O₂-evoked regulation of HIF-1α and NF-κB in perinatal lung epithelium requires glutathione biosynthesis

JOHN J. E. HADDAD AND STEPHEN C. LAND
Oxygen Signalling Group, Tayside Institute of Child Health, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, United Kingdom

Haddad, John J. E., and Stephen C. Land. O₂-evoked regulation of HIF-1α and NF-κB in perinatal lung epithelium requires glutathione biosynthesis. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L492–L503, 2000.—To test the genetic capacity of the perinatal lung to respond to O₂ shifts that coincide with the first respiratory movements, rat fetal alveolar type II (fATII) epithelial cells were cultured at fetal distal lung PO₂ (23 Torr) and then exposed to postnatal (23–76 Torr; mild hypoxic shift), moderate (23–152 Torr; moderate hypoxic shift), or severe (23–722 Torr; severe hypoxic shift) oxygenation. Nuclear abundance and consensus binding characteristics of hypoxia-inducible factor (HIF)-1α and nuclear factor (NF)-κB (Rel A/p65) plus glutathione biosynthetic capacity were determined. Maximal HIF-1α activation at 23 Torr was sustained over the postnatal shift in (Δ) PO₂ and was elevated in vivo throughout late gestation. NF-κB was activated by the acute postnatal ΔPO₂ in fATII cells, becoming maximal with moderate and severe oxygenation in vitro and within 6 h of birth in vivo, declining thereafter. fATII cell and whole lung glutathione and GSH-to-GSSG ratio increased fourfold with a postnatal ΔPO₂ and were matched by threefold activity increases in γ-glutamylcysteine synthetase and glutathione synthase. GSH concentration depletion by l-buthionine-(S,R)-sulfoximine abrogated both HIF-1α and NF-κB activation, with HIF-1α showing a heightened sensitivity to GSH concentration. We conclude that O₂-linked genetic regulation in perinatal lung epithelium is responsive to developmental changes in glutathione biosynthetic capacity.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

PO2 dependence for buffering the production of ROS is in place.

Lung maturation throughout gestation and in the postnatal period each occur within widely different PO2 values. In utero, saccularization of the lung and the functional differentiation of the epithelial lining proceed at a PO2 that lies continuously between 23 and 30 Torr, the O2 transfer capacity of the umbilical vein (13). During parturition, Na+–driven fluid absorption drains the fluid support within the lung, ventilation begins, and luminal end-tidal PO2 rapidly rises to stabilize at within a range of 70–100 Torr. The successful transition from placental to pulmonary gas exchange therefore incurs a four-to-fivelfold relative hyperoxic shift at the epithelial lining of the distal lung that superimposes on hormonally regulated perinatal developmental events such as the expression and production of surfactant (1, 2, 30), expression of ion-transport pathways and their components (24), expansion of gas-exchange surfaces, and alveolarization (45). Despite a late-gestation increase in expressed antioxidant enzymes, the full complement of antioxidant defenses in the fetal lung remains approximately threefold lower than that in the neonatal lung and sixfold lower than that in the adult lung (5, 16). It would therefore appear that the epithelial lining of the perinatal lung possesses a depressed capacity for buffering the production of ROS and, as such, may be acutely responsive to fluctuations in O₂ availability. The foundation is laid for an important redox-linked signaling event unique to the period immediately after the first breath, which may modulate the pattern of gene expression in the epithelial lining of the lung.

The transduction of an O₂ signal to the level of gene expression requires the nuclear translocation and activation of redox-responsive transcription factors over specific ranges of PO2. Hypoxia-inducible factor-1α (HIF-1α) and nuclear factor-κB (NF-κB) are activated by hypoxia and oxidizing signals, respectively, and are therefore functionally poised to coordinate the expression or suppression (i.e., by the loss of transcription factor activity) of genes in response to ΔPO2 regimens in the lung epithelium during the birth transition. The activation of HIF-1α is consistent with its role in coordinating adaptive homeostatic responses to hypoxia by regulating the expression of vascular, glycolytic, and cell cycle regulatory genes in a wide variety of tissues (8, 11, 22), whereas NF-κB, first identified as a factor that regulates the expression of the k light chains
in mouse B lymphocytes (39), is central to the expression of stress response genes involved in modulating the sensitivity of the cell to oxidative injury (12, 36). Thus a molecular switching mechanism that may integrate gene expression with the prevailing P O2 during the transition at birth is in place in the fetal lung epithelium.

To test the concept that the fetal distal lung epithelium is functionally responsive to shifts in O2 availability that are representative of the birth transition and beyond, we derived the following hypotheses: 1) recreation of mild (23—76 Torr), moderate (23—152 Torr), and severe (23—722 Torr) hyperoxic shifts in isolated fetal alveolar type II (fATII) epithelial cells will result in the differential activation of HIF-1α and NF-κB, 2) this activation will be paralleled by an altered redox potential as reflected in the glutathione (GSH-to-GSSG) ratio and glutathione biosynthetic capacity, and 3) the perinatal lung will experience parallel variations in glutathione homeostasis and activation of genetic regulatory factors in response to birth into an O2-rich environment. Our results provide evidence in support of a functional O2-linked, glutathione-buffered O2 signaling pathway that regulates the pattern of gene expression during the transition from placental to pulmonary gas exchange in the distal lung.

MATERIALS AND METHODS

All experimental procedures involving the use of live animals were approved under the Animals (Scientific Procedures) Act, 1986 (United Kingdom).

Primary Culture of fATII Cells

Fetal rats were removed from pregnant Sprague-Dawley rats by cesarean section on day 19 of gestation (term = 22 days), and the lungs were excised, teased free from the heart and upper airway tissue, and finely minced, then washed free of erythrocytes with sterile, chilled Mg2+- and Ca2+-free Hank’s balanced salt solution (0.5 ml/fetus). The cleaned lung tissue was resuspended in 1 ml/fetus of Hank's balanced salt solution containing trypsin (0.1 mg/ml), collagenase (0.06 mg/ml), and DNase I (0.012% wt/vol) and agitated at 37°C for 20 min. The solution was then centrifuged at 100 g for 2 min to remove undispersed tissue, the supernatant was saved to a fresh sterile tube, and an equal volume of Dulbecco’s modified Eagle medium (DMEM) with 10% (vol/vol) fetal calf serum (FCS) was added to the supernatant. After the supernatant was passed through a 120-µm-pore sterile mesh, the filtrate was centrifuged at 420 g for 5 min, the pellet was resuspended in 20 ml of DMEM-FCS, and the cells were placed in a T-150 culture flask for 1 h at 37°C to enable fibroblasts and nonepithelial cells to adhere. Unattached cells were washed three times by centrifugation at 420 g for 5 min each and then seeded onto 24-mm-diameter Transwell clear permeable supports (0.4-µm pore size; Costar) at a density of 5 × 10⁶ cells/filter and allowed to adhere overnight at a fetal distal lung PO2 (23 Torr, ~3% O2-5% CO2). DMEM-FCS was exchanged for 4 ml of serum-free PC-1 medium (BioWhittaker) preequilibrated to a Po2 of 23 Torr at 37°C 24 h later, and the cells were maintained at this Po2 until the experiment. Cultures maintained this way remained viable for at least 96 h (adenylylate energy charge ≥ 0.7; monolayer transepithelial resistance ≥ 250 Ω·cm²).

