Toxicology 325 (2014) 85–95

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Differential *Fmo3* gene expression in various liver injury models involving hepatic oxidative stress in mice

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ARTICLE INFO

Article history:
Received 17 July 2014
Received in revised form 29 August 2014
Accepted 31 August 2014
Available online 2 September 2014

Keywords:
Flavin-containing monooxygenase-3
Hepatotoxicants
Acetaminophen
Alpha-naphthyl isothiocyanate
Bile duct ligation
Carbon tetrachloride
Allyl alcohol
Oxidative stress
Nrf2

ABSTRACT

Flavin-containing monooxygenase-3 (FMO3) catalyzes metabolic reactions similar to cytochrome P450 monooxygenase, however, most metabolites of FMO3 are considered non-toxic. Recent findings in our laboratory demonstrated *Fmo3* gene induction following toxic acetaminophen (APAP) treatment in mice. The goal of this study was to evaluate *Fmo3* gene expression in other diverse mouse models of hepatic oxidative stress and injury. *Fmo3* gene regulation by Nrf2 was also investigated using Nrf2 knockout (Nrf2 KO) mice. In our studies, male C57BL/6J mice were treated with toxic doses of hepatotoxicants or underwent bile duct ligation (BDL, 10 days). Hepatotoxicants included APAP (400 mg/kg, 24–72 h), alpha-naphthyl isothiocyanate (ANIT; 50 mg/kg, 2–48 h), carbon tetrachloride (CCl_4; 10 or 30 μL/kg, 24 and 48 h) and allyl alcohol (AlOH; 30 or 60 mg/kg, 6 and 24 h). Because oxidative stress activates nuclear factor (erythroid-derived 2)-like 2 (Nrf2), additional studies investigated *Fmo3* gene regulation by Nrf2 using Nrf2 knockout (Nrf2 KO) mice. At appropriate time-points, blood and liver samples were collected for assessment of plasma alanine aminotransferase (ALT) activity, plasma and hepatic bile acid levels, as well as liver Fmo3 mRNA and protein expression. Fmo3 mRNA expression increased significantly by 43-fold at 12 h after ANIT treatment, and this increase translates to a 4-fold change in protein levels. BDL also increased Fmo3 mRNA expression by 1899-fold, but with no change in protein levels. Treatment of mice with CCl_4 decreased liver *Fmo3* gene expression, while no change in expression was detected with AlOH treatment. Nrf2 KO mice are more susceptible to APAP (400 mg/kg, 72 h) treatment compared to their wild-type (WT) counterparts, which is evidenced by greater plasma ALT activity. The Fmo3 mRNA and protein expression increased in Nrf2 KO mice after APAP treatment. Collectively, not all hepatotoxicants that produce oxidative stress alter *Fmo3* gene expression. Along with APAP, toxic ANIT treatment in mice markedly increased *Fmo3* gene expression. While BDL increased the Fmo3 mRNA expression, the protein level did not change. The discrepancy with *Fmo3* induction in cholestatic models, ANIT and BDL, is not entirely clear. Results from Nrf2 KO mice with APAP suggest that the transcriptional regulation of *Fmo3* during liver injury may not involve Nrf2.

Abbreviations: Fmo3, flavin-containing monooxygenase-3; APAP, acetaminophen; ANIT, alpha-naphthyl isothiocyanate; BDL, bile duct ligation; CCl_4, carbon tetrachloride; AlOH, allyl alcohol; Nrf2, nuclear factor (erythroid-derived 2)-like 2.

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http://dx.doi.org/10.1016/j.tox.2014.08.013
0300-483X/

1. Introduction

Drug-induced liver injury (DILI) is a significant challenge for both drug development and clinical care. It accounts for more than 50% of all acute liver failure cases in the U.S. (Larson et al., 2005; Lee, 2010). Many chemicals, such as acetaminophen (APAP), carbon tetrachloride (CCl_4) and allyl alcohol (AlOH) have been used to model hepatotoxicity relevant to human exposure. Alpha-naphthyl isothiocyanate (ANIT) and bile duct ligation (BDL) on the other hand are used to model cholestasis, a pathological condition caused by the impairment of hepatic bile flow. While ANIT produces intrahepatic cholestasis, BDL produces extrahepatic cholestasis. With APAP and CCl_4, the parent compound is metabolized by cytochrome P450 (CYP) to generate reactive metabolites, *N*-acetyl-*p*-benzoquinone imine (NAPQI) and trichloromethyl radical ($^{\bullet}CCl_3$), respectively. AlOH in turn is metabolized in the liver by alcohol dehydrogenase to its reactive metabolite, acrolein. The glutathione adduct of acrolein is converted by CYPs to glycidaldehyde. Toxicity resulting from these reactive metabolites is multifactorial and includes lipid peroxidation, generation of oxidative stress, altered cellular redox status and protein adduct formation (Burcham and Fontaine, 2001; Cohen et al., 1997; Jaeschke et al., 2012; Ohno et al., 1985; Tom et al., 1984). During cholestasis resulting from either BDL or ANIT treatment, increase in bile acid concentration stimulates production of reactive oxygen species eventually leading to hepatocellular necrosis and apoptosis (Sokol et al., 1995; Trauner et al., 1998).

The role of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) as a master defense against hepatotoxicity produced by various chemicals has been investigated in several studies. Nrf2 belongs to the cap 'n' collar family of transcription factors that promotes transcription of a battery of cytoprotective genes (Aleksunes and Manautou, 2007; Kensler et al., 2007). Under basal conditions, Nrf2 is largely bound to the cytoskeletal anchoring protein Kelch-like ECH-associated protein 1 (Keap1) also known as cytosolic Nrf2 inhibitor in the cytoplasm. In response to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus. In the nucleus, Nrf2 binds to the GTGACA***GC core sequence of the antioxidant response element (ARE) (Rushmore et al., 1991) and promotes ARE-mediated antioxidant gene expression.

