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PUMA induction mediates acetaminophen-induced necrosis and liver injury

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Abstract

Acetaminophen (APAP) overdose is one of the leading causes of hepatotoxicity and acute liver failure in the United States. Accumulating evidence suggests that hepatocyte necrosis plays a critical role in APAP-induced liver injury. However, the mechanisms of APAP-induced necrosis and liver injury are not fully understood. In this study, we found that p53 up-regulated modulator of apoptosis (PUMA), a BH3-only Bcl-2 family member, was markedly induced by APAP in the mouse livers and in isolated human and mouse hepatocytes. *PUMA* deficiency suppressed APAP-induced mitochondrial dysfunction and release of cell death factors from mitochondria, and protected against APAP-induced hepatocyte necrosis and liver injury in mice. PUMA induction by APAP was p53-independent, and required RIP1 and JNK via transcriptional activation. Furthermore, a small-molecule PUMA inhibitor, administered after APAP treatment, mitigated APAP-induced hepatocyte necrosis and liver injury. Conclusions: Our results demonstrate that RIP1/JNK-dependent PUMA induction mediates APAP-induced liver injury by promoting hepatocyte mitochondrial dysfunction and necrosis, and suggest that PUMA inhibition is useful for alleviating acute hepatotoxicity due to APAP overdose.

Keywords

acetaminophen; liver injury; necrosis; PUMA; mitochondria

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Introduction

Acetaminophen (APAP) is one of the most commonly used drugs for minor aches, pains, and fever. However, APAP overdose can cause severe liver injury and even death, and is the leading cause of drug-induced acute liver failure in the United States.⁽¹⁾ APAP is metabolized in hepatocytes and converted by cytochrome P-450 2E1 (CYP2E1) to N-acetyl-p-benzoquinone imine (NAPQI), which covalently binds to intracellular proteins including mitochondrial proteins to form APAP adducts.⁽²⁾ The formation of APAP adducts in the mitochondria leads to mitochondrial dysfunction, generation of reactive oxygen species (ROS), release of mitochondrial cell death factors such as apoptosis inducing factor (AIF) and endonuclease G (Endo G), resulting in nuclear DNA fragmentation and hepatocyte death.⁽³⁾

Despite numerous studies, the mechanisms of APAP-induced liver injury and hepatocyte death remain to be elucidated. Apoptosis and caspase activation are known to be dispensable for APAP-induced liver injury.^(3, 4) Necrosis is considered as the major mode of cell death in APAP-induced liver injury.^(3, 5) Necrosis is a caspase-independent process characterized by plasma membrane swelling and release of damage associated molecular pattern (DAMP) molecules such as high-mobility group box 1 (HMGB1).⁽⁶⁾ A form of programmed necrosis, known as “necroptosis”, has recently been defined,⁽⁷⁾ which is regulated by receptor-interacting protein kinase 1 (RIP1), RIP3, and mixed lineage kinase domain-like protein (MLKL) upon stimulation by TNF- α or Fas ligand in some cells. Although RIP1 is clearly involved, RIP3 and MLKL appear to be dispensable for APAP-induced necrosis and liver injury.^(8, 9)

c-Jun NH2-terminal kinase (JNK), a member of stress kinases critical for APAP-induced liver injury and cell death, was shown to be activated by RIP1.^(8, 9) Upon RIP1-mediated phosphorylation and mitochondrial translocation,⁽¹⁰⁾ JNK can activate dynamin-related protein 1 (Drp1) to promote mitochondrial fission and dysfunction.⁽⁹⁾ JNK also acts on the Bcl-2 family members to regulate hepatocyte death.⁽¹⁰⁾ However, key mediators of APAP-induced necrosis downstream of RIP1 and JNK have remained unclear.

p53 up-regulated modulator of apoptosis (PUMA) is a pro-apoptotic BH3-only Bcl-2 protein essential for DNA-damage-induced and p53-dependent apoptosis.⁽¹¹⁾ PUMA is induced upon stresses and promotes cell death and tissue injury by binding to and neutralizing Bcl-2, Bcl-X_L, and other pro-survival Bcl-2 family members.⁽¹¹⁾ Our previous studies showed that PUMA is induced by diethylnitrosamine (DEN) in hepatocytes via a JNK-dependent mechanism, which promotes hepatocyte apoptosis, compensatory proliferation, and subsequent hepatocarcinogenesis.⁽¹²⁾ PUMA also has a novel activity of promoting necroptosis, which does not involve Bax/Bak-dependent mitochondrial outer membrane permeabilization (MOMP) and caspase activation.⁽¹³⁾

In this study, we found that PUMA expression is markedly elevated in the livers of APAP-treated mice through a p53-independent but RIP1/JNK-dependent mechanism. PUMA deficiency strongly suppressed APAP-induced and RIP1/JNK-dependent hepatocyte necrosis and liver injury in mice. We further demonstrated that pharmacological intervention using a

small-molecule PUMA inhibitor ameliorated liver damage caused by APAP overdose in mice.

