Dynamic and Coordinated Regulation of KEAP1-NRF2-ARE and p53/p21 Signaling Pathways Is Associated with Acetaminophen Injury Responsive Liver Regeneration

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ABSTRACT

Acetaminophen (APAP) overdose is the leading cause of drug-induced liver injury. Compensatory liver regeneration is crucial for the final outcome of toxicant-induced injury. However, the molecular mechanisms underlying compensatory liver regeneration in mice after APAP-induced liver injury are not completely understood. This study aimed to investigate the role of dynamic and coordinated regulation of Kelch-like ECH-associated protein 1 (KEAP1)–nuclear factor erythroid 2–related factor 2 (NRF2)–antioxidant response element (ARE) and p53/p21 pathways in APAP injury-responsive liver regeneration. We found that mice exhibited massive hepatic toxicity during the first 12 hours after 400 mg/kg APAP treatment, but responsive liver recovery occurred beyond 24 hours as demonstrated by histopathological and biochemical assessments. The expression and nuclear accumulation of NRF2 was increased after APAP treatment. The expression of NAD(P)H-quinone oxidoreductase 1, glutamate-cysteine ligase modifier subunit, and heme oxygenase-1 was inhibited during the first 24 hours and then induced to limit oxidative damage. The content of p53 and its downstream target p21 were significantly increased upon APAP exposure and subsequently decreased to normal levels at 48 hours. Furthermore, levels of cyclin D1, cyclin D–dependent kinase 4, proliferating cell nuclear antigen, and augmenter of liver regeneration at 48 hours were enhanced, suggesting initiation of hepatocyte proliferation and tissue repair. These results demonstrated that dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways was associated with compensatory liver regeneration after APAP-induced acute liver injury.

Introduction

Acetaminophen (APAP), a widely-used analgesic and antipyretic drug, is relatively safe at recommended doses, but an acute or cumulative overdose frequently results in liver injury. Hepatotoxicity induced by APAP is the most common cause of drug-induced acute liver failure in the United States and Great Britain (Larson et al., 2005). APAP toxicity is initiated by accumulation of the toxic reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI), which is generated by cytochrome P450 enzymes (isoforms CYP2E1, 1A2, and 3A) (Patten et al., 1993). NAPQI subsequently triggers hepatic toxicity by covalently binding with cellular proteins and/or by increasing levels of reactive oxygen species (ROS) leading to apoptosis and hepatocellular necrosis (Saito et al., 2010).

Extensive evidence has demonstrated that compensatory liver regeneration is a critical determinant of the final outcome of toxicant-induced injury and dictates survival or death (Anand et al., 2003; Mehendale, 2005). Liver regeneration allows for the replacement of necrotic cells and the full recovery of organ function. It is well known that responsive liver regeneration is governed by a complex signal transduction network involving numerous chemokines, cytokines, growth factors, and nuclear receptors (Fausto et al., 1995, 2006).

The nuclear factor erythroid 2–related factor 2 (NRF2), a master regulator of the antioxidant defense system, mediates a cell survival response. It regulates the expression of a battery of genes encoding intracellular detoxifying enzymes and antioxidant proteins through the antioxidant response element (ARE) (Sykiotis and Bohmann, 2010). ARE-driven detoxification and antioxidant genes include NAD(P)H-quinone oxidoreductase 1 (NQO1), glutamate-cysteine ligase catalytic and modifier subunits (GCLC and GCLM), and heme oxygenase-1 (HO-1) (Jaiswal, 2004; Kobayashi and Yamamoto, 2005). Most recently, it has been reported that augmenter of liver regeneration (ALR) is also a target gene of NRF2 signaling pathway (Dayoub et al., 2013). Under basal conditions, NRF2 interacts with the actin-anchored protein Kelch-like ECH-associated protein 1 (KEAP1) and is localized in the cytoplasm. In response to oxidative/electrophilic stress, NRF2 is released from KEAP1 and translocates into the nucleus, and subsequently activates ARE-responsive gene expression (Kobayashi and Yamamoto, 2005). Several previous studies have demonstrated that activation of the NRF2 signaling pathway serves to protect animals

