Organ-specific contribution of P450 enzymes to bioactivation and acute respiratory tract toxicity of naphthalene

Nataliia Kovalchuk
University at Albany, State University of New York, nkovalchuk@albany.edu

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ORGAN-SPECIFIC CONTRIBUTION OF P450 ENZYMES TO BIOACTIVATION AND ACUTE RESPIRATORY TRACT TOXICITY OF NAPHTHALENE

by

Nataliia Kovalchuk

A Dissertation Submitted to the University at Albany, State University of New York in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

School of Public Health

Department of Environmental Health Sciences

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ABSTRACT

Naphthalene (NA) is an omnipresent air pollutant and respiratory toxicant and a possible human lung carcinogen. NA induces cytotoxicity in airways following bioactivation by CYP (P450) enzymes. The overall goal of this study was to examine the relative contributions of hepatic and pulmonary CYPs to bioactivation, disposition and NA-induced pneumotoxicity in vivo. Substantial differences in the expression and enzymatic activity of P450s occur in various organs in human populations, which could modify susceptibility to NA-induced airway damage. The central hypothesis for this study was that NA has the potential to cause lung toxicity in humans and that the metabolism of NA in both lung and liver influence the consequence of NA exposure on an individual basis. Three projects were undertaken to address this hypothesis.

Project 1 (Chapter 2) utilized a liver-specific P450-reductase (Cpr) null mouse model to test the effects of compromised hepatic microsomal CYP activity on the tissue burden, rates of systemic clearance, and airway toxicity of inhaled NA in vivo at occupationally relevant NA concentrations. The results, which showed a dose-dependent interrelationship between CYP-generated NA metabolites that were formed extrahepatically and the extent of cytotoxic damage of epithelial cells in airways, suggested that, although hepatic P450s contribute to systemic disposition as well as bioactivation of inhaled NA, they are not essential for the pneumotoxicity of inhaled NA.

Project 2 (Chapter 3) explored contributions of hepatic and lung CYPs to the in vivo metabolism and pharmacokinetics of NA in an inhalation exposure model. The results revealed that, in addition to the CYPs encoded by Cyp2abfgs, transgenic human CYP2A13 and CYP2F1, which are expressed in the respiratory tract, and mouse extra-hepatic non-CYP2ABFGS enzymes are capable of bioactivating NA in vivo. Transgenic CYP2A13 and CYP2F1 contributed to
systemic disposition of inhaled NA in mice lacking hepatic P450 activity. The loss of hepatic microsomal CYP activity was associated with redistribution of tissue-stored NA to plasma after termination of inhalation exposure, which prolonged exposure of the target tissue to circulating NA and enhanced target tissue bioactivation in the lung.

Project 3 (Chapter 4) determined directly whether NA metabolites generated by microsomal CYPs in airway Club cells are critical contributors to NA-induced pneumotoxicity. A lung-\textit{Cpr}-null mouse model was utilized, which had site-specific deletion of the \textit{Cpr} gene in a large proportion of airway epithelial cells, and were exposed to NA via the systemic route. The results revealed that NA-induced damage of epithelial cells was focal and mild in lung-\textit{Cpr}-null mice compared to severe exfoliation of epithelial cells in most airways in NA-treated control mice with normal \textit{Cpr} expression. Moreover, the number of proliferating (BrdU-positive) airway epithelial cells was substantially lower in NA-treated lung-Cpr-null mice than in NA-treated control littermates.

Taken together, the results of these studies provide novel insights to the metabolic mechanisms of NA-induced airway toxicity. The findings, while strongly supporting a critical role of lung CYPs in mediating NA-induced airway epithelial damage \textit{in vivo}, indicate potential role of the liver in modulating respiratory toxicity of NA in humans.
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<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Full name</th>
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<tbody>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
</tr>
<tr>
<td>AP-GSH</td>
<td>Acetaminophen-GSH conjugate</td>
</tr>
<tr>
<td>B6</td>
<td>C57BL/6J</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCSP</td>
<td>Club cell secretory protein</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal concentration</td>
</tr>
<tr>
<td>CL</td>
<td>Clearance</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CPR or POR</td>
<td>NADPH-Cytochrome P450 reductase</td>
</tr>
<tr>
<td>CYP or P450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DOX&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Doxycycline-enriched diet</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>F</td>
<td>bioavailability</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione reduced</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>LCN</td>
<td>Liver-&lt;i&gt;Cpr&lt;/i&gt;-null</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LI</td>
<td>Labeling index</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>loxP</td>
<td>Locus X-over P1</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NA</td>
<td>Naphthalene</td>
</tr>
<tr>
<td>NA-d8</td>
<td>Naphthalene-d8</td>
</tr>
<tr>
<td>NAO</td>
<td>Naphthalene-1,2-epoxide</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate reduced</td>
</tr>
<tr>
<td>NA-GSH</td>
<td>Naphthalene-GSH conjugate</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbon</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>rtTA</td>
<td>Reverse tetracycline-controlled trans activator</td>
</tr>
<tr>
<td>S.D.</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>SIM</td>
<td>Selected- ion monitoring</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>tetO</td>
<td>Tetracycline-responsive promoter element</td>
</tr>
<tr>
<td>TWA</td>
<td>Time weighted average</td>
</tr>
<tr>
<td>VOCs</td>
<td>Volatile organic compounds</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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</table>
CHAPTER ONE

INTRODUCTION
NA exposure and toxicity in humans

Naphthalene, which is the simplest member of PAH family, is present ubiquitously in the environment (USEPA, 1986; Witschi et al., 1997; Kakareka et al., 2003). Biomonitoring data reported that naphthols (primary NA metabolites) are detected in urine samples in the general population (both adults and children) of Germany, the USA, Korea, and Portugal (Bouchard M et al., 2001; Preuss R et al., 2004; Li Z et al., 2008; Sul D et al., 2012; Oliveira M et al., 2017). Moreover, NA was also detected at quantifiable levels in the breast milk of nursing Canadian women (Wheeler AJ et al., 2014).

High exposure to NA in occupational settings was reported to occur during mothball manufacturing, phthalate plasticizers production, or working with jet fuel (ATSDR 2005; Chao et al. 2006; Preuss et al., 2003). Urinary naphthol levels were elevated in the post-shift than pre-shift period among asphalt paving employees and US Air Force personnel (Serdar et al., 2004; McClean et al., 2012; Merchant-Borna et al., 2012). These data provide evidences that NA metabolism occurs in humans; but, whether NA-induced toxicity occurs in human respiratory tract in vivo is still not known.

There are no clearcut human epidemiological data on the long-term effects of NA exposure; this was partly due to confounding by co-exposure to other agents (e.g., smoking) that contain NA or related toxicants and partly to the lack of sufficient statistical power. A panel of experts of International Agency for Research on cancer (IARC) classified NA as a Possible Human Carcinogen (group 2B) (IARC, 2002), and the United States Occupational Safety and Health Administration (OSHA) has established a permissible exposure limit (PEL) of 10 ppm for NA. However, all these conclusions were based mainly on carcinogenic findings in the
respiratory tract of laboratory rodents, not humans (Abdo et al., 2001; Long et al., 2003). The concern remains about the relevance of rodent NA-mediated toxic outcome to humans.

**Metabolism of NA (reactive NA metabolites, their detoxification) and toxicity**

Manifestation of toxic effects of any chemical is an interplay between its activation and detoxification processes. P450s are the major enzymes that metabolize NA by converting this relatively inert compound to different toxic metabolites (Fig. 1.1).

**Figure 1.1. Overview of NA metabolism** (modified from Buckpitt AR et al., 2002)

Naphthalene-1,2-epoxide (NAO) is the first reactive intermediate formed by CYPs. NAO can be generated by isolated hepatocytes and amount of effluxing NAO is dependent on substrate
(NA) concentration (Richieri et al., 1987). Moreover, the cytotoxic effects after incubation with NAO were observed in isolated hepatocytes (Buonarati et al., 1989), perfused mouse lung (Kanekal S et al., 1991), isolated murine Club cells (Chichester et al., 1994) and isolated resting human mononuclear leucocytes (Wilson et al., 1995). Early studies demonstrated that NAO can produce dose-dependent depletion of glutathione (GSH) in lung, but not liver or kidney, in mice (Richieri et al., 1988). NAO, a reactive electrophile, is readily conjugated with sulfur nucleophiles. Trapping of NAO by GSH, the most abundant sulfur-containing cellular peptide, was proposed to serve as a marker of NA bioactivation (Buckpitt et al., 1984). The majority (>60%) of urine NA metabolites in mice following NA inhalation (15 ppm, 4-hour exposure session for 7 consecutive days) were derived from conjugation of NAO with GSH (Ayala et al., 2015).

Trapping of NAO by GSH is facilitated by glutathione-S-transferase. Independent of the route of NA exposure, the formed NA metabolites depleted intracellular GSH and caused cytotoxicity in vitro and in vivo in rodents (Greene et al., 2000; West et al., 2000; Plopper et al., 2001; Phimister et al., 2004; Cichocki et al., 2014; Kedderis et al., 2014). Administration of cysteine-glutathione mixed disulfides prevented NA-mediated acute cytotoxic effects (Phimister et al., 2005). Repeated NA exposures augment resistance to its cytoxic injuries in airways. The development of this tolerance to NA involved alterations in metabolizing/detoxifying enzymes and reorganization of epithelial cells in target airway region (Lakritz et al., 1996; Phimister et al., 2004). The mechanism of tolerance to damage in NA-exposed male mice is not fully clear, but it may involve the increase of GSH pool in airway epithelial cells, which would effectively detoxify reactive cytotoxic NA metabolites (Phimister et al., 2004). Induction of gamma-glutamylcysteine synthetase, the first and rate-limiting enzyme in the production of GSH, is
considered as the main driver of the tolerance development in mouse airways (West et al., 2000; West et al., 2002; West et al., 2003). Based on gene expression changes, chronic exposure of F344 rats to NA vapors upregulated signaling pathways related to oxidative stress, regulation of proliferation (cell cycle control, DNA damage response) and GSH homeostasis in the nasal epithelium (Clewell et al., 2014).

Microsomal epoxide hydrolase (mEH), localized mainly in the microsomal fraction of the endoplasmic reticulum, is important for metabolizing a broad range of toxic epoxides (Morisseau et al., 2005). Naphthoquinone is mEH-generated tertiary metabolite of NAO, which can be generated through secondary naphthalene dihydrodiol intermediate by hepatic microsomes (Kitteringham et al., 1996). mEH knockout mice have been generated, which were utilized to examine the contribution of mEH-generated metabolites to the toxicity of benzene and 7,12-dimethylbenz[a]anthracene (Miyata et al., 1999; Bauer et al., 2003). Total amount of NA metabolites generated in microdissected airways of mEH-null mice (both genders) were only one half compared to WT mice, a reduction mainly due to significant decreases in naphthalene dihydrodiol production. However, mEH-null mice were not fully protected from Club cell cytotoxicity induced by inhaled NA, suggesting that mEH-generated metabolites might not contribute significantly to NA-mediated toxicity and possible carcinogenicity (Carratt et al., 2016).

It is difficult to directly monitor transport of reactive NA metabolites (naphthalene-oxide, 1,2-naphthoquinine and 1,4-naphthoquinone) in vivo due to their high reactivity. Quantification of their hemoglobin and albumin adducts in plasma could provide insights into their migration fate. Several experiments revealed that all three of these metabolites form cysteiny1 adducts with both of the aforementioned plasma proteins in a dose-dependent fashion in male F344 rats and
Swiss Webster mice after a single intraperitoneal administration of NA at different doses (Waidyanatha et al., 2002; Troester et al., 2002; Waidyanatha et al., 2008). These studies further confirm that NA metabolites can be generated extrapulmonarily and transported via systemic circulation to the target site of toxicity.

Mechanisms of acute NA-induced toxicity in airways are not fully understood. Early studies suggested that depletion of GSH is the major mechanism of NA cytotoxicity. Later it was demonstrated that GSH depletion in distal airways caused by NA or GSH-depleting agent diethyl maleate (weak electrophile that requires glutathione-S-transferase to lower GSH tissue level) has different effects on Club cells (Phimister et al., 2005). Cells were swollen, blebbed, with disrupted cytoskeleton filaments after exposure to both agents, but only NA-treated cells became necrotic, whereas diethyl maleate-treated cells recovered after stress induced by GSH loss. These results demonstrated that only a combination of GSH depletion and formation of protein adducts with NA metabolites within target cells is critical for NA-induced cytotoxicity. Studies in vitro identified potential protein targets following incubation of liver microsomes or human bronchial epithelial cells with NA or naphthoquinone, respectively (Lame et al., 2003; Isbell et al., 2005). Adducted, 2D-separated proteins were also identified by MALDI MS analysis of epithelial cells from nasal mucosa and liver of mice, rats and monkey after incubation with a relatively high concentration of NA in vitro (DeStefano-Shields et al., 2010; Pham et al., 2012). Moreover, adducted proteins were also detected and identified in mouse airway and nasal olfactory epithelia by proteomics analysis following NA inhalation exposure (Kultz et al., 2015). Most of the identified proteins in these studies overlap and were sulfur-containing in nature with antioxidant (protein sulfide isomerase, heat shock protein, galectin-1, calreticulin) or cytoskeleton (actin, tubulin) properties.
Role of CYP450 in NA metabolism and toxicity

PAHs, which are the most abundant air pollutants, require CYP-mediated bioactivation to exert their toxic effects (Shimada, 2006; Moorthy et al., 2015) and NA is not an exception. CYP-generated NA metabolites are the prerequisite for NA-induced toxic effects.

NA metabolites are formed by naïve human liver microsomes (Tingle et al., 1993), where they are predominantly generated by CYP1A2, 2A6, 2D6 and 3A4 (Cho et al., 2006). However, the ability of hepatic CYPs to bioactivate chemicals is modulated by polymorphism of a given P450 isoform, drug-xenobiotic interactions and hepatic disfunction. The activities of CYP1A2, 2E1 and 3A4 in hepatic microsomal preparations, obtained from patients with cirrhosis, were significantly lower than in healthy subjects when tested with corresponding probe substrates for each CYP (George et al., 1995). Significantly lower in vivo activities of CYP1A2, 2C19, 2D6 and 2E1 were detected in patients with liver disease, compared to healthy subjects, as revealed by using a cocktail of probe substrate drugs, caffeine, mephenytoin, debrisoquine and chlorzoxazone (Frye et al., 2006). Chlorpyrifos-oxon, a metabolite of pesticide chlorpyrifos, was found to significantly alter human hepatic microsomal CYPs activity; the formation of NA metabolites by human CYP1A2, 1B1 and 2B6 was apparently decreased, but that by human CYP2C, 2D and 3A subfamilies was apparently increased (Cho et al., 2007). The impact of an alteration of the activity of hepatic P450s on the pharmacokinetic profile and target tissue toxicity of most environmental pollutants, including NA, in humans remains unknown.

It is well documented that diet can affect the expression of P450s enzymes. The mRNA and protein levels and activity of hepatic CYP2B10 and 3A11 were significantly decreased in mice treated with high-fat diet (Ghose et al., 2011; Chiba et al., 2016; Maximos et al., 2017). Mouse models with little hepatic P450 activity due to hepatocyte-specific deletion of the
NADPH-cytochrome P450 reductase gene (*Cpr*) have been generated (Gu et al., 2003; Henderson et al., 2003). The mouse model generated by the Ding Lab (Gu et al, 2003) has been used to elucidate the contributions of hepatic CYP activity to the *in vivo* disposition and toxicity of many compounds, including acetaminophen (Gu et al., 2005), cyclophosphamide (Gu et al., 2007), chloroform (Fang et al., 2008), 2,6-dichlorobenzonitrile (Xie et al., 2010), methimazole (Xie et al., 2011) and azoxymethane (Megaraj et al., 2014). Moreover, pharmacokinetic studies demonstrated that, in mice, hepatic CYP-generated metabolites are not essential for bioactivation for intraperitoneally administered NA *in vivo* (Li et al., 2011). However, the contribution of CYP activity in liver to airway toxicity mediated by inhaled NA was still unknown.