Materials and Methods

Animals and treatment

All animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh. *PUMA* knockout (KO) (*PUMA*^{-/-}) and *p53* KO (*p53*^{-/-}) mice (Jackson Laboratory) on the C57BL/6J background were previously described,⁽¹²⁾ and were generated from heterozygous intercrosses. Wild-type (WT) littermates from the breeding were used as control mice. Mice were housed in micro-isolator cages in a room illuminated from 7:00 AM to 7:00 PM (12:12-hr light/dark cycle) and were allowed access to water and chow *ad libitum*.

APAP was dissolved in warm saline (0.9 % NaCl; 55°C) and cooled to 37°C before injection. Mice fasted overnight were intraperitoneally (IP) injected with APAP at 250 or 500 mg/kg. Inhibitors, including the RIP1 inhibitors necrostatin 1 (Nec-1) and 7-Cl-O-Nec1 (Nec-1s), the pan-caspase inhibitor z-VAD-fmk (z-VAD), the JNK inhibitor SP600125,⁽¹²⁾ and the PUMA inhibitor (PUMAi; ChemBridge),⁽¹⁴⁾ were dissolved in DMSO into stock solutions, and diluted with 1% DMSO in saline to final concentrations. Inhibitor treatment was performed by IP injection of Nec-1 (10 mg/kg), z-VAD (10 mg/kg), or SP600125 (20 mg/kg) at 2 hr before APAP injection, Nec-1s (5 mg/kg) at 30 min before APAP injection,⁽¹⁵⁾ or PUMAi (10 mg/kg) at 2 hr after APAP injection. To minimize the previously described inhibitory effect of DMSO on APAP-induced liver injury⁽¹⁶⁾, each mouse was injected with inhibitors diluted in 1% DMSO in 100-μl volume. Control mice for the inhibitor experiments, including those with or without APAP treatment, all received injection of vehicle (1% DMSO in 100 μl).

Analysis of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST)

Venous blood of mice was taken from the tail vein at different time points after APAP treatment. Serum ALT and AST levels were measured using the Infinity ALT Kit (Thermo Scientific) and AST Kit (Bioo Scientific), respectively. Briefly, 10-μL serum was added into 100-μL ALT or AST reagent, followed by reading of 340 nm at 37°C using a Wallac Victor 1420 Multilabel Counter microplate reader (PerkinElmer). Each sample was measured in triplicate.

Primary hepatocyte culture

Mouse and human hepatocytes were isolated by a retrograde, non-recirculating perfusion of livers with 0.05% Collagenase Type IV (Sigma Aldrich) as previously described.^(17, 18) Mouse hepatocytes were cultured in Williams' medium E with 10% fetal bovine serum without other supplements for 2 hr to allow for attachment. Human hepatocytes were isolated and cultured according to previously described methods.⁽¹⁹⁾ All human liver specimens were obtained in accordance with the University of Kansas Medical Center Human Subjects Committee approved protocol #13513. Cultured hepatocytes were maintained in a 37°C incubator with 5% CO₂. Hepatocytes were treated in 12-well plates

with APAP diluted in saline, in the presence or absence of the RIP1 or JNK inhibitor diluted in 1% DMSO. Vehicle alone was used as control.

