Role of Cytochrome P450 (CYP)1A in Hyperoxic Lung Injury: Analysis of the Transcriptome and Proteome

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Hyperoxia contributes to lung injury in experimental animals and diseases such as acute respiratory distress syndrome in humans. Cytochrome P450 (CYP)1A enzymes are protective against hyperoxic lung injury (HLI). The molecular pathways and differences in gene expression that modulate these protective effects remain largely unknown. Our objective was to characterize genotype specific differences in the transcriptome and proteome of acute hyperoxic lung injury using the omics platforms: microarray and Reverse Phase Proteomic Array. Wild type (WT), Cyp1a1−/− and Cyp1a2−/− (8–10 wk, C57BL/6J background) mice were exposed to hyperoxia (FiO2 > 0.95) for 48 hours. Comparison of transcriptome changes in hyperoxia-exposed animals (WT versus knock-out) identified 171 genes unique to Cyp1a1−/− and 119 unique to Cyp1a2−/− mice. Gene Set Enrichment Analysis revealed pathways including apoptosis, DNA repair and early estrogen response that were differentially regulated between WT, Cyp1a1−/− and Cyp1a2−/− mice. Candidate genes from these pathways were validated at the mRNA and protein level. Quantification of oxidative DNA adducts with 32P-postlabeling also revealed genotype specific differences. These findings provide novel insights into mechanisms behind the differences in susceptibility of Cyp1a1−/− and Cyp1a2−/− mice to HLI and suggest novel pathways that need to be investigated as possible therapeutic targets for acute lung injury.

Exposure to supraphysiological concentrations of oxygen (hyperoxia) leads to lung injury both in vivo and in vitro. Human patients are exposed to hyperoxia when supplemental oxygen is used for the treatment of critically ill patients with diseases such as acute respiratory distress syndrome (ARDS). Acute respiratory failure due to ARDS has a high mortality1,2. The molecular mechanisms behind acute lung injury (ALI) in this disease process are not well understood. Identification of mechanisms that lead to ALI/ARDS is necessary for the development of new preventive and therapeutic strategies. Hyperoxia is known to contribute to acute lung injury through many molecular mechanisms linked to increased oxidative stress and production of reactive oxygen species3,4. This causes damage to many cellular components such as DNA, lipid and protein, ultimately leading to cell death5.

The cytochrome P450 (CYP) family of enzymes are involved in the metabolism of various exogenous and endogenous compounds6,7. Among them, the CYP1 gene subfamily, regulated by aryl hydrocarbon receptor, is perhaps most relevant to oxygen toxicity4–13. Ours and other groups have investigated the role of the CYP1A subfamily in hyperoxic lung injury. CYP1A1 can be induced by hyperoxia and other specific inducers such as 3-methylcholanthrene in liver and many other organs including the lung14, while CYP1A2 is predominantly expressed in the liver, and is not induced in extra-hepatic tissues even after treatment with inducers. Induction of CYP1A function is protective while inhibition is deleterious in acute hyperoxic lung injury12,15. We have shown that absence of either Cyp1a1 or Cyp1a2 gene in knock-out mice increases the susceptibility to hyperoxic lung injury compared to the respective WT controls16,17. The Cyp1a1−/− and Cyp1a2−/− mice showed greater histological lung injury, inflammation and markers of oxidative stress, hence indicating that both of these proteins are protective in the setting of hyperoxic lung injury. We have previously documented the protective effects of mammalian hepatic CYP1A2 against hyperoxic lung injury18, revealing a critical role for extra-pulmonary organs.

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such as liver in the protection against lung injury by metabolizing potential mediators of ALI including lipid hydroperoxides.

Although we have reported on the phenotype of increased lung injury in mice lacking the gene for Cyp1a1 or Cyp1a2 and the possible underlying mechanism(s)16,17, the differences in the transcriptome and the underlying biological pathways and regulatory networks have not been investigated. The aims of this study were to identify significant differences in the lung transcriptome and proteome between the wild-type (WT), Cyp1a1−/− and Cyp1a2−/− mice in a model of acute hyperoxic lung injury. In this study, we employed a non-biased approach to measure global changes in gene expression in WT, Cyp1a1−/− and Cyp1a2−/− mice following hyperoxia exposure in the lung at 48 h using microarray. We selected the 48 h time point, because most adult mice exposed to hyperoxia survive for 60–90 h with profound lung injury at 72 h of exposure18. Instead of focusing on the later cellular injury phase, we wanted to focus on the initiation phase of hyperoxic lung injury19. We also sought to measure the changes in the lung proteome using reverse phase protein array (RPPA). Furthermore, because hyperoxia is known to cause oxidative DNA damage5,20,21 we determined the levels of pulmonary oxidative DNA adducts in WT, Cyp1a1−/−, and Cyp1a2−/− mice under normoxic as well as hyperoxic conditions.

Results
Role of CYP1A in differential pulmonary gene expression in hyperoxic lung injury. To analyze differences in the pulmonary transcriptome after hyperoxia exposure (>95% O2, 48 hrs) we subjected lung mRNA from exposed and room air controls to microarray analysis. Figure 1A–C shows the volcano plots of the differentially expressed genes (DEGs) (upregulated and downregulated) in WT, Cyp1a1−/− and Cyp1a2−/− mice. The genes shaded in green are common DEGs across the three genotypes whereas the genes represented in red are DEGS exclusive to a genotype. Figures 1D and E show the number of upregulated (UR) and downregulated (DR) DEGs in Cyp1a1−/− and Cyp1a2−/− mice compared to WT mice under room air and after hyperoxia exposure. One hundred fifty three genes (UR:68, DR:85) in room air and 171 genes (UR:99, DR:72) after hyperoxia exposure were differentially expressed in Cyp1a1−/− mice. In Cyp1a2−/− mice, 179 genes (UR:99, DR:80) in room air and 119 genes (UR:30, DR:89) specific after hyperoxia exposure were differentially regulated in the lung compared to WT mice. Table 1 shows the exclusive genes (not shared with the other genotypes) with the highest fold change in WT, Cyp1a1−/− and Cyp1a2−/− mice. Table 2 shows the genes that were downregulated at 48 hrs of hyperoxia exposure in the lung exclusive to a given genotype. The top upregulated and downregulated genes based on fold change, irrespective of the genotype, are shown in supplemental data in Tables S1 and S2. We also determined the genes that were differentially regulated in WT and Cyp1a1−/− or Cyp1a2−/− mice in room air conditions. The genes showing the highest fold change are listed in Tables S3 (upregulated) and S4 (downregulated). One of the listed genes KLF2 is upregulated in Cyp1a1−/− mice and downregulated in Cyp1a2−/− mice compared to WT mice in room air conditions. KLF2 has been identified as an important upstream regulator of the lung transcriptome in a model of neonatal hyperoxic lung injury22. It is important for lung development in the saccular and alveolar phases23. It is also known to be involved in endothelial homeostasis24, vascular barrier function25 and regulation of inflammation26. Baseline differences like above could modulate lung injury upon subsequent exposure to hyperoxia.