Lung is a primary portal-of-entry organ for inhaled pollutants and has a significant capability to generate NA reactive metabolites. Similar extent of cytotoxic injures (vacuolation, exfoliation of damaged cells) was detected in mouse airways after intraperitoneal NA administration and in NA-treated microdissected mouse airways (Plopper et al., 1991; Van Winkle et al., 1996). Moreover, incubation of airway explants with a cytochrome P450 inhibitor piperonyl butoxide (organic compound used in pesticide formulation) prior to NA exposure prevents the NA-induced injury of airway cells (Plopper et al., 1991). Further studies identified that recombinant mouse CYP2F2 has the highest catalytic activity to metabolize NA *in vitro* compared to other PAHs (Shultz et l., 1999; Shultz et al., 2001). Studies *in vivo* using Cyp2f2-null mice further confirmed an important role of CYP2F2 in metabolism and pulmonary toxicity of naphthalene and other environmental pollutants (Li et al., 2011; Cruzan et al., 2012; Shen et al., 2014). Bioactivation of NA to its metabolites by rat 2F4 and recombinant human CYP2F1 has also been observed *in vitro*, but at a much slower rate compared to mouse CYP2F2 (Lanza et al., 1999; Baldwin et al., 2004). Such a species difference in metabolic capacity to bioactivate
NA attributes to different ratio of stereoselective NA-oxides generated in pulmonary CYPs (Buckpitt et al., 1992; Buckpitt et al., 1995; Lewis et al., 2009). Expression of mouse CYP2F2 and human CYP2F1 was detected mainly in the airways (Li et al., 2011; Hukkanen et al., 2002) with much lower levels in humans. CYP2A5, which is a mouse P450 isoform that is expressed mostly in the respiratory tract, can bioactivate NA and is responsible for NA-induced toxicity in mouse nasal olfactory mucosa (Hu et al., 2014). The human ortholog of CYP2A5, CYP2A13, is expressed in human respiratory tract (Hukkanen et al., 2002; Su et al., 2000; Zhang et al., 2007) and efficiently metabolize NA in vitro (Fukami et al., 2008). Even though the protein expression level and activity of human CYP2A13 and CYP2F1 are relatively low, they appeared to mediate the toxicity of inhaled NA in airway epithelial cells, as suggested by data on CYP2A13/2F1-humanized mice (Li et al., 2017).

Environmental pollutants not only are bioactivated by pulmonary expressed P450s, but they may also change the CYP expression level in human airways (Moorthy et al., 2015). Constituents of cigarette smoke downregulate the expression of CYP1 and CYP2A (Shimada, 2017). Expression of CYP2A13 and CYP2F1 at the transcript level was significantly lower in biopsy lung samples from smokers than non-smokers (Thum et al., 2006; Leclerc et al., 2010). Inflammation and preexisting conditions (COPD) also lower the expression level of CYPs, including CYP2F1, in human airways (Berg et al., 2014). Taken together, it is still unknown if NA-induced toxicity could be observed in humans with low CYPs expression and if hepatic CYP-generated metabolites of inhaled NA can mediate the toxicity in the respiratory tract if local NA metabolism by pulmonary CYPs in compromised.

In summary, CYP expression and catalytic activity in lung and liver may vary in an organ-specific manner, in addition to the variations in CYP function due to events that affect
CYP activity in all organs. The impact of an organ-specific change in CYP function on xenobiotic disposition and toxicity \textit{in vivo} is poorly understood, but such knowledge could be critical for understanding the difference in susceptibility of individuals to pulmonary toxicity of respiratory toxicants, such as NA.

**OBJECTIVE AND SCOPE**

The overall goal of this study was to examine the relative contributions of hepatic and pulmonary CYPs to bioactivation, disposition and NA-induced pneumotoxicity \textit{in vivo}. The central hypothesis for this study was that NA has the potential to cause lung toxicity in humans and that the metabolism of NA in both lung and liver influence the consequence of NA exposure on an individual basis. To achieve this goal, the following studies were conducted and reported in this dissertation.

The first study (Project 1) utilized a liver-specific P450-reductase (\textit{Cpr}) null (liver-\textit{Cpr}-null or LCN) mouse model to test the effects of compromised hepatic microsomal CYP activity on the tissue burden, rates of systemic clearance, and airway toxicity of inhaled NA \textit{in vivo} at occupationally relevant NA concentrations. By comparing NA \textit{in vitro} metabolism by hepatic and pulmonary microsomal enzymes, \textit{in vivo} disposition and clearance of inhaled NA, as well as cytotoxic injury of airway epithelial cells, evaluated by high-resolution histopathological analysis, between WT and liver-\textit{Cpr}-null mice, the role of hepatic P450s in pulmonary toxicity, induced by inhaled NA, was determined. The results, which showed a dose-dependent interrelationship between CYP-generated NA metabolites that were formed extrahepatically and
the extent of cytotoxic damage of epithelial cells in airways, suggested that, although hepatic
P450s contribute to systemic disposition as well as bioactivation of inhaled NA, they are not
essential for the pneumotoxicity of inhaled NA.

The second study (Project 2) explored contributions of hepatic and lung CYPs to the in vivo
metabolism and pharmacokinetics of NA in an inhalation exposure mode, examining both
target and non-target tissues of Cyp2abfgs-null mice and CYP2A13/2F1-humanized mice. The
microsomal activity to bioactivate NA in vitro, time-course kinetics of inhaled NA in plasma and
tissues in vivo were compared between Cyp2abfgs-null and CYP2A13/2F1-humanized mice with
either normal or compromised hepatic CYPs activity. The results revealed that, in addition to the
CYPs encoded by Cyp2abfgs, transgenic human CYP2A13 and CYP2F1, which are expressed in
the respiratory tract, and mouse extra-hepatic non-CYP2ABFGS enzymes are capable of
bioactivating NA in vivo. Transgenic CYP2A13 and CYP2F1 contributed to systemic disposition
of inhaled NA in mice lacking hepatic P450 activity. The loss of hepatic microsomal CYP
activity was associated with redistribution of tissue-stored NA to plasma after termination of
inhalation exposure, which prolonged exposure of the target tissue to circulating NA and
enhanced target tissue bioactivation in the lung.

The third study (Project 3) determined directly whether NA metabolites generated by
microsomal CYPs in airway Club cells are critical contributors to NA-induced pneumotoxicity.
A lung-Cpr-null mouse model was utilized, which had site-specific deletion of the Cpr gene in a
large proportion of airway epithelial cells, and were exposed to NA via the systemic route. NA-
induced toxicity in airways was assessed based on routine histopathological analysis and on rate
of cell proliferation, determined using a BrdU incorporation assay. The results revealed that NA-
induced damage of epithelial cells was focal and mild in lung-Cpr-null mice compared to severe
exfoliation of epithelial cells in most airways in NA-treated control mice with normal Cpr expression. Moreover, the number of proliferating (BrdU-positive) airway epithelial cells was substantially lower in NA-treated lung-Cpr-null mice than in NA-treated control littermates. These results suggest that NA has significantly higher carcinogenic potential in individuals with high pulmonary CYPs activity.

Taken together, the results of these studies provide novel insights to the metabolic mechanisms of NA-induced airway toxicity. The findings, while strongly supporting a critical role of lung CYPs in mediating NA-induced airway epithelial damage in vivo, indicate potential role of the liver in modulating respiratory toxicity of NA in humans.
CHAPTER TWO

IMPACT OF HEPATIC P450-MEDIATED BIOTRANSFORMATION ON THE
DISPOSITION AND RESPIRATORY TRACT TOXICITY OF INHALED
NAPHTHALENE
SUMMARY

We determined whether a decrease in hepatic microsomal cytochrome P450 activity would impact lung toxicity induced by inhalation exposure to naphthalene (NA), a ubiquitous environmental pollutant. The liver-Cpr-null (LCN) mouse showed decreases in microsomal metabolism of NA in liver, but not lung, compared to wild-type (WT) mouse. Plasma levels of NA and NA-glutathione conjugates (NA-GSH) were both higher in LCN than in WT mice after a 4-h nose-only NA inhalation exposure at 10 ppm. Levels of NA were also higher in lung and liver of LCN, compared to WT, mice, following exposure to NA at 5 or 10 ppm. Despite the large increase in circulating and lung tissue NA levels, the level of NA-GSH, a biomarker of NA bioactivation, was either not different, or only slightly higher, in lung and liver tissues of LCN mice, relative to that in WT mice. Furthermore, the extent of NA-induced acute airway injury, judging from high-resolution lung histopathology and morphometry at 20 h following NA exposure, was not higher, but lower, in LCN than in WT mice. These results, while confirming the ability of extrahepatic organ to bioactivate inhaled NA and mediate NA’s lung toxicity, suggest that liver P450-generated NA metabolites also have a significant, although relatively small, contribution to airway toxicity of inhaled NA. This hepatic contribution to the airway toxicity of inhaled NA may be an important risk factor for individuals with diminished bioactivation activity in the lung.
INTRODUCTION

Naphthalene (NA) is a ubiquitous contaminant of the environment (USEPA, 1986; Witschi et al., 1997; Kakareka et al., 2003). NA is classified as a Possible Human Carcinogen (group 2B) (IARC, 2002), in part due to its ability to induce nasal tumors in rats and lung tumors in mice in chronic rodent bioassays conducted by the National Toxicology Program (Abdo et al., 1992; Abdo et al., 2001). The current OSHA standard for NA exposure in the workplace is 10 ppm. NA administration injures nonciliated bronchiolar epithelial cells (Club cells) in conductive airways independent of the route of administration in rodents (Plopper et al., 1992a; Plopper et al., 1992b; West et al., 2001).

The mechanism of NA carcinogenicity is not fully understood; but it is clear that cytochrome P450 (P450)-mediated NA bioactivation is essential for NA toxicity, and repeated cycles of NA-induced acute cytotoxicity with subsequent tissue repair are believed to be important initiating events for NA carcinogenicity (Buckpitt et al., 2002; Brusick, 2008). Bioactivation of NA to its reactive metabolite NA-oxide (NAO) by P450 enzymes is the key step in NA-induced cellular damage in airways (Warren et al., 1982; Buckpitt at al., 1983). The reaction of NAO with reduced glutathione (GSH), to produce NA-glutathione conjugates (NA-GSH), is one of major detoxification pathways for the toxicant, and allows NA-GSH to serve as a marker of NA bioactivation in vitro and in vivo (Buckpitt et al., 1984; Richieri aet al., 1988; Buckpitt et al., 1992; Tingle et al., 1993; Wilson et al., 1996). Recent studies have provided further details on the involvement of P450 enzymes in NA bioactivation, including the respective roles of mouse CYP2A5 and CYP2F2 in mediating NA-induced nasal and lung toxicity (Li et al., 2011; Hu et al., 2014). The ability of human CYP2A13/CYP2F1 to bioactivate NA in vivo and mediate NA-induced acute nasal and lung toxicity at occupationally relevant inhalation exposure
levels has also been demonstrated in a CYP2A13/CYP2F1-humanized mouse model (Li et al., 2017). The latter study provides strong supporting evidence for the potential of NA to cause respiratory toxicity in humans.

The aim of this study was to determine whether a decrease in hepatic microsomal P450 activity would impact lung toxicity induced by inhalation exposure to NA; the answer to this question may impact human risk assessment for NA. We hypothesized that a decrease of P450 activity in liver, as would occur in people with liver diseases, will increase the amount of NA available for bioactivation by lung P450s, resulting in the formation of greater amounts of reactive metabolites and more severe damage to the pulmonary airways. The impact of the loss of hepatic NA metabolic activity on systemic NA clearance has been demonstrated previously in mice exposed to intraperitoneally injected NA at relatively high doses (Li et al., 2011). However, the impact of hepatic metabolic disposition on the pharmacokinetics of NA may differ by exposure route; during an inhalation exposure, the respiratory tract would be exposed to NA delivered directly from the air and NA delivered through the blood circulation following absorption.

In the present study, we exposed mice to occupationally relevant doses (5 and 10 ppm) of NA through inhalation, and compared the pharmacokinetics of NA and NA-GSH, and the extent of NA-induced airway cytotoxicity, between wild-type (WT) and liver-Cpr-null (LCN) mice. The LCN mice undergo tissue-specific deletion of the Cpr gene in hepatocytes, which results in tissue-specific abolishment of microsomal P450 activities in the liver (Gu et al., 2003). Thus, we can determine whether a decrease in hepatic microsomal P450 activity would impact lung toxicity induced by inhalation exposure to NA. The LCN mouse model has been previously
utilized to demonstrate the impact of hepatic P450-mediated NA metabolism on the pharmacokinetics of systemically administered NA (Li et al., 2011).

MATERIAL AND METHODS

Chemicals and reagents. NA (CAS# 91-20-3, purity 99%), NA-d$_8$ (CAS# 1146-65-2, purity 99%), GSH(CAS# 70-18-8, purity ≥ 98.0%), β-nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexyl ammonium) salt (NADPH) (CAS#, 100929-71-3, purity ≥ 95.0%), and corn oil (highly refined, low acidity) were purchased from Sigma Aldrich (St. Louis, MO). Acetaminophen-glutathione (AP-GSH) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). NA-GSH standard as a mixture of all four stereoisomers was a generous gift from Drs. Alan R. Buckpitt and Dexter Morin (University of California at Davis, Davis, CA) and was prepared as previously described (Richieri et al., 1987). All solvents (dichloromethane, formic acid, methanol and water) were of analytical grade (Fisher Scientific, Houston, TX). Ingredients for Karnovsky’s fixative were from Tousimis (Rockville, MD).

In vitro assay of NA metabolism. Lung and liver microsomes were prepared from three different batches (each prepared from pooled tissue of 3 mice) of 2-month old, male, LCN and WT mice, as described (Gu et al., 1998). In vitro assay of NA bioactivation was performed as described previously (Shultz et al., 1999); reaction mixtures contained 50 mM phosphate buffer (pH 7.4), NA at a wide range of concentrations added in 2 µL of methanol (0; 0.5; 1.0; 2.0; 5.0; 10.0; 20.0; 50.0; 100.0; 200.0 and 400.0 µM), 10 mM GSH, 0.2 mg/ml of liver or lung microsomal protein, and 0 or 1.0 mM NADPH, in a final volume of 0.2 mL. The reaction was carried out at 37 °C in sealed tubes for 5 min and terminated by the addition of 2 volumes of ice-cold methanol containing 2.5 ng of AP-GSH (internal standard). The resultant mixtures were
centrifuged to remove precipitated proteins, and NA-GSH was quantified in aliquots of the supernatant using LC-MS/MS (see below).

**Animal experiments.** All procedures involving animals were approved by the Wadsworth Center Institutional Animal Care and Use Committee. WT B6 and LCN (Gu et al., 2003) mice from colonies maintained at Wadsworth Center were housed in an acclimatized environment on a 12-h light:dark cycle, and had access to standard rodent chow and drinking water *ad libitum*. Two-month-old male mice were used for experiments.

Nose-only inhalation exposure to HEPA-filtered air (sham-exposure control) or NA vapor was conducted in an Oral Nasal Aerosol Respiratory Exposure System (equipped with a 24-port Jaeger rodent inhalation exposure chamber) (CH Technologies, Westwood, NJ). Mice were acclimatized to the holding tube and exposure chamber (once a day for three days) prior to NA exposure. To generate NA vapor, air was passed through a glass column containing crystalline NA, heated to 52 °C; the vapor was mixed with fresh filtered air to achieve desired average NA concentration in the inhalation chamber. All experiments were started in the morning and consisted of two 2-h exposure periods with a 1-h break in between (added to reduce stress to mice). NA vapor at two different doses was studied, 5 and 10 ppm; the latter dose is an OSHA ([http://www.osha.gov/dts/chemicalsampling/data/CH_255800.html](http://www.osha.gov/dts/chemicalsampling/data/CH_255800.html)) permissible exposure limit for human workers. The 4-h total exposure time was selected to mimic daily occupational exposure.

Concentrations of NA, carbon dioxide (CO₂), carbon monoxide (CO), and oxygen (O₂); relative humidity; and air temperature in the exposure chamber were monitored in real time throughout the exposure using a model IQ-604 Total Volatile Organic Compound (TVOC) Monitor (Graywolf Sensing Solutions, Trumbull, CT), which was pre-calibrated for NA as
recently described (Li et al., 2017). Air flow through each nose port was maintained at approximately 0.3 L/min.

For toxicokinetics studies, blood samples (~20 µL each) from individual mice were collected from the tail vein using heparinized capillary tubes at various time points (0-360 min) after termination of NA exposure. Plasma was prepared by centrifugation of blood samples at 10,000 rpm for 5 min at 4 °C, and was stored in sealed tubes at -80 °C until use. For detection of tissue levels of NA and NA-GSH, mice were placed in fresh air (immediately after termination of NA exposure) for 0, 2, 4 and 20 hours and then euthanized by CO₂ overdose. Lung (lavaged with 1 mL of 1X phosphate-buffered saline (PBS)) and liver were harvested, quick-frozen, and stored in sealed tubes at -80 °C until use.

