ATTENUATION OF HYPEROXIC LUNG INJURY BY THE CYTOCHROME P450 INDUCER ♂-NAPHTHOFLAVONE

HYPOTHESIS

Hyperoxia is used extensively in the treatment of respiratory distress that is commonly observed in pre-term and term infants having pulmonary insufficiency. In premature human infants, the acute lung injury caused by hyperoxic therapies contributes to the development of bronchopulmonary dysplasia (BPD) and also chronic lung disease (CLD) during the newborn and early childhood period. The molecular mechanisms of oxygen-mediated lung injury are not understood presently, but reactive oxygen species (ROS) are likely to play important roles. The central hypothesis of this application is that the upregulation of cytochrome P450 1A (CYP1A) enzymes leads to attenuation of oxygen-mediated lung injury in vivo in rodents. Prevention of BPD and its evolution into chronic lung disease continues to remain elusive despite many therapies and strategies employed currently in the neonatal period. Our research is aimed at elucidating the molecular mechanisms of regulation of CYP1A enzymes by hyperoxia, in relation to hyperoxic lung injury.

STUDY DESIGN, METHODOLOGY, RATIONALE, INNOVATION, AND SIGNIFICANCE OF PROPOSED RESEARCH

The design of the experiment is based on theories surrounding the aryl-hydrocarbon receptor (AHR), its role in regulating the AHR gene battery of enzymes, and the mechanisms of cellular protection conferred by the upregulation of these enzymes. From prior experiments in our laboratory, we have shown that rats pre-treated with the CYP1A inducer 3-methylcholanthrene (3-MC), followed by exposure to a hyperoxic environment for 48–72 hours, show evidence of attenuated lung injury, as compared to vehicle-treated controls. Also, since 3-MC can upregulate CYP1A enzymes for an extended period of time (up to 45 days), we performed experiments involving CYP1A induction by MC and hyperoxia, and our data showed that the CYP1A1, along with other enzymes of the gene battery, continues to confer protection on the cellular architecture of the lungs of the rat. This is evidenced not only by a decrease in pleural effusions of treated mice, but also gross and histopathological evidence of decreased inflammation. We have done hematoxylin and eosin (H&E) staining showing significant preservation of cellular architecture in 3-MC treated rats. Also, we have used the antibody, anti-myeloperoxidase (anti-MPO), to show that there is a decrease in the number of neutrophils invading the tissue.

Furthermore, our current research supports the hypothesis that CYP1A1 is a product of activation of the aryl-hydrocarbon receptor (AHR). Following along that line of reasoning, data from our lab show that when an AHR-knockout mouse is exposed to a hyperoxic environment, there is more lung injury than controls, suggesting that the pathway for prevention of lung injury lies along stimulation of the AHR, and upregulating downstream elements.

To test the aforementioned hypothesis, a mechanistic model is needed. And, although a potent inducer, 3-MC has been implicated as carcinogen, making it limited as a chemical to study in different animal models. It has however, been previously shown that other potently non toxic compounds, such as alpha-naphthoflavones (♂-NF) and beta-naphthoflavones (♀-NF) are also potent inducers of this enzyme system, with ♂-NF being more effective. Some of our preliminary data show that it is possible to recreate the aforementioned model of lung injury attenuation in adult male Sprague-Dawley rats using both ♂-NF and ♀-NF. In some of our first experiments, after ♂-NF and ♀-NF treated rats are exposed to 72 hours of hyperoxia (>95% O2), pleural effusions can be reduced by as much as 50% in ♂-NF treated animals, and as much as 25% in ♀-NF treated animals (Figure 1). Tissue staining with anti-MPO, similar to the mouse model, shows a decreased amount of neutrophils-mediated inflammation. Gross histopathology similarly, correlated with a significant reduction in cellular damage, and exudative debris, with a preservation of the cellular architecture. CYP 1A1 activity was confirmed by measuring ethoxyresorufin O-deethylase (EROD) (CYP1A1) activities (Figure 2), while western blotting was used to assess apoprotein levels, and northern blotting to confirm the presence of RNA. Thus, our results support the hypothesis that elevated CYP1A levels in the inducer treated rats protects animals from oxygen-mediated lung injury. It appears that CYP1A
enzymes attenuate lung injury by destroying ROS produced in the cells. Since some CYP enzymes have been shown to produce ROS in certain model systems, we hypothesize that CYP enzymes of the 1A family are unique and mediate detoxication, rather than activation of ROS. This is the novel aspect of the proposed studies. If our hypothesis turns out to be correct, future studies could lead to the development of novel therapies to prevent/treat lung pathologies in infants undergoing oxygen therapy.