Pathway analysis of DEGs. Biological processes that were enriched but differentially modulated in the transcriptome footprint of the three (WT, Cyp1a1−/− and Cyp1a2−/−) genotypes were identified using Gene Set Enrichment Analysis (GSEA). We focused on pathways that were regulated in opposite directions between the WT and Cyp1a1−/− or Cyp1a2−/− mice. Figure 2 shows the major biological processes for the group of genes that were differentially regulated both in WT, Cyp1a1−/− or Cyp1a2−/− mice (Q < 0.25; Normalized Enrichment Score/NES has opposed signs between either Cyp1a1−/− or Cyp1a2−/− and WT). Pathways that were enriched in opposite direction in WT mice compared to Cyp1a1−/− and Cyp1a2−/− mice were DNA repair and protein secretion (upregulated in WT, downregulated in knock-out) and early estrogen response and hypoxia (downregulated in WT and upregulated in knock-out mice). Among pathways that were differential in Cyp1a2−/− mice compared to WT and Cyp1a1−/− mice are IL-6/JAK/STAT3 signaling which were downregulated and apoptosis and myogenesis, which were upregulated in Cyp1a2−/− mice after 48 hours of hyperoxia exposure.

Upstream transcriptional factors analysis. Next, we sought to analyze the transcription factors (TF) that were responsible for modulating the gene expression changes under hyperoxic conditions in the lung in WT, Cyp1a1−/− and Cyp1a2−/− mice. The top transcription factors driving the expression of upregulated and downregulated genes in WT, Cyp1a1−/− and Cyp1a2−/− mice are shown in Fig. 3. Transcription factors which were preferentially induced or suppressed by hyperoxia and were differentially modulated between WT, Cyp1a1−/− and Cyp1a2−/− mice were determined via GSEA and shown in Fig. 4. Apart from few transcription factors that were regulated in opposite directions between WT and Cyp1a1−/− or Cyp1a2−/− mice (ZEB, ATF6: induced in WT and PPARA, SF1 and USF suppressed in hyperoxia in WT), the changes were similar between WT and Cyp1a2−/− and opposite to those in Cyp1a1−/− mice.

Reverse phase protein array (RPPA). For analysis of the lung proteome we performed RPPA analysis on lung protein samples after 48 hours of hyperoxia exposure and respective room air controls. Figure 5 shows the differences between WT and Cyp1a knockout animals under hyperoxic conditions. We did not see significant differences in protein expression in the Cyp1a2−/− mice exposed to hyperoxia compared to their room air controls. Supplemental Figure 1 shows the differences between hyperoxia exposed and room air controls in each respective genotype. Among the proteins studied, in WT mice, 27 were downregulated and 8 were upregulated compared to room air controls and in Cyp1a1−/− mice, 21 proteins were downregulated and 10 upregulated upon exposure to hyperoxia (Supplemental Figure 1). The protein and corresponding genes differentially expressed between
Figure 1. Hyperoxia exposure of WT, Cyp1a1−/−, and Cyp1a2−/− mice leads to robust yet distinct transcriptomic responses. (A–C) Volcano plots of the differentially expressed genes (DEGs); light green: common DEGs across the three genotypes; red: DEGs exclusive to a genotype. (D) Number of upregulated (UR) and downregulated (DR) DEGs in Cyp1a1−/− and Cyp1a2−/− mice compared to WT mice under room air and after hyperoxia exposure. One hundred and fifty three genes (UR:68, DR:85) specific to room air and 171 genes (UR:99, DR:72) specific to hyperoxia were differentially expressed in Cyp1a1−/− mice. (E) In Cyp1a2−/− mice, 179 genes (UR:99, DR:80) specific to room air and 119 genes (UR:30, DR:89) specific to hyperoxia were differentially regulated in the lung compared to WT mice.
WT and Cyp1a knockout animals under hyperoxic conditions are listed in supplemental data Table S5 and those between hyperoxia exposed and room air controls in each respective genotype are listed in Table S6. Many of the significantly changed proteins detected by the RPPA assay were post-translationally modified, and as such their expression may not have been assessed as significantly changed in the microarray profile.

Real time qPCR validation. Microarray results were verified by qPCR. Instead of choosing targets based solely on fold change for validation, we selected candidate genes from the pathways that were differentially regulated between WT, Cyp1a1−/− and Cyp1a2−/− mice. These included (Nme1, Pcn and Xpc: DNA repair pathway; Hmox1, Ctgf, Slc2a3, Dusp1: response to hypoxia pathway; Cd63, Abca1: protein secretion pathway; Areg from early estrogen response pathway and Trp63 from the apoptosis pathway). This was based on the key insight that small but consistent changes across a large number of genes in a pathway (including genes that do not meet a certain statistical threshold) are more informative than large significant changes in a handful of genes only. We assessed differences in pulmonary gene expression at 24 and 72 h in addition to 48 h to observe the temporal changes related to duration of hyperoxia exposure (Fig. 6). The fold change for each gene was calculated relative to its expression levels in the respective room air controls. We also validated three genes which were commonly upregulated in all three genotypes: Ankrd1, Gdf15 and Nupr1.

The DNA repair pathway genes; Nme1, and Pcn were induced by hyperoxia in WT and Cyp1a1−/− mice compared to room air levels; increasing duration of hyperoxia exposure led to greater fold increases. Interestingly, in Cyp1a2−/− mice, there was decreased expression of these genes compared to WT and Cyp1a1−/− mice. Expression of Xpc was increased in WT mice at 24 and 72 h compared to room air controls but Cyp1a1−/− and

Table 1. Up regulated genes exclusive to a given genotype (Hyperoxia exposed mice compared to room air controls).
Cyp1a2−/− mice showed decreased expression levels compared to WT mice under hyperoxic conditions. Cyp1a2−/− mice exhibited consistently the lowest expression for these genes among the three genotypes.