**NA and NA-GSH detection.** For NA detection, plasma (10 µL) or tissue (lung, liver) homogenates (50 µL) were spiked with NA-d₈ (18 pg for plasma and 12 pg for tissue, in 10 µL of methanol), extracted with dichloromethane (100 µL for plasma and 110 µL for tissue). The organic phase (1 µL injection volume) was analyzed for NA using gas-chromatography mass spectrometry in a splitless injection mode, as previously described (Li et al., 2011), using a Restek Rxi-5ms (30 m x 0.25 mm; 0.25 µm) column (Restek, Bellefonte, PA). The limit for NA detection was 0.8 pmol (on column).

For NA-GSH detection, plasma (10 µL) and tissue homogenate (50 µL) were spiked with an internal standard AP-GSH (2 ng in 10 µL of methanol), and then mixed with methanol (80 and 90 µL, respectively) for protein precipitation. An aliquot of the supernatant (1 µL) was analyzed for NA-GSH using liquid-chromatography mass spectrometry, with a SCIEX 4000 Q-Trap mass spectrometer, as previously described (Li et al., 2011), or with a SCIEX 6500 Q-Trap mass spectrometer (AB-SCIEX, Framingham, MA), as described below.
The 6500 Q-Trap mass spectrometer was coupled to an Agilent 1290 Infinity Series ultra-performance liquid chromatography system (Agilent, Santa Clara, CA) and an Agilent Elipse Plus C18 (2.1 x 50 mm; 1.8 µm) column. Analytes were eluted at room temperature, at a flow rate of 0.2 mL/min, with mobile phases as previously described (Li et al., 2011), using the following program: linear increase from 10%B to 90%B from 0 to 4 min, return to 10%B from 4 to 8 min, and re-equilibration at 10%B for 2 min. The retention times of NA-GSH and AP-GSH were 2.9 and 2.3 min, respectively. The mass spectrometer was operated in positive ion mode using electrospray ionization with the following settings: ion spray voltage, 5500V; temperature, 500°C; curtain gas, 30 psi; ion source gas 1, 22 psi; ion source gas 2, 10 psi; declustering potential, 95 V; and entrance potential, 10 V. Analytes were detected using multiple reaction monitoring (MRM), with the following settings: dwell time, 125 ms; collision gas, medium; collision energy, 30 V; and collision cell exit potential, 10 V. The MRM transitions used for NA-GSH quantification and confirmation were 452/306 and 452/288, respectively. Quantification of the internal standard, AP-GSH, was done using the MRM transition 457/328. The limit for NA-GSH detection was 0.55 pmol (on column). All samples were analyzed in duplicate.

**High-resolution histology and quantitative histopathology.** Mice were exposed to HEPA-filtered air (FA) or NA vapor (5 or 10 ppm) as described above, and sacrificed 20 hours after termination of the exposure, by CO₂ overdose. The lungs were inflated with Karnovsky’s solution as described previously (Van Winkle et al., 2001; Van Winkle et al., 2004; Van Winkle et al., 2017). Fixed lung lobes were embedded in Araldite 502 resin. Lung sections (1-µm thick) were stained with methylene blue azure II. Proximal airways and terminal bronchioles were imaged on an Olympus BH-2 microscope using Adobe Photoshop image capture software. The
morphometric procedures for detection of injured epithelial cells in different airway regions were performed as described elsewhere (Hyde et al., 1990). The volume fractions ($V_v$) were defined by point (P) and intercept (I) counting using a cycloid grid and Stereology Toolbox (Morphometrix, Montpellier, France) for a minimum of 200 points. $V_v$ was calculated using the formula $V_v = P_n/P_t$, where $P_n$ is the number of test points hitting structures of interest (damaged epithelial cells), and $P_t$ is the total points hitting the reference space (epithelial cells). Eight proximal bronchioles or distal airways (at a minimum) from each animal were used to determine morphometric parameters, which were used to calculate the mean and the standard deviation for each exposed group of animals per genotype.

Other methods and data analysis. The Michaelis-Menten kinetic parameters, $K_m$ and $V_{max}$, for in vitro metabolism studies were calculated using GraphPad Prism (GraphPad, San Diego, CA). Student’s t-test was used to analyze the differences between kinetic parameters. The delivered dose of inhaled NA following inhalation exposure was calculated per guidelines (Alexander et al., 2008), using WinNonlin software (Pharsight, Mountain View, CA). Given that bioavailability (F) of NA is unknown after inhalation administration in mice, clearance (CL) was determined as a hybrid parameter $CL/F$. Data from multiple groups were compared using a two-way analysis of variance (ANOVA), followed by Bonferroni’s test for multiple comparisons. $p<0.05$ was considered statistically significant.

RESULTS

Liver-specific impact of hepatic cytochrome P450 reductase gene ($Cpr$) deletion on NA metabolism in vitro. NA metabolism by lung and liver microsomal preparations was compared between WT and LCN mice. The rates of formation of the reactive 1,2-NA epoxide were determined by measuring NA-GSH produced in the presence of added GSH. The rates of
NA-GSH formation were linear with time under the conditions of the assay. As shown in Table 1, catalytic efficiency for the formation of NA-GSH, determined using a broad range of NA concentrations (0.5-400 µM) and saturating amounts of GSH, was 10-fold lower in the liver of LCN mice, compared to that in WT mice; but it was similar between the two mouse strains in the lung.

Table 2.1. Enzyme kinetic parameters for the formation of NA-GSH from NA by lung and liver microsomes from WT and LCN mice.

Liver and lung microsomes were prepared from WT and LCN mice. Reaction mixtures contained 50 mM phosphate buffer, pH 7.4, wide range of NA concentrations (0-400µM), 1.0 mM NADPH, 10 mM GSH, 0.2 mg/ml of liver or lung microsomal protein.

<table>
<thead>
<tr>
<th>Tissue, strain</th>
<th>Km, µM</th>
<th>Vmax, nmol/min/mg of protein</th>
<th>Vmax/Km, ml/mg of protein/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>4.71±0.76</td>
<td>1.10±0.04</td>
<td>0.23</td>
</tr>
<tr>
<td>LCN</td>
<td>5.09±0.89</td>
<td>0.12±0.01*</td>
<td>0.02</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13.6±1.6</td>
<td>1.35±0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>LCN</td>
<td>8.96±1.17</td>
<td>1.27±0.04</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Apparent Km and Vmax values for the microsomal formation of NA-GSH were determined as described in Methods. The results shown represent means ± S.D. of values determined for three separate microsomal samples, each prepared from tissues pooled from three 2-month-old male mice.
Impact of hepatic Cpr deletion on pharmacokinetics of plasma NA and NA-GSH following NA inhalation exposure. NA and NA-GSH were measured in the plasma of WT and LCN mice immediately following the inhalation exposure session (2 h NA, 1 h rest, 2 h NA). As shown in Figure 2.1, after a NA exposure at 10 ppm, the levels of NA and NA-GSH in the plasma of LCN mice were significantly higher than in WT mice at most time-points examined. Pharmacokinetic analysis of the data in Figure 1 indicated ~2-fold higher Cmax values for NA and ~2.5-fold higher Cmax values for NA-GSH in LCN than in WT mice. The AUC values for NA and NA-GSH were respectively ~2 and ~11 times greater in LCN compared to WT mice (Table 2). The rate of NA clearance (Cl/F) in LCN mice was one-half of that in WT mice, coupled with an increased (by ~1.4 fold) plasma elimination half-life (t1/2). These results indicate that the loss of hepatic P450 activity led to increases in systemic bioavailability of inhaled NA. However, the plasma level of NA-GSH was not decreased; in contrast, it was dramatically increased in the LCN mice.

Figure 2.1. Systemic levels of NA and NA-GSH in WT and LCN mice.
Two-month old wild-type (WT) and liver-Cpr-null (LCN) male mice were exposed to 10 ppm of naphthalene (NA) for 4 hours and plasma levels of NA (A) and naphthalene-glutathione
conjugate (NA-GSH) (B) were determined at different time points after termination of NA exposure. Data represent means ± SD (n=4). *, **, ****, p<0.05, 0.01, or 0.0001, respectively, compared to WT mice (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

**Table 2.2. Pharmacokinetic parameters of NA and NA-GSH in the plasma of WT and LCN mice after NA inhalation exposure to 10 ppm.**

Values were derived from data presented in Figure 2.1. Data for plasma represent means±S.D. (n=4 for each group).

<table>
<thead>
<tr>
<th>Analyte, strain</th>
<th>AUC_{0-6h}, µg/ml/min</th>
<th>t_{1/2}, min</th>
<th>CL/F, ml/min</th>
<th>Cmax, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13.2±1.8</td>
<td>111±7</td>
<td>15.8±1.3</td>
<td>76.5±2.6</td>
</tr>
<tr>
<td>LCN</td>
<td>28.6 ±4.5*</td>
<td>158±16*</td>
<td>7.9±1.5*</td>
<td>159±17*</td>
</tr>
<tr>
<td>NA-GSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3.0±0.2</td>
<td>N/A</td>
<td>N/A</td>
<td>153±49</td>
</tr>
<tr>
<td>LCN</td>
<td>33.6±10.5*</td>
<td>N/A</td>
<td>N/A</td>
<td>445±84*</td>
</tr>
</tbody>
</table>

*, p<0.01 (Student’s t-test), compared to corresponding WT mice

N/A, not applicable

**Impact of hepatic Cpr deletion on NA and NA-GSH levels in lung and liver following NA inhalation exposure.** Levels of NA were determined in target (lung) and non-
target (liver) tissues of WT and LCN mice exposed to 5 or 10 ppm of NA vapors. NA was detected in lungs of WT and LCN mice immediately after termination of NA exposure, but it was not detected at other time points examined (2, 4 and 20 hours) (Fig. 2.2A), with an estimated detection limit of ~4.5 ng of NA/g tissue. NA levels in the lungs of LCN mice were higher than in WT mice, at the “0-h” time point, for the 10-ppm (by >5-fold, p < 0.0001) NA dose, with the levels in the 10-ppm NA exposed LCN mice significantly higher (by >3-fold, p < 0.0001) than in the 5-ppm NA exposed LCN mice. NA levels in the lungs of LCN mice also appeared to be higher than in WT mice for the 5-ppm NA dose (by >3-fold), though the difference did not reach statistical significance.

NA was not detected in the livers of WT mice regardless of the NA dose and postexposure time. However, the livers of LCN mice exposed to 5-ppm NA had relatively low, but quantifiable amounts of NA at early time points (0-4 h), whereas those LCN mice exposed to 10-ppm NA had significantly greater amounts of NA (>7-fold, vs the 5-ppm group, p < 0.0001) in their livers at those same time points (Fig. 2.2B). NA was no longer detectable at the 20-h time point, for either NA dose. NA was not detected in any tissue samples from sham (FA)-exposed WT or LCN mice (data not shown).

NA-GSH was detected in lung (Fig. 2.2C) and liver (Fig. 2.2D) of WT and LCN mice at the early (0-h, 2-h and most 4-h) time points after inhalation of 5 or 10 ppm NA vapors, and in the late (20 h) time point after exposure to the high NA dose (10 ppm). NA-GSH levels varied in lungs and livers of WT mice at dose- and time-dependent fashion. Remarkably, NA-GSH levels were elevated in both lung and liver of LCN mice, compared to WT mice, at 2 or 4 h after termination of NA exposure (at 10 ppm, p < 0.0001). A trend of increase was also observed for the same comparisons at 5 ppm. In contrast to the rapid postexposure decline in tissue NA-GSH
levels seen in the WT mice, the rates of decline in the LCN mice were much lower. Additionally, NA-GSH levels were generally higher in the liver than in the lungs of the same animals, at each time point. NA-GSH was not detected in any tissue samples from FA-exposed WT or LCN mice (data not shown).
Figure 2.2. NA and NA-GSH levels in mouse lung and liver after a single 4-hour nose-only inhalation exposure to different doses of NA.

Two-month old wild-type (WT) and liver-Cpr-null (LCN) male mice were exposed to 5 or 10 ppm of naphthalene (NA) for 4 h. Lung (A, C) and liver (B, D) were collected 0, 2, 4 and 20 hours after termination of NA exposure. NA (A, B) and naphthalene-glutathione conjugate (NA-GSH) (C, D) were detected in tissue homogenates. Data represent means ± SD (n=3-5). ****, p<0.0001; *, p<0.05; vs WT, of corresponding dose/time point; &&&&, p<0.0001; &&&, p<0.001; vs 5 ppm, of corresponding genotype/time point (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

Impact of hepatic Cpr deletion on the extents of tissue injury induced by inhaled NA in the airways. NA-induced damage to airway epithelial cells of WT and LCN mice was evaluated at 20 h after termination of exposure, via high-resolution microscopy and quantitative morphometry (Van Winkle et al., 1995). The relative abundance of cytotoxic cells, which show vacuolization, swelling, and/or partial detachment from the basal lamina (Fig. 2.3A, 2.3B), is measured as fraction of damaged cells among total epithelial cells, represented by the parameter “volume fraction” (Vv), which is shown in Figure 2.3C for all treatment groups. NA exposure at either 5 or 10 ppm caused significant increases in the fraction of damaged epithelial cells compared to the FA-treated control groups (p<0.0001), in both proximal and distal airways, with no obvious difference between NA exposure groups or airway regions. However, the fraction of damaged epithelial cells was substantially lower in the LCN mice than in the WT mice (by 36% and 39% in the proximal and distal airways, respectively, at 10 ppm, p<0.01, and by 22% (p=0.12) and 29% (p<0.05) in the proximal and distal airways, respectively, at 5 ppm). Notably,
there were no differences in the average total airway thickness among all groups (not shown).
Figure 2.3. High-resolution histologic analysis of the extent of airway epithelial damage induced by NA inhalation exposure in WT and LCN mice.

Two-month old wild-type (WT) and liver-Cpr-null (LCN) male mice were exposed to 5 or 10 ppm of naphthalene (NA), or to filtered air (FA), for 4 h. Mice were euthanized 20 h after termination of exposure, and lungs were processed for histopathologic analysis as described in Materials and Methods. (A, B) Typical signs of NA-induced epithelial Club cell damage, swelling, vacuolization, and exfoliation in the proximal bronchiole (A) and distal/terminal bronchiolar airways (B), as compared to the normal structures in FA-exposed control mice. Inset in A: enlarged views of swollen cells with darker nuclei staining, which signals cell death, and intracellular vacuoles. Bar = 1 mm. (C), Results of blinded quantitative analysis of the extent of cellular injury in the various groups. The volume fractions (Vv) of damaged epithelial cells are shown as means ± S.D. (n =5 for NA-exposed groups, n=4-7 for FA-exposed groups). Vv was calculated as described in Materials and Methods. All NA-treated samples show significant increase over corresponding FA samples (all p<0.0001; not labeled), *, **, p<0.05 and p<0.01, respectively, significant difference by genotype (LCN vs WT) (two-way ANOVA, followed by Bonferroni’s multiple comparisons test).

DISCUSSION

The impact of hepatic P450-mediated NA metabolism on airway toxicity of NA in an inhalation exposure scenario may include effects on tissue burdens of NA and potentially toxic NA metabolites in the lung and airways. During NA inhalation, the airway epithelial cells are exposed simultaneously to NA aerosols in the airway and NA and NA metabolites arriving from systemic circulation. NA in systemic circulation can be transported to the airway epithelial cells to undergo target tissue bioactivation and cause cytotoxicity, as demonstrated by the induction of
airway cytotoxicity following intraperitoneal NA administration and by vascular perfusion of the lung with NAO (Plopper et al., 1992b). Similarly, NA metabolites, such as NAO, can cause airway Club cell toxicity when administered by vascular perfusion of the isolated lung or in vitro (Kanekal S, 1991; Chichester et al., 1994). However, the sizes of the two pools of NA and of NA metabolites, and their relative contributions to the airway toxicity of inhaled NA, are currently unknown.

Our finding that suppression of hepatic P450 activity can lead to increased bioavailability of inhaled NA in the lung is not unexpected, although experimental confirmation was necessary and the determination of the extent of the increased bioavailability is important for interpreting results of subsequent toxicity study. However, despite the substantial increase in circulating and lung tissue NA levels (Fig. 2.1 and 2.2), the extent of NA-induced acute airway injury, judging from lung histopathology at 20 h following NA exposure, was lower in LCN than in WT mice (Fig. 3). This finding is consistent with the pharmacokinetics of NA-GSH, a biomarker of NA bioactivation. In that regard, the steady-state levels of lung NA-GSH at the termination of the 10-ppm NA exposure (0-hour) were only slightly higher in LCN than in WT mice, but the difference in the apparent half-life was much more noteworthy (Fig. 2.2C). That observation has two implications. One, regarding steady-state NA levels during active exposure, is that the lung tissue levels of NA and circulating levels of NA do not seem to directly translate to levels in airway epithelial cells, which may have a slow extraction of “blood-borne” NA, which may be bound to serum proteins, relative to the rapid absorption of airborne NA. Two, regarding NA bioactivation, is that the greater blood and lung tissue levels of NA do appear to translate to a more sustained exposure of the airway cells to systemically derived NA, and to greater
participation of airway cells in NA bioactivation in the lung, following the termination of active exposure.

