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### Research Article

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## Sheep Lung Cytochrome *P4501A1* (*CYP1A1*): cDNA Cloning and Transcriptional Regulation by Oxygen Tension

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### Abstract

Lung cytochrome P450 activity has been linked to neoplasia and may produce reactive oxidant species and potent arachidonic acid metabolites. In lamb lung, oxygen breathing increases lung P450 activity, and inhibition of lung cytochrome P450 activity reduces oxygen-induced lung injury. The *P4501A1* (*CYP1A1*) isozyme is present in many lung cells, including endothelial cells, and may therefore be involved in the pathogenesis of hyperoxic injury to microvascular endothelium. Therefore, to test the hypothesis that oxygen regulates *P4501A1* gene expression in the lung, we cloned the sheep *P4501A1* cDNA, and examined its regulation by oxygen tension in vivo and in vitro. We found that, in vivo, oxygen breathing significantly increased lung *P4501A1* RNA levels and that this increase preceded the increase in isozyme activity. Oxygen exposure also promptly increased *P4501A1* RNA levels in cultured lamb lung microvascular endothelial cells but not in endothelial cells isolated from the main pulmonary artery or in lung smooth muscle cells. The oxygen-stimulated increase in *P4501A1* RNA levels was not serum dependent, was unaffected by cycloheximide treatment, and could not be mimicked by treatment of the cells with oxygenated medium, conditioned medium, or by chemical oxidants. By nuclear run-on assay in cultured lung endothelial cells, oxygen increased the transcription rate of *P4501A1* by almost fourfold after 90 min of oxygen exposure but had no significant effect on *P4501A1* RNA stability. We conclude that oxygen tension, but not chemical oxidants, increases *P4501A1* gene expression pretranslationally in lung microvascular endothelial cells. We speculate that oxygen induction of P450 activity in these cells may contribute to microvascular injury during oxygen breathing. (*J. Clin. Invest.* 1995. 96:2083–2089.) Key words: pulmonary oxygen toxicity • cytochrome *P4501A1* • gene regulation • endothelial cell • hyperoxia

### Introduction

Oxygen breathing increases the production of reactive oxygen species (ROS)<sup>1</sup> in lung and increases lung microvascular permeability, but the sites and importance of specific ROS production pathways are uncertain (1, 2). Hyperoxia increases both mitochondrial and microsomal oxidant production in lung cells (3), and physiologic studies demonstrate that increased pulmonary microvascular permeability is an early indicator of oxygen injury (4). Several lines of evidence suggest that the pulmonary cytochrome P450 monooxygenases may be involved in hyperoxic lung injury. For example, in reconstituted systems, cytochrome P450 enzymes produce superoxide and hydrogen peroxide due to uncoupling of electron flow during the addition of molecular oxygen to a variety of substrates (5–7). In addition, some mouse strains increase their lung P450 enzyme activity upon exposure to hyperoxia, and these strains are highly susceptible to hyperoxic lung injury compared with strains that do not increase their lung P450 content in hyperoxia (8). In addition, rats given agents which reduce lung P450 content demonstrate decreased susceptibility to oxygen injury (9). Finally, we have shown that oxygen exposure increases lung P450 content in a lamb model of hyperoxic lung injury and that inhibition of P450 with either endotoxin or cimetidine pretreatment reduces pulmonary oxygen toxicity (10, 11).

One of the P450 isozymes in the lung of many species is the *P4501A1* (*Cyp1A1*) isozyme (12–14). Therefore, to understand better the mechanism by which oxygen breathing increases lung P450 activity and to determine which lung cells are responsible for this increase, we cloned the *P4501A1* gene from a lamb lung cDNA library, measured steady state *P4501A1* RNA levels in lung tissue, in primary cultures of sheep lung endothelial cells, and in lung vascular smooth muscle cells. We found that, in vivo, oxygen breathing increased steady state *P4501A1* RNA levels by over fourfold within 12 h of oxygen exposure and that this increase preceded the increase in *P4501A1* enzyme activity. In cultured microvascular endothelial cells (but not in endothelial cells from the main pulmonary artery or in lung smooth muscle cells), oxygen exposure increased *P4501A1* RNA levels within 6 h, and this effect could not be mimicked by exposure of lung cells to chemical oxidants, to oxygenated medium, or to medium conditioned by exposure to hyperoxic cells. Nuclear run-off assays indicated that the oxygen-associated increase in *P4501A1* RNA levels was due in

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1. Abbreviations used in this paper: 3-MC, 3-methylcholanthrene; AhR, aromatic hydrocarbon receptor; ROS, reactive oxygen species; XRE, xenobiotic response element.

part to an increase in *P4501A1* transcription rate. We concluded from these studies that oxygen tension increases lung microvascular *P4501A1* gene expression and speculate that this effect may contribute to the oxidant stress of oxygen exposure.