Among genes involved in response to hypoxia: Hmox1 and Ctgf were induced by hyperoxia with increasing expression with increasing duration of hyperoxia exposure. The induction was very significant in WT mice and to a lesser extent in Cyp1a1−/− mice, and the least induction was observed in Cyp1a2−/− mice. Slc2a3 was induced at 72 h in WT and Cyp1a1−/− mice compared to room air controls but decreased expression was seen in Cyp1a2−/− mice compared to other genotypes. However, expression of Dusp1 was significantly increased in Cyp1a2−/− mice at 24 and 72 h compared to other genotypes and respective room air controls.

We validated Cd63 and Abca1 as part of the protein secretion pathway. Cd63 was induced under hyperoxia in all three genotypes but was most significant in WT mice and the least in Cyp1a2−/− mice. On the other hand, expression levels of Abca1 decreased under hyperoxic conditions in all three genotypes but this was more significant in the Cyp1a1−/− and Cyp1a2−/− mice compared to WT mice at 72 h. Areg (early response to Estrogen pathway) expression was increased at 48 and 72 h in all three genotypes after hyperoxia exposure at 48 and 72 h. Expression was higher in Cyp1a2−/− mice at 48 h but at 72 h, WT mice had higher expression levels compared to Cyp1a1−/− and Cyp1a2−/− mice. Trp63 (Apoptosis pathway) did not show significant change in expression with hyperoxia. The expression was decreased in Cyp1a2−/− mice at 24 h compared to WT mice.

Expression levels of Ankrd1, Gdf15 and Nupr1 were significantly increased in all three genotypes at 48 and 72 h compared to room air controls. At 72 h, the expression was lower in Cyp1a1−/− and Cyp1a2−/− mice compared to similarly exposed WT mice.

There were excellent correlations in fold changes measured by real time PCR and by microarray for the experimentally validated genes, as shown in Table 3. The Pearson Correlation Coefficient for fold change in gene expression was 0.85 for WT, 0.84 for Cyp1a1−/− and 0.78 for Cyp1a2−/− mice.

### Table 2. Down regulated genes exclusive to a given genotype (Hyperoxia exposed mice compared to room air controls).

| Genotype | Gene Symbol | Gene name | Fold change |
|----------|-------------|-----------|-------------|
| WT       | DOCK6       | Dedicator Of Cytokinesis 6 | 0.60         |
|          | INMT        | Indolethylamine N Methyltransferase | 0.59         |
|          | IFITM6      | Interferon induced transmembrane protein 6 | 0.58         |
|          | ATP2A2      | ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2 | 0.57         |
|          | BEX2        | Brain Expressed X-Linked 2 | 0.54         |
|          | ACTC1       | Actin, Alpha, Cardiac Muscle 1 | 0.54         |
|          | SDPR        | Serum deprivation response | 0.51         |
|          | NPPA        | Natriuretic Peptide A | 0.47         |
|          | MYL7        | Myosin Light Chain 7 | 0.40         |
| Cyp1a1−/− | SERPINA1D   | Serpin Family E Member 1 | 0.56         |
|          | TCAP        | Titin-Cap | 0.54         |
|          | APOA2       | Apolipoprotein A2 | 0.53         |
|          | SERPINA1B   | Serpin Family A Member 1 | 0.53         |
|          | IFI27       | Interferon Alpha Inducible Protein 27 | 0.53         |
|          | MB          | Myoglobin | 0.49         |
|          | MUP5        | Major urinary protein 5 | 0.43         |
|          | SLN         | Sarcolipin | 0.41         |
|          | TTR         | Transthyretin | 0.38         |
| Cyp1a2−/− | ALDH1A1     | Aldehyde Dehydrogenase 1 Family, Member A1 | 0.59         |
|          | OGN         | Osteoglycin | 0.59         |
|          | HPGD        | Hydroxyprostaglandin Dehydrogenase 15- (NAD) | 0.58         |
|          | ALAS2       | 5′-Aminolevulinate Synthase 2 | 0.58         |
|          | XLR4A       | X-linked lymphocyte-regulated 4A | 0.57         |
|          | TAX1BP1     | Tax1 Binding Protein 1 | 0.57         |
|          | MT-ND4L     | Mitochondrially Encoded NADH Dehydrogenase 4L | 0.55         |
|          | COL3A1      | Collagen Type III Alpha 1 | 0.48         |
Immunoblot analysis. For validation at the protein level we performed western blots for Rad51 and ERCC1. These genes are both part of the DNA repair pathway and were hence chosen for further validation at the protein level. These results are shown in Fig. 7. Protein expression assessed by western blot assay in whole lung protein from WT, Cyp1a1−/−, and Cyp1a2−/−, and WT mice exposed to room air and after 48 h hyperoxia exposure was measured and quantified using densitometry. Cyp1a2−/− mice had decreased expression of Rad51 and ERCC1 compared to WT or Cyp1a1−/− mice both under room air and after exposure to hyperoxia. Cyp1a1−/− mice had decreased expression of Rad51 and a significantly increased expression of ERCC1 after hyperoxia exposure compared to WT mice.