Notably, NA-GSH is one of several proximal metabolites of the reactive NAO. The others include 1-naphthol, NA-protein adducts, and NA conjugates with other non-protein thiols. The relative abundance of NA-GSH to these other NAO metabolites may be influenced by the availability of GSH, leading to discordance in the levels of NA-GSH detected and the amounts of NAO generated. However, plasma levels of NA and NA-GSH were increased in parallel in the LCN mice, relative to WT mice (Fig. 2.1), indicating that GSH levels were not a limiting factor in NA-GSH formation in this study. Furthermore, there was no significant difference in lung total non-protein thiol levels between WT and LCN mice at the termination of exposure to 5 ppm NA (data not shown). Thus, GSH limitation is unlikely the explanation for the data showing that, while there were substantial differences in the levels of NA in WT and LCN mice, there was virtually no strain-related difference in NA-GSH levels in the livers or lungs of these animals at the end of the NA active exposure period (Fig. 2.2).

Our present finding, that the LCN mouse did not show greater toxicity than WT mice following inhalation exposure to NA, may be explained in part by the low abundance of NA available from the circulation under the occupationally relevant exposure conditions of the present study, relative to NA exposure from the air. The highest levels of NA reached in circulation following inhalation exposure to NA at 10 ppm (<100 ng/ml in WT mice; <200 ng/ml in LCN mice; this study) may be too low to make a notable contribution to lung airway toxicity, compared to levels reached following intraperitoneal injection of NA at a toxic, 300 mg/kg, dose (Cmax at 3-4 μg/ml in WT mice and almost 20 μg/ml in LCN mice) (Li et al., 2011).
On the other hand, the small but significant decrease in LCN vs WT mice in the extent of airway cytotoxicity (Fig. 2.3) suggests a small but definitive contribution by hepatic P450 bioactivation to airway toxicity by inhaled NA. In that regard, previous studies have shown that NAO, the obligatory reactive intermediate of NA, formed intracellularly by isolated mouse hepatocytes, was able to diffuse from cells (Richieri et al., 1987), and cause vacuolization, necrosis and exfoliation of nonciliated bronchiolar epithelial cells in isolated, perfused mouse lung (Kanekal S et al., 1991). Liver-generated NAO is theoretically stable enough (e.g., t\(_{1/2}\) was 10 min in both whole blood and plasma under \textit{in vitro} conditions) (van Bladeren et al., 1984; Kanekal S et al., 1991; Tsuruda et al., 1995) to circulate to the lung and cause damage. It remains to be directly confirmed whether loss of hepatic NA bioactivation leads to significant decreases in circulating or lung tissue levels of these metabolites; but our present data seem to support this notion.

Two different concentrations of NA (5 and 10 ppm) were studied. A dose-response relationship was found in lung NA and NA-GSH levels, with greater tissue burdens found for the higher NA concentration (Fig. 2.2). Curiously, a dose response was not observed for the cytotoxicity indices (Fig. 2.3). It seems unlikely that the NA concentrations employed are already saturating, as there was a clear, dose-dependent increase in bioactivation \textit{in vivo} (NA-GSH formation). One possible explanation for this observation is that the higher concentration caused more rapid cell death during the active exposure, but the method that we used to determine cell injury does not reveal how rapidly the cells were killed. Alternatively, the airway cells that had died prior to the 20 h time point did not contribute to the V\textsubscript{v} (damaged) values, thus making it appear that the two concentrations resulted in a similar extent of tissue injury. A detailed time course study would be needed to detect a dose-related difference in rates of NA-
induced cell death. However, the comparison of cytotoxicity between WT and LCN mice at the 10-ppm concentration is unlikely influenced by a possible difference in rates of cell death, as, unlike the concentration comparisons, the two genotype groups had only slightly different levels of NA-GSH (a biomarker of \textit{in vivo} bioactivation) at the termination of exposure (Fig. 2.2C).

The pharmacokinetic data for plasma NA-GSH following inhalation exposure to NA (Fig. 2.1B, Table 2) differ from previous results with intraperitoneal NA injection, where similar AUC and Cmax values were found between WT and LCN mice; but the T\textsubscript{max} value was slightly increased, probably reflecting the need for NA to accumulate in the lungs before metabolism can occur at a maximal rate, in the LCN mice (Li et al., 2011). In the inhalation exposure model, we could not determine T\textsubscript{max}, or whether a shift in T\textsubscript{max} occurred, but we found that both Cmax and AUC values are greater in LCN than in WT mice (Table 2). This increase in NA-GSH level may reflect the pool of NA-GSH produced by lung and other extrahepatic organs, such as the nasal mucosa, from blood-borne NA (which is much increased in LCN mice) in addition to the pool of NA-GSH formed by lung and nasal mucosa from air-borne NA during first-pass NA metabolism in the airway. Thus, inhaled NA and injected NA are both still efficiently bioactivated \textit{in vivo} in the absence of significant contribution by hepatic microsomal P450s. Notably, the relative contributions of various extrahepatic tissues to circulating NA-GSH in the LCN mice remain to be determined. For the nasal olfactory mucosa, which contains abundant NA-bioactivating P450 enzymes (CYP2A5 and CYP2F2) (Hu et al., 2014), it will be interesting to determine whether the bulk of the produced NA-GSH is excreted to the circulation (thus contributing to plasma levels of NA-GSH) or to the nasal mucus. Additionally, while the data in Figure 2.1B does not reveal whether the rate of NA-GSH degradation was different between WT and LCN mice, the previous pharmacokinetic data for NA-GSH in LCN mice exposed to NA via the intraperitoneal
route (Li et al., 2011) argues against such a possibility. Thus, the apparent persistence of NA-GSH in the plasma of LCN mice after termination of NA inhalation exposure was probably mainly due to continued NA metabolism and NA-GSH formation during the post exposure period.

NA-GSH was present in the liver as well as in the lung, in both WT and LCN mice, although the latter mouse could not produce significant amounts of NA-GSH in the liver via P450-mediated pathways. It is unlikely that the hepatic NA-GSH was produced in the liver by a non-CYP pathway, as a previous study with Cyp2f2-null mice showed that in vivo formation of NA-GSH is largely P450-dependent (Li et al., 2011). Thus, the hepatic NA-GSH must have been produced in the lung and other extrahepatic tissues and then transported to the liver, in the LCN mice.

In conclusion, hepatic microsomal P450 enzymes are not essential for induction of airway toxicity by inhaled NA. Furthermore, although hepatic P450-mediated systemic clearance of inhaled NA had a significant impact on NA levels in the lung and plasma, the increase in lung NA levels because of a functional deficiency of hepatic P450 enzymes did not noticeably impact the extent of NA-induced airway toxicity, at least under the low-dose occupational exposure conditions employed and at the postexposure time point examined here. Conversely, our data further suggest that liver P450-generated NA metabolites have a relatively small, but significant, contribution to airway toxicity of inhaled NA. This hepatic contribution to the airway toxicity of inhaled NA may be an important risk factor for individuals with diminished bioactivation activity in the lung.
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FOOTNOTES

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Authorship Contributions

Participated in research design: Kovalchuk, Van Winkle, Ding

Conducted experiments: Kovalchuk, Kelty, Li, Hartog, Edwards

Contributed new reagents and analytical tools: Zhang, Ding

Performed data analysis: Kovalchuk, Kelty, Van Winkle, Ding

Wrote or contributed to the writing of the manuscript: Kovalchuk, Hartog, Van Winkle, Zhang, Ding
CHAPTER THREE

PHARMACOKINETIC CONSIDERATIONS OF ORGAN CONTRIBUTION TO NAPHTHALENE DISPOSITION AND BIOACTIVATION FOLLOWING INHALATION EXPOSURE
SUMMARY

The extent of xenobiotic toxicity in a portal of entry, extra-hepatic organ is influenced by interplays among environmental exposure, absorption, and systemic exposure, between disposition via the target organ and the liver, and between ultimate toxicants generated locally and those formed by the liver. Given likely variation of each of these factors for a given individual, it is important that we gain a better understanding of such interplays.

In this study, we have examined the relationship among these various factors in the context of NA disposition and bioactivation in mouse models that permit genetic modulation of systemic metabolism of NA by the CYP2ABFGS enzymes, of hepatic metabolism by all microsomal P450 enzymes, or of expression of two human enzymes (CYP2A13 and 2F1) active toward NA metabolism in the respiratory tract. Mice (CYP2A13/2F1-humanized, Cyp2abfgs-null, ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) were exposed to NA via inhalation at an occupationally relevant level for four hours, and the levels of NA and a biomarker of its reactive metabolite (NA-GSH) were measured in plasma, liver, and lung at different post-exposure time points.

The results indicated that the liver is a large source of NA accumulation in ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice during and after inhalation exposure. Large increases in and persistence of plasma NA, redistributed from the liver, and the absolute plasma NA AUC values were consistent with the loss of hepatic P450s activity. The persistence of NA-GSH adducts in mice on LCN background correlated with presence of high levels of NA in plasma and liver. Increases in NA-GSH AUC were observed in “CYP2A13/2F1-humanized and LCN” and ‘Cyp2abfgs-null and LCN’ mice, which reflected the contribution of extra-hepatic, non-CYP2ABFGS enzymes to NA disposition during and after
active inhalation exposure in animals with deficient hepatic P450s. There was also a small but significant difference in plasma NA levels between CYP2A13/2F1-humanized and Cyp2abfgs-null mice, both with compromised hepatic P450s activity, a result suggesting contribution of lung and/or nasal CYP2A13/2F1 to systemic NA disposition. *In vitro* metabolic assay data further supported the idea that respiratory tract expressed CYP2A13 and 2F1 are the major source of NA-GSH formation in mice on LCN background and that these human lung CYPs may have an elevated impact on the risks of NA toxicity in individuals with deficient hepatic CYP function.
INTRODUCTION

One of the limitations of extrapolation of results from animal studies to humans for risk assessment for NA is the presence of species differences in the metabolism of this toxicant. It is not only related to species difference in bioactivation of NA, but also to the unknown contribution of pulmonary and hepatic-generated NA metabolites to airway toxicity.

Metabolism of NA by pulmonary microsomal enzymes has been studied in different species, including humans (Buckpitt et al., 1986; Buckpitt et al., 1987). To better understand the regional specificity and types of airway epithelial cells involved in NA bioactivation, microdissected airway regions and isolated Club cells were used as a source of CYPs proteins (Chichester et al., 1994; Buckpitt et al., 1995). Even though there is an overlap in the CYP isoforms expressed in liver and the respiratory tract, some P450 enzymes are expressed in extra-hepatic tissues to greater extent than in liver (Ding et al., 2003). CYP2F2 is expressed predominantly in mouse airways and has the lowest $K_m$ value to bioactivate NA when studied in vitro using recombinant CYP2F2 protein (Schultz et al., 1999). Experiments in vivo demonstrated that WT mice, pre-treated with a CYP2F2-specific inhibitor (5-phenyl-1-pentyne) prior to NA administration, and Cyp2f2-null mice lost sensitivity to NA-induced pulmonary damage (Verschoyle et al., 1997; Genter et al., 2006; Li et al., 2011). CYP2A5, an enzyme mostly expressed in the nasal olfactory mucosa and the liver, is the main contributor to NA-induced nasal toxicity, as demonstrated by studies using Cyp2a5-null mice (Hu et al., 2014).

Human CYP2F1 and CYP2A13 are enzyme orthologs for mouse CYP2F2 and CYP2A5, respectively. These human enzymes are mainly expressed in the respiratory tract of humans, with negligible expression in other tissues (Carr et al., 2003; Su et al., 2000; Weems et al., 2010). The activity of human CYP2F1 and rat CYP2F4 to bioactivate NA is significantly lower that mouse
CYP2F2. This species difference is a challenge for the extrapolation of animal data to humans. Human recombinant CYP2A13, an enzyme best known for its ability to bioactivate NNK (a known lung carcinogen), efficiently bioactivates NA to naphthols in vitro (Su et al., 2000; Fukami et al. 2008). It was further confirmed that metabolism of inhaled NA by locally expressed CYP2F1 and CYP2A13 results in NA-mediated toxicity in the airway epithelial cells and nasal olfactory mucosa in CYP2A13/2F1-humanized mice (Li et al., 2017).

Microsomal CYPs in liver predominantly contribute to bioactivation and metabolism of many chemicals, both therapeutic drug and environmental toxicants (Gonzalez et al., 1994; Spatzenegger et al., 1995; Nebert et al., 2002; Guengerich et al., 2006; Guengerich PF, 2008). Several hepatic P450 isoforms with ability to bioactivate NA were identified in naïve human liver microsomes (Tingle et al., 1993; Cho et al., 2006). Among them, the most efficient to metabolize NA to 1-naphthol and 2-naphthol (two among three primary NA metabolites) are CYP1A2 and CYP3A4, respectively. CYP2A6 and CYP3A4 have the greatest activity to metabolize dihydrodiol, whereas CYP1A2 and 2D6 are the most effective to generate 1,4-naphthoquinone (Cho et al., 2006). However, activity of hepatic CYP1A and CYP3A can be modulated by various liver diseases (Villeneuve et al., 2004). In addition, it was demonstrated that co-exposure to NA and some pesticides can significantly alter the microsomal CYPs activity in human hepatocytes (Cho et al., 2007). Microsomal activity to metabolize NA was investigated in mouse liver (Li et al., 2011), but specific CYP isoforms with the highest metabolic rate for NA were not identified. It was also demonstrated that, while overall hepatic CYPs activity to bioactivate NA is not essential for NA’s lung toxicity after a single bolus exposure, hepatic CYPs have a significant, though small, contribution to toxicity in airways following NA inhalation exposure at occupationally relevant concentration (10 ppm), as revealed by studies
using liver-Cpr-null mice (Li et al., 2011; Kovalchuk et al., 2017). These data further confirm the dual contributions of hepatic-generated metabolites and pulmonary-generated metabolites for NA-induced toxicity in the respiratory tract of mice, but species difference in activity of locally expressed human CYP2F1 in the airways and CYP2A13 in nasal olfactory mucosa causes difficulties in extrapolation of the mouse data for humans.

In the current study we aimed to dissect the contribution of liver-generated metabolites and metabolites generated by human pulmonary-expressed CYP2A13 and CYP2F1 to bioactivation and disposition of inhaled NA. We exposed CYP2A13/2F1-humanized and Cyp2abfgs-null mice with either normal or compromised hepatic P450s activity to occupationally relevant NA concentration (10 ppm) and examined the disposition of NA and NA-GSH (a marker of NA bioactivation by CYP enzymes) in plasma, liver and lung at various post-exposure times. Our results demonstrated that NA metabolites, generated by CYP2A13 and CYP2F1, and extra-hepatic non-CYP2ABFGS, contribute to systemic NA disposition both during and after termination of inhalation exposure in mice with deficient hepatic P450s activity.

**MATERIAL AND METHODS**

**Chemicals and reagents.** NA (CAS# 91-20-3, purity 99%), NA-d₈ (CAS# 1146-65-2, purity 99%), GSH (CAS# 70-18-8, purity ≥ 98.0%), β-nicotinamide adenine dinucleotide phosphate, reduced tetra(cyclohexyl ammonium) salt (NADPH) (CAS#, 100929-71-3, purity ≥ 95.0%), and were purchased from Sigma Aldrich (St. Louis, MO). Acetaminophen-glutathione (AP-GSH) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). NA-GSH standard as a mixture of all four stereoisomers was a generous gift from Drs. Alan R. Buckpitt and Dexter Morin (University of California at Davis, Davis, CA) and was prepared as previously
described (Richieri et al., 1987). All solvents (dichloromethane, formic acid, methanol and water) were of analytical grade (Fisher Scientific, Houston, TX).

Generation and characterization of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice.