Western blotting

Total proteins were prepared from freshly isolated liver tissues. To analyze individual mice, 300 mg of liver samples from each of at least 3 mice in a treatment group were used to prepare protein lysates. In some cases, pooled samples were prepared by pooling and homogenizing together liver samples (100 mg per mouse) from randomly selected 4 mice in a treatment group. Tissues were minced and homogenized in 1-mL homogenization buffer (0.25 M sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid, 1 mM ethylene glycol tetraacetic acid). After centrifugation at 10,000× g for 10 min, supernatant was collected and analyzed by SDS-PAGE. Western blotting was performed as previously described.⁽²⁰⁾ Antibodies included those from commercial sources that are listed in Supporting Table S1, and rabbit antibodies for hPUMA,⁽²¹⁾ Bid,⁽¹⁷⁾ and APAP adducts (from Dr. Lance Pohl at National Heart, Lung and Blood Institute).⁽²²⁾ Mitochondrial and cytosolic fractions used for western blotting were isolated by differential centrifugation as previously described.⁽²³⁾

Real-time reverse-transcription (RT) Polymerase Chain Reaction (PCR)

Total RNA was isolated from ~100 mg of liver tissue from each treated animal using the Quick-RNA Miniprep Kit (Zymo Research) according to the manufacturer's instruction. cDNA was prepared as previously described.⁽¹²⁾ Real-time RT-PCR was performed on CFX96 Touch Real-Time PCR Detection System (Bio-Rad) with SYBR Green (Thermo Fisher) and primers for *PUMA* (5'-ATGGCGGACGACCTCAAC-3'/5'-AGTCCCATGAAGAGATTGTACATGAC-3') and *GAPDH* (5'-CTCTGGAAAGCTGTGGCGTGATG-3'/5'-ATGCCAGTGAGCTTCCCG TTCAG-3'). PCR cycle conditions were as previously described.⁽¹²⁾ PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Analysis of caspase activity and glutathione (GSH) levels

Caspase activity was measured using the SensoLyte Homogeneous AMC Caspase-3/7 Assay Kit (AnaSpec) following the manufacturer's instructions. Samples were prepared from 50 mg of liver tissue from each animal. The data are presented as relative ratios of fluorescence units and protein concentrations.

GSH levels were determined by using Glutathione Colorimetric Assay Kit (BioVision). Briefly, 100 mg of liver tissue from each mouse in a group were pooled together, and homogenized together with the lysis buffer supplied by the kit. After centrifugation at 8,000× g for 10 min, the supernatant was used to determine the total GSH concentration according to the manufacturer's instructions.

Immunoprecipitation and chromatin immunoprecipitation (ChIP)

Pooled liver tissues (100 mg per mouse) from 4 randomly selected mice in a group were homogenized in 1 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% Nonidet P-40) supplemented with a protease inhibitor cocktail (Roche Applied Sciences).

After centrifugation at 10,000× g for 10 min, the supernatant was harvested and incubated overnight with 2 µg of anti-Drp1 antibody and protein G-agarose beads (Sigma Aldrich). The beads were washed twice with PBS containing 0.02% Tween 20 (pH 7.4), and then boiled in 2× Laemmli sample buffer and subjected to SDS-PAGE and western blotting for Bcl-X_L and Drp1.

ChIP with the c-Jun antibody (Active Motif) was performed using the Chromatin Immunoprecipitation Assay Kit (Millipore) as previously described.⁽²³⁾ The precipitates were analyzed by PCR using primers 5'-CCAGGCCCTTGTCCTGATGTGTAT-3' and 5'-GGCAGGAGGAGCAGCGTGGGGAC-3' to amplify a *PUMA* promoter fragment containing putative AP-1 binding sites. PCR conditions were the same as those previously used for amplifying human *PUMA* promoter.⁽²⁴⁾

Knockdown of RIP1 and JNK using siRNA-expressing adenoviruses

SiRNA sequences for targeting mouse *RIP1* (5'-CTCCATGTACTCCATCACCA-3' and 5'-CCTTCGTTTCCTTCCTCCTCTCTGT-3'), *JNK1* (5'-TGTTGTCACGTTTACTTCTG-3' and 5'-GCAGAAGCAAACGTGACAACA-3'), *JNK2* (5'-GCTCAGTGGACATGGATGAG-3' and 5'-CCGCAGAGTTCATGAAGAA-3'), and control (5'-CCTTCCCTGAAGGTTCCCTCC-3') were individually cloned into the pAdTrace-61 vector (from Dr. Tong-Chuan He at University of Chicago). After recombination with pAdEasy-1 vector in BJ5183-AD-1 electrocompetent cells (Agilent Technologies), equal amount of each individual plasmid for knocking down RIP1, and for knocking down both JNK1 and JNK2 (JNK), were pooled together. High-titer viruses (~10¹¹) were generated in 293 cells as described.⁽²⁵⁾ The titers were determined by counting the numbers of enhanced-RFP-positive cells after infection of 293 cells. To achieve knockdown of RIP1 and JNK in mouse livers, 2-month-old male C57BL/6J mice were injected intravenously (IV) via tail vein with adenoviruses expressing *RIP1*, *JNK*, or control siRNA (5×10⁹ PFU per mouse) for 10 days. Mice were then treated with either APAP (IP, 500 mg/kg) or saline for 6 hr, followed by tissue collection and western blot analysis.