## Methods

**Animal preparation, oxygen exposure protocol, and tissue analysis.** These methods have been described previously (10, 11). Briefly, 2–4-wk-old lambs were anesthetized and vascular catheters were inserted into a limb artery and vein. After 24 h of recovery, the lambs were placed into a wood and Plexiglas chamber which was maintained at 40% humidity and 20°C. Either medical-grade air or 100% oxygen flowed into this chamber at rates of 8–12 liters/min, which was sufficient to maintain ambient  $\text{FIO}_2$  at  $> 0.95$  and  $\text{CO}_2$  tension at  $< 1$  torr. To maintain nutrition and hydration, the lambs were fed commercial infant formula three times per day via orogastric tube ( $150 \text{ cm}^3/\text{kg}/\text{d}$ ), and samples of arterial blood were obtained every 12 h. Lambs were injected with lethal intravenous doses of pentobarbital and lungs were obtained after 12, 24, 48, 72, and 96 h. The left lung was perfused free of visible blood with cold phosphate-buffered saline and frozen at  $-70^\circ\text{C}$  for subsequent microsome and RNA isolation. These protocols were approved by the Vanderbilt Animal Care and Use Committee at Vanderbilt University.

Lung microsomes were isolated from blood-free lung by differential centrifugation (15) and *P4501A1* isozyme activity was estimated as the deethylation of ethoxyresorufin (16). The protein concentration of lung microsomes was measured by the BCA method (17). *P4501A1* activity is expressed as the amount of product per minute per milligram of protein. Total lung RNA was isolated from the left upper lobe of all lambs using the method of Chomczynski (18).

**cDNA cloning.** poly(A)<sup>+</sup> RNA was isolated from the lung of a 3-wk-old mixed breed lamb that had breathed oxygen for 48 h. A cDNA bacteriophage library was constructed using Uni-ZAP XR (Stratagene, La Jolla, CA). The library was screened with a  $^{32}\text{P}$ -labeled (Multi-Prime; Amersham Corp., Arlington Heights, IL) full-length rat *P4501A1* cDNA clone (kindly provided by D. Nebert, University of Cincinnati). After secondary screening, the insert DNA was isolated and subcloned into a plasmid vector (GEMEX-1 DNA; Promega, Madison, WI), restriction-mapped, and sequenced in both directions using both single-stranded (SEQUINASE; United States Biochemical Corp., Cleveland, OH) and cycle sequencing (Circumvent; New England Biolabs Inc., Beverly, MA). Six unique clones were identified, one of which contained the full-length cDNA when compared with the rodent sequence. The sheep cDNA sequence was entered into a DNA analysis program (DNASTAR, Inc., Madison, WI) and compared for sequence homology with other reported genes.

***P4501A1* probes.** A cDNA probe termed 70-2 was used in initial experiments. This consisted of a KpnI-BamHI fragment containing almost the entire cDNA. This cDNA fragment was labeled by multi-priming. Once the cDNA sequence was known, a 30-mer oligonucleotide designed from the 3' untranslated portion of the cDNA was constructed and end labeled. We used this latter probe in *in vitro* studies to reduce the chance of detecting related *P450* RNAs. Both probes gave equivalent results in Northern analysis.

**RNA analysis (Northern and slot blots).** Total lung RNA from lamb lung and from lung endothelial cells obtained after timed exposure to hyperoxia was isolated and quantitated spectrophotometrically. For slot blots, 2  $\mu\text{g}$  of each RNA sample was spotted onto filters using a vacuum apparatus (Schleicher & Scheull, Inc., Keene, NH). For Northern blots, 4  $\mu\text{g}$  of RNA was loaded onto 1% agarose gels and electrophoresed. The RNA was then transferred to nylon membranes overnight. Hybridization conditions for both blotting procedures were as follows. Filters were crosslinked by brief ultraviolet exposure (Stratolinker; Stratagene, La Jolla, CA) and then placed in bags containing 20 ml of prehybridization solution containing 5 ml of  $20 \times \text{SSC}$ , 10 ml formamide, 1 ml 1 M

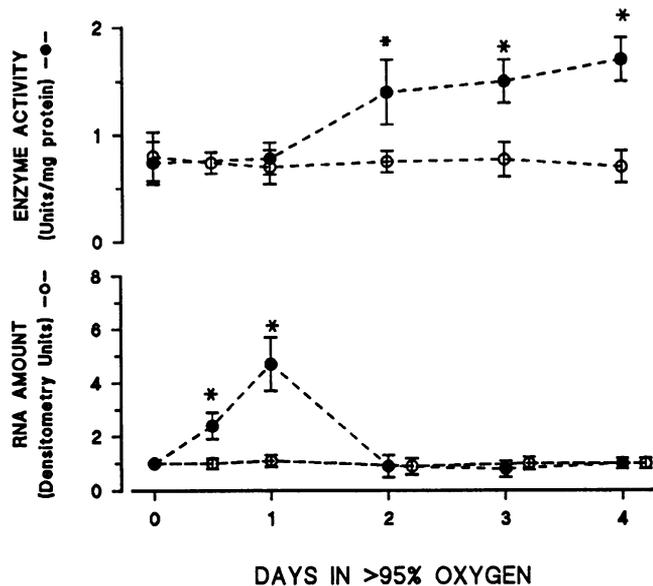
$\text{NaPO}_4$ , 0.2 ml  $100 \times$  Denhardt's solution, and 3 ml double-distilled sterile water. The filters were preincubated for 1 h at  $37^\circ\text{C}$ , then the solution is removed and 10 ml of hybridization solution (four parts prehybridization solution and one part 50% dextran sulfate) is added. Labeled cDNA or oligonucleotide probe ( $\sim 1$  million cpm/ml solution) and an equal volume of salmon sperm DNA (10 mg/ml) were boiled for 5 min and then injected into the bag. The bags were placed in a  $42^\circ\text{C}$  water bath for 20 h. The filters were washed four times at room temperature with  $2 \times \text{SSC}/0.1\%$  SDS for 5 min, then twice at  $55^\circ\text{C}$  with  $0.1 \times \text{SSC}/0.1\%$  SDS for 15 min. The filters were then placed on film for 24–48 h (19). RNA abundance was estimated with a digital scanner and the filters were stripped and reprobbed for the constitutive gene  $\beta$ -actin.

