20 µg/ml), and antipain (20 µg/ml). The lung homogenate was centrifuged at
27,000 g for 10 min. The protein concentration in the supernatant was measured with the Bradford (9) method (Bio-Rad) with BSA as a standard. ELISA was performed as previously described (15). Briefly, microtiter plates were coated with goat polyclonal anti-hTRX (100 µl, at a concentration of 1 µg/ml in PBS). After a wash, nonspecific sites were blocked by adding 10% goat serum in PBS (vol/vol; 200 µl) for 60 min at room temperature. Fifty microliters of standards or samples were added to the microtiter wells, and the plates were incubated at 37°C for 2 h. One hundred microliters of anti-hTRX-Dig were added to each well (1 µg/ml sample buffer). The plates were incubated at 37°C for 1 h. After the wells were washed four times with PBS containing 0.05% Tween 20 (250 µl), the wells were incubated with 100 µl of anti-Dig peroxidase and incubated for 1 h. After a wash, ABTS substrate solution was added, and the plates were incubated at room temperature. During incubation at room temperature, color development was observed from 2 to 20 min. Absorbance was measured at 405 nm in a microplate reader (Molecular Devices).

Quantitation of TRX and TR Activities

TRX activity assay was performed as described by Holmgren and Bjornstedt (24). Briefly, the reaction mixture contained NADPH (200 µM), porcine insulin (80 µM; Sigma), and bovine TR (0.1 µM) in 0.05 M potassium phosphate buffer (pH 7.0) containing EDTA (1 mM) in a total volume of 0.5 ml. The reaction was started by the addition of bovine TR (0.1 µM). TRX activity was calculated as micromoles of NADPH oxidized per minute per milligram of protein at 25°C.

Mammalian TRX reduces 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by NADPH, the reduction of which is catalyzed by TR. The product in the reduction, 5′-thionitrobenzoic acid, is yellow and has an absorbance maximum at 412 nm. We used this reduction of DTNB to determine TR activity in an assay described by Holmgren and Bjornstedt (24). Briefly, the reaction mixture contained NADPH (200 µM), DTNB (5 mM), 1% BSA, and 1% ethanol in potassium phosphate buffer (pH 7.0) containing EDTA (10 mM) in a total volume of 500 µl. The reaction was started by the addition of tissue homogenates. TR activity is expressed as units of absorbance at 412 nm as described by Holmgren and Bjornstedt.

Determination of Redox State of TRX

Carboxymethylation of baboon lung tissue. Carboxymethylation of baboon tissue was done by a modified method of Kralis and Holmgren (27). Briefly, baboon lung tissue (5–20 mg) or lung tissue from lung explant culture was homogenized in 0.5 ml of carboxymethylation buffer (0.1 M Tris·HCl, pH 8.8, 6 M guanidine hydrochloride, and 10 mg/ml of iodoacetic acid) de-aerated in argon for 2 h. After homogenization of the tissue, 5 µl of 10% Triton X-100 were added to tubes, and the tubes were incubated for 1 h at 37°C in the dark. At the end of incubation, samples were centrifuged in a tabletop refrigerated centrifuge (Beckman) for 10 min at 2,500 rpm. The supernatant (0.5 ml) was transferred to PD-10 desalting columns (Pharmacia Biotech) to remove excess guanidine hydrochloride. Protein content was determined by the Bradford (9) method (Bio-Rad).

Western blotting of TRX. Twenty micrograms of carboxymethylated baboon lung homogenate were fractionated on a 15% native polyacrylamide gel (Bio-Rad) along with carboxymethylated hTRX standards, both oxidized and reduced. The protein was transferred to nitrocellulose (Hybond-ECL, Amersham) with a Mini-PROTEAN transfer apparatus (Bio-Rad). Nitrocellulose was washed and incubated with anti-goat IgG to hTRX (American Diagnostica, Greenwich, CT). After being washed, the blot was incubated with goat IgG-horseradish peroxidase conjugate (Pierce) for 1 h at room temperature. Binding of secondary antibody was detected with the Amersham electrochemiluminescence detection system.

Statistical Analysis

Mean values were calculated by averaging data from each experimental group, and the SE of each group was calculated. Means of two groups were compared by two-tailed unpaired t-test, and those from more than two groups were compared by one-way analysis of variance (39). A P value of <0.05 was considered significant.