ABBREVIATIONS: ALR, augmenter of liver regeneration; ALT, alanine aminotransferase; APAP, acetaminophen; ARE, antioxidant response element; AST, aspartate aminotransferase; CDK4, cyclin D–dependent kinase 4; ERK, extracellular signal-regulated kinase; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; GSH, reduced glutathione; HO-1, heme oxygenase-1; JNK, c-Jun N-terminal protein kinase; KEAP1, Kelch-like ECH-associated protein 1; MDA, malondialdehyde; NAPQI, N-acetyl-p-benzoquinone imine; NQO1, NAD(P)H-quinone oxidoreductase 1; NRF2, nuclear factor erythroid 2–related factor 2; PCNA, proliferating cell nuclear antigen; ROS, reactive oxygen species; SIRT1, Sirtuin 1.
against liver injury produced by various hepatotoxicants, including APAP (Chan et al., 2001; Enomoto et al., 2001). Moreover, NRF2 has been shown to enhance cancer cell proliferation and improve the process of liver regeneration after partial hepatectomy (Beyer et al., 2008; Homma et al., 2009). However, whether KEAP1-NRF2-ARE signaling also participates in liver repair following APAP toxicity has not been determined.

p53 is a tumor suppressor that plays an important role in regulating cell growth, DNA repair, and apoptosis (Vogelstein et al., 2000). p53 is activated in response to a wide variety of stress types, which include but are not limited to DNA damage. Activated p53 can inhibit cell proliferation or trigger cell apoptosis, depending on the severity of DNA damage (Bensaad and Vousden, 2005). p21, one of the main effectors of p53, promotes cell cycle arrest and senescence through regulation of cyclin-dependent kinases and regulatory proteins. The importance of p21 for the regulation of liver regeneration was confirmed by several previous studies (Lehmann et al., 2012; Buitrago-Molina et al., 2013). Inhibition of p21 leads to hepatocyte proliferation in mice with severe injury and allows animal survival. It has been observed that APAP exposure induced p53 and p21 expression in C6 glioma cells and in mice (Ray et al., 2001; Chiu et al., 2003; Lee et al., 2006). However, the dynamic change of p53/p21 signal during APAP injury-responsive liver regeneration in mice remains unknown.

Furthermore, it has been shown that acute liver injury induced by moderate doses of APAP is a dynamic process including initiation of injury, progression of injury, and compensatory liver repair (Chiu et al., 2003; Mehdendale, 2005; Bajt et al., 2008). Therefore, we assumed that KEAP1-NRF2-ARE and p53/p21 signaling pathways would be regulated dynamically in a coordinated way during APAP injury-responsive liver regeneration. The aim of this study was to investigate whether the time profile and dynamic regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways was associated with compensatory liver regeneration following APAP-induced liver injury.

Materials and Methods

Reagents. Acetaminophen was obtained from Sigma-Aldrich (St. Louis, MO). Primary antibodies for Western blot analysis against total c-Jun N-terminal protein kinase (JNK), p-JNK, Sirtuin 1 (SIRT1), p-p38, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Cell Signaling Technology (Danvers, MA). Anti-GCLC, anti-p53, and anti-GCLM antibodies were from Santa Cruz Biotechnology (Dallas, TX). All other antibodies including KEAP1, extracellular signal-regulated kinase (ERK), phosphorylated ERK (p-ERK1/2), proliferating cell nuclear antigen (PCNA), cyclin D1, p38, cyclin D–dependent kinase 4 (CDK4), and ALR were provided by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). The secondary antibody was obtained from Cell Signaling Technology (Danvers, MA).

Animals. Male C57BL/6 mice (6–8 weeks old) were obtained from Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China) and maintained under controlled conditions (22–24°C, 55%–60% humidity, and 12-hour light/dark cycle) with free access to standard food and water. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People’s Republic of China (http://www.most.gov.cn). All animal protocols were approved by the Ethics Committee on the Care and Use of Laboratory Animals of Sun Yat-sen University.

All animals were fasted overnight before the experiments, since fasting allows lower doses of APAP to be used and results in less variation of the injury (Jaeschke et al., 2011). APAP solution was made fresh in 0.9% saline at 40 mg/ml, and mice were administered a single dose of 400 mg/kg APAP by intraperitoneal injection. All mice were killed at 0, 6, 12, 24, and 48 hours after APAP treatment. Serum samples and liver tissues were harvested. A portion of the liver was immediately fixed in 10% buffered formalin for histologic analysis and the remaining tissues were flash frozen in liquid nitrogen and stored at −80°C for further use.

Histologic Analysis. Liver tissues fixed in buffered formalin were embedded in paraffin, cut into 3-μm thick sections, stained with H&E, and then used to assess liver damage using a LEICA DM5000B microscope (Leica, Heidelberg, Germany). Three tissues collected at various time points were examined and found to exhibit similar injury for each treatment group at the same time point. Thus, one representative image was presented.