Relative RNA abundance was also estimated using the slot blot method. Results are expressed as relative densitometry units relative to the same filters probed for  $\beta$ -actin.

**Endothelial cell isolation and oxygen exposure protocols.** Endothelial cells from large pulmonary arteries were obtained by gently scraping the cells from large vessels directly into cell culture media (see below). Microvascular endothelial cells and smooth muscle cells were isolated from freshly isolated portions of peripheral blood-free lung of air-breathing lambs using the method of Meyrick et al. (20), except that Dulbecco's modified Eagle medium (DMEM) supplemented with 15 mg/400 ml endothelial growth supplement (Collaborative Biomedical Products, Becton Dickinson, Bedford, MA) was used. In brief, to isolate microvascular endothelial cells, subpleural lung fragments were minced with small scissors and placed in DMEM to which is added 1 mg/ml collagenase type II (Worthington Biochemical Corp., Freehold, NJ). The solution was incubated at  $37^\circ\text{C}$  for 30 min, then spun at  $1,000 \text{ g}$  for 10 min. The supernatant was discarded and the pellet was resuspended in supplemented DMEM containing 5% calf serum (Hyclone Laboratories, Logan, UT), 0.5% penicillin/streptomycin, and 0.1% gentamycin. Aliquots of cells were added to P-100 plates and incubated at  $37^\circ\text{C}$  in 21% air/5% carbon dioxide. Plates were examined daily and fresh medium was added. Cell types were initially identified by morphology, isolated with cloning rings, removed, replated onto plastic P-100 dishes, and covered with DMEM containing 5% fetal calf serum. The cells were incubated in 5%  $\text{CO}_2/95\%$  air at  $37^\circ\text{C}$ . After three to four passages, putative endothelial cells and smooth muscle cells were identified by morphology, subcloned into separate dishes, and also grown on plastic coverslips for immunofluorescent detection of Factor VIII (anti-vWf, anti-FVIII-RAG; and anti-mouse-fluorescein, F(Ab) fragment, both from Boehringer Mannheim Corp., Indianapolis, IN), LDL binding (Lil-Ac-LDL; Biomedical Technologies, Inc., Stoughton, MA), and smooth muscle actin (monoclonal Anti- $\alpha$  smooth muscle actin; Sigma Immunochemicals, St. Louis, MO). Cells which stained positive for Factor VIII and LDL but negative for actin were considered to be endothelial cells and were studied further. Smooth muscle cells were also isolated from lung explants by morphology, subcloned, and tested by immunofluorescence. Cells which were negative for Factor VIII and LDL binding but positive for actin were considered to be smooth muscle cells.

We also performed preliminary studies to determine the effect of ambient oxygen concentration and serum concentration on endothelial cell growth. We placed the cells in serum-free media for 24 h, then added bovine calf serum at concentrations from 0 to 5% and exposed the subconfluent cells to either air or oxygen for 48 h. Then, we discarded the media, pelleted the trypsin/EDTA-treated cells by gentle centrifugation, resuspended them in fresh media, and counted cells in a Coulter counter (Coulter Corp., Hialeah, FL). We found no consistent effect of serum concentration or 48 h of hyperoxia on endothelial cell number. After 48 h, oxygen exposure reduced cell number by  $\sim 15\%$ .

Before oxygen exposure, the near-confluent cells were placed in serum-free media for 24 h. Then, fresh media containing 1% fetal calf serum were added and the cells were exposed to either 5%  $\text{CO}_2/95\%$  air or 5%  $\text{CO}_2/95\%$  oxygen for up to 48 h. Total RNA was isolated and steady state RNA levels were estimated by slot blotting, autoradiog-



**Figure 1.** Effect of breathing > 95% oxygen on lamb lung *P4501A1* enzyme activity (top) and steady state RNA levels (bottom). Note that the significant increase in enzyme activity is preceded by an increase in RNA. Each data point represents the mean  $\pm$  1 SD of values from three to six lambs. An asterisk indicates  $P < 0.02$  compared with air baseline value. Open circles, air-breathing lambs; closed circles, oxygen-breathing lambs.

raphy, and densitometry. *P4501A1* RNA levels are expressed as arbitrary densitometry units relative to actin.

To test whether the effect of oxygen exposure on *P4501A1* gene expression was due to oxidation of media or serum components (21, 22), we bubbled medical grade 100% oxygen into DMEM/1% fetal calf serum for 24 h, then placed this medium on endothelial cells for up to 48 h in a 5% CO<sub>2</sub>/95% air environment. To determine whether the effect of high oxygen tension on *P4501A1* gene expression was due to some soluble factor released by hyperoxic cells, media from cells exposed to 5% CO<sub>2</sub>/95% oxygen for 24 h were removed and applied to fresh endothelial cell cultures and placed in a 5% CO<sub>2</sub>/95% air environment for up to 48 h.

