Research Article

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CHOP is a critical regulator of acetaminophen-induced hepatotoxicity

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Background & Aims: The liver is a major site of drug metabolism and elimination and as such is susceptible to drug toxicity. Drug induced liver injury is a leading cause of acute liver injury, of which acetaminophen (APAP) is the most frequent causative agent. APAP toxicity is initiated by its toxic metabolite NAPQI. However, downstream mechanisms underlying APAP induced cell death are still unclear. Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) have recently emerged as major regulators of metabolic homeostasis. UPR regulation of the transcription repressor CHOP promotes cell death. We analyzed the role of UPR and CHOP in mediating APAP hepatotoxicity.

Methods: A toxic dose of APAP was orally administered to wild type (wt) and *CHOP* knockout (KO) mice and damage mechanisms were assessed.

Results: *CHOP* KO mice were protected from APAP induced damage and exhibited decreased liver necrosis and increased survival. APAP metabolism in *CHOP* KO mice was undisturbed and glutathione was depleted at similar kinetics to wt. ER stress and UPR activation were overtly seen 12 h following APAP administration, a time that coincided with strong upregulation of CHOP. Remarkably, *CHOP* KO but not wt mice exhibited hepatocyte proliferation at sites of necrosis. *In vitro*, large T immortalized *CHOP* KO hepatocytes were protected from APAP toxicity in comparison to wt control cells.

Conclusions: CHOP upregulation during APAP induced liver injury compromises hepatocyte survival in various mechanisms, in part by curtailing the regeneration phase following liver damage. Thus, CHOP plays a pro-damage role in response to APAP intoxication.

Introduction

The liver is a major site for drug metabolism and elimination, and as such is susceptible to drug toxicity [1]. In fact, drug induced liver injury (DILI) has become a leading cause of acute liver failure and transplantation in developed countries [2]. The mechanism of hepatotoxicity associated with most drugs is idiosyncratic and animal models are lacking, making research in this field a challenging task. Only a handful of drugs exhibit type A toxicity, which is characterized by dose dependent tissue damage. Of the various type A hepatotoxic drugs, acetaminophen (N-acetyl-p-aminophenol, APAP) overdose is the leading cause of drug-induced acute liver failure in the western world [3], and one of few hepatotoxic drugs that has been studied in animal models.

APAP toxicity stems from its cytochrome P450-dependent metabolism to N-acetylbenzoquinoneimine (NAPQI). NAPQI is a powerful electrophile and is detoxified by glutathione (GSH). When GSH is depleted, NAPQI accumulates, forming covalent bonds with cysteine groups on hepatocyte macromolecules, thereby interfering with their function [4]. The exact mode of cell death in APAP-induced DILI is still not fully understood. It is clear that APAP toxicity involves massive necrosis of liver parenchyma. However, it is not clear whether apoptosis participates in, and

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Abbreviations: DILI, drug induced liver injury; APAP, acetaminophen (N-acetyl-p-aminophenol); NAPQI, N-acetylbenzoquinoneimine; GSH, glutathione; ER, endoplasmic reticulum; UPR, unfolded protein response; ALI, acute liver injury; ROS, reactive oxygen species; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

what is the exact role of non-parenchymal cells in the pathogenesis of APAP induced liver injury [5,6].

While it was initially postulated that following GSH depletion hepatocytes undergo necrosis due to oxidative stress and mitochondrial damage [7], recent evidence indicates the involvement of pro-apoptotic factors in APAP toxicity, such as Bim and CXCR2 [8]. It was further shown that innate immunity via Toll-like receptors (TLRs), dendritic cells, and autophagy are regulators of liver induced damage following APAP overdose [9–11].

The endoplasmic reticulum (ER) is the major cellular site of protein folding and modification. ER stress occurs when the amount of protein entering the ER exceeds its folding capacity. This imbalance induces a cyto-protective reaction collectively termed the unfolded protein response (UPR) [12]. The mammalian UPR is transduced by three major sensors (IRE1, PERK, and ATF6) that reside in the ER and undergo activation under ER stress conditions. IRE1 and PERK are activated by autophosphorylation, while ATF6 is activated by intra-membrane cleavage, which releases its N-terminal fragment for transcription transactivation. Activated IRE1 splices the mRNA of *XBP1* in a non-canonical fashion, yielding the potent transcription factor XBP1s [13,14]. Activated PERK phosphorylates eIF2α, inducing selective translation of ATF4 and transcription of *ATF3* and CCAAT-enhancer-binding protein homologous protein (*CHOP*) in a sequential manner [15].

Activation of the UPR leads initially to attenuation of protein synthesis and protein translocation into the ER, thus preventing further accumulation of misfolded proteins. This initial step is followed by an increase in the capacity of the ER to handle unfolded proteins. If the stress is not relieved in a timely fashion, cell death is triggered in an intricate mechanism that involves caspase activation, calcium leakage from the ER and mitochondrial damage [16]. One of the main elements in the program of ER stress mediated apoptosis is CHOP, a transcription repressor that is activated downstream of the PERK and IRE1 pathways of the UPR. Once activated, CHOP translocates to the nucleus, inhibits the expression of anti-apoptotic genes, and activates pro-apoptotic genes [17]. CHOP expression in the liver has been demonstrated to respond to various types of stress modules, such as LPS and CCl_4 treatment [18,19]. Moreover, deletion of *CHOP* protects mice from various liver-specific challenges, such as diet-induced steatohepatitis, bile duct ligation, and alcohol intoxication [20–22]. Because CHOP is not restrictively regulated by the UPR, it is not clear whether ER stress is also involved in these types of injury. Nonetheless, these studies implicate CHOP as a factor that mediates liver damage following diverse types of stress responses.