RESULTS

Effect of Fetal Gestational Age and Air Breathing in the Newborn on Expression of Baboon Lung TRX and TR mRNAs

There was no change in expression of TRX or TR mRNA in fetal baboon lungs during the final third of gestation (125–175 days; data not shown). Thus there was no evidence for developmental regulation of TRX or TR gene expression in the low oxygen tension of the late-gestation fetal environment. A significant increase in lung TRX, but not in TR mRNA occurred by day 2 of life in air-breathing term newborn baboons compared with that measured in late-gestation (175-day) fetal control lungs (Fig. 1).

Effect of Birth and Respiratory Distress on Lung TRX and TR mRNAs

To investigate the effect of oxygen breathing on TRX and TR gene expression, premature newborn baboons were exposed to oxygen as described in METHODS. Lung TRX mRNA was significantly elevated in 140-day-gestation newborns receiving either 100% oxygen or PRN oxygen for 6 or 10 days. The mean inspired oxygen concentration for animals in the PRN group was 45% on day 6 and 30% on day 10. Although TRX mRNA was
In contrast to the situation with TRX, 140-day newborns breathing PRN oxygen did not have significant elevations in lung TR mRNA at age 6 (mean 45% oxygen) or 10 days (mean 30% oxygen; Fig. 2B). On the other hand, newborns exposed to 100% oxygen for 6 or 10 days did have significant elevations in lung TR mRNA (Fig. 2B). Newborns at 24 h of age in the 140-day PRN and 100% oxygen groups had lung TR mRNA levels similar to those seen at 6 days of age in the respective groups (data not shown). Again, there were too few such animals for statistical analysis. In 125-day animals given PRN oxygen, elevations in lung TRX mRNA comparable to those observed in 140-day animals exposed to 100% oxygen persisted after 6, 10, 14, and 17 days (data not shown). Similarly, TR mRNA increased after 24 h and remained elevated in 6-day PRN and 10-day PRN oxygen animals compared with gestational control animals.

Effect of Oxygen on TRX mRNA Expression in Fetal Lung Explant Culture

To isolate the acute effects of hyperoxia from other early postnatal factors in respiratory distress syndrome such as barotrauma and bacterial colonization, a fetal lung explant culture system was employed. Exposure to 95% oxygen in Denver (~80% oxygen at sea level) for 24 h caused an increase in TRX (Fig. 3A) and TR mRNAs (Fig. 3B) relative to the levels of these mRNAs during continued culture in 1% oxygen. The relative increases in TRX and TR mRNAs between 1 and 95% oxygen were fairly constant among the various gestational ages evaluated (125–175 days), and the capacity to increase these messages in response to hyperoxia was also retained in lung tissue explants from adult baboons (data not shown). To evaluate the rate of response of gene expression of the TRX system to hyperoxia, we exposed lung explants to either 1 or 95% oxygen for 1, 2, or 4 h. As demonstrated in Fig. 3C, increases in TRX and TR mRNAs occurred within 4 h. This demonstrates the relatively rapid induction of the TRX system in response to hyperoxia.

Effect of Oxygen on TRX Protein and TRX and TR Activities In Vivo

On day 6 of life, TRX protein was increased in lungs of 140-day premature newborn baboons exposed to 100% oxygen as determined by ELISA (Fig. 4A). In these studies, lungs of 140-day premature newborn baboons exposed for 10 days to 100% oxygen also had increased TRX protein levels. However, there was no significant difference between 6- or 10-day 100% oxygen exposures. In addition, TRX protein activity was increased significantly in both the 6-day PRN and 6-day 100% oxygen groups compared with the gestational control group (140 days) as determined by insulin reduction assay (P < 0.005; Fig. 4B). There was no significant difference between the 6-day PRN and 6-day 100% oxygen groups. In addition, similar increases in TR activity were observed in the lungs of 6-day PRN and 6-day 100% oxygen newborns compared with those