Mild, moderate, and severe Po2 regimens were re-created with four variable O2-Po2 incubators (Biotech Galaxy) preset to fetal distal lung Po2 (23 Torr, ~3% O2-5% CO2), early postnatal alveolar Po2 (76 Torr, ~10% O2-5% CO2), mild hyperoxia (152 Torr, ~21% O2-5% CO2), and severe hyperoxia (722 Torr, ~95% O2-5% CO2). After at least 24 h of culture at 23 Torr, the cultures were placed into an equivalent volume of PC-1 medium that had been previously equilibrated (24 h) to each Po2 for 4 h before extraction as detailed in Cell Harvesting and Nuclear Protein Extraction.

Cell Harvesting and Nuclear Protein Extraction

Nuclear extracts were prepared from monolayers of fATII cells as detailed elsewhere (43), with minor modifications. The filters were washed twice in 5 ml of ice-cold O2-preequilibrated PBS, and the cells (1–2 × 10⁶Po2 regimen) were collected and centrifuged at 420 g for 5 min at 4°C. Nuclei were released by resuspending the pellet in 250 µl of buffer A containing (in mM) 10 Tris·HCl (pH 7.8), 10 KCl, 2.5 NaHPO4·2H2O, 1.5 MgCl2·6H2O, 1 Na2VO4, 0.5 dithiothreitol (DTT), and 0.4 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) and 2 µg/ml each of leupeptin, pepstatin A, and aprotinin. The suspension was left in ice for 10 min followed by a 45-s homogenization at a moderate speed. The nuclei were collected by centrifuging the slurry at 4,500 g for 5 min at 4°C and resuspending the nuclei in 100 µl of buffer B [buffer A adjusted to (in mM) 20 Tris·HCl (pH 7.8), 420 KCl, and 20% (vol/vol) glycerol]. The nuclei were then lysed at 4°C for 30 min with gentle agitation, the debris was cleared by centrifugation at 10,000 g for an additional 30 min at 4°C, and the supernatant was frozen in liquid nitrogen and stored at −70°C until used. In all cases, protein content was determined by the Bradford method with BSA as a standard.

Whole lung extraction. Lungs were excised from prenatal rats (gestational days 19 and 21; n = 6 each), postnatal rats (days 0, 1, 2, 3, 4, and 6; n = 4–6 each), and adults (8 wk), rinsed once in saline, and frozen in liquid N2 followed by storage at −70°C. Total protein was extracted by homogenizing 50–100 mg of tissue in a suitable volume of a buffer (1:40 wt/vol) containing (in mM) 20 HEPES (pH 7.5), 0.2 EDTA, 1.2 NaH2PO4, 1.5 MgCl2, 2.5 NaH2PO4, 100 NaCl, 5 DTT, and 1 AEBSF and 10 µg/ml of leupeptin (Na2VO4, DTT, AEBSF, and leupeptin were added before extraction). Tissue debris was formed into pellets by centrifugation at 10,000 g for 30 min at 4°C, and the supernatant was mixed with an equal volume of the same extracting buffer supplemented with 40% (vol/vol) glycerol. For glutathione and adenylylate determinations, 50–100 mg of excised tissue were homogenized in 10 volumes of 7% perchloric acid (PCA) at 4°C for 1 min. The homogenate was centrifuged at 10,000 g for 30 min, and the supernatant was frozen in liquid nitrogen and stored at −70°C. On the day of use, the samples were neutralized with a known volume of 3 M K2CO3, centrifuged as above, and analyzed as detailed in Western Analysis and Electrophoretic Mobility Shift Assays. Cytosolic extracts for the assessment of antioxidant enzyme activities were prepared by homogenizing the whole lung in 1 ml of buffer A (as above) followed by centrifugation at 4,500 g for 5 min at 4°C. The supernatant was frozen in liquid nitrogen and kept at −70°C until used. The pellet was resuspended in 0.5 ml of buffer B and processed for nuclear extraction as detailed above.

Western Analysis and Electrophoretic Mobility Shift Assays

Nuclear proteins (20–25 µg) were resolved by SDS-PAGE with a 7.5% separating phase at room temperature at 150 V for 1 h. After electrophoretic transfer onto nitrocellulose, each
membrane was washed in Tris-buffered saline (TBS; 20 mM Tris·HCl, pH 7.6, and 500 mM NaCl) followed by blocking for 1 h at room temperature in TBS plus 0.1% (vol/vol) Tween 20 (TBS-T) with gentle agitation. After three washes in TBS-T, the membranes were incubated with either monoclonal anti-HIF-1α IgG (1:200; Novus Biologicals) or polyclonal anti-NF-κB p65 (Rel A) IgG (1:500; Santa Cruz Biotechnology) antibodies in TBS-T overnight at 4°C. Primary conjugates were visualized on film with an anti-rabbit IgG-biotinylated antibody coupled with streptavidin-horseradish peroxidase enhanced chemiluminescence (ECL, Amersham Life Sciences). Western detection of β-actin was used as a semiquanitative internal control for lane loading.