A low toxic APAP dose causes nuclear accumulation of Nrf2 in mouse liver, which is accompanied by increased expression of Nrf2 dependent cytoprotective genes such as heme oxygenase-1 (*Hmox1*), NAD(P)H:quinone oxidoreductase-1 (*Nqo1*) and glutamate cysteine ligase catalytic subunit (*Gclc*) (Aleksunes et al., 2005, 2006a; Bauer et al., 2000; Chiu et al., 2002; Goldring et al., 2004). Similar results have been reported with ANIT, BDL, CCl_4, AlOH and which are the other models of hepatic oxidative stress used in the present study (Aleksunes et al., 2005, 2006b; Liu et al., 2013; Randle et al., 2008; Tanaka et al., 2009). On the other hand, Nrf2 KO mice are more susceptible to APAP-induced liver injury compared to their wild-type counterparts (Chan et al., 2001; Enomoto et al., 2001). Likewise, Nrf2 KO mice are also more susceptible to CCl_4- and AlOH-induced hepatoxicity compared to wild-type mice (Liu et al., 2013). However, Nrf2 KO mice do not exhibit any difference in susceptibility to either BDL or ANIT treatment (Tanaka et al., 2009; Weerachayaphorn et al., 2012). This response is attributed to the adaptive compensatory changes involving nuclear transcription factors, including Fxr, Shp, Pxr and Hnf1α, efflux bile acid transporters, altered GSH levels and bile flow rates in Nrf2 KO mice (Tanaka et al., 2009; Weerachayaphorn et al., 2012). Collectively, the models of hepatic injury selected for the current study not only result in hepatic oxidative stress but also activate the Nrf2–Keap1 regulatory pathway.

Despite *Fmo3* being considered non-inducible, studies with aryl hydrocarbon receptor (AhR) agonists in mice revealed liver *Fmo3* gene induction (Celius et al., 2008, 2010). A recent gene array analysis performed in our laboratory also demonstrated *Fmo3* gene induction in the APAP autoprotection mouse model (mice receiving a low hepatotoxic APAP dose that become resistant to a subsequent higher APAP dose) (O'Connor et al., 2014). Unlike with AhR agonists that result in marginal increases in Fmo3 protein expression in mouse liver, we showed significant increases in Fmo3 protein levels by 15-fold in APAP autoprotected mice (Rudraiah et al., 2014). *Fmo3* induction by other hepatotoxicants that produce oxidative stress is not currently known.

In human liver, transcription factors regulating constitutive *FMO3* expression as well as those involved in developmental expression pattern have been extensively studied (Klick and Hines, 2007; Klick et al., 2008; Shimizu et al., 2008). Because the mammalian FMOs were considered non-inducible by xenobiotics (Cashman and Zhang, 2002; Krueger and Williams, 2005), the transcriptional regulation of FMO involving stress-activated transcription factors or receptors that bind ligands and interact with DNA was not studied as other forms of regulation. Thus, little is known about the transcriptional regulation of *Fmo3* in response to toxicant exposure. Recently, Celius et al. (2010) showed that the Fmo3 mRNA up-regulation by 3-methylcholanthrene (3MC) and benzo(a) pyrene (BaP) but not TCDD in Hepa-1 cells is mediated by p53 and its binding to a p53-response element in the promoter region of *Fmo3* (Celius et al., 2010).

Differentially expressed genes in the APAP autoprotection model were further analyzed using Causal Reasoning Engine (CRE), a recently developed computational platform (O'Connor et al., 2014). CRE analysis provides hypotheses on the upstream molecular events that best explain gene expression profiles based on prior biological knowledge. CRE analysis of differentially expressed genes in APAP autoprotection study supports an induction of the Nrf2 pathway (O'Connor et al., 2014). Additionally, the 5′-flanking region of the mouse *Fmo3* contains multiple copies of the ARE (Celius et al., 2008). Therefore, the purpose of the present study was to investigate liver *Fmo3* gene expression under oxidative stress conditions involving activation of the Nrf2–Keap1 regulatory pathway. Mice were dosed with hepatotoxicants APAP (400 mg/kg, 24–48 h), ANIT (50 mg/kg, 2–48 h), CCl_4 (10 or 30 μL/kg, 24 and 48 h) or AlOH (30 or 60 mg/kg, 6 and 24 h) or underwent sham surgery or bile duct ligation (10 days). Doses selected for hepatotoxicants are based upon the previous studies conducted in our laboratory resulting in oxidative stress and tissue injury. The inclusion of multiple time-points following hepatotoxicants exposure enabled comprehensive characterization of temporal changes in *Fmo3* in relation to injury and recovery. Further, in order to investigate whether Nrf2 mediates *Fmo3* gene expression, Nrf2 KO mice were employed. APAP was used as a model toxicant in the Nrf2 KO mice study. From these experiments, it is concluded that not all hepatotoxicants that produce oxidative stress in mice induce liver *Fmo3* gene expression. Toxic ANIT treatment, along with the previously demonstrated APAP treatment, markedly increases *Fmo3* gene expression. While the BDL increases Fmo3 mRNA expression, the protein levels do not change. The APAP treatment induces *Fmo3* gene expression in Nrf2 KO mice liver suggesting that the transcriptional regulation of *Fmo3* might not involve Nrf2

2. Materials and methods

2.1. Chemicals

Acetaminophen, alpha-naphthyl isothiocyanate, carbon tetrachloride, allyl alcohol, propylene glycol and corn oil were purchased from Sigma–Aldrich (St. Louis, MO). All other reagents were of reagent grade or better and commercially available.