Histology and immunostaining analyses

Tissue processing and immunostaining were performed as previously described.⁽¹²⁾ Briefly, liver tissues from the treated mice were fixed in 10% formalin for 24 hr followed by processing and paraffin embedding. Sections (5 µm) of paraffin-embedded tissues were subjected to deparaffinization and antigen retrieval (boiling for 10 min in 0.1 M citrate buffer, pH 6.0/1 mM EDTA). Non-specific antibody binding was blocked by using 20% goat serum at room temperature for 1 hr. Hematoxylin and eosin (H&E) staining was performed as described.⁽¹²⁾ Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining was conducted with ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore) according to the manufacturer's instructions. Active caspase 3 and HMGB1 staining was performed with rabbit anti-cleaved caspase 3 (Cell Signaling) and anti-HMGB1 (Abcam), respectively, at 1:100 dilution in a humidified chamber at 4°C overnight. Signals were visualized by Alexa 488 conjugated goat-anti-rabbit secondary antibodies (Invitrogen) at 1:1000 dilution for 1 hr at room temperature, with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride; Vector Laboratories) for nuclear counterstaining. Images were acquired

using an Olympus BX51 system microscope equipped with SPOT camera and SPOT Advanced 5.1 software. TUNEL, HMGB1 and H&E staining signals were quantified by NIH Image J software (<https://imagej.nih.gov/ij/>).

Statistical analysis

Statistical analysis was performed using GraphPad Prism V software. Data are presented as means $\pm$ SD. Statistical significance was calculated using the Student's *t*-test, except for the results of survival experiments, which were analyzed by the Log-Rank test. $P < 0.05$ was considered significant.

Results

PUMA is induced in APAP-induced liver injury

To study the role of Bcl-2 family proteins in APAP-induced liver injury, WT C57BL/6J mice fasted overnight were treated with 250 mg/kg of APAP by IP injection. APAP treatment led to highly escalated serum ALT and AST activities in a time-dependent manner, with the highest levels detected at 24 hr post treatment (Fig. 1A). At this time point, typical features of liver injury and centrilobular cell necrosis were detected by H&E staining (Supporting Fig. S1A) and TUNEL staining of broken DNA ends (Supporting Fig. S1B) in the livers of APAP-treated mice. We therefore chose to use 250 mg/kg of APAP for most subsequent experiments.

PUMA protein and mRNA were markedly induced within 24 hr in a time-dependent manner in the livers of APAP-treated WT mice (Fig. 1B, C), which correlated with the increased serum ALT/AST levels (Fig. 1A). PUMA was also induced by APAP in mouse primary hepatocytes (Fig. 1D), and in human primary hepatocytes in a dose-dependent manner (Fig. 1E). In contrast, transient induction of Bax and Bim, depletion of Bid, Noxa and Bcl-X_L, increased tBid as previously shown,⁽²⁶⁾ and unchanged Mcl-1 were detected in the APAP-treated mouse livers (Supporting Fig. S1C), and human primary hepatocytes (Supporting Fig. S1D). Furthermore, strong PUMA induction was also detected in mice treated with 500 mg/kg of APAP for 6 hr, which correlated with more acute liver injury relative to that induced by 250 mg/kg of APAP (Supporting Fig. S2A-C).

We then used immunostaining to analyze PUMA expression in the livers of APAP-treated mice. PUMA was found to be expressed in a punctate pattern mixed with a slightly diffused pattern in the cytoplasm of hepatocytes at 6 and 24 hr after APAP treatment (Fig. 1F). Centrilobular areas with the most cell loss at 24 hr had much stronger PUMA induction compared to areas without cell loss (Fig. 1F). PUMA expression was not observed in the livers of untreated mice and APAP-treated C57BL/6J *PUMA* KO (*PUMA*^{-/-}) mice (Fig. 1F), verifying the specificity of PUMA antibody used for immunostaining. These results indicate that the induction of PUMA correlates with the pathological changes and may play an important role in APAP-induced necrosis and liver injury.