Lung oxidative DNA adducts analysis in WT, Cyp1a1−/− and Cyp1a2−/− mice in acute hyperoxic lung injury. Oxidative DNA adducts were analyzed in the lungs by 32P-postlabeling. The 8,5′-Cyclo-2′-deoxyadenosine (cA) adducts that we measured in this study represent one of the major oxidative DNA lesions formed in DNA by hydroxyl radical attack on 2′-deoxyadenosine, followed by intramolecular cyclization between C5 and C8. Specifically, we measured levels of the dinucleotides AcA, GcA, CcA, and TcA, since they have been proposed as novel biomarkers of oxidative DNA damage, and were robustly increased under conditions of oxidative stress in vivo and in vitro. Figure 8(A) shows the typical profile of non-polar (AcA) and polar (GcA, CcA, and TcA) oxidative DNA lesions. In WT mice and Cyp1a1−/− mice, the total adducts were significantly
augmented after 72 h of hyperoxia (Fig. 8b) and the levels were higher in Cyp1a1−/− mice compared to WT mice. On the other hand, the Cyp1a2−/− mice showed increased total adduct levels at room air and 24 h compared to similarly exposed WT mice, and the total adduct levels in the Cyp1a2−/− mice decreased at the 48 and 72 h time points (Fig. 8b).
Figure 5. Hyperoxia-exposed Cyp1a1−/− and Cyp1a2−/− mice are characterized by robust and distinct proteomic changes compared to hyperoxia-exposed WT. Reverse Phase Protein Analysis (RPPA) was used to generate proteomic profiles of WT, Cyp1a1−/− and Cyp1a2−/− mice exposed to hyperoxia. Differential protein expression was evaluated using parametric t-test (significance for fdr-adjusted q-value < 0.25). For a uniform visual representation, expression of each protein were z-score transformed; e.g. for each protein, values were first mean-centered across all samples, then further divided by the standard deviation across all samples. A. Proteomic changes of Cyp1a1−/− mice compared to WT mice exposed to hyperoxia B. Proteomic changes of Cyp1a2−/− mice compared to WT mice exposed to hyperoxia.
In regard to individual adducts, WT mice did not exhibit significant changes after hyperoxia exposure (Fig. 8c–f). In Cyp1a1−/− mice, the AcA adducts were significantly higher at 72 compared to room air. Upon comparison with WT mice, Cyp1a1−/− mice had higher levels of AcA and CcA adducts at room air and after 24 and 72 h of hyperoxia exposure (Fig. 8c,d). GcA and TcA adduct levels were higher in Cyp1a2−/− mice at room air and 24 h compared to WT mice. The GcA and TcA adducts were markedly attenuated after 48–72 h of hyperoxia in Cyp1a2−/− mice. Some additional spots were formed in the DNA of mice both in room air and hyperoxic conditions. We do not know the identity of these modifications, and we will characterize these compounds in future studies.

**Discussion**

The key findings of this study were the identification of differentially regulated molecular pathways in hyperoxic lung injury that may explain the susceptibility of Cyp1a1−/− and Cyp1a2−/− mice to acute lung injury in this model. Differences in the lung transcriptome have been described in hyperoxic lung injury 32–34 but the role of (CYP)1A enzymes in modulating the lung transcriptome under hyperoxic conditions using knock-out animal models has not been previously reported. We have shown previously that hepatic CYP1A2 protects against hyperoxic lung injury by decreasing lipid peroxidation and oxidative stress in vivo17, thus revealing a critical protective role for extra-pulmonary organs such as liver. F2-isoprostanes/isofurans that are formed during hyperoxia exposure may undergo detoxification by CYP1A2 to non-toxic metabolites. Through in vitro experiments with recombinant human CYP1A2, we demonstrated the formation of a...
dinor metabolite from PGF2-α showing that F₂-isoprostanes are endogenous substrates for CYP1A2. In Cyp1a2−/− mice, these compounds would accumulate, be transported to the systemic circulation, and lead to increased lung injury. Using a similar methodology, we also showed that CYP1A1 has a protective role in part due to CYP1A1-mediated decrease in the levels of reactive oxygen species-mediated lipid hydroperoxides e.g. F₂-isoprostanes/isorurans, leading to attenuation of oxidative damage. Compounds such as omeprazole, a proton pump inhibitor and beta-naphtoflavone, a flavonoid have been shown to attenuate hyperoxic lung injury by inducing CYP1A1.

We reported differential gene expression in the lungs after hyperoxia exposure for 48 hours and identified genes that are differentially expressed unique to each of the genotypes as well as those that are common. We analyzed differences in gene expression at the 48 h time point because most animals displayed severe lung injury by 72 h, and many died between 60–90 h of exposure. We focused on pathways that were regulated in opposite directions by hyperoxia in WT and Cyp1a1−/−/− and Cyp1a2−/−/− mice. Table 3 shows the fold change in gene expression for selected genes with Real time PCR and Microarray.

### Table 3. Table showing Fold change in gene expression for selected genes with Real time PCR and Microarray.

| Gene Name                          | Genes   | PCR (Fold change from room air) | Microarray (Fold change from room air) |
|------------------------------------|---------|---------------------------------|----------------------------------------|
|                                    |         | WT     | Cyp1a1−/− | Cyp1a2−/− | WT     | Cyp1a1−/− | Cyp1a2−/− |
| Growth differentiation factor 15   | GDF15   | 161.6  | 186.5    | 141.4     | 19.85  | 18.19     | 18.49     |
| Ankyrin repeat domain 1            | ANKRD1  | 17.63  | 21.07    | 13.52     | 6.49   | 10.22     | 8.63      |
| Nuclear protein 1, transcriptional regulator | NUPR1 | 14.25  | 18.12    | 16.56     | 13.41  | 11.31     | 15.19     |
| CD63 molecule                      | CD63    | 1.82   | 2.23     | 1.85      | 1.96   | 2.01      | 1.74      |
| ATP binding cassette subfamily A member 1 | ABCA1 | 0.8    | 0.61     | 0.44      | 0.84   | 0.82      | 0.74      |
| XPC complex subunit, DNA damage recognition and repair factor | XPC | 0.92   | 1.17     | 0.73      | 0.86   | 0.89      | 0.77      |
| NME/NM23 nucleoside diphosphate kinase 1 | NME1 | 2.48   | 2.71     | 1.82      | 1.92   | 1.96      | 1.73      |
| Proliferating cell nuclear antigen | PCNA    | 1.49   | 1.72     | 1.19      | 1.04   | 1.00      | 0.92      |
| Solute carrier family 2 member 3   | SLC2A3  | 0.92   | 1.49     | 0.74      | 0.91   | 1.13      | 0.83      |
| Tumor Protein P63                   | TRP63   | 0.97   | 1.32     | 0.7       | 0.95   | 1.03      | 1.00      |
| Amphiregulin                        | AREG    | 8.04   | 9.54     | 17.17     | 3.90   | 4.36      | 7.72      |
| Connective tissue growth factor     | CTGF    | 8.81   | 6.71     | 5.94      | 5.47   | 3.33      | 5.04      |
| Heme oxygenase                      | HMOX1   | 3.54   | 3.43     | 2.25      | 1.60   | 1.99      | 1.48      |
| Dual specificity phosphatase 1      | DUSP1   | 1.68   | 1.76     | 9.08      | 0.79   | 0.91      | 2.00      |

