Research Article

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Removal of acetaminophen protein adducts by autophagy protects against acetaminophen-induced liver injury in mice

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Background & Aims: Acetaminophen (APAP)-induced liver injury is the most frequent cause of acute liver failure in the US and many other countries. Metabolism of APAP results in formation of APAP protein adducts (APAP-AD) in hepatocytes and triggers mitochondrial dysfunction and necrosis. However, the mechanisms for how APAP-AD are removed from hepatocytes remain unknown.

Methods: Mice or primary hepatocytes were treated with APAP. APAP-AD were determined by immunoblot, immunostaining and high pressure liquid chomatography with electrochemical detection analysis.

Results: We found that APAP-AD were detected at 1 h, peaked at approximately 2 h, declined at 6 h and almost full removed at 24 h post treatment with APAP in mouse livers and in primary mouse hepatocytes. APAP-AD displayed a punctate pattern and were colocalized with GFP-LC3 positive autophagosomes and Lamp1 positive lysosomes in APAP-treated primary hepatocytes. Moreover, isolated autophagosomes and autolysosomes from APAP-treated mouse livers contained APAP-AD, suggesting autophagy may selectively remove APAP-AD. APAP-AD were detected in both detergent soluble and insoluble pools in APAP-treated mouse livers and hepatocytes. More importantly, pharmacological inhibition of autophagy by leupeptin or chloroquine increased whereas induction of autophagy by Torin 1 decreased serum APAP-AD levels in APAP-treated mice, which correlated with alanine aminotransferase levels and liver necrosis. Furthermore, SQSTM1/p62, an autophagy receptor protein, was recruited to APAP-AD. Adenovirus-mediated shRNA knockdown of SQSTM1/p62 led to increased APAP-AD and necrosis in primary hepatocytes.

Conclusions: Our data indicate that APAP-AD are removed though selective autophagy. Pharmacological induction of autophagy may be a novel promising approach for treating APAP-induced liver injury.

Lay summary: Acetaminophen overdose can form acetaminophen protein adducts and mitochondria damage in hepatocytes resulting in liver injury. Activation of autophagy-lysosomal degradation pathway can help to remove acetaminophen protein adducts. Pharmacological induction of autophagy may be a novel promising approach for treating APAP-induced liver injury.

Keywords: Acetaminophen; Autophagy; p62/SQSTM1; Acetaminophen protein adducts; Liver injury.

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Abbreviations: AP, autophagosome; APAP, acetaminophen; APAP-AD, acetaminophen protein adducts; AMAP, 3′-hydroxyacetanilide; ALT, alanine aminotransferase; CQ, chloroquine; CYP2E1, cytochome P450 2E1; GSH, glutathione; HPLC-ED, high pressure liquid chomatography with electrochemical detection; LC3, microtubule-associated protein light chain 3; Leu, leupeptin; Ly, lysosome; NAPQI, N-acetyl-p-benzoquinone imine; mTOR, mechanistic target of rapamycin; NAC, N-acetylcysteine; PI, propidium iodide.

Introduction

Acetaminophen (APAP) is a safe drug at therapeutic levels, but an overdose can cause severe liver injury and even acute liver failure in animals and man [1]. It is well established that the formation of a reactive metabolite, presumably N-acetyl-p-benzoquinone imine (NAPQI), which causes the depletion of cellular glutathione (GSH) and APAP protein adduct (APAP-AD) formation, is critical for the initiation of toxicity [1–4]. Initially, it was hypothesized that protein binding directly causes cell death [5]. However, this hypothesis was later refined by suggesting that critical proteins within cells were being adducted. Despite substantial efforts and some success in identifying APAP-AD, consequences of protein binding on certain enzyme activities in the cell could not explain the massive cell necrosis observed after APAP overdose [6,7]. However, it was recognized that APAP overdose causes mitochondrial dysfunction, which is critical to trigger hepatocyte necrosis and subsequent liver injury [8–10]. The effects on mitochondria are thought to be initiated by APAP-AD formation. This hypothesis originated from the comparison between APAP, where mitochondrial APAP-AD and toxicity were observed, and 3′-hydroxyacetanilide (AMAP), a non-hepatotoxic regioisomer of APAP, which shows similar overall protein binding in the mouse liver but no adducts in mitochondria [11]. More importantly, similar to the mouse model, APAP-AD are formed in patients after APAP overdose [12], and we demonstrated that mitochondrial damage is an important feature of APAP-induced liver injury in patients [8,13]. Thus, APAP-AD formation is the critical initiation event for mitochondrial damage, which is vital for the amplification of cell death signaling events that determine

APAP-induced necrosis and liver injury [14]. Therefore, timely removal of APAP-AD and damaged mitochondria to maintain normal mitochondrial function and provide sufficient ATP is a critical process necessary for the recovery of mice and humans from APAP intoxication [15,16]. Despite the importance of APAP-AD formation, how hepatocytes remove APAP-AD is unknown.

Cells can respond and adapt to various forms of stress, which may limit the extent of cell death. One adaptation and protective mechanism to control cellular stress, protein homeostasis and maintain mitochondrial quality is autophagy. Autophagy is a highly conserved intracellular degradation pathway, which is activated in response to adverse environmental conditions, such as the deprivation of nutrients or growth factors, as a survival mechanism [17,18]. Autophagy protects cells by selectively removing toxic protein aggregates and damaged mitochondria [19–21]. We previously demonstrated that APAP activates autophagy in primary cultured hepatocytes and in mouse livers to remove damaged mitochondria [21–23]. However, whether APAP-induced autophagy can also help remove APAP-AD is not known. Therefore, our goal in the present study was to assess the role and mechanisms of APAP-induced autophagy in removal of APAP-AD in primary cultured mouse and human hepatocytes as well as in a mouse model.

