Hydroxia-induced changes in estradiol metabolism in postnatal airway smooth muscle

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CONSIDERABLE EVIDENCE LINKS supplemental oxygen exposure in preterm and term neonates to the development of neonatal lung diseases, such as bronchopulmonary dysplasia (BPD) and asthma. There is a known sex predilection for BPD, but the underlying mechanisms are not clear. We tested the hypothesis that altered, local estradiol following hyperoxia contributes to pathophysiological changes observed in immature lung. In human fetal airway smooth muscle (fASM) cells exposed to normoxia or hyperoxia, we measured the expression of proteins involved in estrogen metabolism and cell proliferation responses to estradiol. In fASM cells, CYP1A1 expression was increased by hyperoxia, whereas hyperoxia-induced enhancement of cell proliferation was blunted by estradiol. Pharmacological studies indicated that these effects were attributable to upregulation of CYP1A1 and subsequent increased metabolism of estradiol to a downstream intermediate 2-methoxyestradiol. Microarray analysis of mouse lung exposed to 14 days of hyperoxia showed the most significant alteration in CYP1A1 expression, with minimal changes in expression of five other genes related to estrogen receptors, synthesis, and metabolism. Our novel results on estradiol metabolism in fetal and early postnatal lung in the context of hyperoxia indicate CYP1A1 as a potential mechanism for the protective effect of estradiol in hyperoxia-exposed immature lung, which may help explain the sex difference in neonatal lung diseases.

ever, the underlying mechanisms for such differences are not clear. Maternal steroids as well as endogenous fetal/neonatal steroids may play a role. At birth, fetal estradiol, the major circulating estrogen, decreases by 100-fold (19) and is replaced by endogenous production. Hepatic and pulmonary production of sex steroids is recognized, where both organs contain enzymes responsible for metabolism of estradiol (3). However, hepatic enzyme activity develops slowly through the postnatal period (3, 13). Therefore, pulmonary sex steroid production and metabolism may be more important in the early postnatal period, with consequent autocrine/paracrine effects that lead to observed sex differences in neonatal lung disease, especially in the setting of hyperoxia.

Although cellular and molecular mechanisms underlying BPD are likely complex (5, 15), on the basis of the knowledge that pulmonary estrogen synthesis occurs and that estrogen is a potent mitogen (18), we hypothesized that altered local estradiol metabolism following hyperoxia contributes to changes in developing lung. We utilized a recently characterized, age-appropriate in vitro model of human fetal airway smooth muscle (fASM) cells to examine the relationship between hyperoxia and local estradiol metabolism.

MATERIALS AND METHODS

Materials. 17β-Estradiol (E2), 2-methoxyestradiol (2-ME), 1-aminobenzotriazole (1-AB), platelet-derived growth factor (PDGF), and hydrogen peroxide (H2O2) were from Sigma (St. Louis, MO). Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) unless otherwise noted. Caspase 9 p35, CYP19 (P450 aromatase), cytochrome P450 1A1 (CYP1A1), cytochrome P450 1B1 (CYP1B1), catechol-O-methyl transferase (COMT), estrogen receptor-β (ERβ), estrogen receptor-α (ERα), proliferating cell nuclear antigen (PCNA), cleaved caspase 3, and GAPDH were from Cell Signaling Technology (Beverly, MA).

fASM cells. Briefly, fASM cells were obtained and enzymatically dissociated from unidentified airways of deceased 18–20-wk-gestation fetuses (courtesy of MRC-Wellcome Trust HDBR Group, Newcastle University and University College London; approved by Ethics Committees in the UK, and considered exempt by Mayo Institutional Review Board) (14, 17). Cells were grown in standard culture conditions using DMEM F12 (phenol red free) with 10% FBS. Experiments were performed in cells serum deprived for 24 h in 96-well or 60-mm plate formats. Hyperoxia was limited to 50% based on a recent study showing toxicity at >70% oxygen.