In furthering our research goals and aims, our group’s investigation concerns the upregulation of cytochrome P450 isoforms and its connection to the aryl-hydrocarbon receptor (AHR). It has been proposed that the AHR is the pivotal protein in the expression of a gene battery of cytoprotective enzymes, such as CYP 1A1, 1A2, and superoxide dismutase. Our group previously has shown that AHR-knockout mice are very susceptible to lung injury after exposure to a hyperoxic environment. We would like to extend this idea by investigating the presence of a gene battery and its relation to the aryl-hydrocarbon receptor in the rat model. We would however, like to expose the AHR-knockouts to hyperoxic environments and analyze the activities of CYP 1A1, 1A2, and superoxide dismutase and compare this to the wild-type rat model. This experiment would establish the necessity of the presence of AHR and its downstream elements, similar to that of the mouse.

As mentioned before, we are concerned with a possible mechanism as to the cytoprotective nature of this enzyme gene battery. Others have implicated the presence of several compounds in the intracellular environment that are responsible for initiating a cascade of events leading to cellular damage. One of the newer ideas in free radical molecular biology include theories of isoprostanes and their possible involvement in the development of lung disease, in particular, with respect to the generation of ROS. It is currently unclear as to whether or not there is a causal relationship between these molecules and the resultant pulmonary complications, or if they are a by-product of cellular degradation by ROS. However, we would like to include this as a second stage of our proposed research project.

**Design of Specific Aim 1:** To test the hypothesis that pre-treatment of adult rats with the CYP1A inducers or NF will result in alleviation of lung injury caused by subsequent hyperoxic exposures, and that hepatic and/or pulmonary CYP1A enzymes contribute to the beneficial effects. Seventy-two male Sprague-Dawley rats will be randomized into 3 groups of 24 each. One group will be treated with NF (93 mol/kg), a non-genotoxic CYP1A inducer, once daily for 4 days. The second group will be treated with NF (300 mol/kg), once daily for 4 days. The last (control) group will be given the vehicle corn oil (CO). One day after inducer withdrawal, 6 animals from each group will be maintained in room air, while the
remaining 18 animals will be exposed to hyperoxia for 24, 48 or 60 h. Hepatic and pulmonary CYP1A1/1A2 protein expression (apoprotein and catalytic activities) will be analyzed in 4 individual animals. Phase II and other antioxidant enzyme activities (i.e. GST-\( \mu \)-, NQO1, UDPGT, GPs, SOD, and catalase) will also be determined in lungs and livers of these animals. We will determine the levels of ROS-mediated lipid peroxidation products (i.e., F2-isoprostanes and isofurans\(^3\)) in a portion of lung tissues of the animals. In addition, pleural effusions and body weight/lung weight ratios, as indicators of lung injury will be determined. In the remaining 2 animals from each group, histological assessment of lung injury will be performed by H&E staining as well as by quantifying number of neutrophils by immunohistochemistry.

**Design of Specific Aim 2:** We have two sets of experiments in this group. In Specific Aim 2a, 72 rats will be randomized into 3 groups of 24 each. The rats will be administered an AHR antagonist 3’4’-dimethoxyflavone (DMF)\(^10\) i.p. at doses 0 mg/kg (vehicle control), 2 mg/kg, and 4 mg/kg. Six animals from each treatment group will be maintained in room air, and the remaining 18 animals will be exposed to hyperoxia. Four animals from each of the hyperoxia groups will be sacrificed at 24, 48, and 60 h, and hepatic and pulmonary CYP1A1/phase II/antioxidant enzyme expression and lipid peroxidation products will be determined. Histological analyses of lung injury (described above) will be studied in 2 individual animals from each group. Before the start of the experiment, we will do pilot studies to ensure that DMF inhibits AHR-mediated gene activation in vivo at the doses mentioned above. We will use MC as the inducer to establish the dose of DMF that is required to inhibit CYP1A1 gene induction.

In Specific Aim 2b, 2 month-old male 48 wild type or 48 AHR-null animals will be divided into 2 groups of 24 each. One group will receive \( \beta \)-NF (93 \( \mu \)mol/kg) once daily for 4 days and the other group will receive the vehicle CO. Six animals from each group will be maintained in room air, while the remaining 18 animals will be exposed to hyperoxia for 24, 48, or 72 h. Hepatic and pulmonary CYP1A1/phase II/antioxidant enzyme expression and lipid peroxidation products will be determined. Histological analyses of lung injury (described above) will be studied in 2 individual animals from each group.