Materials and methods

Antibodies and reagents

Antibodies used in the study were p62 (Abnova), β-Actin (Sigma), GAPDH (Cell Signaling), Lamp1 (Developmental Studies Hybridoma Bank, Iowa City, IA), and the rabbit anti-APAP-adducts antibody was kindly provided by Dr. Lance Pohl (National Heart, Lung and Blood Institute) [24]. The rabbit polyclonal anti-LC3 antibody was described previously [25]. The secondary antibodies and other reagents were described in the Supplementary materials and methods.

Animal experiments

Wild-type mice were maintained in a C57BL/6 background. All animals received humane care and the procedures were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center. Mice were either given saline (i.p.) or APAP (500 mg/kg, i.p.). Mice were sacrificed at 0.5, 1, 2, 6 and 24 h. In some experiments, mice were treated with chloroquine (CQ, 60 mg/kg, i.p.) or leupeptin (Leu, 40 mg/kg, i.p.) simultaneously with APAP (500 mg/kg) for 3 and 6 h, respectively. For post treatment experiments, mice were either given saline or APAP (500 mg/kg, i.p) for 2 h followed by administration of vehicle (4% methyl-β-cyclodextrin in saline, i.p.) or Torin 1 (2 mg/kg, dissolved in 4% methyl-β-cyclodextrin in saline, i.p.) for another 6 h. Liver injury was assessed by the determination of serum alanine aminotransferase (ALT) activity and hematoxylin and eosin staining of liver sections as we described previously [22]. Total liver lysates were prepared using Radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl (lauryl) sulfate).

Acquisition of human samples

Blood samples were obtained from patients admitted to the University of Kansas Hospital in Kansas City, Kansas following APAP overdose and from healthy volunteers, as previously described [13].

Hepatic GSH content and APAP protein adducts measurement

GSH was measured using a modified Tietze assay, as described [26]. APAP-cysteine (APAP-Cys) adducts were measured by high pressure liquid chomatography (HPLC) with electrochemical detection (ED) as we described previously [23]. Namely, sample dialysis was replaced with a rapid gel-filtration method to remove free APAP and APAP-GSH prior to digestion and analysis. Protein levels were determined using a standard bicinchoninic acid assay, and adducts were normalized to total protein in the liver homogenates.

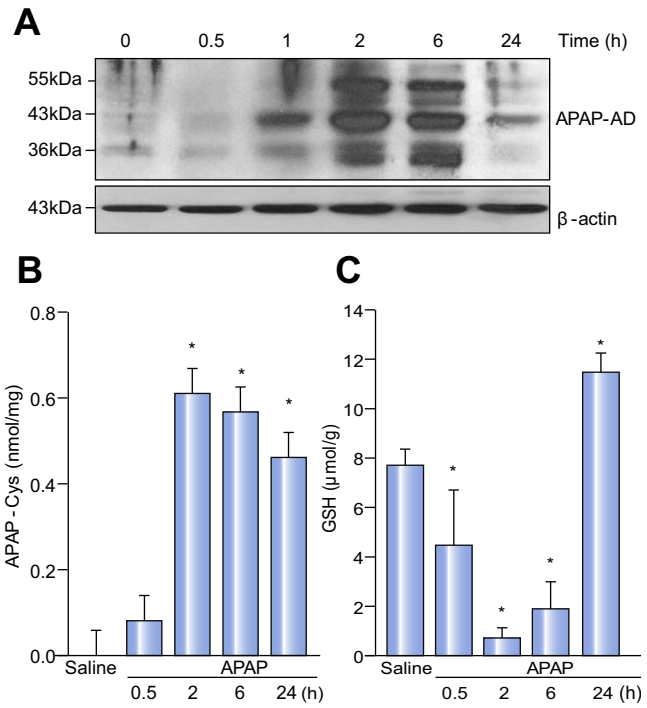

Fig. 1. The dynamic change of APAP-AD levels in mouse livers. Male C57BL/6J mice were treated with APAP (500 mg/kg) for different time points and pooled liver lysates from 3–4 mice of each group were subjected to Western blot analysis (A) or HPLC-ED analysis (B) for APAP-AD (means ± SE, n = 3–4). (C) Mice were treated as in (A) and total hepatic GSH contents were determined and data are presented as means ± SE (n = 3–5). *p <0.05. One-way ANOVA analysis with Scheffé's post hoc test.

Primary hepatocytes culture

Murine and human hepatocytes were isolated by a retrograde, nonrecirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma) as described previously [27,28]. All cells were maintained in a 37 °C incubator with 5% CO_2.

Immunoblot analysis for autophagy markers and APAP-AD from human blood samples

Twenty micrograms of serum proteins from healthy human volunteers and APAP-exposed humans were subjected to immunoblot analysis for APAP-AD and LC3. Human blood samples were obtained from APAP overdose patients and healthy volunteers as described previously [13].

Cellular fractionation for autophagosomes and lysosomes, preparation for detergent soluble and insoluble liver fractions, immunoblot assay, electron microscopy and fluorescence confocal microscopy were described in detail in Supplementary materials and methods.

Statistical analysis

Experimental data were subjected to Student *t* test or one-way analysis of variance analysis with Scheffé's post hoc test where appropriate.