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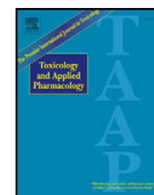

Analysis of changes in hepatic gene expression in a murine model of tolerance to acetaminophen hepatotoxicity (autoprotection)

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ABSTRACT

Pretreatment of mice with a low hepatotoxic dose of acetaminophen (APAP) results in resistance to a subsequent, higher dose of APAP. This mouse model, termed APAP autoprotection was used here to identify differentially expressed genes and cellular pathways that could contribute to this development of resistance to hepatotoxicity. Male C57BL/6J mice were pretreated with APAP (400 mg/kg) and then challenged 48 h later with 600 mg APAP/kg. Livers were obtained 4 or 24 h later and total hepatic RNA was isolated and hybridized to Affymetrix Mouse Genome MU430_2 GeneChip. Statistically significant genes were determined and gene expression changes were also interrogated using the Causal Reasoning Engine (CRE). Extensive literature review narrowed our focus to methionine adenosyl transferase-1 alpha (MAT1A), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), flavin-containing monooxygenase 3 (Fmo3) and galectin-3 (Lgals3). Down-regulation of MAT1A could lead to decreases in S-adenosylmethionine (SAMe), which is known to protect against APAP toxicity. Nrf2 activation is expected to play a role in protective adaptation. Up-regulation of Lgals3, one of the genes supporting the Nrf2 hypothesis, can lead to suppression of apoptosis and reduced mitochondrial dysfunction. Fmo3 induction suggests the involvement of an enzyme not known to metabolize APAP in the development of tolerance to APAP toxicity. Subsequent quantitative RT-PCR and immunochemical analysis confirmed the differential expression of some of these genes in the APAP autoprotection model. In conclusion, our genomics strategy identified cellular pathways that might further explain the molecular basis for APAP autoprotection.

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Introduction

Although acetaminophen (APAP) is one of the most commonly used over-the-counter analgesic and antipyretic agents in the world, it accounts for more than 50% of all acute liver failure cases in the U.S. and Great Britain (Larson et al., 2005). For this reason, there is concern that has prompted extensive research aimed at elucidating the mechanism of APAP hepatotoxicity and how it may be prevented. Due to the similarities in injury and recovery between rodents and humans, rodent models have proven useful in studying signaling pathways involved in APAP hepatotoxicity (Park et al., 2005).

One experimental approach to modulate APAP hepatotoxicity in rodents is through auto/heteroprotection. Autoprotection is defined as resistance to toxicant re-exposure following acute, mild injury with the same toxicant, whereas heteroprotection is achieved when different toxicants are used for pretreatment and treatment. Carbon tetrachloride (CCl_4) is one compound that has been used extensively as a chemical model of autoprotection. Mehendale and co-workers speculated in the early 1990s that compensatory hepatocellular proliferation is of critical importance to CCl_4-induced autoprotection (Thakore and Mehendale, 1991). This hypothesis was supported by use of the antimitotic agent colchicine, which blocked autoprotection by preventing hepatocellular proliferation after the initial dose of CCl_4, demonstrating that compensatory cell division following initial dosing with CCl_4 is at least in part, responsible for the heightened tolerance and ability of the liver to recover from toxicant re-exposure (Rao and Mehendale, 1991).

Abbreviations: APAP, acetaminophen; ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; CFB, clofibrate; i.p., intraperitoneal; Fmo3, flavin-containing monooxygenase 3; Lgals3, galectin-3; H_20_2, hydrogen peroxide; MAT1A, methionine adenosyl transferase-1 alpha; Mrp, multidrug resistance-associated protein; MMLV-RT, moloney murine leukemia virus reverse transcriptase; NFE2L2 or Nrf2, nuclear factor (erythroid-derived 2)-like 2; PPAR, peroxisome proliferator activated receptor; qRT-PCR, quantitative Real-Time Polymerase Chain Reaction; Vnn1, vanin 1; WT, wild type.

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Our laboratory has previously conducted studies using chemical treatments and conditions that reduce the severity of APAP toxicity in mice (Aleksunes et al., 2008a; Moffit et al., 2007b). Peroxisome proliferators such as clofibrate (CFB) are known to diminish APAP toxicity in mice (Manautou et al., 1994). Using knockout mice, we determined that protection by CFB is reversed in the absence of the PPARα receptor, demonstrating that its activation is necessary for hepatoprotection (Chen et al., 2000). Gene array analysis on livers from these rodents identified vanin-1 as a gene of interest. Vanin-1 (Vnn1) mRNA is significantly up-regulated in wild-type mice exhibiting protection from APAP toxicity, but not in PPARα-null mice (Moffit et al., 2007b). Increases in Vnn1 expression augment the levels of hepatic cystamine, which is an antioxidant capable of protecting against APAP hepatotoxicity (Miners et al., 1984; Moffit et al., 2007b). This increase in cystamine may explain why CFB protects the mouse liver from APAP toxicity. Vnn1 also modulates immune function by contributing to the extravasation of inflammatory cells to sites of injury (Meghari et al., 2007).