Animal breeding and genotype analysis. Cyp2abfgs<sup>−/−</sup> (Cyp2abfgs-null), CYP2A13/2F1-TG<sup>+/−</sup>/Cyp2abfgs<sup>−/−</sup> (CYP2A13/2F1-humanized), and liver-Cpr-null (LCN) mice, all on C57BL/6 background, were obtained from breeding stocks maintained at Wadsworth Center. Progeny of Cyp2abfgs-null and liver-Cpr-null (LCN) mice were further intercrossed with CYP2A13/2F1-humanized mice to generate ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice. Detailed breeding lineage is presented in Fig.3.1A. All pups from breeding were genotyped using tail DNA for Cre transgene, the loxP sites in the Cpr gene, Cyp2f2<sup>+</sup>, Cyp2abfgs<sup>−</sup>, and/or the CYP2F1<sup>+</sup> alleles. Representative results of genotyped alleles are shown in Fig.3.1B.

Western immunoblot analysis. Microsomes from liver of liver-Cpr-null (LCN), Cyp2abfgs-null, ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice were prepared as described previously (Ding et al., 1990). Microsomal proteins (10 µg) were separated in freshly prepared 10% polyacrylamide-SDS (sodium dodecyl sulfate) gel and transferred into a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA). After blocking the membrane with 5% dry non-fat milk in TBST (150 mM NaCl, 0.1% Tween-20, 50 mM Tris-Cl, pH 7.4) for 10 minutes at room temperature, the membrane was probed overnight at 4°C with following antibodies: rabbit anti-rat cytochrome P450 reductase (Enzo Life Science, Plymouth, PA) and rabbit anti-human calnexin (GenScript, Piscataway, NJ) as a marker protein for the endoplasmic reticulum. Following triplicate washing of the membrane in TBST, membrane was incubated with anti-rabbit horseradish peroxide-conjugated secondary antibody.
for 60 minutes at room temperature. After triplicate washing, the protein bands were visualized using the Molecular Imager® Gel Doc™ XR+ System with Bio-Rad® Image Lab™ software (Bio-Rad Laboratories, Inc., Hercules, CA).

**Assay for NA bioactivation in vitro.** The rate of NA-GSH formation was measured to evaluate the microsomal metabolic activation of NA following the previously described protocol (Shultz et al., 1999). In brief, microsomes were prepared from individual livers, pooled lung or olfactory mucosa tissues of male 2-3-month old ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice. Reaction mixtures contained 50 mM phosphate buffer (pH 7.4), different NA concentrations (2, 10 or 100 µM, added in 1 µL methanol), 10 mM GSH, 1 mM NADPH, different concentrations of microsomal proteins (0.2 mg/mL for liver, 0.25 mg/mL for olfactory mucosa and 1 mg/mL for lung) in a final volume 0.1 mL. The reaction was carried out in a capped glass tubes at 37°C for 10 minutes for liver, 30 minutes for olfactory mucosa and 60 minutes for lung microsomal proteins and quenched by adding 0.2 mL of ice-cold methanol containing 5 pg of internal standard AP-GSH. The resultant mixtures were centrifuge twice to remove precipitated proteins and aliquots of supernatants were injected into UPLC-MS/MS for analysis as previously described (Kovalchuk et al., 2017). Reaction mixtures with omitted NADPH, GSH, NA or boiled microsomes were analyzed as negative controls.

**Animal experiments.** Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. Two- to three-month old CYP2A13/2F1-humanized, Cyp2abfgs-null, ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ male mice were used for the study.

NA inhalation exposure settings were the same as described elsewhere in details (Kovalchuk et al., 2017). Mice were exposed to a single 4-hour session of 10 ppm NA or HEPA-

For plasma toxicokinetic studies, blood samples were collected via tail vein at various time points (0, 1, 4, 8 and 20 hours) after termination of NA exposure. For tissue kinetics studies mice of all four genotypes were exposed simultaneously for each time point. Lung and liver of NA- or filtered air-exposed mice were collected (0, 2, 6 and 20 hours of post-exposure), quick-frozen and stored in sealed tubes at -80°C for further analysis. Concentrations of NA and NA-GSH in plasma and homogenates from lung and liver were detected as described (Kovalchuk et al., 2017).

**Other methods.** The bicinchoninic acid assay was used to quantify of total protein concentration (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard. Pharmacokinetic parameters (area under the curve (AUC), elimination half-live (t1/2), clearance) were calculated using the WinNonlin software (Pharsight, Mountain View, CA). Statistical significance of differences between studies groups in various parameters was examined using GraphPad Prism (GraphPad, San Diego, CA).

**RESULTS**

**Generation and characterization of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice.**

‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice were progeny of successive intercrosses between Cyp2abfgs<sup>-/-</sup> (Cyp2abfgs-null), liver-<i>Cpr</i>-null (LCN) and further CYP2A13/2F1-TG<sup>+/+</sup>/Cyp2abfgs<sup>-/-</sup> (CYP2A13/2F1-humanized) mice (Fig 3.1).
Figure 3.1. Generation of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice.
(A) Breeding lineage to generate CYP2A13/2F1+/−-humanized mice with either normal or compromised hepatic CYP-mediated metabolism. The original CYP2A13/2F1+/−-humanized (CYP2A13/2F1+/−/Cyp2abfgs−/− with normal hepatic CYPs activity) mice were intercrossed with Alb-Cre+/−/Cprlox/lox/Cyp2abfgs−/− (compromised CYP-mediated metabolism in hepatocytes). (B) Representative results of a conventional PCR to identify presence of amplicons of Cre, Cpr, Cyp2f2, Cyp2abfgs-null and CYP2F1 alleles in founder strains and their progenies.

In ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice, the expression of Cre recombinase is driven by rat albumin promoter resulting in hepatocyte-specific deletion of Cpr gene between exons 3-15 (Wu et al., 2003) and significantly lower expression of respective protein in liver (Fig.3.2).

Figure 3.2. Expression of hepatic CPR protein in founder strains and their progenies.

Livers from naïve LCN (1), Cyp2abfgs-null (2), ‘Cyp2abfgs-null and LCN’ (3) and ‘CYP2A13/2F1-humanized and LCN’ (4) male mice were used for microsomal preparation and immunoblot analysis as described in Material and Methods. Microsomal proteins were analyzed using anti-CPR polyclonal antibody and calnexin as a loading control.
A genomic fragment with 12 mouse Cyp genes (all Cyp2a, Cyp2b, Cyp2f, Cyp2g and Cyp2s subfamilies) on chromosome 7 was deleted in ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice. This deletion resulted in the absence of corresponding encoded mouse CYP2 proteins (data not shown). Among other P450s, CYP2A13 and CYP2F1 proteins are expressed in the respiratory tract of humans (Raunio et al., 1999; Hukkanen et al., 2002; Bernauer et al., 2006). The ‘CYP2A13/2F1-humanized and LCN’ mice have predominant localization of CYP2A13 in olfactory mucosa and CYP2F1 in the airways (Li et al., 2017).

‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice are normal in growth, development, general appearance, daily activity and reproduction. Body and organ weights (lung, kidney, brain, heart) of 2-month-old adult ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’, besides liver weight, are similar to WT mice (data not shown). Enlargement of liver is observed in adult ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ male mice (1.98±0.33 g and 2.12±0.18 g, respectively) and is consistent with a previously published report on liver weight for LCN mice of similar age and gender (2.04±0.49 g) (Gu et al., 2003).

**Microsomal activity for NA bioactivation in different tissues in vitro.** The rates of NA-GSH formation in incubation mixtures with microsomal proteins from individual livers, and pooled lung or olfactory mucosa, from ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice were compared at different NA concentrations (2, 10 or 100 µM) (Fig.3.3). Rates of NA-GSH formation in different organs for both genotypes have the following rank order: olfactory mucosa > liver > lung, at either 10 or 100 µM NA concentration. Lung and liver microsomal activities were similar at the lowest tested NA concentration (2 µM), and were
significantly lower than in olfactory mucosa (p<0.0001) in both ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)
Figure 3.3. Metabolic activation of naphthalene by liver, lung and olfactory mucosa microsomes of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice in vitro.

Rates of NA-GSH formation were determined as described in Material and Methods. Reaction mixtures contained 50 mM phosphate buffer (pH 7.4), 10 mM GSH, 1 mM NADPH, different concentrations of microsomal proteins (0.2 mg/mL for individual liver, 0.25 mg/mL for pooled olfactory mucosa and 1 mg/mL for pooled lung) and different NA concentrations 2 µM (A), 10 µM (B) or 100 (C) µM. The values represent mean±S.D. (n=3-4). **** p<0.0001 statistically significant by genotype; ^, ^, ^^^^p<0.05, p<0.01, p<0.0001 statistically significant by tissue compared to nasal mucosa for each genotype respectively; & p<0.05 statistically significant between lung and liver for corresponding genotype (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

Genotype-dependent difference in ability to bioactivate NA in vitro was observed in microsomes from olfactory mucosa and lungs, but not liver, where the human transgenes were not expressed. Lung microsomes of ‘CYP2A12/2F1-humanized and LCN’ mice had higher ability to bioactivate NA than ‘Cyp2abfgs-null and LCN’ mice by 40.2% and 47.7% at 10 and 100 µM concentrations, respectively. This genotype-dependent difference was more prominent in microsomal activity from olfactory mucosa at all tested NA concentrations (from 3.9- to 6.1-fold higher in “CYP2A13/2F1-humanized and LCN” than “Cyp2bfgs-null and LCN” mice). Taken together, these results confirm a significant region-specific difference within the respiratory tract to bioactive NA in “CYP2A13/2F1-humanized and LCN” mice, as was reported for the founder CYP2A13/2F1-humanized strain in our previous study (Li et al., 2017).
Impact of hepatic Cpr deletion on pharmacokinetics of NA and NA-GSH in plasma following NA inhalation exposure in CYP2A13/2F1-humanized and Cyp2abfgs-null mice.

Levels of NA and NA-GSH were measured in plasma of CYP2A13/2F1-humanized, Cyp2abfgs-null, ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice at multiple time points, starting immediately after termination of a single 4-hour exposure to 10 ppm NA. Levels of NA in plasma of mice with compromised hepatic CYPs activity (LCN background, namely ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) were significantly higher (p<0.001) compared to corresponding controls with normal hepatic CYPs activity (namely CYP2A13/2F1-humanized and Cyp2abfgs-null) during first eight hours after termination of NA exposure (Fig.3.4). NA level in plasma continued to increase in ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice after termination of active NA inhalation exposure. The trend in NA elevation was similar between the two mouse strains on LCN background; however, observed NA elevation was statistically significant during first four hours after NA exposure termination, compared to 0-hour time point, only for ‘Cyp2abfgs-null and LCN’ mice (p<0.05). There was a small, but statistically significant, difference (p<0.05) in NA plasma level between ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice at 4 and 8 hours after termination of NA exposure (Fig. 3.4.C). NA plasma level in mice on LCN background fluctuated during first eight hours after NA exposure termination and never dropped below the value at the 0-hour time point.

Levels of NA in plasma of CYP2A13/2F1-humanized and Cyp2abfgs-null mice were significantly lower (p<0.001) in all examined post-exposure time points compared to corresponding mice on LCN background. Unlike the relatively steady plasma NA levels during first eight hours after NA exposure termination in mice on LCN background, levels of this
toxicant in plasma of mice with normal hepatic CYPs activity (CYP2A13/2F1-humanized and Cyp2abfgs-null) declined rapidly during the first 4 hours after termination of NA exposure (Fig. 3.4).
Figure 3.4. Levels of NA and NA-GSH in plasma after a single 4-hour nose-only exposure to 10 ppm NA.

Two-month old CYP2A13/2F1-humanized, ‘CYP2A13/2F1-humanized and LCN’ (A), Cyp2abfgs-null and ‘Cyp2abfgs-null and LCN’ (B) male mice were simultaneously exposed to 10 ppm of naphthalene (NA) for 4 hours and plasma levels of NA and naphthalene-glutathione conjugate (NA-GSH) were determined at different time points after termination of NA exposure. Levels of NA and NA-GSH in plasma of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ male mice are depicted in a separate panel C. Data represent means ± SD (n=3-4). *, ***, ****, p<0.05, 0.001, or 0.0001, respectively, (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

The levels of NA-GSH in plasma immediately after termination of NA inhalation exposure was similar among all four different genotypes with either normal or compromised hepatic CYPs activity (Fig. 3.4). Level of NA-GSH declined rapidly in plasma of CYP2A13/2F1-humanized and Cyp2abfgs-null mice and was not detected at 8 and 20 hours after NA exposure termination. In contrast, level of NA-GSH was steady during first eight hours of post-exposure in ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice, which coincided with steady NA level in their plasma during the same post-exposure time period (Fig. 3.4.). Moreover, ‘CYP2A13/2F1-humanized and LCN’ mice have a trend of lower NA-GSH, consistent with plasma NA levels, than ‘Cyp2abfgs-null and LCN’ mice.

Toxicokinetic analysis of the data from Figure 3.4 (Table 3.1) revealed ~2.8 fold higher Cmax values for NA in mice with compromised hepatic CYPs activity (‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) than in mice with normal hepatic CYPs
activity (CYP2A13/2F1-humanized and Cyp2abfgs-null, respectively). The AUC values for NA were ~36-fold higher in ‘CYP2A13/2F1-humanized and LCN’ than CYP2A13/2F1-humanized mice and ~66-fold higher in ‘Cyp2abfgs-null and LCN’ than Cyp2abfgs-null mice. There was ~1.7-fold increase (p<0.05) in AUC for NA values in ‘Cyp2abfgs-null and LCN’ than ‘CYP2A13/2F1-humanized and LCN’ mice. The rate of NA clearance (Cl/F) was significantly decreased in ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ (~ 64-fold and ~65.7-fold respectively) compared with CYP2A13/2F1-humanized and Cyp2abfgs-null mice accordingly. In addition, plasma elimination half-life (t\(_{1/2}\)) was substantially increased in ‘CYP2A13/2F1-humanized and LCN’ (~18-fold) and ‘Cyp2abfgs-null and LCN’ (~12-fold) than in Cyp2A13/2F1-humanized and Cyp2abfgs-null mice respectively. The AUC for NA-GSH values were increased by ~7.8-fold in ‘CYP2A13/2F1-humanized and LCN’ compared with CYP2A13/2F1-humanized and ~5.7-fold in ‘Cyp2abfgs-null and LCN’ than Cyp2abfgs-null mice. These results emphasize that 1) hepatic CYP activity is not essential for bioactivation of inhaled NA; 2) there is an important contribution by extra-hepatic non-Cyp2abfgs enzymes to disposition of inhaled NA during an inhalation exposure and in the post-exposure period in mice with deficient hepatic CYPs activity.

Table 3.1. Toxicokinetic parameters of NA and NA-GSH in plasma of Cyp2abfgs-null, CYP2A13/2F1-humanized, ‘Cyp2abfgs-null and LCN’ and ‘CYP2A13/2F1-humanized and LCN’ mice following a single 4-hour nose-only inhalation exposure to 10 ppm of NA.

<table>
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<tr>
<th>Analyte, strain</th>
<th>AUC(_{0-20h}), µg/ml/min</th>
<th>t(_{1/2}), min</th>
<th>CL/F, ml/min</th>
<th>Cmax, ng/ml</th>
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<tr>
<td>NA</td>
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<tr>
<td></td>
<td>Cyp2abfgs-null</td>
<td>CYP2A13/2F1-humanized</td>
<td>‘Cyp2abfgs-null and LCN’</td>
<td>‘CYP2A13/2F1-humanized and LCN’</td>
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<tr>
<td></td>
<td>4.2±0.9</td>
<td>4.6±3.9</td>
<td>277.4±43.6****</td>
<td>166.8±16.4&amp;&amp;.****</td>
</tr>
<tr>
<td></td>
<td>3.5±1.4</td>
<td>3.2±1.1</td>
<td>42.72±11.4</td>
<td>57.0±38.6</td>
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<td>72.3±17.7</td>
<td>121.7±77.5</td>
<td>1.1±0.3*</td>
<td>1.9±0.2****</td>
</tr>
<tr>
<td></td>
<td>80.3±26.5</td>
<td>67.6±20.2</td>
<td>226.2±43.2****</td>
<td>191.5±48.7****</td>
</tr>
</tbody>
</table>

NA-GSH

<table>
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<tr>
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<th>CYP2A13/2F1-humanized</th>
<th>‘Cyp2abfgs-null and LCN’</th>
<th>‘CYP2A13/2F1-humanized and LCN’</th>
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<td>5.2±1.1</td>
<td>3.2±2.6</td>
<td>29.6±4.2*</td>
<td>25.5±8.1*</td>
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<tr>
<td></td>
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<td>25.7±14.3</td>
</tr>
<tr>
<td></td>
<td>48.0±20.1</td>
<td>51.6±18.4</td>
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</tbody>
</table>

Results presented as mean±SD (n=3-4 per group).