Electrophoretic mobility shift assays (EMSAs) were conducted with the following radiolabeled deoxyoligonucleotide sequences purchased from Genosys: HIF-1α (consensus sequence underlined), W-18, 5′-GCCCTACGTGCTGCTCA-3′ (3-bp missense control, M-18, 5′-GCCCTAAAGCTGCTCTCA-3′); and NF-κB, W-22, 5′-AGTTGAGGAGGAGTTCCCATGAGC-3′ (1-bp missense control, M-22, 5′-AGTTGAGGAGGAGTTCCCATGAGC-3′). After end labeling with polynucleotide kinase (Boehringer Mannheim) purifying and annealing probes, identical amounts of radioactivity (2 × 10^6 counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl counts/min) were added to the binding reactions containing 1–5 µg of fATII cell nuclear extracts in a final volume of 40 µl

L-Buthionine(S,R)-Sulfoximine Pretreatment

L-Buthionine(S,R)-sulfoximine (BSO) selectively inhibits the biosynthesis of reduced glutathione (GSH) by irreversibly blocking the activity of γ-glutamylcysteine synthetase (γ-GCS) (18). A stock solution of BSO (1,125 µM) was prepared in deionized water and stored at 4°C. FATTI cells, cultured at 23 Torr as in Primary Culture of FATTI Cells, were pretreated for 24 h with BSO [0 (control), 1, 10, or 50 µM] before the cells were shifted for 4 h to each new PO2. In all cases, the final concentration of BSO was adjusted with PC-1 medium pre-equilibrated to the corresponding PO2. After each treatment, the cells were processed for nuclear and cytosolic extraction as in Cell Harvesting and Nuclear Protein Extraction.

Metabolite and Enzyme Activity Determinations

Adenylate energy charge (4) was used as an index of cellular metabolic competence according to the formula [(ATP concentration + 0.5ADP concentration)/total adenylate concentration], with the concentrations of ATP, ADP, and AMP (total adenylate) determined spectrophotometrically (7). GSH concentrations were also determined spectrophotometrically in neutralized PCA extracts by following the glyoxylase-catalyzed production of S-lactyl-GSH at 240 nm in a 1-mL volume containing 790 µl of phosphate buffer (25 mM KH2PO4 and 25 mM K2HPO4, pH 6.8), 150 µl of 1% BSA, 10 µl of sample, 10 µl of glyoxylase-I (1 mg/ml), and 40 µl of methylglyoxal (0.1 M). Oxidized glutathione (GSSG) was determined in the same cuvette by the addition of 1 mg/ml of glutathione reductase (GSSG-RD) and 8 µl of 12 mM β-NADPH and then by following the change in absorbance at 340 nm (7). Control cuvettes contained the same content of buffer but with a matched sample volume of deionized water. The same supernatant was used to determine the content of nicotinamide (NADPH/H+ and NADP+1) by glutamate dehydrogenase and glucose-6-phosphate dehydrogenase reactions, respectively (7). The protein content of each PCA precipitate was redissolved in 1 M NaOH and determined as above, enabling the results to be expressed as micromoles per milligram of protein.

The assay conditions for determining activities of glutathione biosynthetic enzymes are detailed below. In each case, specific activity is expressed as units per milligram of protein where 1 unit of enzyme activity is the amount that catalyzes the formation of 1 µmol product/min. All assays were conducted at 30°C.

Glutathione peroxidase. Glutathione peroxidase (GSH-PX; EC 1.11.1.9) was determined with the method previously described (29). Briefly, cytosolic extracts were incubated in PBS buffer containing 5 mM EDTA, 10 mM NAD(P)H, 100 U/ml of GSSG-RD, 1.125 M NaN3 (a catalase inhibitor), and 150 mM GSH in a final volume of 1 ml. The enzymatic reaction was initiated by the addition of 100 µl of 2 mM H2O2 (30% H2O2, 10.15 M; the molar concentration of H2O2 was calculated with the coefficient value 0.0394 cm^-1·mM^-1 at 240 nm), and the linear rate of conversion of NADPH/H+ to NADP+ at 340 nm between 10 and 5 min after initiation of the reaction was followed.

GSSG-RD. GSSG-RD (EC 1.6.4.2) was determined with the method previously described (31) with minor modifications. The rate of oxidation of NAD(P)H by GSSG at 30°C was used as a standard measure of enzymatic activity. The activity of GSSG-RD was measured by monitoring the rate of formation of NADP+ at 340 nm between 0 and 5 min after addition of the sample.

γ-GCS. γ-GCS (EC 6.3.2.2) was determined with the method previously described (37). The reaction mixture (1 ml) contained Tris·HCl (100 mM, pH 8.2), sodium l-glutamate (10 mM), Na2ATP (5 mM), sodium phospho(enol)pyruvate (2 mM), KCl (150 mM), NADH (0.2 mM), pyruvate kinase (5 U, bovine heart type III), and lactate dehydrogenase (10 U, rabbit heart type I). The reaction was initiated by adding the sample, and the rate of NAD+ formation was followed at 340 nm.

Glutathione synthase. Glutathione synthase (GS; EC 6.3.2.3) was assayed in a reaction mixture containing Tris·HCl (100 mM, pH 8.2, at 30°C), KCl (50 mM), L-γ-glutamyl-L-α-amino butyric acid (5 mM), ATP (10 mM), glycine (5 mM), MgCl2 (20 mM), EDTA (2 mM), and sample (added last) in a final volume of 0.1 ml. Added to this was 0.02 ml of 10% sulfosalicylic acid and 0.9 ml of a buffer containing phospho (enol)pyruvate (0.5 mM), NADH (0.2 mM), pyruvate kinase (1 U), MgCl2 (40 mM), KCl (50 mM), and K2HPO4 (250 mM, pH 7.0). The reaction was initiated with 1 U of lactate dehydrogenase, and the rate of NAD+ formation was followed.

Statistical Analysis

Experimental results are expressed as means ± SE. Statistical analysis was performed with one-way analysis of variance with SigmaStat 2.0, followed by post hoc Tukey's test to
Table 1. Adenylate high-energy phosphate homeostasis and energy charge ratio under different Po2 values in fATII cells

<table>
<thead>
<tr>
<th>Po2</th>
<th>Adenylate Phosphate Content, nmol/mg protein</th>
<th>Energy Charge Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[ATP]</td>
<td>[ADP]</td>
</tr>
<tr>
<td>152 Torr</td>
<td>79.04 ± 15.07</td>
<td>25.29 ± 2.96*</td>
</tr>
<tr>
<td>23 Torr</td>
<td>43.04 ± 6.75</td>
<td>26.43 ± 6.58</td>
</tr>
<tr>
<td>23 → 76 Torr</td>
<td>33.12 ± 5.81</td>
<td>11.92 ± 4.15*</td>
</tr>
<tr>
<td>23 → 152 Torr</td>
<td>48.56 ± 4.12</td>
<td>15.64 ± 6.03†</td>
</tr>
<tr>
<td>23 → 722 Torr</td>
<td>48.41 ± 13.04</td>
<td>20.64 ± 10.44</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 experiments/group. fATII, fetal alveolar type II; [ATP], [ADP], and [AMP], concentration of ATP, ADP, and AMP, respectively; BSO, L-buthionine-(S,R)-sulfoximine; 152 Torr, early postnatal alveolar Po2; 23 Torr, fetal distal lung Po2; 23 → 76, 23 → 152, and 23 → 722 Torr, mild, moderate, and severe hyperoxic shifts, respectively. fATII cells were exposed to oxidative stress for 4 h at 37°C. Significantly different from [ATP] at corresponding Po2: *P < 0.05; †P < 0.01. Significantly different from [ATP] at 152 Torr: ‡P < 0.05; §P < 0.01.

determine significance among treatments. The a priori level of significance at 95% confidence was accepted at P < 0.05.