Fig. 2. Effect of birth and respiratory distress on lung TRX and TR mRNAs. A: effect of oxygen exposure on TRX mRNA standardized to 28S rRNA. Lung tissue was obtained from 140-day gestational control or fetal premature baboons delivered by cesarean section and exposed to various oxygen tensions as described in METHODS. Tissue was homogenized in GITC and processed for total RNA, and TRX mRNA was determined by hybridizing total RNA with cDNA probes for TRX as described in METHODS. GC, 140-day fetal premature baboons (n = 5) delivered and immediately killed and lung tissue processed for RNA isolation; 6d pm O2 and 10d pm O2, 140-day fetal premature baboons (n = 3/group) delivered and ventilated with 95% oxygen for 6 and 10 days, respectively, in critical care unit and killed after this treatment; 6d 100% O2 and 10d 100% O2, 140-day fetal premature baboons (n = 4/group) delivered and ventilated with 100% oxygen administered continuously for 6 and 10 days, respectively, in critical care unit and killed after this treatment. Blot was stripped and retreated with 28S rRNA. Quantitative densitometric data are shown. * Significant difference from GC.
Effect of Oxygen on TRX and TR Activities in Lung Explant Culture

To isolate the effect of oxygen on lung TRX and TR protein activities, we exposed explants of fetal lungs of 125, 140, and 175 days gestation to 1 or 95% oxygen for 24 h and processed for total RNA. Total RNA was hybridized with cDNA probes for human TRX (A), TR (B), and 28S rRNA (28S; A and B). Lanes 1–3, explants exposed to 1% oxygen; lanes 4–6, explants exposed to 95% oxygen. C: effect of oxygen on TRX and TR mRNAs shown by acute time course in fetal lung explant explants cultured as described in METHODS.

Effect of Oxygen on TRX Protein in Vivo

Lung tissue was obtained from 140-day gestational control and fetal premature baboons delivered by cesarean section and exposed to various oxygen tensions for 6 days as described in METHODS. Tissue was homogenized in homogenization buffer, and ELISA was performed in duplicate as described in METHODS. B: effect of oxygen on TRX activity in vivo. Lung tissue was obtained from 140-day gestational control and fetal premature baboons delivered by cesarean section and exposed to various oxygen tensions for 6 days as described in METHODS. Tissue was homogenized in homogenization buffer, and TR activity assay was performed in duplicate as described in METHODS. A412, absorbance at 412 nm. *Significant increase in TRX protein (A), TRX activity (B), and TR activity (C) compared with GC.
48 h. TRX activity was determined by insulin reduction assay. There was no significant difference in the TRX activity of more premature fetal 125- or 140-day explants exposed to 1 or 95% oxygen. However, there was a significant increase in TRX activity in explants from near-term, 175-day fetal lungs exposed to 95% oxygen compared with those incubated in 1% oxygen (Fig. 5A). In addition, increases in TR activity were observed in fetal lung explants exposed to 95% oxygen compared with 1% oxygen exposure as determined by DTNB reduction assay (Fig. 5B). In contrast to TRX, TR activity was significantly increased in 95% oxygen in explants from 125- or 140-day baboons. However, in the near-term animals (175 days), the increase in TR activity was not significant.

Effect of Actinomycin D on TRX and TR Increase by Oxygen in Lung Explant Culture

To determine whether the increase in TRX and TR mRNAs after exposure to oxygen was mediated at the transcriptional level, we incubated lung explants in 1 or 95% oxygen with actinomycin D (1 µg/ml), an inhibitor of transcription. As shown in Fig. 6, actinomycin D abolished increases in both TRX and TR mRNAs by 95% oxygen in lung explants. These results indicate that the increases in TRX and TR mRNAs caused by elevated oxygen tension occur at the level of transcription.

Effect of Oxygen on Redox State of TRX In Vivo and in Lung Explant Culture

Because the biological effects of TRX are mediated predominantly by its reduced form, we sought to determine the redox status of TRX in respiratory distress as well as in lung explant cultures exposed to variable oxygen tension. Because hTRX contains five cysteine residues, these cysteines can be present in different states of oxidation, including dimerized TRX via Cys73 oxidation (21). Electrophoretic analysis by SDS-PAGE of freshly prepared hTRX shows a mixture of five bands of apparent molecular masses ranging from 8.1 to 11 kDa (21). Air oxidation results in the disappearance of some bands and causes formation of a 23-kDa band due to formation of TRX dimer. In Fig. 7, lane 1, hTRX was oxidized by exposure to air, showing a high-molecular-mass band representing the homodimer (21) as well as smaller oxidized TRX bands. As demonstrated in Fig. 7, lanes 3–5, oxygen exposure increased the relative proportion of oxidized-to-reduced TRX levels in near-term infant baboons exposed to PRN or 100% oxygen. Increased oxidized TRX levels were also
observed in lung explants exposed to 95% oxygen (Fig. 7, lanes 6–8). In addition, TRX dimers were also detected in explants exposed to various concentrations of oxygen. Because these explants were exposed to air during shipment before being cultured, there could have been increased oxidation of TRX, resulting in the formation of dimers. Samples obtained in vivo did not show dimer formation. In Fig. 7, lane 3, 140-day fetal baboon lungs showed a comparable distribution of oxidized and reduced TRX. In Fig. 7, lane 4, after 6-day PRN oxygen in vivo, most of the total TRX was found to be in the oxidized state. In Fig. 7, lane 5, 140-day newborn baboon lungs exposed to 95% oxygen in vivo showed increased TRX protein, and again, most of the TRX present was in the oxidized state. Similar results were obtained in the 140-day lung explant culture. Explants exposed to 1% oxygen showed a similar TRX distribution as that of fetal baboons in vivo, whereas 95% oxygen caused a shift in the TRX redox balance toward the oxidized state. These observations suggest that there may be a limitation in availability of reduced TRX during the evolution of neonatal respiratory distress in premature lungs.