N.A. – not applicable.

Values were derived from data presented in Fig3.2.

statistically significant between:

‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ (&&& p<0.001),

CYP2A13/2F1-humanized and ‘CYP2A13/2F1-humanized and LCN’, or

Cyp2abfgs-null and ‘Cyp2abfgs-null and LCN’ (****p<0.0001, *** p<0.001, *p<0.05).

Impact of hepatic Cpr deletion on NA and NA-GSH levels in lung following NA inhalation exposure in CYP2A13/2F1-humanized and Cyp2abfgs-null mice. Levels of NA
and NA-GSH were determined in lungs of mice with normal (CYP2A13/2F1-humanized and Cyp2abfgs-null) and compromised (‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) hepatic CYPs activity at different time point after 10 ppm NA inhalation exposure. Level of NA immediately after termination of NA inhalation exposure was 3.9-fold higher in ‘CYP2A13/2F1-humanized and LCN’ than in CYP2A13/2F1-humanized and 3.5-fold higher in ‘Cyp2abfgs-null and LCN’ than in Cyp2abfgs-null mice (Fig. 3.5A-B). NA level in lungs was higher than in plasma at 0-hour post-exposure time by 3.8-fold in CYP2A13/2F1-humanized, by 4.8-fold in Cyp2abfgs-null, by 5.3-fold in ‘CYP2A13/2F1-humanized and LCN’ and by 5.8-fold in ‘Cyp2abfgs-null and LCN’ mice. In addition, there was a trend of higher NA level in lungs of Cyp2abfgs-null than CYP2A13/2F1-humanized mice regardless of their hepatic CYPs activity (33% and 23% higher in mice with normal and compromised hepatic NA bioactivation, respectively). Level of NA in lungs of CYP2A13/2F1-humanized and Cyp2abfgs-null mice decreased rapidly and was not detectable at the 6-hour post-exposure time point. In contrast, NA level in lungs of mice with compromised hepatic CYPs activity (‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) remained relatively steady during first two hours after NA exposure termination and eventually decreased to non-detectable levels at the 20-hour post-exposure time (Fig.3.5C). These results suggest that lungs of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ were exposed to greater amount of NA for much extended time that lungs of the respective controls (CYP2A13/2F1-humanized and Cyp2abfgs-null) following termination of NA inhalation exposure.

NA-GSH levels in lungs were nearly identical in all groups of NA-exposed mice immediately after exposure termination despite significant difference in NA levels in plasma and tissues between mice with normal (CYP2A13/2F1-humanized and Cyp2abfgs-null) and
compromised (‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) hepatic CYPs activity. Levels of NA-GSH in lungs decreased significantly (p<0.01) at the 2-hour post-exposure time in mice with normal hepatic CYPs activity, but were steady in mice with compromised hepatic CYPs activity (Fig. 3.5).

Levels of NA-GSH adducts were not detected at the 6-hour post-exposure time in CYP2A13/2F1-humanized and Cyp2abfgs-null, in contrast to the ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice, in which NA-GSH levels became non-detectable only at the 20-hour post-exposure time point. Thus, compromised hepatic CYP-mediated metabolism of inhaled NA resulted in persistence of both NA and NA-GSH levels in lungs, a trend that is similar to NA and NA-GSH levels in plasma.
Figure 3.5. Levels of NA and NA-GSH in lung after a single 4-hour nose-only exposure to 10 ppm NA.
Two-month old CYP2A13-humanized, ‘CYP2A13/2F1-humanized and LCN’ (A), Cyp2abfgs-null and ‘Cyp2abfgs-null and LCN’ (B) male mice were exposed to 10 ppm of naphthalene (NA) for 4 h. Lungs were collected 0, 2, 6 and 20 hours after termination of NA exposure. NA (first column) and naphthalene-glutathione conjugate (NA-GSH) (second column) were detected in tissue homogenates. Levels of NA and NA-GSH in lung homogenates of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ male mice are depicted in a separate panel C. Data represent means ± SD (n=3-5). ***, p<0.001; **, p<0.01; between different genotypes; ^^^^, p<0.0001; ^, p<0.01; ^p<0.05; for corresponding time point for each genotype (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

**Impact of hepatic Cpr deletion on NA and NA-GSH levels in liver following NA inhalation exposure in CYP2A13/2F1-humanized and Cyp2abfgs-null mice.** Deletion of Cpr gene and the respective protein (redox partner for CYP enzymes) in hepatocytes had a significant impact on NA levels in the liver of NA-exposed mice. The NA level in this tissue was 20-fold higher in ‘CYP2A13/2F1-humanized and LCN’ than CYP2A13/2F1-humanized mice and 26.5-fold higher in ‘Cyp2abfgs-null and LCN’ than Cyp2abfgs-null mice immediately after termination of 4-hour inhalation exposure (10 ppm) (Fig 3.6A-B). Moreover, NA level was 33% higher in liver of ‘Cyp2abfgs-null and LCN’ compared with ‘CYP2A13/2F1-humanized and LCN’ mice (Fig. 3.6.C) at 0-h post-exposure time. NA level was higher in lungs than in liver by 40% in Cyp2abfgs-null mice and by 20% in CYP2A13/2F1-humanized mice immediately after the inhalation exposure. In contrast to the results observed in mice with normal hepatic CYPs activity, NA predominantly accumulated in liver of mice with compromised hepatic CYPs activity during post-exposure time. NA level was 4.5-fold higher in liver than in lung in
‘Cyp2abfgs-null and LCN’ and 3.4-fold higher in ‘CYP2A13/2F1-humanized and LCN’ mice at 0-hour post-exposure time. NA levels in liver of mice with normal hepatic CYPs activity (CYP2A13/2F1-humanized and Cyp2abfg-null) declines rapidly and was not detected 6 hours after NA exposure termination. In contrast, relatively steady level of this toxicant was found in mice with compromised hepatic CYPs activity (‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’) during 6 hours of post-exposure time with a trend of higher NA level in ‘Cyp2abfgs-null and LCN’ mice. These results revealed that deletion of hepatic Cpr gene significantly impact the disposition of inhaled NA and liver serves as a NA reservoir even after termination of NA inhalation exposure.

Level of hepatic NA adducts (NA-GSH) was ~59% higher in Cyp2abfgs-null than in ‘Cyp2abfgs-null and LCN’ and 61% higher in CYP2A13/2F1-humanized than in ‘CYP2A13/2F1-humanized and LCN’ mice at 0-hour time point (Fig. 3.6A-B). Deletion of Cpr gene in hepatocytes leads to persistence of NA and NA-GSH levels in plasma and tissues.
Figure 3.6. Levels of NA and NA-GSH in liver after a single 4-hour nose-only exposure to 10 ppm NA.
Two-month old CYP2A13-humanized, ‘CYP2A13/2F1-humanized and LCN’ (A), Cyp2abfgs-null and ‘Cyp2abfgs-null and LCN’ (B) male mice were exposed to 10 ppm of naphthalene (NA) for 4 h. Livers were collected 0, 2, 6 and 20 hours after termination of NA exposure. NA (first column) and naphthalene-glutathione conjugate (NA-GSH) (second column) were detected in tissue homogenates. Levels of NA and NA-GSH in liver homogenates of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ male mice are depicted in a separate panel C. Data represent means ± SD (n=3-5). ****, p<0.0001; between different genotypes; ^^^^^, p<0.0001; ^^, p<0.01; ^p<0.05; for corresponding time point for each genotype (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

**DISCUSSION**

This study examined the contribution of hepatic-generated NA metabolites and NA metabolites generated by respiratory tract-expressed human CYP2A13 and CYP2F1 to the overall bioactivation and disposition of inhaled NA.

Disposition of inhaled NA has a unique pattern in CYP2A13/2F1-humanized and Cyp2abfgs-null mice with compromised hepatic P450s activity, compared to these mice with normal hepatic P450s activity. Concentration of NA declined rapidly in plasma, lung and liver after inhalation exposure termination in CYP2A13/2F1-humanized and Cyp2abfgs-null mice with normal hepatic P450s activity. Whereas the concentration of NA in plasma of ‘CYP2A13/2F1-humanized and LCN’ and ‘Cyp2abfgs-null and LCN’ mice was significantly higher and further increased after termination of NA exposure, indicating the redistribution of NA from liver to plasma and other organs. In line with this scenario, mice with compromised hepatic CYPs activity also have higher tissue levels of NA and showed slower post-exposure elimination from lung and liver than mice with normal hepatic P450s activity.
Levels of NA adducts (GSH-trapped stereoisomers of NA-oxide) in all examined matrices were independent of hepatic CYPs activity when the levels are measured immediately after termination of NA inhalation exposure. Time-course of NA-GSH during post-exposure period was consistent with that of NA levels in plasma and tissues: adduct levels declined quickly in mice with normal hepatic P450s activity, but persisted in CYP2A13/2F1-humanized and Cyp2abfgs-null on LCN background. The results from the Cyp2abfgs-null mice on LCN background further indicated that, besides human CYP2A and CYP2F, other extra-hepatic non-Cyp2abfgs isoforms can efficiently bioactive NA during and after inhalation exposure.

Our results further suggest that microsomal CYPs activity in nasal olfactory mucosa is an important site of NA bioactivation in an inhalation exposure scenario when hepatic P450s activity is compromised. Metabolites of inhaled NA, presumably generated by nasal CYPs (mainly human CYP2A13), can enter systemic circulation from the local site of their formation and attribute to elevated NA-GSH level in plasma, but maybe not lung or liver, in “CYP2A13/2F1-humanized and LCN” mice. Thus, small, but significant, increase in NA adducts was observed in the plasma of “CYP2A13/2F1-humanized and LCN” mice, compared to Cyp2abfgs-null on LCN background, which demonstrates the contribution of respiratory, CYP2A13- and CYP2F1-generated, metabolites of inhaled NA to systemic NA-GSH levels.

Residual hepatic P450s activity might be the minor contributors to the total pool of NA metabolites, judging from in vitro results in this study, even at high NA internal doses in vivo. This residual ability to bioactive NA in mice on LCN background might come from hepatocytes with intact CPR or mitochondrial CYPs. Several researchers reported the bioactivation of dioxin and B(a)P (another member of the PAH family) by mitochondrial CYPs (Genter et al., 2006;
Bansal et al., 2014). The metabolic activity of hepatic and pulmonary mitochondrial CYPs to bioactivate NA is worth to examine in future studies.

In summary, the results of this study provide evidence that bioactivation and kinetics of inhaled NA is modified in CYP2A13/2F1-humanized mice if hepatic P450s activity is compromised. Humans with high activity of pulmonary-expressed CYPs and compromised P450s metabolism in liver will be exposed to high and more persistent levels of NA even after termination of inhalation exposure, and might be at higher risk of NA-induced damage of airway epithelial cells. The toxicity of inhaled NA to epithelial cells in nasal mucosa and airways will be examined in the future studies. Polymorphism of human CYP2A13 and 2F1 should also be studied to investigate how genetic variations of these genes can affect NA-induced cytotoxicity in the respiratory tract. As the majority of inhalation exposures in the real world occurs in mixture, it would be worthwhile to pursue and investigate the regulation, expression and activity of CYP2A13 and CYP2F1 by common air pollutants and drugs.

ACKNOWLEDGEMENT

We thank Drs. Alan Buckpitt and Dexter Morin from University of California at Davis for providing NA-GSH standards.

FOOTNOTES

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Studies described in this chapter are included in a manuscript under preparation. The authors are Nataliia Kovalchuk, Qing-Yu Zhang from Department of Environmental Health Sciences, School of Public Health, State University of New York at Albany, Wadsworth Center, New York State Department of Health, Xinxin Ding from College of Nanoscale Science, SUNY Polytechnic Institute, Albany, NY, and Laura Van Winkle from UC Davis, Davis, CA.

Authorship Contributions

Participated in research design: Kovalchuk, Zhang, Van Winkle, Ding

Conducted experiments: Kovalchuk

Contributed new reagents and analytical tools: None

Performed data analysis: Kovalchuk, Ding

Wrote or contributed to the writing of the manuscript: Kovalchuk, Zhang, Van Winkle, Ding
CHAPTER FOUR

IMPACT OF PULMONARY CYPs ON NA-INDUCED ACUTE PULMONARY DAMAGE
SUMMARY

Naphthalene (NA) is an omnipresent air pollutant and respiratory tract toxicant. NA induces cytotoxicity in airways following bioactivation by CYP (P450) enzymes. Previous studies suggested that liver and lung are both capable of bioactivating NA in vitro and in vivo. However, direct evidence for a specific role of pulmonary P450 enzymes in mediating NA-induced airway toxicity, particularly upon exposure to NA via the systemic route, is still lacking.

The aim of this study was to examine the specific contribution of pulmonary CYPs to airway toxicity of NA using a lung-Cpr-null mouse. We predicted that lung epithelial cells that lack Cpr expression, which would have little microsomal P450 activity, would be resistant to NA-induced cytotoxicity. Cre-mediated deletion of Cpr in the lung-Cpr-null mouse occurred in a substantial proportion of the Club cells, which, before undergoing Cpr deletion, had high ability in the bioactivation of NA. Under the conditions used, doxycycline-induced deletion of Cpr occurred in 66% of Club cells in proximal airways and 86% of Club cells in distal airways, based on the results of a dual immunofluorescence study. Levels of NA and NA-GSH (a biomarker of NA bioactivation) were similar in the plasma of lung-Cpr-null mice and their control littermates after administration of a single bolus dose of NA (200 mg/kg). Histological examination of airways revealed that NA-induced damage of epithelial cells is focal and mild in lung-Cpr-null mice compared to severe exfoliation of epithelial cells in most airways in NA-treated control mice to those of control littermates. Moreover, the number of proliferating (BrdU-positive) airway epithelial cells was 4-fold lower in NA-treated lung-Cpr-null mice than in NA-treated control littermates. These results confirm that CYPs in the lung can mediate NA-induced airway epithelial damage in vivo.
INTRODUCTION

The lung is a primary target organ for toxicity of many chemicals either inhaled or derived from systemic circulation. The lung also has the ability to bioactivate pro-carcinogens (VOCs, N-nitrosamines) because of *in situ* expression of necessary CYP enzymes (Hukkanen et al., 2001; Ding et al., 2003; Bernauer et al., 2006). Whereas the airway epithelial cells include almost 40 different cell types that varies along the proximal-distal axis, the xenobiotic-metabolizing enzymes predominantly localized in non-ciliated (Club, former Clara) cells and type II alveolar cells (Chichester et al., 1991; Fanucchi et al., 1997). Studies with styrene, a chemical with similar metabolic and toxicity profiles to naphthalene, demonstrated an ability of Club cells alone to bioactivate styrene and its downstream metabolites, which cause cellular injury (Carlson GP, 2000; Harvilchuck et al., 2006; Harvilchuck et al., 2009). Isolated Club cells and lung explants from microdissected airways are fully capable to bioactivate NA and cause acute Club cell injury in mice and monkey without involvement of hepatic CYPs-generated NA metabolites (Chichester et al., 1994; Buckpitt et al., 1995; Van Winkle et al., 1996; Boland et al., 2004). These *in vitro* studies demonstrated the importance of *in situ* NA metabolism and cytotoxicity in airways, however, the role of pulmonary CYP-mediated NA metabolism in NA-induced pulmonary toxicity *in vivo* is not fully understood. In this study we test the hypothesis that pulmonary microsomal CYP enzymes play an important role in NA metabolic activation and subsequent deleterious effects in airway epithelial cells.

One of the approaches to investigate the contribution of CYP isoforms, expressed in an organ of interest, to CYP-mediated metabolism and toxicity is to utilize mouse model(s) with conditional deletion of CPR (a redox partner for all microsomal CYP enzymes) in this organ. We previously used liver-*Cpr*-null mouse models with abolished hepatic microsomal CYPs activity
to study the role of liver-generated NA metabolites to toxicity in airways following NA inhalation exposure at occupationally relevant concentrations (Chapter 2). To further explore the role of NA metabolism by pulmonary CYPs to damage of airway epithelial cells, the previously generated lung-\textit{Cpr}-null mouse model was utilized (Weng et al., 2007). This unique mouse model (CCSP-\textit{rtTA}^{+/+}/\textit{tetO}-\textit{Cre}^{+/+}/\textit{Cpr}^{lox/lox}) has an expression of reverse tetracycline transactivator (\textit{rtTA}) in the majority of Club cells without targeting other cell types in the lung. Expression of \textit{Cre} recombinase is induced after binding of \textit{rtTA} to the \textit{TetO} promoter in the presence of doxycycline and causes the selective deletion of \textit{Cpr} gene between exons 3-15 in airway Club cells. This mouse model on A/J background was used earlier to study the role of pulmonary CYPs in metabolism and tumorigenesis of NNK (4-(methylnitrosamino)-1-(2-pyridyl)-1-butanone), a known tobacco procarcinogen (Weng et al., 2007).