Biochemical Analysis. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured to indicate APAP-induced liver injury and/or liver recovery. ALT and AST activities of serum obtained at 0, 6, 12, 24, and 48 hours after APAP treatment were determined using a Beckman Synchrone CX5 Clinical System and a commercial reagent kit (Kefang Biotech, Guangzhou, China) according to the manufacturer’s protocol. To assess oxidative stress induced by APAP, levels of reduced glutathione (GSH), hydrogen peroxide (H2O2), and malondialdehyde (MDA) were also determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western Blot Analysis. Western blot analysis was performed as described in our previous report (Chen et al., 2014). Briefly, total liver or nuclear protein extracts were prepared from frozen liver tissues, and protein concentration was determined using the bichinchoninic acid method (Thermo Scientific, Rockford, IL). Equivalent amounts of protein extracts were separated via SDS-PAGE and then blotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Phosphorylated proteins were blocked with 5% bovine serum albumin in Tris-buffered saline, and other proteins with 5% nonfat milk in Tris-buffered saline. Membranes were incubated overnight at 4°C with primary antibodies, followed by secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Protein-antibody complexes were detected using an electrochemiluminescence kit (Engreen Biosystem, Beijing, China) and exposed to an X-ray film (GE Healthcare, Piscataway, NJ). The intensity of protein bands was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. Data are presented as the mean ± S.E.M. Statistical analysis was performed by one-way analysis of variance with Bonferroni’s multiple comparison test using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). P value less than 0.05 was considered statistically significant.

Results

APAP-Induced Liver Injury Is Followed by Compensatory Liver Regeneration in Mice. In this study, 400 mg/kg APAP treatment resulted in massive hepatic toxicity as revealed by H&E staining of liver sections (Fig. 1A) and increased serum ALT and AST activities (Fig. 1B). Centrilobular necrosis, clearly evident at 6 hours after APAP treatment, peaked between 12 and 24 hours. Compared with the first 24 hours, much less hepatocellular injury and necrosis was observed at 48 hours, but was not yet completely resolved. To assess liver injury after APAP treatment, time courses of serum ALT and AST activities were measured over 48 hours. The ALT and AST levels were increased in parallel to the area of hepatic necrosis. The ALT and AST activities were significantly elevated with a peak at 12 hours after APAP dosing, subsequently reduced at later time points, and then tended toward baseline levels by 48 hours as previously described (Bajt et al., 2008). These observations indicated that APAP-induced acute liver injury mainly occurred during the first 12 hours following APAP challenge. Furthermore, APAP treatment caused a significant decrease in total and mitochondrial GSH levels in livers at 6 hours and 12 hours after APAP treatment, suggesting an excess NAPQI generation leading to GSH depletion (Fig. 1C). However, levels of GSH were increased beyond 12 hours and restored at 48 hours. Levels of mitochondrial H2O2 and total liver MDA were rapidly elevated after APAP challenge (Fig. 1, D and E). Following the maximum levels at 6 hours, H2O2 and MDA levels subsequently fell and returned to normal by 48 hours, which reflected decreased oxidative stress. On the other hand, JNK activation is known to be a critical step in the intracellular signaling related to APAP-induced liver injury, along with activation of...
ERK1/2 (Gunawan et al., 2006; Saito et al., 2010). As expected, Western blot analysis of total and phosphorylated JNK indicated rapid activation of JNK in APAP-treated mice within 6 hours after APAP challenge (Fig. 2). However, the level of JNK phosphorylation drastically declined in a time-dependent manner at later time points and was low or undetectable at 48 hours. Elevated p-JNK levels were consistent with increased p-c-Jun levels, suggesting that p-JNK was correlated with enhanced activity. ERK1/2 was also significantly activated with an early peak of activation.

Fig. 1. APAP-induced liver injury was followed by compensatory liver regeneration in mice. (A) H&E-stained liver sections, (B) serum ALT and AST activities, (C) total liver and mitochondrial GSH levels, (D) mitochondrial $H_2O_2$ levels, and (E) total MDA levels from APAP-treated mice over a time course of 0–48 hours after 400 mg/kg APAP challenge. Data are expressed as the mean ± S.E.M. ($n = 5$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus 0 hour, #* $P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus 48 hours.
at 6 hours when mice were exposed to APAP. Interestingly, ERK2 (p42) appeared to be preferentially activated over ERK1 (p44) upon APAP exposure. Similarly to p-JNK, p-ERK1/2 levels declined to basal levels by 48 hours. Additionally, p38 mitogen-activated protein kinase was phosphorylated and active in normal liver but rapidly inactivated between 6 and 24 hours after APAP treatment and reactivated at 48 hours.