**Data Analysis and Interpretation:** Should \( \beta \)-NF-treated animals show increased expression of CYP1A1 and decreased lung injury and inflammation in hyperoxia, it would lend further credence to the hypothesis that CYP1A1 plays a beneficial role in hyperoxic lung injury. We expect animals exposed to hyperoxia 1 day after \( \beta \)-NF withdrawal, when CYP1A1 levels would be high, to be less susceptible to hyperoxic lung injury than those exposed to vehicle-treated animals. If hepatic CYP1A enzymes are involved in the protective effects of \( \beta \)-NF, which induce hepatic and pulmonary CYP1A enzymes, then animals given \( \beta \beta \)-NF + hyperoxia will not be more tolerant to hyperoxia than animals exposed to hyperoxia alone. Determination of phase II/antioxidant enzyme activities would also be important in data interpretation. Should any of the antioxidant enzymes be persistently elevated by \( \beta \)-NF, and then we would interpret the data to conclude that these enzymes may also contribute to the protection by \( \beta \)-NF against hyperoxic lung injury. Levels of ROS will provide important information regarding mechanisms of hyperoxic lung injury. Since we hypothesize that elevated CYP1A levels protect against oxidative damage by detoxifying lipid peroxidation products, we will estimate the levels of F2-isoprostanes/ isofurans in lungs of animals exposed to CYP1A modulators and determine whether protection against lung injury correlates with diminished levels of these compounds and other ROS (e.g., \( \text{H}_2\text{O}_2 \)) in vivo. Experiments with DMF will clarify the role of the AHR in the protective effects of \( \beta \)-NF. If our hypothesis is correct, then DMF-pretreated animals will be more susceptible to hyperoxic injury than those exposed to hyperoxia alone. Similarly, AHR-null animals will be more susceptible to oxygen-mediated lung injury than similarly exposed wild type animals. Furthermore, if AHR-mediated induction of CYP1A1 were critical, then \( \beta \)-NF would protect wild type but not AHR-null animals from hyperoxic lung injury. Thus, the proposed studies should provide novel mechanistic insights into the role of CYP1A1 in hyperoxic lung injury.

**Methodology:** Liver or lung microsomes will be isolated by the differential centrifugation technique.\(^11\) EROD (CYP1A1) and MROD (CYP1A2) assays are performed essentially according to the method of Pohl and Fouts, as detailed in our publication.\(^9\) All the antibodies are commercially available. NQO1 and
GST assays towards 1-chloro-2,4-dinitrobenzene and cumene hydroperoxide will be determined in the cytosol by spectrophotometry. Catalase, GP, UDPGT, and SOD\textsuperscript{12-14} will be assayed according to published procedures. Western blotting, Northern blotting, and RT-PCR will be performed as reported before\textsuperscript{5}. Levels of F\textsubscript{2}-isoprostanes and isofurans in lung tissues of animals under normoxic and hyperoxic conditions will be measured in lung tissues using commercially available kits.

Lung injury will be evaluated by measurement of lung weight/body weight ratios, pleural effusions, measurement of extravascular lung waters and by histology.\textsuperscript{15} Rats or mice will be anesthetized with an overdose of pentobarbital sodium i.p, and one of the lobes of the right lung will be fixed in situ with 10\% buffered formalin, pH 7.4, using a constant inflation pressure of 20 cm\textsubscript{H}2O. Following processing, tissues will be embedded in paraffin and sectioned at 4 \textmu m on a rotary microtome (Leitz 1512). For routine histology to assess lung morphology and injury, sections will be stained with Hematoxylin and Eosin (H&E). Lung inflammation will be evaluated by counting the number of neutrophils or by performing immunohistochemistry with anti-MPO or anti-neutrophil antibody.