A mouse model of APAP autoprotection has been established in our laboratory to investigate the role and regulation of hepatobiliary drug transporters during development of resistance to APAP hepatotoxicity. We have determined that APAP autoprotection in mice is not due to differences in bioactivation or detoxification of APAP (Aleksunes et al., 2008a). These studies focused on the differential expression of members of the multidrug resistance-associated protein (Mrp) superfamily and their role in APAP autoprotection. These proteins are ATP-dependent membrane transporters responsible for the efflux of chemicals from the liver. mRNA and protein expression of the sinusoidal efflux transporter Mrp4 is elevated following APAP pretreatment, and its increased expression is localized to hepatocytes in centrilobular areas where compensatory cellular proliferation following pretreatment with mildly hepatotoxic doses of APAP is confined (Aleksunes et al., 2008a). Our studies also showed that colchicine treatment following administration of the priming dose of APAP reverses tolerance to hepatotoxicity, much like in the CCl_4 model. The reversal in tolerance by colchicine is associated with a lack of induction in Mrp4 gene and protein expression that is usually seen with APAP. This suggests that Mrp4 expression is increased in proliferating hepatocytes as a mechanism for efflux of toxic by-products and to lower the chemical burden on hepatocytes, which in turn should lead to faster and more efficient recovery from APAP re-exposure (Aleksunes et al., 2008a).

While a role for Mrp4 in APAP autoprotection is well supported, we were interested in identifying other molecular pathways that might also contribute to the development of resistance to APAP hepatotoxicity resulting from pre-treatment to this toxicant. Therefore, C57BL/6J liver samples from our previous APAP autoprotection study (Aleksunes et al., 2008a) were subjected to gene array analysis. Statistically significant genes were analyzed individually and using the Causal Reasoning Engine (CRE) to gain further insight into the molecular mechanisms of autoprotection. CRE is a recently developed computational platform that provides hypotheses on the upstream molecular events that best explain gene expression profiles by interrogating prior biological knowledge (Enayetallah et al., 2011). Indeed, this approach did identify additional mechanisms that might further explain the molecular basis for autoprotection.

Methods and materials

Chemicals. Acetaminophen, propylene glycol, and colchicine were purchased from Sigma-Aldrich (St Louis, MO). Zinc formalin was obtained from Fisher Scientific (Pittsburgh, PA). All other reagents were of reagent grade or better.

Animals. 10–12-week old male C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Upon arrival, the mice were acclimated for one week. The mice were housed in a 12-h dark/light cycle in a temperature and humidity controlled environment. The mice were fed laboratory rodent diet 2018 (Harlan Teklad, Madison, WI) *ad libitum*.

Dosing regimen 1: Following overnight fast, mice were treated with APAP (400 mg/kg) in 50% propylene glycol or vehicle; then, 48 h later, APAP and vehicle pretreated animals were treated with either APAP (600 mg/kg) in 50% propylene glycol or vehicle (5 mL/kg i.p.). Liver and plasma were collected 4 or 24 h later.

Dosing regimen 2: Following overnight fast, mice were treated with APAP (400 mg/kg) in 50% propylene glycol or vehicle (5 mL/kg i.p.). Liver and plasma were collected 2, 4, 8, 12, 24 and 48 h later.

Dosing regimen 3: Following overnight fast, mice were treated with APAP (400 mg/kg) in 50% propylene glycol or vehicle (5 mL/kg i.p.). To block compensatory hepatocyte proliferation, 2 mg/kg colchicine or vehicle (saline; 5 mL/kg i.p.) was given 24 and 49 h later. A second dose of APAP (600 mg/kg) or vehicle (50% propylene glycol, 5 mL/kg i.p.) was administered 48 h after the initial APAP dose. Plasma and livers were collected 24 h after the second dose of APAP.

All animal studies were performed in accordance with the National Institute of Health standards and the Guide for the Care and Use of Laboratory Animals. This work was approved by the University of Connecticut's Institutional Animal Care and Use Committee.

Gene array analysis. Snap frozen liver samples were sent to Gene Logic (Gaithersburg, MD) where RNA was isolated and hybridized to the MU430_2 GeneChips. Affymetrix Cel files were imported using Gene Spring software (Agilent, Santa Clara, CA) and data were Robust Multichip Average (RMA) normalized. Chip normalization was to the 50th percentile and gene normalization was to control samples. Variances were assumed equal and Welch's *t*-tests were performed on the data. A p-value cut-off for significance of 0.05 was used, as was a fold change of ± 2.

Interrogation of the gene expression changes using the Causal Reasoning Engine (CRE). CRE is a recently developed computational platform that provides hypotheses on the upstream molecular events that best explain gene expression profiles by interrogating prior biological knowledge. The CRE model is similar to that of Pollard et al. (2005) using novel statistical measures and biological modeling tools (Chindelevitch et al., 2012). Briefly, CRE generates hypotheses with predicted polarity based on a number of statistical measures, including Enrichment and Correctness testing. For Enrichment we used Fisher's exact test to evaluate the significance of a hypothesis, whereas Correctness tests the direction of the hypothesis (predicted decreased or increased). For example, a hypothesis of MAT1A is a CRE prediction of change in MAT1A enzymatic activity where a relatively significant number of the observed gene expression changes are known to be downstream of MAT1A, and the polarity of such gene expression changes supports a decreased directionality. In the CRE, the MeSH tag cloud is a visual approach that provides insight into the context of the generated hypotheses. Evidence for a particular molecular hypothesis is linked to one or more references in the literature. Most articles are indexed in Medline with Medical Subject Headings (MeSH terms) that describe key aspects of the article (*e.g.*, “cardiac arrhythmia,” “blood,” “diabetes,” and “liver”). For each MeSH term the number of occurrences in the set of articles supporting the selected hypotheses is counted and compared to the general frequency of occurrence of the particular MeSH term in all articles underlying the causal network and statistical significance assessed by the Fisher's exact test. To visualize the results, a MeSH term tag cloud is displayed in which font size corresponds to statistical significance.

Alanine aminotransferase (ALT) assay. Plasma ALT activity was determined as an indicator of liver injury. Infinity ALT reagent (Thermotrace, Melbourne, Australia) was used according to the manufacturer's protocol.

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