**Dynamic Regulation of KEAP1-NRF2-ARE Pathway Is Related to APAP Injury-Responsive Liver Regeneration.** The expression levels of proteins involved in KEAP1-NRF2-ARE pathway were measured to explore whether KEAP1-NRF2-ARE signaling pathway participated in compensatory liver regeneration following APAP-induced acute liver injury. The results showed that the level of KEAP1 protein was downregulated between 6 and 24 hours after APAP treatment but upregulated at 48 hours (Fig. 3). Treatment with 400 mg/kg APAP in mice significantly induced NRF2 expression with a peak at 12 hours and facilitated nuclear accumulation of NRF2 with a peak at 6 hours as previously described (Goldring et al., 2004; Copple et al., 2008). The protein expression of NRF2 target genes, including *Ho-1*, *Nqo1*, *Gclm*, *Gclc*, and *Alr*, in APAP-treated mice was also examined using Western blotting. Compared with basal levels observed in uninjured livers, APAP exposure first reduced HO-1 protein levels between 6 and 24 hours, followed by a marked induction at 48 hours. GCLM levels remained unchanged during the first 12 hours after APAP challenge, and then declined significantly at 24 hours. The expression of NQO1 was reduced at 6 hours and 12 hours. Both NQO1 and GCLM levels were upregulated at 48 hours after APAP treatment. Additionally, a biphasic regulation of ALR by APAP was also observed, including inhibition phase from 6 to 24 hours and induction phase between 24 and 48 hours during APAP injury-responsive liver regeneration. However, the protein expression of GCLC presented a single-phase upregulation over a time course of 0–48 hours following APAP challenge. The present data implied that NRF2 target genes were suppressed at the early stage of APAP-induced liver injury but upregulated at a later stage.

**Dynamic Regulation of p53/p21 Signaling Pathway Is Associated with Compensatory Liver Regeneration Following APAP-Induced Liver Injury.** To explore whether p53/p21 signaling was associated with liver repair after APAP-induced liver injury in mice, p53 and p21 expression was analyzed. p53 was normally maintained at low levels in uninjured livers. As expected, APAP treatment induced p53 expression with the peak p53 at 6 hours and 12 hours (Fig. 4). Beyond 12 hours, p53 levels were markedly reduced. Similar to p53, p21 showed a significant induction at 6 hours after APAP exposure followed by a time-dependent decline at later time points, and returned to normal levels at 48 hours. Treatment with APAP
resulted in an inhibition of SIRT1 expression in a time-dependent manner. Furthermore, the expression of cyclin D1, CDK4, and PCNA in APAP-injured livers was measured. Treatment with APAP resulted in a decrease in cyclin D1 and PCNA levels. However, there was a further significant decrease of cyclin D1 protein expression at 24 hours followed by an increase to baseline levels by 48 hours. PCNA levels were also increased at 48 hours compared with other time points at early stages but not completely restored to normal levels observed in uninjured livers. The protein expression of CDK4 was unchanged between 0 and 24 hours after APAP overdose, but significantly upregulated at 48 hours. Detailed analysis of cell proliferation-associated protein levels over a time course of 0 to 48 hours revealed that the mice experienced transient impairment of hepatocyte proliferation at the early stage of APAP toxicity, and this inhibition was overcome by 48 hours, leading to compensatory liver regeneration.

Discussion

APAP overdose is the leading cause of drug-induced liver injury and a significant public health concern. Previous studies have revealed that compensatory tissue repair following toxicant challenge plays a crucial role in the final outcome of liver injury, either recovery from injury or injury progression leading to liver failure (Mehendale, 2005; Bajt et al., 2008). The current study evaluated the role of dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 pathways in APAP injury-responsive liver regeneration.

The results showed that the area of hepatic necrosis and activities of ALT and AST increased significantly during the first 12 hours, suggesting initiation and progression of liver injury, and subsequently declined at later time points, reflecting regression of injury. In addition, a decline of oxidative stress was observed, as demonstrated by increased hepatic and mitochondrial GSH levels and decreased mitochondrial H2O2, liver MDA, p-JNK, p-c-Jun, and p-ERK1/2 levels beyond 12 hours. p38 mitogen-activated protein kinase, involved in the liver regeneration networks, was rapidly inactivated after APAP overdose and then reactivated at 48 hours, which was supported by previous observations related to the regenerating liver after partial hepatectomy (Liao et al., 2004). These results suggest that compensatory liver regeneration occurs at later stage of liver injury induced by APAP (Fig. 5).