Western analysis. fASM cells were exposed to normoxia or 50% hyperoxia for 24 h, harvested, and processed for Western analysis using standard techniques. Far-red fluorescence secondary antibodies (LI-COR Biosciences, Lincoln, NE) were used to visualize bands on
a LI-COR OdysseyXL system, quantified using densitometry, and normalized to GAPDH.

**Cell proliferation.** Previously described techniques for assessment of cell proliferation were used (14). Briefly, ~5,000 fASM cells/well were plated in a 96-well plate, allowed to adhere overnight, and then incubated with 0.5% serum media for 24 h. Cells were then treated (vehicle, E2, 2-ME, 1-AB, and PDGF-BB; see RESULTS) for 24 h under normoxic or 50% hyperoxic conditions. Media were aspirated, and Invitrogen CyQuant NF Cell Proliferation kit (Invitrogen, Carlsbad, CA) was used to determine fluorescence values on a Flexstation 3 microplate reader (Molecular Devices, Sunnyvale, CA; excitation 480 nm/emission 530 nm). Proliferation values were normalized for the initial numbers of cells as previously described (14) and also recalculated as percentage of values at normoxia.

**CYP1a1 activity.** A CYP1a1-Luciferin-CEE P450-Glo Assay kit (Promega, Madison, WI) was used. Specifically, ~20,000 serum-starved fASM cells/well were exposed to normoxia or 50% hyperoxia for 24 h. As a positive control, cells were treated with 10 μM 3-methylcholanthrene. Resultant luminescence was measured on a Turner Biosystems Veritas Microplate luminometer (Promega).

**Reactive oxygen species measurement.** Serum-starved fASM at 50,000 cells/well were incubated with 5 μM CM-H2DCFDA (Life Technologies, Grand Island, NY), a general oxidative stress fluorescent dye indicator, at ambient temperature for 45 min. After baseline reading, cells were treated with estradiol, 2-ME, or H2O2 (positive control). Fluorescence at indicated time points was measured using the Flexstation 3 microplate reader at 488 nm/525 nm with a 515-nm cutoff for 5 min. Data were normalized to baseline readings.

**In vivo mouse model: tissue preparation and mRNA expression profiling.** The neonatal mouse hyperoxia model was recently described (9). Briefly, with Mayo Clinic Institutional Animal Care and Use Committee approval (no. A41708), timed pregnant ICR mice were maintained on an ad libitum diet of standard chow and water. Within 12 h of birth, pups were randomized into groups: hyperoxia (80% oxygen) or room air (21% oxygen) for 14 days. At the appropriate time, mice were killed by CO2 narcosis, and lungs were excised. Total RNA from frozen lung samples was isolated using standard techniques (9). mRNA expression profiling was performed using the Affymetrix GeneChip Mouse Genome 430 2.0 Array (>45,000 transcripts) and analyzed using the Partek Genomics Suite 6.4 software, normalized with the Robust Multichip Average Algorithm, and converted to log2 values, and logarithmic data were statistically analyzed as previously described (9). The resultant mRNA microarray data have already been submitted to the Gene Expression Omnibus database (accession no. GSE25286).

**Statistical analysis.** Array data were analyzed using the Partek Genomics Suite software. ANOVA was used to determine significance between time points. After correction for multiple comparisons, genes were determined to be significantly regulated if their differential P value was <0.001 between groups. Fold changes were calculated between the treatment groups and controls. Cellular experiments were performed using three to four fetal airway samples. Statistical comparisons for cellular experiments were made using SigmaPlot (SYSTAT, San Jose, CA), and statistical significance was established at P < 0.05. Values are means ± SE.