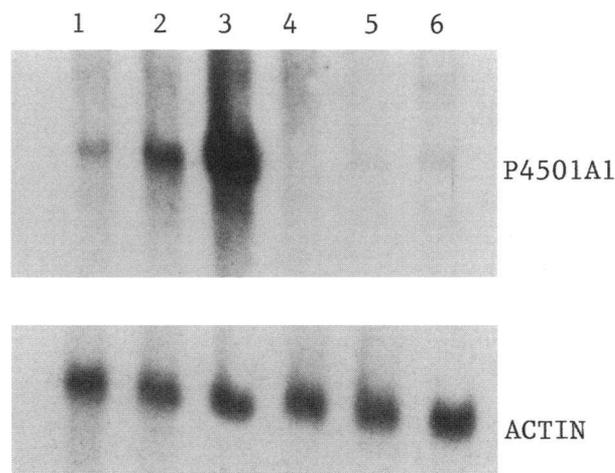
To determine whether new protein synthesis was required for the hyperoxic effect, we added cycloheximide (Sigma Immunochemicals) (1.25  $\mu$ g/ml) to the media before exposing the cells to hyperoxia.

To determine whether chemical oxidants could mimic the effect of oxygen tension, we added either *tert*-butyl hydroperoxide (Sigma Immunochemicals) or hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ) in concentrations between 10 and 300  $\mu$ M and grew these cells in 5% CO<sub>2</sub>/95% air for up to 48 h. Finally, we treated cultured cells with 3-methylcholanthrene (3-MC) (Sigma Immunochemicals) (0.1–1.0 mM), a known inducer of *P4501A1* at the transcriptional level (23).

To determine whether oxygen exposure increased *P4501A1* gene expression at the transcriptional level, we isolated endothelial cell nuclei after 90 min of exposure to either air or 95% oxygen and performed a nuclear run-on assay (24). As controls, DNA fragments from Bluescript plasmid, actin, and  $\alpha$ -tubulin were used, and transcription rate was estimated by scanning autoradiographs with a digital scanner.

To determine whether oxygen exposure influenced *P4501A1* RNA stability, lung endothelial cells were exposed to either air/5% CO<sub>2</sub> or 95% oxygen/5% CO<sub>2</sub> for 6 h, then actinomycin-D (0.005 mg/ml media) and isolated RNA were added every 3–6 h for 18 h. RNA was isolated and *P4501A1* levels were quantitated by slot blots and densitometry, then graphed on semi-log paper. Results were fitted to a log-linear curve.

**Statistics.** To assess the effects of air or oxygen exposure on



**Figure 2.** Representative Northern blot of total lung RNA from oxygen-exposed lambs. Lanes 1–6 depict RNA from individual lambs who breathed oxygen for 0 (air), 12, 24, 48, 72, and 96 h (4  $\mu$ g RNA per lane). The top row depicts the single 2.7-kb band identified as *P4501A1*; the bottom row is the same filter stripped and reprobed for  $\beta$ -actin.

*P4501A1* enzyme activity and RNA amount, we used one-way ANOVA followed by multiple range testing. To assess the effects of serum concentration, oxygenated and conditional media, and treatment with *tert*-butyl hydroperoxide, hydrogen peroxide, cycloheximide, and 3-MC, we compared steady state RNA levels to air-exposed control flasks after 24 h of treatment using an unpaired *t* test connected for multiple comparisons (25). In the table and figures, the results are the mean  $\pm$  1 SD for three to six independent experiments. For drug treatment studies, the effect of the highest concentration of drug which did not affect cell viability is presented. All experimental results were confirmed in at least two different experiments; each experiment was performed at least three times.

## Results

**Oxygen tension increases lung *P4501A1* RNA levels and enzyme activity in vivo.** Using a heterologous *P4501A1* cDNA probe, we found that oxygen exposure significantly increased *P4501A1* enzyme activity and this increase was preceded by a fourfold increase in steady state RNA levels within 24 h (Fig. 1). It is noteworthy that the increase in *P4501A1* RNA occurs 48 h before any physiologic or histologic evidence of lung injury (10, 11). In lamb lung tissue, a single RNA species of  $\sim$  2.6 kb was identified (Fig. 2). Oxygen breathing had no effect on actin RNA levels (Fig. 2).

**Description of sheep *P4501A1* cDNA (Fig. 3).** Our clone of the sheep *P4501A1* cDNA was 2,586 bp in length and contained 105 bp of the 5' untranslated region before two in-frame translation start sites. The 3' untranslated region is 924 bp in length from the TAG translation stop site to the polyadenylated tail. The 1,557 bp cDNA open reading frame contains a consensus sequence for a heme-iron binding site at the expected region of the deduced protein sequence from amino acid 457 to 466 (26). Homology comparison shows that the sheep cDNA coding region is 85% homologous to human 1A1, 82% to rat 1A1, and 74% to human 1A2.