While Nagy *et al.* observed the induction of ER stress following APAP administration [23,24], a recent study by Hur *et al.* did not observe any signs of UPR activation [25]. Both studies administered APAP by i.p. injection, a route not relevant to the clinical use of the drug. Here we followed UPR activation following oral administration of APAP. ER stress and UPR activation were observed as a late event in the cascade of responses activated by APAP and coincided with CHOP upregulation. Furthermore, *CHOP* knockout, both *in vitro* and *in vivo*, conferred a survival advantage, possibly mediated by increased proliferation. Our data implicates CHOP as a critical regulator of APAP toxicity.

Materials and methods

Mice and induction of APAP toxicity

C57BL/6J and *CHOP* knockout mice (on a C57BL/6J background) were purchased from Jackson Laboratories. Mice harboring a conditional floxed allele of *XBP1* were crossed to the Alb-Cre expressing strain to obtain a liver specific knockout of *XBP-1* (Alb-Cre/*XBP-1*f/f). Animal care and experiments were approved and conducted in accordance with the Hebrew University-Hadassah Animal Authority guidelines. Male mice 10–12 weeks old, 24–26 g were fasted for 10 h at night, and then administered 500 mg/kg acetaminophen commercial drops or vehicle by intragastric gavage. Both APAP and vehicle were provided by the manufacturer (Novimol™ drops, CTS, Israel) ensuring the proper control. The mice were kept fasted for another 4 h, and then allowed food *ad libitum*. Mice were sacrificed 24 h after APAP administration. Control mice were treated i.p. with tunicamycin (Fermentek, Jerusalem, Israel) injection of 1 mg/kg (volume of 20 μl/1 mouse). Following sacrifice, liver samples were immediately harvested into liquid nitrogen and kept at −80 °C for protein and RNA extraction. A portion was fixed in 4% formaldehyde for 24 h, and then kept in 70% ethanol or in 30% sucrose for immunohistochemistry.

Reagents and antibodies, Western blot procedures, luciferase live imaging, large T immortalization of primary hepatocytes and their *in vitro* challenge with APAP are described in the Supplementary information.

Liver enzyme assessment

Serum was collected, and stored at −20 °C. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using Reflotron sticks (Roche Pharmaceuticals, Israel), with a Reflotron reader.

RT-PCR and quantitative RT-PCR

Total cellular RNA was isolated with Trizol reagent (Prio-Lab, Jerusalem, Israel) according to the manufacturer instructions. For reverse transcription, 0.5 μg of total RNA was transcribed using the cDNA synthesis kit (Applied Biosystem, CA, USA). Aliquots of 1 μl cDNA were subjected to 35 cycles of PCR amplification. *Actin* or *Ubc* were used as internal control. The primer sequences are shown in Table 1 (each primer pair was designed to span an intron). Negative controls included the amplification of samples without a prior RT reaction.

Quantitative RT-PCR was performed in the PCR 7900HT Real-Time PCR System (Applied Biosystems) using SYBR Green mix (Applied Biosystems).

Immunohistochemical staining

Liver samples were fixed in 4% neutral-buffered formalin and embedded in paraffin. 5 μM sections were dewaxed and hydrated through graded ethanol dilutions then cooked in citrate buffer (pH 6, 10 mM) in a pressure cooker at 115 °C for 3 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide followed by washing. The sections were then incubated with the indicated antibodies. Secondary Ab exposure was performed using the Mach 3 Rabbit tool kit (Biocare Medical, CA, USA) following the manufacturers' instructions. All sections were counterstained with hematoxylin and were visualized using an Olympus light microscope. Quantification was performed by visualizing the slides on an Olympus BX61 microscope, photography was performed on Photometric CoolSnap ES and analysis was done using Ariol SL-50 Applied Imaging 3.1.270 software.

CYP2E1 activity assay

Microsomes were prepared from liver hepatocytes. Briefly, liver samples were homogenized in cold homogenizing buffer (Tris 0.1 M, EDTA 10 mM, KCl 0.15 M), using motor driven homogenizer, and were centrifuged (12,500*g*, 15 min). Supernatants were ultra-centrifuged (105,000*g*, 70 min) and the pellet was suspended in cold pyrophosphate buffer (Sodium pyrophosphate 0.1 M, pH 7.4, EDTA 10 mM). Samples were ultracentrifuged again (105,000*g*, 45 min) and pellets were homogenized and kept in microsomes buffer (Tris 0.05 M pH 7.5, EDTA 10 mM, glycerol 20%).

Cyp2e1 activity: microsomes (20 μg) were placed in Reaction buffer (KPi 0.1 μM pH 7.4, p-nitrophenyl 0.4 mM). Reaction was induced using NADPH (1 mM) for 60 min, and stopped using 30% tricholoroacetic acid. Blanks had no