\textit{Significance:} Hyperoxia is used extensively for the treatment of pulmonary insufficiency, as is encountered in prematurely born infants and in individuals with adult respiratory distress syndrome (ARDS). However, considerable evidence links oxygen to the development of neonatal diseases, i.e. chronic lung disease, termed BPD\textsuperscript{13} and retinopathy of prematurity (ROP).\textsuperscript{16} BPD is the major source of morbidity and mortality in prematurely born infants, and primary oxidant injury caused by exposure to hyperoxia is a major risk factor in the etiology of BPD. The adverse effects of hyperoxia have been studied extensively in experimental animals.\textsuperscript{11, 17-21} Exposure of experimental animals to hyperoxia causes lung damage\textsuperscript{22}, and similar injury may occur in human patients undergoing supplemental oxygen therapy. The molecular mechanisms responsible for oxygen toxicity are not completely understood, but it is logical that lung injury should be mediated by ROS-mediated oxidation and/or peroxidation of lung lipids, proteins, or other molecules.\textsuperscript{11, 17-21, 23} Because ROS have been implicated in the manifestation of many lung diseases such as BPD, ARDS, PPHN, COPD, etc, which taken together, account for the leading causes of death in Western societies, the proposed research, which focuses on mechanisms of lung injury by hyperoxia, should be beneficial in developing strategies for the prevention/treatment of these diseases. Additionally, the proposed research, which focuses on the regulation of CYP1A enzymes by hyperoxia relation to lung dysfunction, may aid future therapies in providing protection from oxidant stress injuries in sick pre-term and term infants and adults undergoing oxygen therapy. Current research is directed towards the role of prevention CLD, exploring new approaches for antioxidant administration, manipulation of endogenous antioxidants and other pharmacologic strategies (e.g., CYP1A inducers) to minimize lung injury. Preliminary studies from our laboratory have shown that pretreatment of rats or mice with CYP1A1 inducers such as MC or \textgreek{b}-NF is protective against acute lung injury induced by hyperoxia. Thus, the proposed research is likely to lead to development of novel therapeutic interventions (e.g., using flavonoids) in patients with lung diseases such as BPD.

Thus, the proposed research is likely to contribute to development of new interventions in the prevention/treatment of pulmonary diseases such as BPD, ARDS, pulmonary hypertension, etc, in humans, and the goals of the proposed research will support the mission of the American Academy of Pediatrics, which is to prevent and treat pediatric diseases in humans.

**PRINCIPAL INVESTIGATOR**

The principal investigator, Anuj Sinha, will do all data collection, including rat surgery and measurements of pleural effusions, measurement of the activities of enzymes, Western and Northern blotting of experimental animal tissues.
OVERALL PROJECT GOALS AND SPECIFIC AIMS

The Specific Aims of the proposal are as follows:

1. To test the hypothesis that pre-treatment of adult rats with the CYP1A inducer BNF will result in alleviation of lung injury caused by subsequent hyperoxic exposures, and that hepatic and/or pulmonary CYP1A enzymes contribute to the beneficial effects. Rats will be pre-treated with vehicle, naphthoflavone (NF), naphthoflavone (NF), which induces CYP1A1 in lung, but not liver, followed by exposure to hyperoxia for selected time points. Catalytic activities and protein expression of CYP1A1/1A2, phase II enzymes [e.g., glutathione S-transferase (GST), NADPH quinone reductase (NQO1), UDP glucuronyl transferase (UDPGT)], and other antioxidant enzymes (e.g., superoxide dismutase (SOD), glutathione peroxidase, and catalase), will be determined in lung and liver. Levels of ROS-mediated lipid peroxidation products (i.e., F2-isoprostanes and isofurans) will also be studied in lung tissues, as indexes of oxidative stress in vivo. In addition, extent of lung injury and inflammation will be determined.

2. To test the hypothesis that the AHR mechanistically contributes to the beneficial effects of the CYP1A inducers in modulating hyperoxic lung injury. Rats will be pre-treated with the AHR antagonist DMF, followed by exposure to hyperoxia for selected time points. In the second set of experiments we will expose wild type and AHR knockout mice that had been pre-treated with vehicle or BNF, followed by exposure to hyperoxia for selected time points. Expression of CYP1A, phase II/antioxidant enzymes, and parameters of lung injury will be studied.

The overall project goal is to eventually characterize a possible mechanism associated with the development of bronchopulmonary dysplasia and chronic lung disease in the newborn premature infant. Once a possible mechanism has been elucidated, it will become easier to develop novel therapies for the treatment for this disease with significant morbidity in infants.

BUDGET

The following is a brief summary of supplies needed for experimentation based on prior experiments in our group:

- $1,500.00 Animals
- $3,500.00 Chemicals and Reagents

Budget Justification: The project entails use of rats and mice. We require rats for the entire project. We have already have a breeding colony for AHR-null mice. We will need to maintain at least 20 cages for the proposed experiments. We request $1,500.00 for animals’ costs for the whole project.

Chemicals and reagents: The project entails use of many techniques such as Northern hybridization, RT-PCR, western blotting, histopathology, enzyme assays, etc. We will need to purchase kits for RT-PCR, isotopes for Northern assays, antibodies for Western blots and immunohistochemistry, substrates for enzyme assays, gases for hyperoxia exposures, reagents for histological analyses. A total of $3,500.00 is requested for whole project.