**Oxygen tension increases lung *P4501A1* in lamb lung microvascular endothelial cells.** As shown in Fig. 4, oxygen expo-

P4501A1  
\* \* \* S E Q U E N C E \* \* \*

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1  ATTCCTTGGG  GCCTCCCTGG  ATCTTCTTTG  GGGTCCCTGG  CTGCAGAGAC  CACTCTTCCC
61  AGCCCAAGTC  TGCACCCCTC  CACAGCCACA  GCCACCTTGG  AATCTATGTC  GATCAGTGTG
121  TCTGTGTTTG  GACTCCCCAT  CCCCATCTCG  CCTCTCTGTC  CTCTGCTGTC  CTCTGCTGTC
181  TTCTGCTCTG  TATTCTGGGT  GGTCAGGACC  TGGCGGCTCT  GGGTCCCTCA  AGGCCTGAAG
241  AGTCCACCAG  AGCCCTGGGG  CTGGCCCTGC  ATGGGGCAGC  TGCTGACCTT  GGGGAAGAAC
301  CCACACCTGG  TTCTGTGCGA  ACTGAGCCAG  GCCTATGGGG  ACCTGCTGCA  GATCCGCATT
361  GGCTGCACAC  CCGTGTCTGG  CCGAGCCGSC  CTGACACCA  TCOCGAGCC  CCGTGTGGGG
421  CAGGCGATG  ATTTCAAGGG  CCGGCCACAC  CTCTACAGCT  TCACTTTGTT  CTCTGACCGG
481  CAGAGACGTA  CCTTCAACCC  AGACTCTGGA  CCGGTNTGGG  CTGCCCGGG  ACGCCCTGGC
541  CAGAATGCC  TGAAGAATTT  CTCCACCGCC  TCAGACCCGG  CATCCTTGTG  CTCTGCTGTC
601  CTGGAAGAGC  ATGTGAGCAA  GGAGGCTGAG  TACCTCCTGG  GCAAGTTCCA  GGAGCTGATG
661  TCAGGGCCTG  GCGCTTCGA  CCCCACAGG  TATGTAGTGG  TCTCAGTGGC  CAATGTATC
721  TGTGCCATAT  GCTTTGGCCG  GGGCTATGAC  CACGATGACC  AAGAGTTTCT  TAGCCCTATC
781  AACCTGAGTA  ATGAGTTTGG  GGAGATAACT  GCCTCCGGGA  ACCCAGCTGA  CTTTCATCCCT
841  GTCTCCGTT  ACCTGCCCAA  CACTGCCCTG  GACTCTTCA  AGGACCTGAA  TCGGAGGTTT
901  TACCTCTTGG  TACAGAAGAT  AGTCAAGGAA  CACTATAAAA  CGTTTGAGAA  GGCTCACATC
961  CCGGACATCA  CAGACAGCCT  GATTGAGCAC  TGTCAGGATA  AGAGGCTGGA  CGAAGATGCC
1021  AATATCCAGC  TGTCCGATGA  GAAGATCATT  AATGTCTGTA  TGGACCTCTT  TGGGGCCGGG
1081  TTGACACAG  TCACAATGTC  CCATTTCTCT  GAGCCCTCTG  TACTGCTGTA  CAGCCCCAGC
1141  GGTCAGAAA  AAGATTCAGG  AGGAGCTGGA  CACAGTGAAT  GGCAGGGGG  GGTGCCCCA
1201  GCTCTGAC  AGAOCCTAGC  TGCCCTATTT  GGAGCCCTTC  ATCCGTGAG  CCTTCCGACA
1261  CTCTCCTTTC  GTCCCTTCA  CCATCCACA  CAGTACCACA  AGAGACCA  ATTTGAATGG
1321  CTTTTACATC  CCGAAGGGGG  NCTGTGTCTT  TGTGAACCG  TGGCAGATCA  ACCATGGCA
1381  GAGCTATGAG  GAGGATCCAT  CTGAGTTCGG  GCCAGAACC  TTCTCCTACT  CTGATGGCAC
1441  GCTCAACAAA  GTACTGAGTG  AGAAGTGAT  TATTTTCGGC  TTGGGCAAGC  GGCAGTGCAT
1501  TGGTGAATC  ATTCGCCCTG  TGGAGGCTTT  TCTCTCTCTG  GCCATCTGAT  TGCATCAGGT
1561  GAAATTCAC  GTGACCCCGG  GTGTGAAGTT  GGACATGACC  CCTTGTATG  GGCCTGACAT
1621  GAAGCACGCC  CCGTGTGAGC  ACTTTCAGTT  GGCATCCGC  TCTTGGGGA  GCGTCTGCA
1681  GCCTAGACAG  CCGATGTGCT  CTTGGGGGAC  AGATAGCCCT  GCCCCTGTCT  GGGCAGCCAG
1741  GCCAGGGGCT  AGCCAGGGGA  GCCTAAAACC  CGCAGACATT  GTTGGGCTGA  TTTTTTGTCT
1801  GTCTGAGCTA  TGGCAAGCT  TGAGGGATCA  TACCTGCCCC  CCAATCCGTA  CTGTGCTTTC
1861  TGCAACATGA  CCACAGATAG  CAGACACATC  AGAGGCCACA  CAGGAGCTGA  TGGAGCCCTT
1921  CTGAAGTGT  GCTGAAAGGG  CTAGAGGATC  CTTAGGCCCT  TGGAAACCTC  TAAAGAGGTT
1981  TTGGGAAGCC  CCGTGGCCCG  GCCACAGGC  TGGTTGGCTT  TCCATGGAGG  CTGACTGGCT
2041  TGAGAAACAT  TCAGACAGG  TCACAGCAGG  GCCTGACCAA  TCTCTTTGAC  AGTTGGAGGC
2101  CACTGAGACT  GAAGGAAGA  GGCAGTCCAG  AATACTGACA  TGGAGTGTAT  CTCCCTGCTT
2161  AAACAAGACT  GAGCAATCTG  ACCACTAGGG  TCTGGGACAC  TCTGCTGTG  AGATGACCTT
2221  CCTCTCTGCA  GGCAGGGGTG  GGGCTGCTGC  CTGGCCTTGT  TCCTGTCCAG  GGAGGCTGA
2281  GAATCTGTGT  CTAACAGAG  CAGAGAGCAG  AGGGAGGGCC  CAAATCCAGG  CACCAGGACT
2341  GGATCAGAG  GGAGGGGTG  GTAATCGAGT  CACTTTCTAC  TGCACTCTCT  TTCAATATAC
2401  CCTGTGTAA  GTCTTTAAA  AATTTTAGCC  TGCATGTGAT  TTGCATGTTT  GTATTTACCA
2461  TATATGAGAG  CTTAAGACA  GCTTATTGAG  AGCCATGAG  AAAATTTCAA  CCGAGTATC
2521  CAGAAATGTG  TAGGAAATAT  CTATCTACCT  GAGCTAAATA  AAGATATTAT  TCAGAAAAA
2581  AAAAA