**Figure 7.** Effect of hyperoxia on Rad 51 (A) and ERCC1 (B) protein expression in WT, Cyp1a1−/− and Cyp1a2−/− mice in room air and after 48 h of hyperoxia. For western blotting, lung whole protein (20 μg of protein) from individual animals (n = 4/group) exposed to room air or to hyperoxia for 48 h. For loading controls, the membranes were stripped and incubated with antibodies against β-actin, followed by electrochemical detection of bands. Two way ANOVA was used to assess statistical significance in protein expression among genotypes and the effect of hyperoxia. Significant differences from room air controls are represented by **p < 0.01 and ***p < 0.001. Significant differences in expression compared to levels in WT mice is represented by #p < 0.05, ##p < 0.01 and ###p < 0.001. Significant differences in expression compared to levels in Cyp1a1−/− mice is represented by δp < 0.05, δδp < 0.01 and δδδp < 0.001.
directions between the WT and Cyp1a1−/− or Cyp1a2−/− mice. DNA repair and protein secretion pathways were upregulated in WT and downregulated in knock-out, whereas the reverse was the case for early estrogen response and hypoxia pathways.

DNA Repair Pathway. Reactive oxygen species (ROS) generated during hyperoxic conditions can cause damage to many cellular components including DNA, which has been reported to contribute to the development of diseases such as cancer and cardiopulmonary diseases including ARDS. DNA damage and induction of DNA repair genes has been reported in the lung in WT, Cyp1a1−/−, and Cyp1a2−/− mice that were maintained in room air (RA) or exposed to 24, 48, and 72 hours of hyperoxia. The total cA values were derived from the addition of the individual adduct values. Two way ANOVA was used to assess statistical significance in adduct levels among genotypes and the effect of hyperoxia. Significant differences in adduct levels between WT and Cyp1a1−/− or Cyp1a2−/− mice is represented by *p < 0.05, **p < 0.01 and ***p < 0.001. Significant differences between normoxic (RA) and hyperoxic animals are represented by *p < 0.05, **p < 0.01, and ***p < 0.001.

Figure 8. Pulmonary 8,5′-cyclo-2′-deoxyadenosine (cA) oxidative DNA adducts in WT Cyp1a1−/−, and Cyp1a2−/− mice exposed to hyperoxia. (a) Representative thin-layer chromatography maps displaying bulky oxidative DNA adducts. Polar and non-polar oxidative DNA adducts were detected by 32P-postlabeling, as described in Materials and Methods. Panels (b–f) show quantitative analyses of total cA, AcA, CcA, GcA, and TcA adducts in the lung in WT, Cyp1a1−/−, and Cyp1a2−/− mice (n = 3/group) that were maintained in room air (RA) or exposed to 24, 48, and 72 hours of hyperoxia. The total cA values were derived from the addition of the individual adduct values. Two way ANOVA was used to assess statistical significance in adduct levels among genotypes and the effect of hyperoxia. Significant differences in adduct levels between WT and Cyp1a1−/− or Cyp1a2−/− mice is represented by *p < 0.05, **p < 0.01 and ***p < 0.001. Significant differences between normoxic (RA) and hyperoxic animals are represented by *p < 0.05, **p < 0.01, and ***p < 0.001.
response by coordinating DNA replication with DNA repair. This pathway was downregulated in the knock-out animals under pathway analysis, which could explain the diminished DNA repair and increased cell death, and in turn it might lead to increased lung injury in these animals as, described before. Cyp1a2−/− mice showed significantly decreased expression of genes (at the mRNA and the protein level) in this pathway including Xpc (Xeroderma Pigmentosum, Complementation Group C), which neutralizes oxidative DNA damage by playing a role in nucleotide excision repair. Cyp1a1−/− mice showed decreased protein levels of Rad51 compared to WT mice after exposure to hyperoxia. Rad51 plays a major role in DNA double-strand break repair and prevents genomic instability and the generation of tumorigenic mutations. However, there was increased expression of ERCC1 protein (Excision Repair Cross-Complementation Group 1) in Cyp1a1−/− mice after hyperoxia exposure compared to WT mice and room air controls. This gene is involved in recombinational DNA repair and in the repair of inter-strand crosslinks. Total adducts were significantly increased in Cyp1a1−/− mice compared to WT mice and room air controls, hence the increase in ERCC1 could have been in response to the increased adduct formation in these mice.

**Protein Secretion Pathway.** The candidate genes in the protein secretion pathway that were further evaluated using PCR were: Cd63 and Abca1 (ATP Binding Cassette Subfamily A Member 1). CD63 belongs to the tetraspanin family of cell-surface proteins. They mediate signal transduction events and play a role in the regulation of cell development, activation, growth and motility. Cd63 was induced under hyperoxia in all three genotypes but was most increased in WT mice and the least in Cyp1a2−/− mice. Doyle et al. showed that CD63 is essential for leucocyte recruitment by endothelial cells. It could thus play a pro-inflammatory role in this model of acute lung injury. ABCA1 is a member of the ABC1 subfamily. This protein functions as a cholesterol efflux pump in the cellular lipid removal pathway. In LPS induced acute lung injury, this protein was found to be protective and decreased inflammation. It was downregulated in hyperoxia exposed mice in all three genotypes but the decreases were more pronounced in the Cyp1a1−/− and Cyp1a2−/− mice compared to WT mice at 72 h.