**DISCUSSION**

The data presented here indicate that the principal proteins of the TRX system, TRX and TR, are not developmentally regulated in the prenatal period, but instead, their expression appears to be controlled primarily by ambient oxygen tension postnatally. This pattern of expression differs considerably from a number of classic antioxidant enzymes (17, 25, 43, 46), although it is clear that oxygen can play an important role in modulating expression of some of these in the newborn (25). Not only are the mRNAs encoding both TRX and TR elevated in response to oxygen in vivo, but TRX protein and both TRX and TR activities also increased in the lungs of newborn baboons exposed to oxygen. At 6 days after birth in 140-day-gestation animals, the increase in both messages and activities occurred to a comparable extent in both 100% oxygen-exposed animals who were destined to develop BPD (12, 13) and those exposed only to PRN oxygen who do not acquire chronic lung disease. In 100% oxygen-exposed newborns, elevation of lung TRX and TR mRNAs persisted even after 10 days, whereas TRX mRNA expression declined again in PRN oxygen-treated animals as the inspired oxygen tension was weaned. Although only a small number of newborns were available for study at this time point, newborn baboons exposed to 100% or PRN oxygen for only 24 h also had a strong elevation in both messages. Elevation of TRX and TR mRNAs in response to elevated oxygen tension occurred in premature newborns of each gestational age (125 and 140 days). Spontaneously delivered term newborns had increased lung TRX mRNA, but not TR mRNA, in response to air breathing alone. Thus pulmonary TRX and TR gene expression do not appear developmentally regulated during the final third of gestation, and upregulation of these mRNAs in response to oxygen is unimpaired in newborns throughout this interval.

In contrast to upregulation of mRNA, data obtained from fetal lung explants cultured in different oxygen concentrations indicated that there was a gestational spectrum of TRX protein activity expression in response to hyperoxia in vitro. Specifically, lung tissue from the most premature baboon fetus studied (125 days, approximately equivalent to a 24- to 26-wk human fetus) did not increase TRX protein in response to 95% oxygen over this interval. Lung tissue from animals of 140 days gestation showed a nonsignificant tendency to increase TRX protein, whereas explants from animals approaching term gestation (175 days) showed a substantial elevation in TRX activity in response to hyperoxia. Besides facilitating evaluation of the effects of acute exposures, the explant system allows the isolation of the effect of oxygen tension from other factors such as bacterial or fungal colonization or infection, inflammation, exogenous cytokines, and baro-
trauma that may be present in the intubated, artificially ventilated newborn. Data obtained from newborns in vivo after 6 days of exposure indicated that, by this time point, expression of TRX activity in response to elevated oxygen tension in both 100% and PRN oxygen-exposed groups was not impaired in 140-day animals. However, the various postnatal factors listed above might have contributed to the elevation of TRX expression in vivo at any of these gestational ages. Considerable caution is necessary when attempting to extrapolate in vitro findings to the in vivo situation. However, based on the data from the explant system, it appears that the TRX protein response to hyperoxia in lungs of the most premature newborns could be delayed.