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Figure 3. Lamb lung *P4501A1* cDNA sequence. Boxes indicate translation start and stop sites; a putative heme-binding domain is underlined. See text for details.

sure significantly increased *P4501A1* gene expression within 6 h and was maximal after 24 h; oxygen exposure had no effect on actin or  $\alpha$ -tubulin RNA levels. As shown in Fig. 5, there was no effect of serum concentration, oxygenated media, or media harvested from hyperoxic cells on *P4501A1* RNA levels. Cycloheximide also had no effect on basal *P4501A1* gene expression and did not effect the response to oxygen exposure. Treatment of the cells with either the chemical oxidant *tert*-butyl hydroperoxide or hydrogen peroxide, over a range of concentrations (10–300  $\mu$ M) that did not affect cell viability, had no effect on steady state RNA levels. As expected, treatment of microvascular endothelial cells with 3-MC (a known inducer of *P4501A1* transcription) at concentrations up to 1 mM significantly stimulated *P4501A1* gene expression. In two separate experiments, the effect of hyperoxia on 1A1 RNA accumulation was unaffected by changing the serum concentration from 0 to 5% (data not shown). The combination of 3-MC (1 mM) and hyperoxia resulted in a sixfold increase in *P4501A1* levels after 24 h of exposure.

*P4501A1* RNA was barely detectable in pulmonary artery endothelial cells and in lung vascular smooth muscle cells after 5–7 d of autoradiography; no effect of hyperoxia could be detected (data not shown).

The effect of oxygen exposure on *P4501A1* transcription rate is shown in Table I. Oxygen exposure had no effect on transcription of the  $\alpha$ -tubulin or actin genes, but after 90 min of oxygen exposure the rate of initiation of new *P4501A1* transcripts increased 3.6-fold relative to air exposure ( $P < 0.01$ ).

Finally, oxygen tension had no effect on *P4501A1* RNA stability. The  $t_{1/2}$  under air conditions was  $6.8 \pm 1.4$  and  $5.9 \pm 1.6$  h under hyperoxic conditions (NS,  $n = 3$ ,  $r = 0.97-0.99$ ).

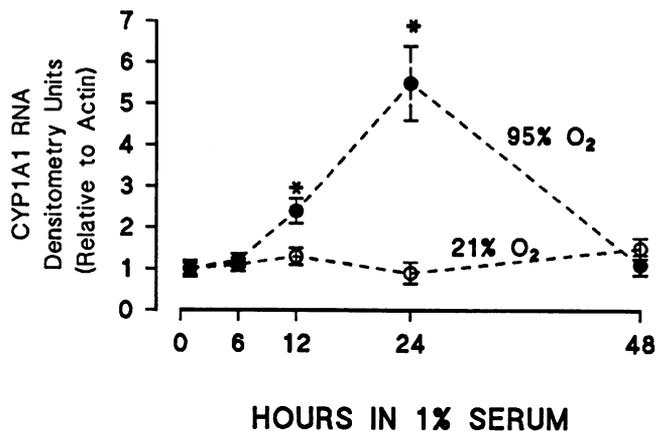


Figure 4. Oxygen exposure increases *P4501A1* RNA levels in sheep lung microvascular endothelial cells. Each point represents the mean  $\pm$  1 SD of cells obtained from at least four individual lambs at each time. \*  $P < 0.01$ .