RESULTS

Cellular Energy Charge in fATII Cells Exposed to Oxidative Stress

Cells remained metabolically competent throughout the period of preexperimental culture at 23 Torr and on exposure to each ΔPo2 (Table 1), with no notable change in cellular energy charge. However, reoxygenation of hypoxic cultures induced a modest increase in ATP concentration (not significant), whereas the ADP and AMP concentrations remained static. Also shown in Table 1 is the static cellular energy charge of cell cultures pretreated with BSO (50 μM), which rules out any nonspecific toxicity of BSO. To further confirm this fact, the total protein content of cells pretreated with 1, 10, and 50 μM BSO was determined to be not significantly different (P > 0.05) from that in control cultures (data not shown). In addition, alveolar pretreatment with BSO (50 μM) has been shown to intervene specifically at the level of the cell cycle events (such as with p53) and the level of factors that are key components of the signaling pathways governing apoptosis (such as with Bax and Bcl-2 protooncogenes), suggesting specificity rather than necrotic toxicity (J. J. E. Haddad, R. E. Olver, and S. C. Land, unpublished observations).

Immunoblot Western Analysis for HIF-1α and NF-κB

Manipulation of O2 availability alters the nuclear abundance of both HIF-1α and NF-κB but in opposing directions. HIF-1α showed a clear nuclear accumulation in cells maintained without perturbation at 23 Torr over those maintained static at 152 Torr. When exposed to a ΔPo2 of 23 → 76 Torr, nuclear abundance of this protein was reduced but still remained significantly greater than that in oxygenated cultures (Fig. 1A), and, indeed, HIF-1α was not detectable in the nuclei of cells exposed to a ΔPo2 greater than this range. Figure 1B shows compiled densitometric data from at least four separate experiments in reference to the abundance of β-actin in each lane. The nuclear abundance of NF-κB increased with the elevation in Po2 beyond 76 Torr, becoming maximal on a shift toward moderate (23 → 152 Torr) and severe (23 → 722 Torr) hyperoxia (Fig. 1C). Compiled experimental data showing densitometric analysis of NF-κB abundance in reference to β-actin are shown in Fig. 1D.

Analysis of HIF-1α and NF-κB Consensus Sequence DNA Binding Activity

To monitor activated DNA consensus binding at various fetal to neonatal Po2 values, EMSAs were
performed for HIF-1α and NF-κB (Fig. 2, A and C, respectively). HIF-1α binding activity was determined with the probe W-18, which encodes a 5-base hypoxia response element from the erythropoietin promoter. In parallel with the increase in the nuclear abundance of HIF-1α in fATII cells maintained at 23 Torr or shifted from 23 to 76 Torr was a marked increase in HIF-1α DNA consensus binding activity, which diminished at higher PO2 values (Fig. 2, A and B). Similarly, NF-κB binding activity was determined with the probe W-22, which contained the NF-κB consensus sequence from the human immunodeficiency virus long-terminal repeat. Mild to severe hyperoxia (ΔPO2 of 23 → 76, 23 → 152, and 23 → 722 Torr) induced NF-κB binding activity (Fig. 2, C and D), with maximal activation at a ΔPO2 of 23 → 722 Torr and paralleled the appearance of this transcription factor in the nucleus.

In separate experiments, specificity of the transcription factor-oligonucleotide complex formation noted in Fig. 3 was tested by 1) incubation of nuclear samples with either M-18 or M-22, 2) addition of unlabeled W-18 or W-22 at 100-fold molar excess to labeled oligonucleotides, and 3) supershift analysis with nuclear extracts that had been preincubated with antibodies specific to HIF-1α or NF-κB (p65) before addition of the appropriate probe. Figure 3A shows the maximal activation of HIF-1α at 23 Torr and the retarded supershift complex with a shift from 23 to 76 Torr. The specific binding of HIF-1α is abolished with M-18 and the addition of 100-fold competitor. Figure 3B shows the activation of NF-κB and the indicated supershift. Similarly, NF-κB binding was diminished with M-22 or 100-fold competitor.

Western Analysis of Cell Cultures Pretreated With BSO

fATII epithelial cells pretreated with BSO show a dose-dependent attenuation in the nuclear abundance of both transcription factors that correlates with the reduced GSH content (Table 2) within the ΔPO2 regimens that stimulated their maximal activity. Western immunoblotting revealed the effect of BSO (1, 10, and 50 µM) on HIF-1α (Fig. 4A) and NF-κB (Fig. 4C) nuclear abundance kinetics. Ratio analysis by densitometry relative to β-actin is given for HIF-1α at 23 Torr and at a ΔPO2 of 23 → 76 Torr (Fig. 4B) and for NF-κB at a ΔPO2 regimens of 23 → 76, 23 → 152, and 23 → 722 Torr (Fig. 4D).