The reduced form of TRX is responsible for most of the known biological functions of the protein. A number of parallel observations can be made with regard to the studies of the redox state of lung TRX in the in vivo studies from fetal and newborn baboons and in the fetal baboon lung explant culture system. First, the proportion of reduced TRX is considerably greater in fetal lung tissue and in fetal lung explant tissue incubated in 1% oxygen than is present at higher oxygen tensions in vivo or in vitro. Second, the oxidized dimeric form of TRX was detectable at the highest oxygen tension both in vivo and in vitro. The TRX active site is blocked by dimer formation, and the dimer is not a substrate for TR (5). Third, it is apparent that expression of TRX protein both in vivo and in the explant system is elevated under both high and low oxygen tensions relative to that noted at more physiological ambient levels of oxygen present in postnatal life (21%). Although the effects of hyperoxia have not been described, elevation of expression of both TRX and TR mRNAs by hypoxia has been reported previously (5). Thus the lung TRX redox status, in addition to the expression of the protein and its reductase, are dynamically modulated by oxygen tension.

The potential sources of variability within the data deserve discussion. Within the fetal lungs and in the explant culture system, variability often tended to be small. Explant cultures were always short term (≤48 h), whereas most of the in vivo data were derived from animals exposed for longer durations (6–10 days or more). Samples from animals who died suddenly from causes such as pneumothorax after shorter exposures (<48 h) were few, and statistical comparisons of these data with the longer-exposure duration groups were not possible. Samples from fetal animals near term and after delivery at term also were relatively few in number. Given that multiple groups of investigators shared tissues from each animal, sample sizes (in mg of tissue) also were sufficiently small that repeated assays on the same tissues were generally not possible, especially from lungs of smaller, more premature animals. In addition, other forms of sampling variation were possible. Within PRN and 100% oxygen-exposed groups, the degree of illness tended to be relatively homogenous among 140-day animals such that PRN oxygen animals usually were recovering from respiratory distress by age 6–10 days, whereas 100% oxygen-exposed animals had increasingly severe respiratory distress and BPD. Within the latter group, the lungs of BPD animals usually showed alternating areas of hyperinflation and atelectasis (12, 13), which can affect tissue ventilation, perfusion, and oxygenation. In addition, areas of edema, hemorrhage, and/or inflammation can also be present, and these, too, tend to increase with duration of illness. These types of disease involvement in the lung are not altogether uniform. Although all samples were obtained from peripheral lung, they were also subject to developmental differences in cell types present at different gestational ages.

Despite the potential variation in results that might have resulted from local or regional differences, certain discrepancies in the data would not appear likely on these bases. For example, within the 140-day PRN oxygen-exposed group, lung TR activity showed a much greater increase (Fig. 4C) than lung TR mRNA (Fig. 2B; age 6 days for both). These animals had been exposed to higher oxygen concentrations, especially during the first 2–3 days of life, and it is possible that elevated TR mRNA and protein activities had occurred earlier in response to those oxygen tensions, with persistence of the elevated TR activity. However, such findings also open the possibility that posttranscriptional factors could contribute to elevated TR expression in hyperoxia. The same possibility can be acknowledged regarding TRX expression, although we tended to find relatively parallel changes in TRX mRNA and activity.

Elevation of expression of TRX and TR by oxygen could be beneficial through 1) direct and indirect antioxidant effects of TRX and TR (6, 7, 16, 23, 30, 44, 50), 2) supply of reducing equivalents to TRX-dependent peroxidases (peroxiredoxins) (11, 33), and/or 3) activation of manganese superoxide dismutase gene expression (14). Perhaps more importantly, the TRX system ensures the maintenance of reduced thiol groups essential for activity of numerous enzymes. Among these, eNOS (35, 36), itself upregulated, at least in part, by oxygen at birth (8), plays a key role in pulmonary vasodilatation during the perinatal transition (1). In addition, the TRX system can reactivate sulfhydryl-containing enzymes that are inactivated by oxidative stress (10, 16, 23, 32, 36, 44, 50) such as the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (16, 30). Elevation of glycolysis in lungs appears to be an essential component of adaptation to oxidative stress (2–4, 41). The potential importance of the TRX system in adaptation to hyperoxia is also indicated by transcriptional upregulation of gene expression for TRX (34) and TRX-linked thiol peroxidase (26) in response to oxygen in bacterial species. Moreover, in eukaryotes, proteins containing the TRX active site have been detected in the mitochondrion (45), nucleus (28), plasma membrane (49), and extracellular space (38), and two of these appear to be unique proteins (28, 45). Taken together, these data suggest that increased expression of proteins of the TRX system by oxygen could have an important protective role in the mammalian lung.
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REFERENCES