Further results clearly demonstrated that treatment with 400 mg/kg APAP resulted in KEAP1 inhibition and NRF2 upregulation between 6 and 12 hours. Despite the marked NRF2 nuclear translocation occurring after APAP challenge, none of four NRF2 target genes was significantly induced during this period. In contrast, HO-1, NQO1, and GCLM levels were downregulated during the first 24 hours. Translocation of NRF2 was uncorrelated with unchanged and/or decreased expression of these NRF2 downstream target genes, suggesting that...
there were some delays between NRF2 nuclear translocation and its target gene induction and protein translation. However, during the later stage of liver injury, the protein expression of detoxification and antioxidant genes \( \text{Ho-1, Nqo1, Gclc, and Gclm} \) was significantly induced to promote liver repair. Glutamate cysteine ligase, composed of a catalytic (GCLC) and a modifier (GCLM) subunit, catalyzes the rate-limiting step in GSH biosynthesis. Thus increased GSH levels at the later stage were correlated with significantly upregulated GCLC and GCLM expression. Our data indicated that two stages, including induction and repression phases in expression of KEAP1 and NRF2 target genes, were present during APAP-induced liver injury in mice. The KEAP1-NRF2-ARE signaling pathway may regulate an adaptive response to protect against oxidative damage following APAP challenge.

In addition, ALR was identified to promote liver regeneration after partial hepatectomy (Polimeno et al., 2011) and to augment hepatocyte proliferation when the regenerative process was primed (Francavilla et al., 1994). ALR may exhibit a beneficial effect via NRF2 pathway on compensatory liver regeneration following APAP-induced liver injury.

On the other hand, the expression of p53 and p21 was significantly induced after APAP challenge and subsequently downregulated to normal levels at 48 hours. SIRT1 was reported to regulate acetylation of p53 on Lys382 residue and p53-dependent apoptosis (Cheng et al., 2003). Very recently, SIRT1 has been identified as a regulator of the regenerative response in the liver through farnesoid X receptor and mammalian target of rapamycin signaling pathway (García-Rodríguez et al., 2014). Whether SIRT1 expression is affected by APAP is not yet reported. In the present study, a decrease in SIRT1 levels followed by elevated p53 and p21 levels was observed upon APAP exposure. Additionally, JNK phosphorylation induced by APAP can activate and stabilize p53, leading to reduced ubiquitination and proteasomal degradation, as previously described (Fuchs et al., 1998). Our observations showed a substantial overexpression of p53 and p21 during the first 24 hours, which may be the reason for the inhibition of cell cycle activation as indicated by decreased cyclin D1 and PCNA expression. Levels of p53 and p21 declined between 24 and 48 hours, but cyclin D1, CDK4, and PCNA expression rapidly increased during this period, which allowed regeneration to proceed in APAP-treated mice. A previous study showed that the expression of cyclin D1 mRNA and PCNA protein showed a significant increase at 24 hours following 200 mg/kg APAP treatment (Bajt et al., 2008), which is different from our observations. This can be explained by the difference in APAP doses, since tissue repair is a toxicant dose-dependent dynamic process (Mehendale, 2005). With each increment in APAP dose, a corresponding delay in the onset of tissue repair would be observed. APAP overdose resulted in a strong and robust induction of p53/p21 in mice with acute liver injury during the early time points, which would subsequently impair liver regeneration. When injury regression occurred at later time points, levels of p53 and p21 were returned to baseline. Together, these data indicate that the degree of liver injury is related to the strength of p53/p21 induction in the liver and its effect on hepatocyte proliferation. Recently, a correlation between NRF2 and p53 in response to oxidative stress has been reported (Faraonio et al., 2006; You et al., 2011; Chen et al., 2012). In this study, at the early stage of liver injury caused by APAP, high levels of ROS may induce p53 expression and activation, which would subsequently inhibit a battery of cytoprotective genes mediated by NRF2, such as \( \text{Ho-1, Nqo1, Gclm, and Alr} \).
and promote cell cycle arrest and apoptosis. Under low levels of ROS at the later stage, normal levels of p53/p21 could induce expression of antioxidant genes such as NRF2 target genes to protect against ROS-mediated damage and restore cell cycle progression. Collectively, our results suggest that dynamic and coordinated regulation of KEAP1-NRF2-ARE and p53/p21 signaling pathways is associated with compensatory liver regeneration following APAP-induced liver injury in mice.

**Authorship Contributions**

**Participated in research design:** Bi, Huang, Fan.

**Conducted experiments:** Fan, Chen, Tan, Zeng, Yi, Wang.

**Contributed new reagents or analytic tools:** Yo, Wang, Hou.

**Performed data analysis:** Fan, Bi.

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