**RESULTS**

**Estradiol metabolism proteins in fASM cells.** To characterize changes in estradiol metabolism in human fASM cells with hyperoxia, we examined protein expression of the four primary enzymes involved, CYP19 (aromatase), CYP1a1, CYP1b1, and COMT, as well as the two primary estrogen receptors, ERα and ERβ (Fig. 1A) following normoxia vs. 50% hyperoxia exposure. Only CYP1a1 expression was affected by hyperoxia, with increased expression after 24 h (Fig. 1B), correlating with increased CYP1a1 enzyme activity (Fig. 1C).

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![Image](http://example.com/fig1.png)

**Fig. 1.** Comparison of protein expression in human fetal airway smooth muscle (fASM) cells. A: protein expression of metabolizing enzymes as well as estrogen receptors was unchanged with hyperoxia exposure. CYP1b1, cytochrome P450 1B1; COMT, catechol-O-methyl transferase; ERα, estrogen receptor-α; ERβ, estrogen receptor-β. B: CYP1a1 expression by Western analysis (normalized for GAPDH) was significantly increased following 24 h of 50% hyperoxia exposure. C: CYP1a1 enzyme activity, as detected by luminescence (RLU, relative light units), is increased in hyperoxia. 3-Methylcholanthrene (3-MC), a known inducer of CYP1a1 expression, is included as a positive control. Values are means ± SE. *Significant effect of hyperoxia (P < 0.05).
Proliferation of fASM cells. Hyperoxia significantly enhanced proliferation of fASM cells (Fig. 2A). Under normoxic conditions, clinically relevant concentrations of E2 significantly increased proliferation of fASM cells in a dose-dependent manner to levels comparable to that induced by the mitogen PDGF (Fig. 2). In contrast, under hyperoxic conditions, E2 did not increase proliferation, indeed even reducing proliferation to below normoxic baseline (Fig. 2). On the basis of maximum proliferation induced at 100 pM E2 under normoxia, subsequent experiments were conducted at this concentration. Proliferation measurements using CyQuant were also confirmed by increased expression of PCNA in the presence of E2 in normoxia but with no change in expression when E2 was added in hyperoxia (Fig. 2B).

2-ME effects on fASM proliferation. CYP1a1 is responsible for metabolism of E2 to 2-ME. Because hyperoxia increased CYP1a1, we next determined whether failure of E2 to increase proliferation in hyperoxia was attributable to increased CYP1a1 activity. First, we determined the effect of exogenous 2-ME and found decreased proliferation even under normoxic and hyperoxic conditions (Fig. 2C). Separately, inhibition of CYP1a1 using 1-AB (100 μM) resulted in E2 increasing fASM proliferation in the presence of hyperoxia (Fig. 2D).

2-ME effects on apoptosis. On the basis of our results of lack of proliferation by E2 in hyperoxia-exposed cells, and previous reports that 2-ME has apoptotic activity, we explored whether apoptosis was induced by 2-ME resulting from CYP1a1 using 1-AB (100 μM) resulted in E2 increasing fASM proliferation in the presence of hyperoxia (Fig. 2D).

mRNA changes with hyperoxia in mouse model. We have previously reported (9) that, with hyperoxia, gene expression of well-known biomarkers of BPD, such as TGF-β, IGFl, and fibroactin-1, are increased, whereas expression of VEGF-α is decreased. Furthermore, expression of p21 is increased, suggesting hyperoxia-induced arrest of proliferation. To further confirm changes in sex steroid pathways, the same six mRNA related to estrogen handling (receptors, synthesis, and metabolism) were selected for evaluation (Table 1) and compared between groups. Overall, after 14 days of hyperoxia, increased expression for two of the six mRNA (ERα and CYP1B1) was observed, whereas COMT showed no change, and the remaining mRNA showed decreased expression. Of all the changes with hyperoxia, only CYP1a1 reached statistical significance after correction for multiple comparisons, showing an approximately fivefold reduction after 14 days (Table 1).