In this study, doxycycline-treated lung-\textit{Cpr}-null mice and their control littermates, all on a C57BL/6 (B6) background, were treated with NA at a toxic dose to cause damage of airway epithelial cells. The extent of NA-induced toxicity was evaluated by histological analysis of lung sections and monitoring of cellular proliferation in airways following NA-induced injury. The impact of target-tissue loss of CYPs activity on systemic kinetics of NA and NA-GSH clearance \textit{in vivo} was also determined. The results of these studies are expected to provide evidence regarding whether individuals with normal hepatic CYPs activity and low activity of pulmonary CYPs (reported in human lung) will be protected from the NA-induced damage of airway epithelial cells.
MATERIAL AND METHODS

Mouse breeding and doxycycline treatment. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center. The lung-\(Cpr\)-null mouse line was regenerated according to the breeding lineage presented the Fig.4.1A. The \(Cpr^{\text{lox/lox}}\) (Wu et al., 2003) mice were obtained from breeding stock maintained at Wadsworth Center, CCSP-rtTA\(^{+/+}\) (B6. Cg-Tg(Scgb1a1-rtTA)1Jaw/J, catalog number is 006232) and tetO-Cre\(^{+/+}\) (B6.Cg-Tg(tetO-cre)1Jaw/J, catalog number is 006234) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All founder and progeny were tested on presence of the loxP sites in the \(Cpr\) gene, CCSP-rtTA and tetO-Cre transgenes by conventional PCR using tail DNA. The typical results of PCR are presented in Fig.5.1B. Inducible deletion of \(Cpr\) gene in airway Club cells of lung-\(Cpr\)-null (CCSP-rtTA\(^{+/+}\)/tetO-Cre\(^{+/+}\)/\(Cpr^{\text{lox/lox}}\)) mice was initiated by doxycycline administered as a pelleted diet (625 mg/mg; Envigo Teklad Diets, Madison, WI) starting from E0 (through the dam) until the age of 2 months according to the previously reported protocol Fig.5.1C (Hokuto et al., 2004).

Dual immunofluorescence analysis. All immunofluorescence analyses were performed on ~5-\(\mu\)m sections of lung tissue. First, the slides with lung sections were initially incubated in 0.2% osmium tetroxide for 5 minutes to block tissue autofluorescence. The slides were then deparaffinized by double xylene washes and rehydrated in descending grades of ethanol (100%, 90%, 80% and 70%), followed by 1X PBS. Antigens were retrieved by incubating the slides in TE buffer (pH 9.0) for 40 minutes in 96\(^{\circ}\)C waterbath. After cooling to room temperature and washing with 1X PBS, the non-specific binding of the antibodies on the slides was blocked by a mixture of 10% normal goat serum and 1% bovine serum albumin in 1X PBS for 1hour.
Double immunofluorescence was performed following sequential 3-step protocol. First, the slides were incubated with the first primary anti-POR antibody (rabbit polyclonal, dilution 1:500, Abcam) in a humidified chamber overnight at 4°C following incubation of washed slides with the first secondary Alexa-Fluor®488-conjugated antibody (goat anti-rabbit, dilution 1:200, Abcam) at room temperature for 1 hour. Second, the slides were blocked with second blocking solution (10% normal rabbit serum and 1% bovine serum albumin) for 1 hour. Third, the slides were incubated with the second primary anti-uteroglobin antibody (rabbit polyclonal, dilution 1:1500, Abcam) for 1 hour following 1-hour incubation with the second secondary Alexa-Fluor®594-conjugated antibody (goat anti-rabbit, dilution 1:200, Abcam). The nuclei were counterstained with DAPI and the slides were mounted in a VectorShield antifade medium (Vector Laboratories, Burlington, CA), protected with a coverslip and stored in the dark at 4°C until viewing. For negative controls, the slides were incubated with a normal goat serum instead of primary antibodies.

Fluorescence images were obtained using a Nikon 50i microscope (Nikon Inc., Melvile, NY) at the Wadsworth Center Advanced Light Microscopy Core. The images were captured with a Spot CCD camera (Diagnostic Imaging, Sterling Heights, MI) separately using Quad-Fluor filter settings B-2E/C for Alexa Fluor®488, UV-2E/C for Alexa Fluor®594 and G-2E/C for DAPI to prevent bleed-through effects. The images were imported into an ImageJ program (https://imagej.nih.gov/ij/) where the separate red, green and blue images were merged and labeled.

The quantification of CPR-positive cells was performed separately in proximal and distal airways by counting cells with positive green (Alexa Fluor®488) signals in cytoplasm and cells with positive blue (DAPI) signal in nuclei. The number of identified positive cells obtained from
9-10 microscopic fields for each mouse were normalized to total number of analyzed cells and expressed in percentage.

To evaluate targeted CPR deletion in Club cells, epithelial cells in different airway regions that were simultaneously positive for CPR labeled with Alexa Fluor®488 and CCSP labeled with Alexa Fluor®594 were counted. The number of double positive cells (CPR+ and CCSP+) was counted in 4-5 microscopic fields per mouse, normalized to total number of cells counted and expressed in percentage.

**Pharmacokinetic study of NA and NA-GSH in lung-Cpr-null mice.** Control littermates (CCSP-rtTA+/Cprlox/lox or tetO-Cre+/Cprlox/lox) and lung-Cpr-null mice (CCSP-rtTA+/tetO-Cre+/Cprlox/lox) both fed with doxycycline diet (see above) were used in this study. A single dose of NA at 200 mg/kg dose (dissolved in corn oil) was administered intraperitoneally. Blood samples were collected from a tail vein of an individual mouse at different time intervals (15, 30 minutes and 1, 2, 4, 6 and 10 hours) after NA dosing via heparin-coated capillary tubes. After centrifugation of plasma samples at 9,000 g for 8 minutes at 4°C, plasma samples were stored at -80°C for further analysis. Methods for NA and NA-GSH detection were as previously described (Kovalchuk et al, 2017).

**Histological analysis of NA-induced airway damage in lung-Cpr-null mice.** Doxycycline-treated lung-Cpr-null mice and their control littermates were treated with a single dose (intraperitoneal injection) of 200 mg/kg of naphthalene or corn oil (vehicle). The lungs of treated mice were collected 24 hours after an injection by intratracheal instillation of 10% neutral buffered formalin at a constant pressure of 20-25 cm of fixative. After a one-hour fixation, the trachea was ligated and the fully inflated lung was placed in a large volume of the same fixative. The lungs were removed from fixative ~48 hours after necropsy and placed into 70% ethanol for
paraffin embedding. Tissue sectioning and staining with hematoxylin and eosin (H&E) were performed at the Wadsworth Center Histopathology Core facility. Images of paraffin-embedded lung sections (~5µm thickness) were obtained using a Nikon 50i light microscope (Nikon Inc., Melville, NY) equipped with a digital camera at the Wadsworth Center Advanced Light Microscopy core facility.

The assessment of the damage was evaluated by an investigator blinded to the treatment groups. The following previously established criteria (Li et al., 2011) were used for semi-quantitative analysis of NA-induced damage in distal airway epithelia: “negative” (normal bronchiolar epithelium), “focal” (partial detachment of bronchiolar epithelia from basement membrane) and “severe” (detachment of bronchiolar epithelia, denuded basement membrane). Percentage of damaged distal airways as \( \frac{\text{number of damaged airways (separately for each grade)}}{\text{total number of airways examines}} \times 100\% \) was obtained after examination of 25-30 airways per each mouse.

**Labeling of proliferating cells in airways following acute NA-induced injury in lung-Cpr-null mice.** Lung-Cpr-null mice and their control littermates kept on doxycycline-enriched diet were administered either 200 mg/kg NA or corn oil (vehicle) via intraperitoneal route as a single dose. Lungs were collected 48 hours (2 days) after treatment as described in details above as well as small intestine (positive control). One hour prior to euthanasia mice were injected with BrdU at 50 mg/kg in PBS.

Serial unstained sections (~ 5 µm thickness) of mouse lung tissues were evaluated for the presence of BrdU using immunohistochemistry. Antigen retrieval was performed as outlined by the supplier (Abcam, Cambridge, MA) with some modifications, as described (Van Winkle et al., 1995; Lawson et al., 2002). In brief, deparaffinized and rehydrated lung sections were digested in 2 N hydrochloric acid at 60 °C for 15 min and then neutralized in 0.1 M sodium borate buffer,
pH 9.0, for 10 min. Sections were exposed to 0.05% proteinase (in TE buffer, pH 8.0) at 37 °C for 10 min followed by blocking with 3% hydrogen peroxide in methanol. The lung sections were then incubated with the primary antibodies: biotin-labeled, prediluted anti-BrdU (Abcam) at a 1:2 further dilution in PBS, for 2 hours at room temperature. The sections were further incubated with a streptavidin-horse radish peroxidase-conjugate (Abcam, Cambridge, MA). Antigen-antibody complexes were visualized with 3,3’-diaminobenzidine as the substrate; dark-brown nuclei were identified as positive staining. Negative control sections were either from mice not treated with BrdU, or from BrdU-treated animals, but incubated with PBS in place of the primary antibodies. Sections of small intestine of BrdU-treated animals were used as a positive control.

The number of cells with BrdU-labeled nuclei was counted digitally, using an Nikon Eclipse 50i microscope (Nikon, Tokyo, JP) equipped with a QICAM color digital camera (Surrey, BC, Canada) that is linked to a computer running the Q-capture Pro 7 software (Media Cybernetics, Rockville, MD). Cells with brown nuclear staining were considered as positive and the numbers were counted in distal conducting airways (a diameter ≤ 200 µm) in five random microscopic fields per lung section at ×200 magnification. BrdU labeling index (LI) was determined by dividing the number of BrdU-positive cells by the number of total epithelial cells (sum of BrdU-positive and –negative cells) and multiplying by 100% (Hotchkiss et al., 1997). Data are presented as means±S.D. of four to five mice per group.

Other methods. Data from multiple groups were compared using a two-way analysis of variance (ANOVA) following Bonferroni post-hoc test to correct for multiple comparisons. p<0.05 was considered statistically significant.
RESULTS

Expression of CPR and CCSP in airways of lung-Cpr-null mice. The lung-Cpr-null mice were regenerated following the lineage presented in Fig.4.1. They were normal compared to their control littersmates independent of their diet status in gross morphological features, development, fertility, behavior. Body and organ weight (lung, liver) were similar between lung-Cpr-null mice and their control littersmates, both doxycycline-treated and untreated. Genotype frequency among pups from doxycycline-treated breeders was 27% for tetO-Cre\textsuperscript{+/−}/CCSP-rtTA\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox} (29/108), 26% for CCSP-rtTA\textsuperscript{+/−}/tetO-Cre\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox} (28/108), 24% for CCSP-rtTA\textsuperscript{+/−}/tetO-Cre\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox} (26/108) and 23% for CCSP-rtTA\textsuperscript{+/−}/tetO-Cre\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox} (25/108) mice with no significant difference between genotypes (p>0.05, Chi-square test), suggesting absence of embryonic lethality.
tetO-Cre\textsuperscript{+/−}/CCSP-rtTA\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox}

**Figure 4.1. Generation of lung-\textit{Cpr}-null mice on a C57BL/6 (B6) background.**

The original CCSP-rtTA\textsuperscript{+/−} and tetO-Cre\textsuperscript{+/−} on B6 background were crossed with Cpr\textsuperscript{lox/lox} to generate lung-\textit{Cpr}-null mice (CCSP-rtTA\textsuperscript{+/−}/tetO-Cre\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox}) (A). Schematic representation of CRE-mediated deletion of \textit{Cpr} gene in Club cells in presence of doxycycline in airways of lung-\textit{Cpr}-null mice (B). Results of conventional PCR for presence transgenes in founder and progeny strains (C).

To address the efficacy of \textit{Cpr} gene deletion in the respiratory tract, we examined the expression of CPR protein in airway epithelial cells of 2-month-old lung-\textit{Cpr}-null mice kept
either on regular or DOX+ diets, and their DOX+ control littermates. CPR protein was readily detectable in epithelial cells of proximal and distal airways in lung-Cpr-null mice fed with regular mouse chow and DOX+ control littermates (Fig. 4.2A-B). Consistent with previous observations, the loss of CPR expression was detected in a small number of airway epithelial cells (Fig. 4.2A-B) in lung-Cpr-null mice kept on regular mouse diet (Weng et al., 2007). Such findings are in agreement with former results on “leakiness” of the tetO-Cre transgene expression even in absence on doxycycline in tetO-Cre+/- founder strain (Perl et al., 2002; Perl et al., 2005).
Figure 4.2. Efficient deletion of CPR in airway epithelial cells.

Lung-Cpr-null mice were kept either on regular or doxycycline-enriched diet, and control littermates were kept on doxycycline diet. Lung sections were obtained from naïve 2-month old males and stained with antibodies against CPR and counterstained with DAPI. Numbers of CPR-positive epithelial cells in proximal (A) and distal (B) airways was counted in 9-10 random ×200 microscopic fields (3-4 mice in each group). The scale bar is 50 µm. Percentages of cells are presented as mean±S.D. for CPR positive cells (C) ****p<0.0001 statistically significant by diet status, &&&&p<0.0001 statistically significant by genotype (two-way ANOVA followed by Bonferroni’s test for multiple comparisons). Arrows point to CPR-negative epithelial cells in airways.

Number of CPR-positive cells was drastically decreased along the airway tree (by 3.7- and 3.9-fold in proximal airways, 5.0- and 5.3-fold in distal airways depending on diet status and genotype, respectively) in DOX-treated lung-Cpr-null mice consistent with the efficient deletion of Cpr gene by the Cre recombinase (Fig. 4.2C).
Deletion of the Cpr gene in triple transgenic lung-Cpr-null mice (tetO-Cre\textsuperscript{+/−}/CCSP-
rtTA\textsuperscript{+/−}/Cpr\textsuperscript{lox/lox}) was driven by a 2.3 kb rat CCPS promoter to target the Cre-mediated
recombination in Club cells and type II alveolar cells in airway epithelia. Percentage of double-
positive CPR\textsuperscript{+/−}/CCSP\textsuperscript{+} epithelial cells was higher in proximal than distal airways by 2-fold in
lung-Cpr-null mice kept on regular diet, 1.86-fold in DOX+ control littermates (Fig. 4.3A-D).
The percentage of dual CPR\textsuperscript{+/−}/CCSP\textsuperscript{+} cells was similar in proximal and distal airways (16.4% and
12.12%, respectively) in both airway regions in DOX+-treated lung-Cpr-null and significantly
lower (p<0.0001) compares to other control groups (Fig. 5.3C-D). Expectedly, number of CPR\textsuperscript{−}/
CCSP\textsuperscript{+} cells was substantially higher in DOX+-treated lung-Cpr-null mouse (p<0.0001) with
2.53-fold increase in only CCSP-positive cells in distal than proximal airways. These results
further confirm that conditional doxycycline-induced deletion of Cpr gene occurs predominantly
in Club cells, which have known difference in regional expression along the airway tree in mice
(Pack RJ et al., 1981; Plopper CG et al., 1980; Plopper CG et al., 1992).
Figure 4.3. Targeted deletion of CPR in airway epithelial Club cells.

Lung-Cpr-null mice were kept either on regular or doxycycline-enriched diet, and control littermates were kept on doxycycline diet. Lung sections were obtained from naïve 2-month old males and were stained with antibodies against CPR, CCSP and counterstained with DAPI. The number of double-positive CPR and CCSP epithelial cells was counted in proximal (A) and distal (B) airways using 4-5 random ×200 microscopic fields (3-4 mice in each group). Percentages of cells are presented as mean±S.D. for double positive CPR and CCSP cells in proximal (C) and distal (D) airways. The scale bar is 50 μm. ****p<0.0001 statistically significant by diet status, &&&&p<0.0001 statistically significant by genotype (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

Acute NA-induced toxicity in airways of lung-Cpr-null mice.

NA and NA-GSH levels in plasma were detected and quantified at different time intervals after 200 mg/kg NA administration. Pharmacokinetic profiles for these two analytes in plasma (Fig. 4.4) were similar between doxycycline-treated lung-Cpr-null mice and their control
littermates. Levels of NA in plasma were comparable between two strains and indicate that the lungs of NA-treated lung-\textit{Cpr}-null mice and their controls kept on doxycycline diet were exposed to similar internal NA dose. Whereas similar NA-GSH levels in plasma confirm that the loss of CPR expression in Club cells in airways of doxycycline-treated lung-\textit{Cpr}-null mice does not contribute to systemic clearance of peritoneally administered NA.