**Response to Hypoxia Pathway.** DUSP1 (Dual Specificity Phosphatase 1) protein has intrinsic phosphatase activity, and specifically inactivates mitogen-activated protein (MAP) kinase. DUSP1 expression is increased under conditions of increased oxidative stress and apoptosis. In this study, DUSP1 expression was significantly increased in Cyp1a2−/− mice, which may be indicative of increased oxidative stress in these animals. This was consistent with our previous report of increased oxidative stress in the lungs of Cyp1a2−/− mice when exposed to hyperoxia compared to WT mice. Hmox1 (Heme oxygenase 1) was up regulated in both WT and Cyp1a1−/− and to a significantly lesser extent in Cyp1a2−/− mice after hyperoxia exposure. Previous investigators have reported up regulation of this gene in the lung under hyperoxic conditions. Both protective and deleterious effects of its induction have been reported. This could be because of cell-specific effects or linked to the byproducts of Hmox1 mediated reaction including free iron or carbon-monoxide. Ctgf (Connective tissue growth factor) mRNA was decreased in the knock-out mice and increased in the WT mice after hyperoxia exposure in this model. Increase in CTGF expression in WT mice has been reported in hyperoxia models and in models with bleomycin induced pulmonary fibrosis where it is thought to have pro-fibrotic effect. Slc2a3 (or GLUT3: Glucose transporter type 3) is involved in transmembrane transport of glucose and is up regulated in hypoxic conditions possibly though the HIF-1alpha pathway. Expression was increased at 72 h in WT and Cyp1a1−/− mice compared to room air controls, but decreased expression was seen in Cyp1a2−/− mice compared to other genotypes.

**Early Estrogen Response pathway.** The early estrogen pathway was also differentially modulated between the genotypes; downregulated in WT, but upregulated in the knock-out animals. This pathway comprises of genes that define early response to estrogen. Caldon et al. proposed that estrogen receptor signaling may compromise effective DNA repair and cellular apoptosis in favor of proliferation. In this study, we showed increased activation of the DNA repair pathway in WT mice but this was less in the knock-out mice corroborating the hypothesis above. We looked into Amphiregulin (Areg) expression by PCR. It was significantly increased in the lungs of Cyp1a2−/− mice compared to WT mice but all genotypes showed increased expression following hyperoxia exposure. Amphiregulin is known to mediate estrogen signaling. It also increases TGF-β mediated lung fibrosis by functioning as a ligand for EGF receptor. Expression of Areg was increased in the newborn lung exposed to chronic hyperoxia and may have a pro-inflammatory effect in HLI as noted in other diseases.

**Apoptosis pathway.** Trp63 or p63 is a transcription factor of p53 gene family involved in cell differentiation and response to stress. It also functions as a master regulator of epidermal development. Trp63 regulates p53 function and has been shown to have pro-survival function by antagonizing p53 activity. In our study, we found significant down-regulation of this gene in Cyp1a2−/− mice indicating its potential role in the increased lung injury phenotype observed in these mice. Induction in Ankrd1 and Gdf15 expression under hyperoxic conditions has been reported in WT mice. Overexpression of Ankrd1 (Ankyrin repeat domain 1) has been shown to enhance apoptosis through the p53 pathway. Gdf15 (Growth Differentiation factor 15) is a member of the TGF-β superfamily and is widely distributed in mammalian tissues. It is a stress-responsive cytokine and we have previously shown that it has a pro-survival and anti-oxidant role in hyperoxia in vitro. Nupr1 (Nuclear Protein 1, Transcriptional Regulator) is a stress activated protein known to regulate apoptosis, cell cycle and regulation of TGF-β activity. Induction of Nupr1 by hyperoxia has been reported in the newborn mouse lung exposed to hyperoxia and in the newborn retina following hyperoxia exposure.

The fact that a significant level of bulky oxidative DNA adducts were formed in room air conditions suggests that even during normoxic conditions, ROS can be produced at levels that result in DNA damage. The increase
in total adducts levels after 24–72 h of hyperoxia in WT animals (Fig. 8) was probably due to increased oxidative stress induced by oxygen. The higher levels of GcA and TcA adducts in WT mice suggests that these bases are more susceptible to oxidative attack that adenosine or cytosine. The presence of adducts after as early as 24 h of hyperoxia exposure supports the hypothesis that oxidative DNA adducts mechanistically contribute to lung injury. Our observation that in mice lacking the Cyp1a1 gene the total adducts were significantly augmented after 72 h of hyperoxia (Fig. 8D) supports the idea that CYP1A1 plays a role in the detoxification of the adduct precursors. Since Cyp1a1−/− mice display increased lung injury compared to the WT mice86, we postulate that oxidative DNA injury has a causative role in lung injury.

Although the Cyp1a2−/− mice were even more susceptible to hyperoxic lung injury than Cyp1a1−/− mice87,88, the fact that the Cyp1a2−/− mice showed significantly higher levels of total oxidative DNA adducts under room air conditions than those in WT mice (please compare Fig. 8E and C) suggests that CYP1A2 plays an important role in the detoxification of the adduct precursors. As these adducts are may have already reached physiological saturation, higher oxygen levels as seen at 24–72 h after hyperoxia may not have resulted in increased oxidative DNA damage in these animals. Interestingly, the levels of oxidative DNA lesions were attenuated in Cyp1a2−/− mice after prolonged hyperoxia (Fig. 8F–I), and this could be due to enhanced DNA repair of these specific nucleotides in vivo, but further experimental proof is needed to substantiate this idea.

The differences in lung proteome as analyzed with the RPPA methodology showed that there were greater number of proteins differentially expressed between WT and Cyp1a1−/− than WT and Cyp1a2−/− mice. These differences may underlie the different lung injury phenotypes observed in these animals. For example: mTOR (mechanistic Target of Rapamycin), was downregulated in both WT and Cyp1a1−/− mice compared to room air controls in hyperoxia. Inhibition of the mTOR pathway has been shown to increase hyperoxia-induced lung injury by increasing autophagy94. Similarly expression of VEGFR2 was decreased in both WT and Cyp1a1−/− mice. Decreased expression of this gene under hyperoxic conditions has been reported previously95. Among the upregulated proteins were phosphorylated ACC1; Acetyl-CoA carboxylase (2.22 fold in WT and 1.22 fold in Cyp1a1−/− mice) that catalyzes the ATP-dependent carboxylation of acetyl-CoA to produce malonyl-CoA, the pivotal step in the fatty acid synthesis pathway. Phosphorylation inhibits the activity of ACC1 and is observed in conditions when the energy status of the cell is low. Protein expression of phosphorylated AMPK (S'-prime-AMP-activated protein kinase) is also increased in both WT and Cyp1a1−/− mice, which is the activate form of this protein, and leads to downstream phosphorylation and inhibition of ACC1 as was observed in our study96,97.