**Fig. 2. Proliferation of human fASM cells.** Cellular proliferation of human fASM cells in the presence of estradiol (E2) (A), 2-Methoxyestradiol (2-ME) (C), or CYP inhibitor, 1-a-minobenzotriazole (1-AB) (D), was evaluated using a fluorescent CyQuant assay (see MATERIALS AND METHODS). A: Under normoxic conditions, E2 significantly increased fASM proliferation even at low concentrations of 100 pM-1 nM to extents comparable to that induced by the mitogen PDGF. However, exposure to 50% hyperoxia for 24 h prevented E2-induced proliferation but did not affect PDGF influences. B: separately, estradiol exposure increased proliferating cell nuclear antigen (PCNA) expression consistent with increased cell proliferation. C: in contrast to E2, the metabolite 2-ME did not affect fASM cell proliferation and, if anything, blunted proliferation at high concentrations, regardless of the oxygen concentration. D: inhibition of CYP1a1 with 1-AB restored the effect of E2 on proliferation in the presence of hyperoxia. Data were performed in triplicate and expressed as means ± SE. *Significant effect for hyperoxia. #Significant effect for E2 or 2-ME effect (P < 0.05).
A rapid report of oxygen species (ROS) generation in fASM exposed to hyperoxia, E2, and 2-ME. A: changes in protein expression of active caspase 3 and procaspase 9 with estradiol treatment under hyperoxic conditions reflect an increase in apoptosis. These results were reversed with the addition of the CYP1a1 inhibitor 1-AB, suggesting that the metabolite 2-ME contributes to the increase in apoptosis. ROS generation can contribute to increased apoptosis. The addition of 2-ME to fASM does not show any increase in ROS; rather, 2-ME showed a dose-dependent decrease in ROS generated (B and C). fASM under hyperoxic conditions exposed to E2 or with the CYP1a1 inhibitor 1-AB also supports the observation that 2-ME does not increase ROS (C). Data were performed in triplicate and expressed as means ± SE. # Significant effect for hyperoxia, * significant effect for normoxia (P < 0.05).

**DisCUSSION**

In this study, we tested the hypothesis that altered local metabolism of E2 with hyperoxia contributes to changes in developing lung. Our studies using fASM exposed to hyperoxia demonstrated the relative stability in expression of enzymes responsible for estrogen metabolism with the exception of CYP1a1, which metabolizes E2 to 2-ME. Importantly, examination of the potential role of CYP1a1 in human fASM cells shows that, although E2 is normally proproliferative, its effect is lost in the presence of hyperoxia because of increased CYP1a1 levels but restored when CYP1a1 is inhibited. Overall, these data suggest that local estrogen metabolism may be important in altered cellular proliferation that occurs with hyperoxic injury in the newborn lung.

CYP1a1 is mainly an extrahepatic enzyme present in rodent and human lungs, intestines, placenta, and kidneys and is involved in metabolism of a number of compounds such as E2. CYP1a1 first metabolizes E2 to the unstable intermediate 2-hydroxyestradiol, which is further processed into the more stable 2-ME (via COMT). On the basis of data showing no change in COMT expression with hyperoxia, we focused our attention on the effects of E2 and 2-ME. To understand the cellular impact of altered CYP1a1 expression in human tissue and its contribution to E2 effects on proliferation, we used fASM cells. Our studies indicate that E2 normally increases proliferation in fASM, whereas 2-ME is largely without effect until much higher concentrations that are likely nonphysiological. However, when exposed to moderate levels of hyperoxia, which upregulates CYP1a1, the proproliferative effect of E2 is lost, consistent with the idea that CYP1a1 is involved in modulating E2 effects on proliferation. A previous study using newborn rats showed that induction of CYP1a1 attenuates hyperoxic lung injury (6), suggesting a protective role for CYP1a1. Our work here suggests a mechanism by which induction of CYP1a1 can attenuate hyperoxic lung injury, namely the inhibition of the proproliferative effect of E2. An additional mechanism may be the resultant increased generation of the antiproliferative intermediate 2-ME; however, the large 2-ME concentrations at which proliferation effects were observed at least in human fASM cells make this mechanism less likely.