Fig. 2. DNA consensus binding analysis for HIF-1α (A and B) and NF-κB (C and D) nuclear extracts (1–5 µg). A and C: representative electrophoretic mobility shift assays (EMSA) for HIF-1α and NF-κB activation states, respectively, over range of ΔPO2 regimens. Probe contained no nuclear samples. FP, free probe; NS, nonspecific. B and D: densitometric analysis of each retarded band for HIF-1α and NF-κB, respectively. Values are means ± SE of 4 independent experiments. Significant difference relative to intensity of band obtained at 152 Torr: *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Effects of hypoxia, reoxygenation, and hyperoxia on DNA binding activity of HIF-1α (A) and NF-κB (B). Lanes 1–3, bands for 23 Torr, 23 → 152 Torr plus mutant, and 23 → 722 Torr plus mutant, respectively. Arrows, positions of respective specific antibody-complexed supershifts. SS, supershift; C, 100-fold competitor; M, mutant; A, antibody; N, none. Data are representative of 5 separate experiments. Addition of mutant oligonucleotide M-18 and 100-fold competitor completely abolished binding of HIF-1α. Addition of mutant oligonucleotide M-22 and 100-fold competitor completely abolished binding of NF-κB.
Analysis of HIF-1α and NF-κB DNA Binding Activity With BSO Pretreatment by EMSA

The effect of BSO (0, 1, 10, and 50 µM) on DNA binding activity is shown for HIF-1α and NF-κB in Fig. 5, A and C, respectively. Exponential decay of the binding activity of either HIF-1α or NF-κB is evident with increasing concentrations of BSO. Figure 5, B (HIF-1α) and D (NF-κB), shows histogram analysis of the dose-response curve.

Oxidizing events in the cytosol are buffered, in part, by GSH-PX-catalyzed oxidation of 2GSH = GSSG, with the generation of NADP⁺ (Fig. 6 A). Because this reaction is readily reversible, the following criteria were used to indirectly assess intracellular redox status in fATII cells and the whole lung: 1) GSH reduction state [(GSH concentration - GSSG concentration)/GSSG concentration], 2) reduced (NADPH/H⁺) and oxidized (NADP⁺) forms of nicotinamide, and 3) assessment of glutathione biosynthetic enzyme activity. Figure 6, B and C, shows the variations of glutathione and nicotinamide, respectively, under each PO₂ regimen. GSH reaches an apparent maxima over PO₂ = 76 and PO₂ = 152 Torr and, despite a decline, is persistently elevated at PO₂ = 722 Torr tension over cultures that had not been exposed to a D PO₂ (23 and 152 Torr). The ratio of GSH to GSSG varies with treatments as follows: 5:1, 12:1, and 3:1 for a D PO₂ of 23 = 76, 23 = 152, and 23 = 722 Torr, respectively. With increasing abundance of GSH, NADPH/H⁺ levels dropped over the same O₂ range, which reflects the NADPH/H⁺ dependency of GSSG-RD (Fig. 6, A and C). Variations in glutathione pool expressed as percentages of the control value (152 Torr) are illustrated in Fig. 6 B.

Table 2. Glutathione homeostasis under different PO₂ values in fATII cells

<table>
<thead>
<tr>
<th>PO₂</th>
<th>Total Glutathione Content, µmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µM BSO</td>
<td>1 µM BSO</td>
</tr>
<tr>
<td>152 Torr</td>
<td>128.5 ± 10.5</td>
</tr>
<tr>
<td>23 Torr</td>
<td>145.2 ± 11.2</td>
</tr>
<tr>
<td>23=76 Torr</td>
<td>421.8 ± 25.7</td>
</tr>
<tr>
<td>23=152 Torr</td>
<td>320.8 ± 21.3</td>
</tr>
<tr>
<td>23=722 Torr</td>
<td>227.5 ± 18.9</td>
</tr>
<tr>
<td>10 µM BSO</td>
<td>102.8 ± 11.5</td>
</tr>
<tr>
<td>10=27 Torr</td>
<td>324.7 ± 14.2</td>
</tr>
<tr>
<td>10=142 Torr</td>
<td>269.4 ± 11.4</td>
</tr>
<tr>
<td>10=722 Torr</td>
<td>179.7 ± 13.1</td>
</tr>
<tr>
<td>50 µM BSO</td>
<td>46.6 ± 3.7†</td>
</tr>
<tr>
<td>50=50 Torr</td>
<td>84.3 ± 4.9‡</td>
</tr>
<tr>
<td>50=282 Torr</td>
<td>51.3 ± 4.3‡</td>
</tr>
<tr>
<td>50=10 Torr</td>
<td>65.9 ± 6.6‡</td>
</tr>
<tr>
<td>50=152 Torr</td>
<td>23.2 ± 2.7‡</td>
</tr>
<tr>
<td>60 µM BSO</td>
<td>21.4 ± 2.8‡</td>
</tr>
<tr>
<td>60=722 Torr</td>
<td>27.5 ± 3.2‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7, 5, 6, and 6 experiments for 0, 1, 10, and 50 µM BSO, respectively. fATII cells were pretreated with BSO for 24 h at 37°C. Total glutathione content = GSH + 2GSSG. Significantly different from 0 µM BSO (control; without BSO pretreatment): *P < 0.05; †P < 0.01; ‡P < 0.001.

Redox State in fATII Cells at Various PO₂ Values

Oxidizing events in the cytosol are buffered, in part, by GSH-PX-catalyzed oxidation of 2GSH = GSSG, with the generation of NADP⁺ (Fig. 6 A). Because this reaction is readily reversible, the following criteria were used to indirectly assess intracellular redox status in fATII cells and the whole lung: 1) GSH reduction state [(GSH concentration - GSSG concentration)/GSSG concentration], 2) reduced (NADPH/H⁺) and oxidized (NADP⁺) forms of nicotinamide, and 3) assessment of glutathione biosynthetic enzyme activity. Figure 6, B and C, shows the variations of glutathione and nicotinamide, respectively, under each PO₂ regimen. GSH reaches an apparent maxima over PO₂ = 23 = 76 and 23 = 152 Torr and, despite a decline, is persistently elevated at PO₂ = 23 = 722 Torr tension over cultures that had not been exposed to a D PO₂ (23 and 152 Torr). The ratio of GSH to GSSG varies with treatments as follows: 5:1, 12:1, and 3:1 for a D PO₂ of 23 = 76, 23 = 152, and 23 = 722 Torr, respectively. With increasing abundance of GSH, NADPH/H⁺ levels dropped over the same O₂ range, which reflects the NADPH/H⁺ dependency of GSSG-RD (Fig. 6, A and C). Variations in glutathione pool expressed as percentages of the control value (152 Torr) are illustrated in Fig. 6 B.
one homeostasis for cell cultures pretreated with BSO is given in Table 2, where the depletion degree is summarized for the dose-response curve at various PO2 values.