We also highlighted the various transcription factors, which could have been differentially regulated between the genotypes in hyperoxic lung injury. TF genes are usually not significantly up- or downregulated in microarray experiments. Their activity is mainly regulated at the level of ligand binding or at the posttranscriptional level. One example is: C/EBPβ (cat/enhancer binding protein), which was induced in WT and Cyp1a2−/− mice, but suppressed in Cyp1a1−/− mice. The (C/EBP) family of proteins are transcription factors that respond to extracellular signals and regulate cell proliferation and differentiation98. Increased expression of C/EBPβ has been reported in rats exposed to hyperoxia in vivo99. Ramsay et al. showed that hyperoxia exposure resulted in increased expression of the C/EBP beta isoform: liver-inhibiting protein (LIP), which decreases the expression of CCSP (Clara cell secretory protein) which is protective under conditions of oxidant lung injury80. Increased expression of the C/EBPα isoform (Fig. 8E) suggests that CYP1A2 plays an important role in the detoxification of the adduct precursors. As these adducts are may have already reached physiological saturation, higher oxygen levels as seen at 24–72 h after hyperoxia may not have resulted in increased oxidative DNA damage in these animals. Interestingly, the levels of oxidative DNA lesions were attenuated in Cyp1a2−/− mice after prolonged hyperoxia (Fig. 8F–I), and this could be due to enhanced DNA repair of these specific nucleotides in vivo, but further experimental proof is needed to substantiate this idea.

In conclusion, we present the changes in the mouse pulmonary transcriptome and proteome after hyperoxia exposure at the 48 h time point in WT, Cyp1a1−/− and Cyp1a2−/− mice. We identified DEGs involved in apoptosis, DNA repair and estrogen response pathways that may explain the differences in susceptibility of Cyp1a1−/− and Cyp1a2−/− mice to HLI. We also highlighted the differences in the DNA repair pathway and highlighted involved genes and proteins that could explain the differences in DNA injury observed in the knock-out animals. These findings provide novel insights into the mechanisms involved in HLI and suggest new pathways that need to be investigated as possible preventative and/or therapeutic targets against acute lung injury in humans.

**Methods**

**Animals.** Approval for this study was obtained from the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine (Protocol number AN-907). All experiments were performed in accordance with relevant guidelines and regulations. Care of animals in research met the highest contemporary standards as per the 8th edition of the guide for the care and use of laboratory animals and other IACUC protocols.

C57BL/6J wild type (WT) mice were obtained from Charles River laboratories (Wilmington, DE) and creation of the Cyp1a1(−/−) knockout mouse line, which was on C57BL6 background, has been described before81. Creation of the Cyp1a2(−/−) knockout mouse line, has been previously described82. These mice, which were on a mixed background (B6/SV129) were cross-bred into the C57BL/6J background by back-crossing for 12 generations, resulting in Cyp1a2(−/−) on >99% B6 background. Eight to ten week old mice were maintained at Texas Children’s Hospital animal facility and used for the study. They were fed standard mice food and water ad libitum and maintained in a 12 h day/night cycle.

**Oxygen exposure.** Adult male mice (8–10 week-old) were maintained in either room air (21% oxygen) or exposed to hyperoxia (95–100% oxygen) using pure O2 at 51/min for 48 hrs in a sealed Plexiglass chamber, as reported previously83. We measured the oxygen concentration in the plexiglass chamber by an analyzer (Getronics, Kenilworth, New Jersey). After hyperoxia exposure, the animals were anesthetized with 200 mg/kg of sodium pentobarbital (i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. The lungs tissues were harvested for further analysis.
RNA isolation. We used 3 animals per genotype per treatment group. The samples were not pooled. Total RNA from frozen lung samples was isolated using the mirNeasy kit as per the manufacturer's standard protocols (Qiagen, Valencia, CA, USA). Sample concentration was assayed using a Nanodrop-8000 (Thermo Scientific, Wilmington, DE, USA) and quality checks were done using the Nanodrop spectrophotometer and the Agilent Bioanalyzer. 250 ng of total RNA was reverse transcribed, and microarray hybridization performed using the Illumina Gene Expression MouseWG-6 v2.0 Expression BeadChip Kit at the Laboratory for Translational Genomics at Baylor College of Medicine. The transcriptome profile data was quartile-normalized by the Bioconductor lumi package82. RNA quality parameter were as follows: The 260/280 and 260/230 ratios needed to be greater than 1.8. Further the RNA Integrity Number (RIN) was analyzed using the Agilent Bioanalyzer. The samples needed to have RIN values of 7–10 and with a range of 1–1.5.

Data analysis. We used 3 biological replicates in each group. The groups were: (1) Room air-WT, (2) Room air-Cyp1a1−/−, (3) Room air-Cyp1a2−/−, (4) Hyperoxia-WT, (5) Hyperoxia-Cyp1a1−/− and (6) Hyperoxia-Cyp1a2−/−.

Microarray Data Analyses

The Lumi package82 implemented in the R statistical software83, version 2.14.1, was used to perform quality control of the signal intensity data on the transcript probes, background adjustment, variance stabilization transformation, and rank invariant normalization. A detection p value cutoff of 0.01 was required for the normalized intensities to consider a transcript as detected. Differentially Expressed Genes (DEG) were selected following the t-test comparing the groups of interest. The genes were considered to be differentially expressed for fdr-adjusted q-value < 0.2 and the linear fold change ≥1.25 or < 0.8. The volcano plots highlighting the DEGs were generated based on log2 fold change and –log10 p-values for each comparison, using the R statistical system. A graphical representation of the DEGs was generated in form of heatmaps of mean-centered normalized expression values and employing the euclidean distance metric the and average clustering method; the R statistical software was used for heatmap generation.

Pathway enrichment and Transcription Factor Analysis. Rank file for each comparison was created based on the log2 fold change for each gene. We next employed Gene Set Enrichment Analysis (GSEA) methodology27 and software, against the Molecular Signature database (MSigDB) compendium of gene sets84. Gene Set Enrichment Analysis first finds an aggregate gene set score (termed enrichment score/ES) then runs 1000 permutations to establish a background distribution for ES. The ratio between ES and the average ES is termed Normalized Enrichment Score (NES). GSEA essentially determines whether a key component of a pathway or biological process gene set is significantly enriched in up-regulated genes (NES > 0, fdr-adjusted Q-value < 0.25) or in down-regulated genes (NES < 0, fdr-adjusted Q-value < 0.25). An established and fertile paradigm for hypothesis generation is that if the NES for a pathway in comparisons stemming from two different treatments are significant but having opposite signs, then the treatments might direct the pathways in opposite directions. The pathway collections KEGG, Reactome, Hallmark, and GOBP (Gene Ontology Biological Processes) were used to determine enriched pathways. We also used a compendium of putative transcription factor targets based on the TRANSFAC database to identifying enriched transcription factors in the transcriptome footprints analyzed. Overrepresentation (ORA) method was used to identify the key transcription factors modulating gene expression in our experiment (hypergeometric distribution; p < 0.05).