**Figure 4.4. NA and NA-GSH levels in plasma of lung-\textit{Cpr}-null mice at various time intervals after administration of 200 mg/kg of NA.**

Two to three-month old males of lung-\textit{Cpr}-null (n=3) and their control littermates kept on doxycycline-enriched diet (n=3) were administered 200 mg/kg NA as a single intraperitoneal injection. NA (left panel) and NA-GSH (right panel) were detected and quantified in plasma obtained from individual mice at various time intervals after NA dosing as described in Material and Methods. Data presented as mean±SD.

**Semi-quantitative histopathological assessment of acute NA-induced damage in airways.** Acute naphthalene-mediated injury in airway epithelial cells is readily detectable 24 hours after its peritoneal administration (Plopper et al., 1992; Van Winkle et al., 1995). We used
previously established scoring system to evaluate the extent of NA-induced damage in airway epithelial cells (Li et al., 2011) with an example for each score presented in Fig 4.5A. Upon examination of lung sections in a blinded-to-treatment fashion, pathological changes were observed in distal airways of lung-Cpr-null mice and their control littermates 24 hours after administration of a single dose of 200 mg/kg NA, but not detectable in vehicle control-treated groups (Fig 4.5B). While epithelial cells were detached from basement membrane in almost all distal airways in NA-treated control littermates, focal and slight disintegration of a few epithelial cells was observed, and in only ~35-40% airways, in NA-treated lung-Cpr-null mice (Fig 4.5C).
Figure 4.5. Histopathological analysis of NA-induced epithelial damage in airways of lung-
*Cpr*-null mice.

Two- to three-month old lung-*Cpr*-null male mice and their control littermates (n=4-5 per group) were administered a single IP injection of 200 mg/kg of naphthalene (NA) or corn oil (vehicle). Lungs were collected 24 hours after the treatment and 25-30 airways per mouse in random ×200 microscopic fields were examined. Grades for evaluation of airway epithelial damage (A), representative images of damaged distal airways in paraffin-embedded H&E stained lung sections (B) and percentage of damaged distal airways (C) are depicted. Arrows point to epithelial cells partially detached from basement membrane in distal airways. Scale bar is 50 µm.

*Cellular proliferation in airways of lung-*Cpr*-null mice following NA administration.*

BrdU incorporation was low in epithelial cells in distal conducting airways of lung-*Cpr*-null mice and their control littermates kept on doxycycline-enriched diet (Fig. 4.6A). BrdU labeling index (LI) was significantly higher (p<0.001) in airway epithelial cells of control littermates, and have only a positive trend in lung-*Cpr*-null mice following NA administration comparing to sex- and genotype-matched controls at 2-day post-treatment. Moreover, there was a significant
genotype-dependent difference (p<0.0001) in number of BrdU-positive cells between NA-treated lung-Cpr-null mice and their control littermates.

All these results strongly support the hypothesis that pulmonary P450s play a significant role in acute NA-mediated toxicity in airway epithelial cells.
Figure 4.6. BrdU labeling index in airways of lung-Cpr-null mice followed NA administration.

Two to three-month old lung-Cpr-null mice and their control were administered a single bolus dose of either 200 mg/kg NA or corn oil via intraperitoneal route. Lungs were collected 2 days after treatment. Representative images of BrdU-positive cells (arrows) in distal airways are shown (A). The proportion (%) of BrdU-positive cells (B) was quantified as described in Material and methods. Results represent means±S.D. of four mice per each group. Scale bar is 50 µm. &&&&p<0.0001 is significantly different by mouse genotype, ****p<0.0001 statistically significant by treatment (two-way ANOVA followed by Bonferroni’s test for multiple comparisons).

DISCUSSION

Deletion of floxed Cpr gene is mediated by activation of two transgenes in presence of doxycycline in the regenerated lung-Cpr-null mouse (CCSP-rtTA<sup>+</sup>/tetO-Cre<sup>+</sup>/Cpr<sup>lox/lox</sup>). The CPR protein is detectable in virtually all airway cell types without noticeable region difference in naïve control groups (doxycycline-treated control littermates and lung-Cpr-null mice fed with regular mouse diet). The CRE-mediated recombination in presence of doxycycline results in efficient deletion of Cpr gene in airway epithelial cells in lung-Cpr-null mice. Presence of rat CCSP promoter targets the Cpr deletion in Club cells and type II alveolar cells. In doxycycline-treated lung-Cpr-null mouse the Cpr deletion occurred in 66% of Club cells in proximal airways and 86% of Club cells in distal airways. Such a mosaic pattern of doxycycline-induced deletion is reported by other groups that use CCSP-rtTA<sup>+</sup>/tetO-Cre<sup>+</sup> mice with different floxed genes of interest (Perk et al., 2005; Mucenski et al., 2005). Partially, it could be attributed to heterogeneity
of Club cells reported in rodents (Ji et al., 1995). Small portion of Club cells is distinguished from most Club cells by low expression of phase I enzymes, but not CCSP protein (Reynolds et al., 2010).

Xenobiotic-metabolizing enzymes expressed in target tissues are believed to modulate in situ toxicity, but do not significantly contribute to systemic clearance of administered toxicants. The results of plasma kinetic studies of NA and biomarker of its biactivation NA-GSH of parenterally administered NA in lung-Cpr-null mice support this notion and are in agreement with results for NNK and its metabolite NNAL in previously published study utilizing the same mouse model (Weng et al, 2007). The internal NA dose available for bioactivation by pulmonary P450s was higher after bolus intraperitoneal administration compared to NA concentration following inhalation exposure (Li et al., 2017, Kovalchuk et al., 2017) and was independent of mouse genotypes used in this study.

Epithelial cells in distal airways are the target sites of NA-induced toxicity following intraperitoneal route of NA administration and such sensitivity correlates with the expression of CYPs enzymes in Club cells in this region (Plopper et al., 1992). Among CYP enzymes expressed in the respiratory tract, CYP2F2 has the higher catalytic efficiency to bioactivate NA in vitro (Shultz et al., 1999). The critical role of mouse CYP2F2 enzyme localized within Club cells was demonstrated by the previous finding of resistance to NA-induced toxicity in Cyp2f2-null mice in vivo (Li et al., 2011). Residual activity of CYPs in the airway, found in lung-Cpr-null mice due to mosaic deletion of Cpr gene, causes focal disintegration of a few epithelial cells in a small portion of distal airways based on histopathological analysis. The observed slight NA-induced toxicity in lung-Cpr-null mice could be mediated by circulating NA metabolites generated by other extrapulmonary and/or hepatic microsomal CYPs, but the results suggest that
their contribution is small compared to metabolites generated locally in Club cells. These results overall support our hypothesis that pulmonary CYPs are critical to exert NA-mediated cytotoxicity in airways.

Club cells serve not only as sites of metabolic activation of toxicant within a lung, but they also serve as progenitor cells. Several studies have investigated factors that contribute to Club cell regeneration in airways following NA injury in different mouse models (Stripp et al., 1995; Park et al., 2006; Kida et al., 2008, Rawlins et al., 2009; Ustiyan et al., 2012). To monitor cellular proliferation during tissue regeneration and repair, BrdU-positive nuclei in airway epithelial cells are also enumerated after administration of naphthalene (Van Winkle et al., 1995; Lawson et al., 2002) or styrene (Cruzan et al., 2012). In the absence of CYP2F2 bioactivation of styrene, a toxicant with metabolism and pulmonary toxicity similar to naphthalene, the styrene-induced damage, judging from BrdU incorporation into airway epithelial cells, was not reported in Cyp2f2-null mice (Cruzan et al., 2012). BrdU-positive nuclei in epithelial cells in airways of lung-Cpr-null mice after NA administration in our study were slightly increased, but did not reach statistical significance when compared to vehicle-treated controls. These results further confirm that NA bioactivation by microsomal pulmonary CYPs is a key event in NA-induced injury of airway epithelia.

Based on the results of the present study, we conclude that pulmonary CYPs play a critical role for NA bioactivation and subsequent injury of airway epithelia by pulmonary-generated NA reactive metabolites. Low activity of pulmonary CYPs, as reported in human lung, in combination with normal hepatic P450 activity, will be protective against acute NA-induced damage of airway epithelial cells. The risk of toxicity is not clear for other lung toxicants (3-methylindole, trichloroethylene), to which humans are widely exposed via smoking or in
occupational settings. Lung-\textit{Cpr}-null mouse model would be a useful tool to investigate the contribution of pulmonary CYPs to metabolism, toxicity and carcinogenic potential of these inhaled toxicants in lungs in a cell-specific fashion.

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**FOOTNOTES**

The results of this study were submitted to present at the annual Experimental Biology meeting 2018.
CHAPTER FIVE

CONCLUSIONS AND SIGNIFICANCE
The overall goal of this study was to examine the contribution of hepatic and pulmonary CYPs to bioactivation, disposition and NA-induced toxicity in airways. We examined the relationships between NA environmental and systemic exposure, its regional bioavailability controlled by local or systemic disposition, ultimate NA toxicants generated locally or in the liver, and NA-induced toxicity in airway epithelial cells. Using Cyp-tissue-specific null and CYP-humanized mouse models we demonstrated 1) the impact of hepatic CYPs on bioactivation and disposition of inhaled NA in mice with pulmonary-expressed human CYP2A13 and CYP2F1 (chapter 3); 2) the possible contribution of hepatic CYP-generated metabolites to epithelial cell injury in airways following NA inhalation exposure (chapter 2); and 3) the dominant role of NA metabolites generated by pulmonary P450s to NA-induced cytotoxic damage and carcinogenic potential in airways (chapter 4). These studies provided new knowledge on systemic factors and in vivo mechanisms that modify the risks of lung toxicity in NA-exposed individuals.

CYP-mediated metabolism of naphthalene has been studied extensively. The key question remain unanswered is whether the NA-induced damage to airway cells occur due to NA toxic metabolites generated locally or formed in the liver and transported via systemic circulation to the target site of toxicity. Previous studies demonstrated that toxic NA metabolites can form hemoglobin and albumin adducts in mice and rats in vivo (Waidyanatha et al., 2002; Waidyanatha et al., 2008), but origin of these metabolites were unknown. The contribution of hepatic P450-generated metabolites to disposition and toxicity of drugs and carcinogens was investigated using liver-Cpr-null mouse model (have an ablation of CYPs activity irrespective of isoform due to deletion of Cpr – redox partner of all CYP enzymes) (Gu et al., 2005, Gu et al., 2007). Bioactivation of NA occurs in liver-Cpr-null mice (Li et al., 2011) implying that hepatic P450s are not essential to generate NA metabolites, but the extent of NA-mediated
pneumotoxicity in these mice was never investigated. Moreover, there are limited studies that investigate acute NA-induced injury of airway epithelial cells, but not metabolic aspects associated with it, following inhalation exposure (West et al., 2001), which a predominant route of widespread NA exposure in human populations. Our studies using liver-Cpr-null mice not only further confirmed that CYP-generated metabolites of inhaled NA are produced extrahepatically, but also demonstrated dose-dependent response of airway epithelial cells to NA-induced injury (chapter 2). These findings imply that NA-mediated pneumotoxicity occurs without significant contribution of hepatic CYP-generated metabolites even at levels that are half of established standards at occupational settings (10 ppm).

Epidemiologic data on NA toxicity in humans is not available. Extrapolation of animal data to humans are challenging due to species difference in catalytic activity of CYP enzymes between rodents and human, and unknown impact of CYP-generated metabolites from different organs to NA-mediated pneumotoxicity. Physiologically based pharmacokinetic (PBPK) model has been recently developed to predict possible absorption, distribution, metabolism and possible pulmonary toxicity of NA in humans (Campbell et al., 2014). Input data on NA bioactivation in humans, as a key event in NA-induced airway injury, were extrapolated from microsomal metabolic activity obtained from rats and monkeys (a human surrogate). Metabolites of inhaled NA generated locally in the respiratory tract of humans (portal of entry organ in real world scenario) in addition to NA metabolites derived from systemic circulation can intensify the NA-induced damage in airways. Human CYP2A13 and CYP2F1 are expressed in the nasal olfactory mucosa and lung, and are capable of bioactivating NA in vitro with lower efficiency compared to mouse orthologous enzymes. Toxicity of inhaled NA in nasal and airway epithelia at occupationally relevant concentration (10 ppm) was demonstrated in CYP2A13/2F1-humanized
mice (have global knockout of all Cyp2a, 2b, 2f, 2g, and 2s genes, but expression of human CYP2A13/2F1 transgene in the respiratory tract to eliminate species interference in important CYPs activity) (Li et al., 2017). Our studies further examined the impact of hepatic P450-generated NA metabolites on kinetics and disposition of inhaled NA in CYP2A13/2F1-humanized mice either with normal or compromised CYPs activity in liver (chapter 3). Consistent with the results in chapter 2, hepatic CYPs are not required to bioactivate inhaled NA, but contribute to its systemic clearance. Due to the slower catalytic efficiency of human CYP2A13 and CYP2F1 compared to mouse CYP2F2 to bioactivate NA, and deletion of Cyp2abfgs gene cluster and respective proteins that participate in NA metabolism, the kinetics and disposition of inhaled NA has unpredicted patterns in CYP2A13/2F1-humanized mice with compromised hepatic P450s activity. In particular, 1) NA is accumulated in liver during a single NA inhalation exposure (10 ppm) and is redistributed to other organs after its termination resulting in extended “secondary” exposure of lungs to NA from systemic circulation. 2) Bioactivation of inhaled NA occurs, and hepatic microsomal P450s and CYP2ABFGS enzymes are not required to perform this function. 3) Human respiratory-expressed CYP2A13 and CYP2F1 contribute to systemic disposition of inhaled NA only when hepatic P450s activity is compromised. These data suggest that exposure to NA will be extended in humans with deficient hepatic metabolism and they might be at higher risk of developing NA-induced acute injury in their airways. The impact of hepatic CYP-generated metabolites of inhaled NA to cytotoxic airway damage in CYP2A13/2F1-humanized mice with compromised P450s activity is worth to explore in future studies. These results will provide further experimental evidence for predicting NA pneumotoxicity in humans with impaired hepatic microsomal CYPs activity and help to further validate the developed PBPK models.
Activity of pulmonary microsomal proteins to bioactivate NA is well established in vitro, but their overall contribution to NA-mediated toxicity in airway epithelia is not known. Besides CYP2F, other CYP isoforms (CYP1A, 2E1, 3A4/5) are expressed in the respiratory tract and have capability to bioactivate NA. Among diverse airway epithelial cells, Club cells are enriched in microsomal P450 proteins. The best strategy to examine the role of pulmonary CYP metabolism in toxicity is to block CYPs activity in a cell-specific fashion. Mouse model with deletion of Cpr gene in Club cells has been generated (Weng et al., 2007). The Cre-lox mediated deletion of Cpr gene occur in majority of Club cells irrespective of their regional localization in airways. Lung-Cpr-null mouse model was utilized previously to examine the role of CYP metabolism in the target tissue to toxicity of tobacco-specific nitrosamine NNK (a known human carcinogen). Using the same mouse model we demonstrated that 1) NA level in systemic circulation is not always a good predictor of NA-induced pneumotoxicity after acute exposure; 2) pulmonary-expressed CYPs are critical to acute injury of airway epithelial cells and their subsequent proliferation after NA exposure. These data suggest that NA metabolites generated by pulmonary CYPs are the main contributors to NA-mediated cytotoxicity in airways. Further studies are desired to confirm that subsets of airway epithelial cells with low CYPs expression are protected against acute injury induced by inhaled NA.

In summary, the results of this project support the central hypothesis that hepatic CYPs activity contribute to the sensitivity to acute NA-induced toxicity. The major findings of this work are presented in Fig. 5.1. We provide experimental evidence that NA metabolites, generated locally by pulmonary CYPs, are critical for acute NA-induced toxicity and proliferation of injured epithelial cells in airways. Even though the hepatic CYPs are not required for bioactivation of inhaled NA, they are essential for NA clearance in vivo and determine the
total exposure through systemic circulation. The plasma level of NA might be used as a biomarker of exposure, whereas NA-GSH could serve as a biomarker of NA bioactivation in future epidemiological studies to account for human variability in NA metabolism and better predict the risk of adverse NA-induced effects in airways on an individual basis.

Figure 5.1. Summary of the major findings of the present research
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