Activity of Enzymes Involved in Glutathione Homeostasis

Glutathione enzyme activities were examined in control, fetal lung O2, postnatal lung O2, and mild and severely hyperoxic cultures (Table 3). fATII cells exposed to fetal distal lung PO2 (23 Torr) showed maximum activity of all enzymes investigated except for γ-GCS where the activation peak occurs between 23 → 76 and 23 → 152 Torr, although its activity is significant in comparison to the control value (P < 0.05). GSH-PX, which catalyzes the reduction of H2O2 and certain lipid peroxides, had its activity significantly increased at 23 Torr (P < 0.01 compared with normoxia). The activity of this enzyme steadily declined with increasing fetal to neonatal ΔPO2 regimes but remained significant at 23 → 76 Torr (P < 0.05). GSSG-RD activity increased maximally at 23 Torr (P < 0.05), remained significant at 23 → 76 Torr (P < 0.05), and steadily declined to a minimum at 23 → 152 and 23 → 722 Torr. γ-GCS and GS showed similar trends in their activities. Additionally, γ-GCS maintained a plateau between 76 and 152 Torr before declining in activity, yet remaining significant relative to the baseline levels recorded in normoxia. GS activity was maximum at 23 Torr (P < 0.01), significant at 23 → 76 Torr (P < 0.05), and declined at higher tensions. A significant correlation by sigmoidal regression was found between GSH synthetic activity of γ-GCS and the total GSH pool increase observed over fetal to neonatal ΔPO2 regimes (Fig. 7). There was no correlation with other enzymes involved in maintaining intracellular levels of glutathione.

Glutathione Homeostasis and Enzyme Activities in Pre- and Postnatal Lung Development

Glutathione homeostasis was assessed in pre- and postnatal lungs obtained at various time intervals. GSH levels in prenatal lungs (gestation days 19 and 21) were significantly lower than those in postnatal lungs (2–6 h) where a maximum was observed relative to gestation day 19 (Fig. 8A). GSSG showed opposite kinetics to GSH variations in the prenatal lung (Fig. 8B), with a minimum observed in the late postnatal period (days 2–6). The content of GSSG at each gestation period was significantly lower than that of GSH (P < 0.01), with an average GSH-to-GSSG ratio of ~50:1 (Fig. 8, A and B). GSSG concentration on gestation days 19 and 21 and 1–6 h postnatally was significantly higher than that in the late postnatal period (days 1–6).

<table>
<thead>
<tr>
<th>PO2</th>
<th>GSH-PX</th>
<th>GSSG-RD</th>
<th>γ-GCS</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>152 Torr</td>
<td>0.70 ± 0.15</td>
<td>0.58 ± 0.08</td>
<td>0.09 ± 0.01</td>
<td>0.71 ± 0.12</td>
</tr>
<tr>
<td>23 Torr</td>
<td>2.89 ± 0.56†</td>
<td>1.90 ± 0.32*</td>
<td>0.13 ± 0.02*</td>
<td>1.79 ± 0.11†</td>
</tr>
<tr>
<td>23 → 76 Torr</td>
<td>1.53 ± 0.25*</td>
<td>1.19 ± 0.22*</td>
<td>0.22 ± 0.04*</td>
<td>1.36 ± 0.15*</td>
</tr>
<tr>
<td>23 → 152 Torr</td>
<td>0.40 ± 0.14</td>
<td>0.14 ± 0.05</td>
<td>0.23 ± 0.03*</td>
<td>0.69 ± 0.18</td>
</tr>
<tr>
<td>23 → 722 Torr</td>
<td>0.85 ± 0.03</td>
<td>0.10 ± 0.02*</td>
<td>0.16 ± 0.02*</td>
<td>0.90 ± 0.17</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 experiments/group. GSH-PX, reduced glutathione peroxidase; GSSG-RD, oxidized glutathione reductase; γ-GCS, γ-glutamylcysteine synthase; GS, glutathione synthase. fATII cells were exposed to oxidative stress. Significantly different from activity at 152 Torr: *P < 0.05; †P < 0.01; ‡P < 0.001.
The activity of GSH-PX increases steadily to a plateau that is maximally sustained between days 0 and 1 after birth compared with those in the adult and on day 4 after birth (Table 4). This activity was highly significant on gestation day 19 ($P < 0.001$). The activity of GSSG-RD showed opposite kinetics, with minimal activity observed on day 0 (3 h) after birth. This activity regained a modest increase on days 0 (6 h) and 1, although it was still significantly lower compared with that in the adult.

$\gamma$-GCS activity started to increase on gestation day 19 and reached a maximum on day 0 (3–6 h) after birth. The same trend was observed with the activity of GS. The elevation in GSH concentration in the postterm lung in the early postnatal period compared with that in the preterm lung was significantly correlated with the activities of $\gamma$-GCS ($R = 0.85; P < 0.05$; Fig. 9A) and GS ($R = 0.81; P < 0.05$; Fig. 9B).

### Transcription Factor DNA Binding Activity in Pre- and Postnatal Lung Development

The DNA binding analysis of HIF-1$\alpha$ (Fig. 10A) indicated maximal activity on gestation day 19, which declined during the early postnatal period. DNA binding analysis of NF-κB (Fig. 10B) indicated activation in the early postnatal period, reaching an apparent peak 3–6 h after birth, which then declined to a still detectable level on postnatal days 1 and 4. The specificity of the corresponding binding of HIF-1$\alpha$ or NF-κB was determined by the addition of mutant (M-18 and M-22), 100-fold competitor, and a retarding band supershift formed by the specific immunoglobulin (data not shown).

### DISCUSSION

Genetic responses to hypoxic or hyperoxic stresses are regulated, in part, by transcription factors in which redox-dependent activation, nuclear translocation, and DNA consensus binding are the rate-limiting determinants of the species and expression intensity of genes that sustain cellular functional integrity under either...

### Table 4. Activity of enzymes involved in glutathione metabolism and antioxidant defense mechanisms in neonatal lungs at various stages of development

<table>
<thead>
<tr>
<th>EnzymeActivity, U/mg protein</th>
<th>GSH-PX</th>
<th>GSSG-RD</th>
<th>$\gamma$-GCS</th>
<th>GS</th>
</tr>
</thead>
<tbody>
<tr>
<td>G19</td>
<td>0.14±0.01†</td>
<td>0.04±0.001</td>
<td>0.15±0.01†</td>
<td>0.88±0.03</td>
</tr>
<tr>
<td>3 h</td>
<td>0.20±0.03†</td>
<td>0.02±0.001†</td>
<td>0.23±0.01†</td>
<td>1.33±0.04†</td>
</tr>
<tr>
<td>6 h</td>
<td>0.21±0.01†</td>
<td>0.03±0.001*</td>
<td>0.24±0.01†</td>
<td>1.18±0.06†</td>
</tr>
<tr>
<td>Day 1</td>
<td>0.19±0.02†</td>
<td>0.03±0.01*</td>
<td>0.12±0.01</td>
<td>0.72±0.03†</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.07±0.02</td>
<td>0.04±0.01</td>
<td>0.11±0.01</td>
<td>0.70±0.05</td>
</tr>
<tr>
<td>Adult</td>
<td>0.07±0.01</td>
<td>0.05±0.001</td>
<td>0.10±0.01</td>
<td>0.65±0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 experiments/group. G19, gestational day 19; 3 h, 6 h, day 1, and day 4, time after birth. Significantly different from activity of adult lung: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. 