## Discussion

We found that oxygen exposure in vivo significantly increases steady state lung *P4501A1* RNA levels and enzyme activity in the lamb model of acute pulmonary oxygen toxicity. In this well-characterized model, oxygen breathing causes no net increase in lung antioxidant enzyme concentrations or in lung glutathione levels. In addition, there are no histologic or physiologic abnormalities in the lung until 60–72 h of oxygen breathing. After 72 h of hyperoxia, lung microvascular permeability to water and protein increases and the animals develop pulmonary edema and fatal respiratory failure (10, 11). Therefore, it is likely that the early increase in *P4501A1* RNA levels and the subsequent increase in isozyme activity in the hyperoxic lung are the result of increased expression by lung cells in situ and not due either to the influx of inflammatory cells or to proliferation of resident lung cells.

A direct effect of oxygen tension on a specific lung cell population is also shown by our in vitro data. In cultured lamb lung microvascular endothelial cells, oxygen exposure significantly increased *P4501A1* RNA levels by increasing transcription rate; RNA stability was unaffected by oxygen exposure. In these endothelial cells, oxygen-stimulated expression occurred as early as 6 h, was not serum dependent, did not require protein synthesis, and could not be mimicked in vitro by hyperoxic media, conditioned media, or by two chemical oxidants. We interpret these data to indicate that oxygen tension regulates *P4501A1* gene expression at the transcriptional level in the lung, and in particular, in the lung microcirculation.

For our in vitro experiments, we serum starved the cells for 24 h then added 1% bovine calf serum at the start of exposure to normoxic or hyperoxic conditions. Serum contains detectable catalase activity, and it is possible that the response to hydrogen peroxide or *tert*-butyl hydroperoxide was influenced by this serum effect. However, there was no effect of these oxidants on *P4501A1* RNA levels even after 24–48 h and over a wide range of concentrations approaching toxicity. Moreover, the accumulation of *P4501A1* RNA after 24 h of hyperoxia was unaffected by serum concentrations which varied from 0 to 5% (data not shown). These findings make it unlikely that the cells

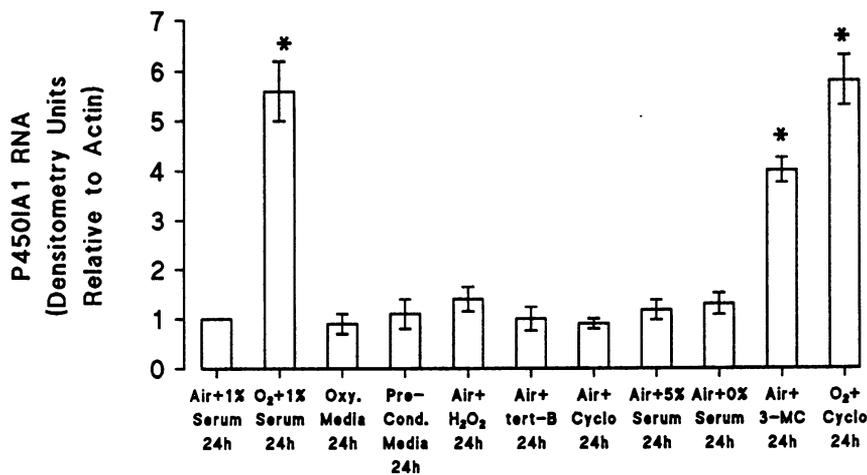


Figure 5. Effect of oxygenated media, conditioned media, serum concentrations, chemical oxidants, cycloheximide, and 3-MC on *P4501A1* RNA levels in sheep lung microvascular endothelial cells. Except for the hyperoxic cells, cells were treated for 24 h with each agent and kept in a 5% CO<sub>2</sub>/21% oxygen environment. Abbreviations and doses: H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide (0.1 mM); tert-B, tert-butylhydroperoxide (0.1 mM); Cyclo, cycloheximide (1.25 mg/ml media); 3-MC, 3-methylcholanthrene (1 mM). Each bar represents the mean ± 1 SD; \*P < 0.02 compared with air control.

were protected from oxidant stress by the antioxidant properties of serum. We normalized our RNA results to  $\beta$ -actin expression which showed good interassay reproducibility and single clear bands on Northern blots (Fig. 2), and its abundance was unaffected by hyperoxia or by treatment with chemical oxidants.

Although regulation of eukaryotic gene expression by hypoxia (27) and by chemical oxidants (28) has been described, transcriptional regulation of gene expression by hyperoxia is rare (29). In the mouse lung, the surfactant protein B gene is increased by hyperoxia (30). Oxygen-induced lung injury in rabbits has also been shown to increase expression of a gene which encodes the tissue inhibitor of metalloproteinases, but this effect requires 96 h of oxygen exposure; by that time, there is histologic evidence of substantial inflammation and type II cell hyperplasia (31).

The mechanisms by which high oxygen tension regulates gene transcription are unknown. Hyperoxia could act by globally stimulating ROS production, which would then interact with *trans*-acting factors which bind to the antioxidant response element (32), or by influencing redox-sensitive genes like *fos*, *jun* (33), or NF- $\kappa$ B (34). However, because neither tert-butyl hydroperoxide nor hydrogen peroxide induced sheep 1A1 gene expression even in concentrations which approached cell toxicity, our data thus far suggest that oxygen is inducing *P4501A1* transcription by a mechanism other than ROS production. An alternative explanation is that hyperoxia is triggering a latent capacity for transcriptional activation in a constitutive factor, such as the oxidant-modified oxyR protein described in *Escherichia coli* (35). Another possibility is that hyperoxia is modi-

fying a metalloprotein in lung microvascular endothelial cells which in turn directly or indirectly interact with an as yet undefined DNA regulatory element; this mechanism has been proposed for hypoxic regulation of the erythropoietin gene expression (36). We are now isolating the flanking regions of the sheep *P4501A1* gene and preparing fusion gene constructs to test these possibilities.