This work also represents the first attempt to evaluate the effects of the estradiol intermediate 2-ME in human fetal airway. The antiproliferative effects noted with 2-ME generation via COMT, which upregulates CYP1a1, the proproliferative effect of E2 is lost, consistent with the idea that CYP1a1 is involved in modulating E2 effects on proliferation. A previous study using newborn rats showed that induction of CYP1a1 attenuates hyperoxic lung injury (6), suggesting a protective role for CYP1a1. Our work here suggests a mechanism by which induction of CYP1a1 can attenuate hyperoxic lung injury, namely the inhibition of the proproliferative effect of E2. An additional mechanism may be the resultant increased generation of the antiproliferative intermediate 2-ME; however, the large 2-ME concentrations at which proliferation effects were observed at least in human fASM cells make this mechanism less likely.

This work also represents the first attempt to evaluate the effects of the estradiol intermediate 2-ME in human fetal airway. The antiproliferative effects noted with 2-ME generation via upregulation of CYP1a1, as well as with direct treatment, led us to examine whether 2-ME induces apoptosis. Indeed 2-ME mediates apoptosis through caspase activation and not ROS generation and offers additional insight for how sex steroids may modulate lung development in the context of hyperoxia.
What remains unknown is the mechanism by which hyperoxia upregulates CYP1a1. One potential mechanism may be indirect regulation via miRNA. In our previous study using mouse lung (9), we found that, at 14 days postnatal, hyperoxia induces a change in miR-10b, which happens to be one of the miRNAs regulating CYP1a1 expression in mouse. What remains to be determined is whether other miRNAs are involved or whether more upstream mechanisms such as ROS induced by hyperoxia play a role.

We have previously used the murine hyperoxia model (9) to study the pathological arrest of lung development in BPD because hyperoxic exposure of newborn mouse pups recapitulates human BPD histopathology (1, 22). In human fASM, hyperoxia was found to increase CYP1a1 at 24 h, whereas in the murine hyperoxia model (albeit whole lung), hyperoxia significantly reduced CYP1a1. It is evident from previous studies that there are species differences in expression profiles of CYP1a1 under normoxic conditions (3), which may explain the divergent results found in this study. Another potential reason for the discrepancy is the fact that the mice were exposed to 80% oxygen, whereas fASM were exposed to 50% for a shorter duration (24 h vs. 14 days). Indeed, it is possible that alterations in CYP1a1 expression are dependent on oxygen concentration and duration, as well as the stage of lung development. In our previous work when CYP1a1 expression was examined over the course of mouse lung development, there is variability in CYP1a1 expression depending on developmental stage (10). It is obviously difficult to systematically match ages in human vs. mouse models. Further study is required to explore the relationships between CYP1a1, lung development, and hyperoxia in these complementary models.

The novel finding of the upregulation of CYP1a1 in fASM by hyperoxia may lend insight into the etiology of sex differences in BPD. Although there are currently no data in the developing lung per se, a previous study in adult smokers found decreased levels of lung CYP1a1 in males (16). If a similar sex difference pattern were to hold true in neonates, lesser upregulation of CYP1a1 in male babies in the presence of hyperoxia may make them more susceptible to development of BPD.

In conclusion, our work contributes several novel themes. First, we demonstrate that estrogen metabolism via CYP1a1 can occur in the lung. Previous work has shown CYP1a1 expression in airway epithelial, airway alveolar, and airway endothelial cells (7) and its presence in vascular smooth muscle cells (11). No other studies to date have discussed metabolism in the neonatal lung or more specifically fASM. Estradiol synthesis and metabolism in nongenadal tissue may be of less importance in the adult because of mature hepatic enzyme activity as well as gonadal contributions in regulation of sex steroid levels. However, in the neonate, local production of estradiol may play a greater role and thus be relevant to sex differences in the development of neonatal lung disease induced by insults such as hyperoxia.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES

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