---

**Fig. 7.** Sigmoidal correlation with best subset regression of total glutathione synthesis on $\gamma$-glutamylcysteine synthetase ($\gamma$-GCS) synthetic activity over fetal to neonatal $\Delta P_O_2$ values in FATII cells. Data are means ± SE of 4 independent measurements run in duplicate.

**Fig. 8.** Glutathione homeostasis in whole lungs isolated on days 19 and 21 of gestation (G19 and G21, respectively) and in early postnatal period. Values are means ± SE of 5 duplicate measurements taken from nonsiblings. A: GSH concentration. Significant difference compared with G19: *$P < 0.05$; **$P < 0.01$. Line fit by pseudo-Voigt analysis shows peak of GSH in postnatal period is between 3 and 6 h. B: GSSG concentration. Significant difference compared with G19: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Rational regression parameter I shows gradual decline in GSSG from G19 into early postnatal period.
stress condition (8, 20). Because the O2 history of distal lung epithelial development is restricted to 23 Torr in utero, we reasoned that the fourfold increase in \( P_{O2} \) that accompanies the first breath at birth would constitute a substantial O2 signaling event that would mediate a change from hypoxic to oxidative (aerobic) forms of genetic regulation.

In fATII epithelial monolayers maintained in the steady state at a fetal distal lung PO2 (23 Torr), HIF-1\( \alpha \) showed a high level of nuclear translocation and activation that persisted when the cultures were exposed to a sustained shift toward early postnatal distal lung PO2 (76 Torr). Neither nuclear translocation nor consensus sequence binding were detected in cells cultured in the steady state at 152 Torr or exposed to a \( \Delta P_{O2} \) of 23 = 152 Torr or beyond. Work conducted by others (47) on adult ferret lungs and lung epithelial cell lines has determined that HIF-1\( \alpha \) cellular abundance is graded between 0.5 and 30 Torr, becoming maximal at the lower end of this range over 2–8 h. Our results suggest that the activity of HIF-1\( \alpha \) resides over a wider spectrum of PO2 in fATII epithelial cells that incorporates both fetal and early postnatal alveolar PO2 values, an assertion that is supported by our observations that HIF-1\( \alpha \) nuclear abundance and consensus binding activity are present in the whole lung preterm, is sustained through the early postnatal period, and is lost thereafter. Because the activation pathway for HIF-1\( \alpha \) is favored by a reducing environment (i.e., lowered ROS production) coupled with deoxyferroheme conformation, muted ubiquitinization, proteasome degradation, phosphorylation, and aryl hydrocarbon nuclear translocator-dependent nuclear translocation (35), this finding presents the intriguing possibility that one or more of these components serves to effect a rightward shift in \( O_2 \) dependency (i.e., raise the Michaelis-Menten constant) of HIF-1\( \alpha \) activation in the perinatal lung. Although the functional significance of this remains to be demonstrated, it seems that the sensitivity of HIF-1\( \alpha \) activation pathways to subambient PO2 values is heightened during birth.

Nuclear abundance and consensus binding of NF-\( \kappa B \) were not detected in fATII epithelial cells under steady-state culture at either 23 or 152 Torr; however, the activity of this transcription factor was powerfully induced by shifts from fetal to early postnatal PO2 (76 Torr) and into moderate (152 Torr) and severe (722 Torr) hyperoxia, suggesting that this is primarily responsive to acute changes in PO2, which, at the lower...
end of the range, are coincident with early postnatal PO2. As with HIF-1α, the profile of NF-κB activation in the whole lung follows the expected change in PO2 at birth, becoming maximally active in the initial 24 h of the postnatal period and falling thereafter. Although NF-κB activation is well established as an early response to oxidative stress in the lung (26, 36), a study (32) based on fetal distal lung epithelial cultures indicated that there is a strong association among moderate hyperoxia (i.e., PO2 > 152 Torr), ROS production, and NF-κB-regulated expression of epithelial Na+ channels, pointing to the importance of this pathway as a modulator of developmental events in the lung at birth. When taken together with HIF-1α data, the activity profiles of both transcription factors appear sufficiently tuned to mediate the changeover from hypoxic to oxidative forms of genetic regulation at PO2 values that are within the range of those expected with the first breaths at birth.

The signal transduction components that link the availability of O2 to the activation of these transcription factors are poorly defined but are broadly believed to hinge on the free abundance of oxidants (i.e., ROS) in the cytosol (15, 28). In the case of HIF-1α, for example, posttranslational stability, nuclear translocation by the aryl hydrocarbon nuclear translocator, and consensus DNA binding are coupled with O2-associated changes in both conformation and activity of a ferroheme-containing protein, believed to express peroxide generation via NADPH oxidase-type activity (3, 23). Hypoxic cessation of peroxide production mediates HIF-1α stabilization, nuclear translocation, and gene expression (21, 42). The activation pathway for NF-κB, on the other hand, requires the disassociation from its cytosolic inhibitory subunit IκB, an event that requires phosphorylation but which is favored by oxidizing conditions (12, 28, 34). Clearly, the extent to which cells express antioxidant defenses bears consequence for the efficiency with which an O2- or redox-linked signal can be transmitted from environment to effector protein.