The pulmonary expression of *P4501A1* enzyme activity has been linked to arachidonic acid and xenobiotic metabolism and to lung cancer (37–40). The *P4501A1* isozyme is structurally and catalytically similar to *P4501A2*, but this latter gene is not appreciably expressed in lung (37, 38). We cloned the *P4501A1* cDNA from a sheep lung cDNA library, and its sequence shares greater homology with human 1A1 than human 1A2. In addition, our oligonucleotide 1A1 probe was designed from the 3' untranslated portion of the lamb lung 1A1 cDNA, making detection of other P450 RNA species unlikely. The unambiguous resolution of this possibility would require cloning of both the sheep 1A2 and 1B2 isozymes. However, we believe that we have cloned the sheep *P4501A1* cDNA.

*P4501A2* and perhaps 1B1 isozymes can metabolize ethoxyresorufin, and if either isozyme was present in sheep lung it would have contributed to the metabolic activity depicted in Fig. 1.

The transcriptional regulation of the *P4501A1* gene has been studied in detail (39, 40). The 5' flanking region of *P4501A1* contains multiple DNA sequences termed the xenobiotic response element (XRE) which permits transcriptional activation by phenolic hydrocarbons (37) which bind a cytosolic transcription activator termed the aromatic hydrocarbon receptor (AhR) (41). The endogenous ligand for this receptor is unknown, but the AhR and related nuclear binding proteins (42) result in transcriptional activation of *P4501A1* by many polycyclic hydrocarbons including 3-MC and dioxins. In lamb lung microvascular endothelial cells, treatment with 3-MC increased steady state *P4501A1* RNA levels by almost fourfold, suggesting that the AhR and its cofactors are present in these cells and that the sheep *P4501A1* gene contains at least one XRE. In human keratinocytes, *P4501A1* is transcriptionally induced by subjecting the cells to physical stresses; physical stress also induced the transcription of other genes regulated by the AhR and XRE, suggesting that endogenous AhR ligands may be involved (43).

Table I. Effect of Oxygen Tension on *P4501A1* Gene Transcription\*

Gene	Air	95% Oxygen	O <sub>2</sub> /Control
<i>P4501A1</i>	24.2 ± 3.1	87.6 ± 6.3 <sup>†</sup>	3.6 ± 0.3 <sup>†</sup>
$\alpha$ -tubulin	32.1 ± 5.0	20.5 ± 8.2	0.64 ± 0.2
$\beta$ -actin	40.1 ± 5.2	38.6 ± 6.4	0.96 ± 0.1

\* Densitometry units corrected for PGEM control; n = 3–4; <sup>†</sup> P < 0.02. Microvascular endothelial cells were exposed to air or 95% oxygen for 90 min, then nuclear run-on assay was performed as described.

Whether hyperoxia is acting as an environmental stress in lamb lung endothelial cells remains to be elucidated. In our study, it is possible that hyperoxia is increasing the availability of an endogenous ligand for the AhR. Recent studies have also suggested that the antioxidant response element and XRE are similar but the proteins which bind to them are not closely related (44).

It is noteworthy that the effect of oxygen tension on *P4501A1* gene expression was not evident in pulmonary artery endothelial cells or in lung smooth muscle cells. Whether the large vessel endothelial cells de-differentiate in culture or constitutively lack the relevant transcription factors is unknown, but our results provide another example of the site-specific nature of gene expression in pulmonary endothelial cells (45). Therefore, we conclude that the oxygen-associated increase in lung *P4501A1* enzyme activity in vivo is strongly associated with overexpression in pulmonary microvascular endothelial cells. A physiologic role for *P4501A1* expression in the pulmonary microcirculation is unknown, but our whole animal studies clearly indicate that the induction of expression by hyperoxia is associated with microvascular injury in vivo. Additional evidence that *P4501A1* is linked to lung injury comes from the recent report of a *P4501A2* knock-out mouse. *P4501A2* is not present in mouse lung, but surprisingly pulmonary expression of the 1A1 isozyme was very high and the phenotype was lethal in > 99% of homozygotes; death was associated with pulmonary hypoplasia (46).

Our findings may have clinical relevance for patients undergoing oxygen therapy. If *P4501A1* enzyme activity is inducible in lung cells by oxygen and if microsomal oxidant production is increased, this would contribute to the oxidant stress of oxygen exposure, particularly in endothelial cells which are highly vulnerable to oxidant injury. In these cells, even small increases in ROS production could initiate peroxidation chain reactions adjacent to the endoplasmic reticulum if unopposed by antioxidants. Hyperoxic induction of *P4501A1* activity might also increase the production of potent epoxides from arachidonic acid which might further amplify hyperoxic lung injury. A better understanding of the enzymatic sources of ROS production in the lung could lead to the development of new strategies to reduce hyperoxic lung injury. Our findings may also provide further stimulus to understand the mechanisms by which ambient gases regulate gene expression.

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