RPPA profiling and analysis. Reverse phase protein array analysis (RPPA) was performed in the Functional Proteomics RPPA core facility at the MD Anderson Cancer Center. In brief, cell lysates were serially diluted by increasing two-fold ratios for 5 successive dilutions (ranging from undiluted to 1:16 dilution) and arrayed on nitrocellulose coated slides in an 11 × 11 format. Samples were probed with antibodies by tyrainide-based signal amplification approach and visualized by DAB colorimetric reaction. Slides were scanned on a flatbed scanner to produce 16-bit tiff image. Spots from tiff images were identified and the density was quantified by Array-Pro Analyzer. Overall protein expression was normalized by integrating all dilution curves for all samples and all antibodies, using the Supercurve algorithm, implemented in the R statistical system85. Normalized linear values were analyzed for differential proteins following the same comparison scheme as in microarray dataset. Significance was assessed using a parametric t-test followed by Benjamini-Hochberg multiple hypothesis testing correction; we considered changes significant for Q < 0.25. A graphical representation in form of heatmaps for significantly changed proteins was generated using mean centering of the data and hierarchical clustering based on the Euclidean distance in the R statistical system. For a uniform visual representation of the RPPA results heatmap, expression of each protein was z-score transformed; e.g. for each protein, values were first mean-centered across all samples, then further divided by the standard deviation across all samples.

Real time qPCR validation. A subset of genes was validated by quantitative real-time PCR (qRT-PCR) to validate the microarray results. We selected genes from the pool of DEGs in which were differentially regulated among the three genotypes. RNA (50 ng), isolated as above, was subjected to quantitative TaqMan RT-PCR using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene-specific primers purchased from life science technologies (Table S7). 18S was used as the reference gene. Quantitative values were obtained from the threshold PCR cycle number (Ct) at which the increase in signal was associated with an exponential growth for PCR product becomes detectable. Relative mRNA levels for chosen target genes were normalized to 18S content. Relative expression levels of each target gene were calculated according to the equation, 2−ΔCt, where ΔCt = Ct target gene − Ct 18S gene.
Western Blot analysis. For western blotting, lung whole protein (20 μg of protein) from individual male animals (n = 4/group) exposed to room air or to hyperoxia for 48 h, was prepared as mentioned in the RPPA section and protein concentration was measured using the. Primary antibody against Rad51 (Cell Signaling; Cat#8875S; dilution 1:1000) and ERCC-1 (Santa Cruz; Cat#sc-17809; dilution 1:300) were purchased. Primary antibodies were used at a concentration of 1:1000 and secondary antibody at a concentration of 1:3000; β-actin was used as the loading control, followed by electrochemical detection of bands. The statistical analysis of densitometric values was done using Students t-test and p value < 0.05 was considered significant.

Measurement and analysis of lung oxidative DNA adducts. DNA (10 μg) was enzymatically degraded to normal (Np) and modified (Xp) deoxyribonucleoside 3′-monophosphates, as well as dinucleotides containing 3′-terminal ca (Np-cA) with micrococcal nuclease and spleen phosphodiesterase at pH 6.0 and 37 °C for 3.5 h. After treatment of the mixture with nuclease P1 to specifically convert the four normal Nps to nucleosides, the modified mononucleotides (Xp) or dinucleotides (Np-cA) were converted to 5′,32P-labeled derivatives (pXp or pNp-cA) by incubation with carrier-free [γ-32P]ATP and polynucleotide kinase. Chromatographic conditions were as previously described28,29. Briefly, radioactively labeled digests were applied to modified PEI-cellulose thin layers and chromatographed overnight (15–16 h) with solvent 1 (2.8 M sodium phosphate, pH 5.2) (D1), to purify bulky adducts. Labeled adducts retained in the lower (2.8 × 1.0 cm) and central (2.8 × 1.0 cm) sections of the D1 chromatogram were briefly autoradiographed on Cronex 4 X-ray film and then contact-transferred to individual acceptor sheets and resolved by two-dimensional TLC. The non-polar L fractions were separated with solvents 2 (2.12 M lithium formate, 3.75 M urea, pH 3.35) and 3 (0.4 M sodium phosphate, 0.25 M Tris–HCl, 4.25 M urea, pH 8.2), and the polar C fractions with solvents 2 and 4 (1.0 M sodium phosphate, pH 6.0), in the first and second dimensions, respectively. An additional development in the second dimension was performed with solvent 5 (1.0 M NaH2PO4, pH 6) in order to reduce background on C maps. 32P-labeled I-compounds were visualized by screen-enhanced autoradiography at −80 °C using Kodak XAR-5 films or with the aid of an InstantImager (Perkin Elmer, formerly Packard Instruments). Radioactivities of TLC fractions from individual animals were determined with the aid of an InstantImager. Appropriate blank count rates were automatically subtracted by the instrument from sample values. The extent of covalent DNA modification was estimated by calculating relative adduct labeling (RAL) values from corrected sample count rates, the amount of DNA assayed (expressed as pmol DNA monomer units or DNA-P), and the specific activity of [γ-32P]ATP according to

\[
\text{RAL} = \frac{\text{DNA modification(s) [cpm]}}{\text{DNA-P [pmol]} \times \text{Spec. act}_{\text{ATP}} [\text{cpm/pmol}]}
\]

Quantitative data represented minimum estimates because 100% adduct recovery presumably was not achieved.

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Author Contributions
K.L., B.M. and C.C. wrote the main manuscript, analyzed data and prepared the figures and illustrations S.M. and C.C. performed the bioinformatics analysis, interpretation and prepared figures and illustrations. W.J., L.W., X.C., G.Z. and A.V. generated and analyzed experimental data, all authors reviewed the manuscript.

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