To determine the redox-buffering capacities that accompany HIF-1α and NF-κB activation, intracellular levels of glutathione and its cofactor nicotinamide were determined under each ΔPO2 regimen. The ROS scavenging function of glutathione (L-γ-glutamyl-L-cysteinylglycine), the major intracellular nucleophile in mammalian cells (27, 44), centers on the oxidation of the cysteine thiol moiety of two molecules of GSH to produce GSSG (Fig. 6A), a reaction catalyzed by GSH-PX. GSSG is enzymatically recycled to 2GSH by GSSG-RD through an energy-consuming reaction that is dependent on the availability of NADPH/H+ as an electron acceptor. Consequently, GSH-to-GSSG ratios serve as a correlative index of the oxidative potential within the cytosol. Our studies point toward an association between each ΔPO2 regimen and an increased total glutathione pool in which the absolute concentration of GSH is elevated four- to fivefold over that in cells maintained in steady-state culture at either 23 or 152 Torr. The kinetics of GSH variation over fetal to neonatal PO2 values showed maxima at ΔPO2 regimes of 23→76 and 23→152 Torr, which matched the minima recorded for NADPH/H+ under the same conditions, and as expected, an increase in NADP+ content was reported with ascending ΔPO2 regimes that are significantly higher than the concentration of NADPH/H+.

Because GSH acts as a powerful intracellular redox buffer, changes in its overall abundance may modulate redox-linked signaling events within the cell (38). Interestingly, the observed elevation in GSH from 23 Torr was accompanied by a significant reduction in the nuclear abundance and consensus binding of HIF-1α and an opposing elevation in NF-κB over fetal to neonatal ΔPO2 regimes and beyond. These results suggest that the transcription factor activation profiles we observed for HIF-1α and NF-κB over the birth transition complement the changes in the reducing potential of the glutathione pool and the establishment of GSH as the predominant redox form. Experimental depletion of the glutathione pool by dose-dependent inhibition of γ-GCS with BSO resulted in the stepwise inactivation of both transcription factors under each activating ΔPO2 value. Intriguingly, although HIF-1α appeared maximally active at 23 Torr in a low total glutathione environment (145 μmol/mg protein; Table 2), its activity was substantially more responsive to glutathione depletion compared with all activated ranges of NF-κB explored when the control glutathione content was two- to threefold greater. Although we cannot infer from these data alone that the effect is specifically ROS dependent, it appears clear that maintenance of the glutathione pool and, by inference, the shuttling of electrons between reductant and oxidant components of this pathway are prerequisites for transcription factor activation under any given O2 profile. We are currently investigating the molecular mechanisms by which this activation may be coupled to glutathione homeostasis and lung cellular survival or death in the event of electrophilic or oxidative tissue injury.

The substantial changes in the GSH-to-GSSG ratio observed in response to upward ΔPO2 values in the fATII monolayer culture model and the similarity of this response to changes in the glutathione pool in whole lung over the perinatal period may occur via glutathione reduction-oxidation cycling or de novo synthesis. GSSG recycling to 2GSH is catalyzed by NADPH-dependent GSSG-RD, the greatest detectable activity of which coincided with the fetal to neonatal range of PO2 values in fATII cells and the late-gestational phase of lung development. We have observed that GSSG-RD blockade with 1,3-bis-(2-chloroethyl)-1-nitroso urea leads to a substantial accumulation of GSSG at the expense of GSH in fATII monolayers exposed to a ΔPO2 of 23→76 Torr (Haddad et al., unpublished observations), which would tend to suggest that this pathway is operative during moderate PO2 values. However, the lowered activity of this enzyme over hyperoxic ΔPO2 regimes, coincident with reduced GSH-PX activity, indicates that the importance of glutathione recycling via this route is restricted to perinatal PO2 values. The pathway through which de novo synthesis of GSH from glutamate,
glycine, and cysteine proceeds is rate limited by the activities of γ-GCS and GS, both of which exhibit elevated activities at fetal to early neonatal PO₂ values in fATII cells and correlate positively with the increase in the GSH pool. Moreover, the activities of both γ-GCS and GS are highly elevated in the whole lung, particularly in the early postnatal period, again coincident with the increase in postnatal PO₂. De novo synthetic pathways are clearly important routes for establishing GSH as the dominant nucleophile in the newly ventilating lung. Taken together, the marked elevations in GSH concentration and enzyme activity involved in glutathione homeostasis and synthesis (GSH-PX, GSSG-RD, γ-GCS, and GS), redox cycling (GSH-PX and GSSG-RD), and antioxidant defense (GSH-PX) over the fetal to neonatal ΔPO₂ and in the early perinatal period underscore the importance of the glutathione biosynthetic pathway as an adaptable component of respiratory antioxidant defenses critical for surviving birth (16).

Functionally, the relationship among O₂, glutathione biosynthesis, and transcription factor activity bears important implications for the pattern of cellular survival and alveolarization on exposure to O₂-linked stresses. Perturbations in glutathione homeostasis in the lung epithelium have been implicated in several pathological conditions such as idiopathic pulmonary fibrosis (10), respiratory distress syndrome (9, 25), and cystic fibrosis (33). In a number of cellular models, depletion of GSH accelerates the onset of apoptosis on exposure to oxidants (6), an effect that can be reversed by experimental maintenance of GSH content by inhibiting GSH synthesis. Given the importance of the redox state in the development of respiratory distress syndrome, the relationship between the oxidant stress in the lung and both the decrease in GSH and the increase in GSSG is critical for understanding the pathology of this condition.

In conclusion, we provide evidence that both abundance and activation kinetics of HIF-1α and NF-κB in the fetal distal lung epithelium are differentially responsive to changes in O₂ availability over the shifts in PO₂ that occurs during inhalation of the first breath. This is coincident with a substantial O₂-linked increase in the capacity of the tissue to engage in glutathione biosynthesis and redox shunting that may effectively form a feedback mechanism governing redox-linked signaling events. This O₂-responsive characteristic of the perinatal epithelium highlights PO₂ as an important modulator of events crucial to the transition from placental- to pulmonary-based modes of gas exchange and thus bears consequence for the clinical treatment of pediatric respiratory disorders that require O₂ therapy.

We thank Helen Murphy for expert technical assistance in isolating and preparing fetal alveolar type II epithelial cell cultures.

This work was supported by Medical Research Council and Anonymous Trust grants to S. C. Land.

J. J. E. Haddad is recipient of a George J. Livanos Prize PhD Scholarship.

This work was presented at the semiannual meeting of The Physiological Society in Manchester, UK (19).

Address for reprint requests and other correspondence: S. C. Land, Oxygen Signalling Group, Tayside Institute of Child Health, Ninewells Hospital and Medical School, Univ. of Dundee, Dundee DD1 9SY, UK (E-mail: s.c.land@dundee.ac.uk).

Received 12 August 1999; accepted in final form 26 October 1999.

REFERENCES