PUMA deficiency suppresses APAP-induced liver injury

We then investigated the functional role of PUMA in APAP-induced liver injury. Compared to WT mice, *PUMA* KO markedly suppressed the increases in serum ALT/AST levels and hepatocyte loss in response to 250 mg/kg APAP treatment for 24 hr (Fig. 2A-C). *PUMA* KO also protected mice from liver injury following 250 mg/kg APAP treatment at 36 hr, a time point when the serum ALT/AST levels had declined compared to those at 24 hr (Supporting Fig. S3A, B). However, similar levels of APAP adduct formation, CYP2E1 expression, and glutathione (GSH) depletion were detected in the livers of WT and *PUMA* KO mice (Supporting Fig. S3C, D), indicating unaffected APAP metabolism. Furthermore, *PUMA* KO suppressed the acute liver injury induced by 500 mg/kg of APAP at 6 hr (Supporting Fig. S3E, F), and also improved the survival of mice at 72 hr (Fig. 2D). These results suggest a critical role of PUMA in APAP-induced liver injury and hepatocyte death.

PUMA mediates APAP-induced hepatocyte necrosis and mitochondrial dysfunction

We then investigated the mechanism by which PUMA promotes liver injury and hepatocyte death in response to APAP treatment. Consistent with previous studies,^(3, 4) little caspase activation was detected in the livers of APAP-treated WT and *PUMA* KO mice (Supporting Fig. S4A-C). Caspase inhibition by z-VAD did not influence the protective effects of *PUMA* KO on APAP-induced liver injury (Supporting Fig. S4D, E). TUNEL staining, which was unaffected by caspase inhibition, was markedly reduced in the livers of APAP-treated *PUMA* KO mice (Fig. 3A). HMGB1, a nuclear protein that binds to chromatin, is known to be released from nuclei of cells undergoing necrosis.⁽⁶⁾ HMGB1 cytoplasmic translocation was detected by immunostaining in the livers of APAP-treated WT mice, which was blocked in *PUMA* KO mice (Fig. 3B). These results suggest that PUMA mediates APAP-induced liver injury by promoting necrosis.

Further analysis revealed that upon APAP treatment, PUMA was accumulated in the mitochondria and cytoplasm of mouse hepatocytes (Fig. 3C,E). *PUMA* KO suppressed APAP-induced mitochondrial fission detected by COX IV immunostaining (Fig. 3C and Supporting Fig. S5A). Transmission electron microscopy (TEM) identified damaged and swollen mitochondria in the hepatocytes of WT mice, which were absent in those of *PUMA* KO mice (Fig. 3D). Consistent with the involvement of mitochondria-mediated necrosis,^(8, 9) APAP-induced cytosolic release of AIF and Endo G, as well as mitochondrial translocation of Drp1, were abrogated in *PUMA* KO livers (Fig. 3E). In contrast, *PUMA* KO did not affect the induction and mitochondrial translocation of JNK, Bax, Bim, tBid, and the expression of Bcl-X_L (Fig. 3 E and Supporting Fig. S5B), but modestly reduced cytosolic release of cytochrome *c* (Fig. 3E), which may reflect mitochondrial membrane permeabilization in necrosis⁽²⁷⁾. The residual cytochrome *c* release is likely related to slight induction of PUMA-independent cell death, which does not contribute significantly to liver injury (Supporting Fig. S4A-E). Furthermore, immunoprecipitation analysis revealed that APAP treatment causes PUMA-dependent dissociation Bcl-X_L and Drp1 in the livers (Fig. 3F), suggesting PUMA mediates APAP-induced necrosis by relieving Drp1 from Bcl-X_L, leading to mitochondrial fission and dysfunction.

RIP1/JNK-dependent, but p53-independent PUMA induction in APAP-induced liver injury

PUMA was originally identified as a transcriptional target of the tumor suppressor p53 in DNA-damage-induced apoptosis.⁽¹¹⁾ APAP treatment only slightly increased p53 in WT mice (Fig. 4A). *p53* KO did not affect the induction of PUMA protein and mRNA (Fig. 4A, B), or suppressed APAP-induced serum ALT/AST activities (Fig. 4C), centrilobular necrosis (Fig. 4D), TUNEL staining (Supporting Fig. S6A), and HMGB1 cytosolic release (Supporting Fig. S6B). Therefore, APAP-induced liver injury and hepatocyte necrosis are mediated by the induction of PUMA through a p53-independent mechanism, which was also suggested by other studies.⁽²⁸⁾

RIP1, a key kinase involved in programmed necrosis, was shown to be involved in APAP-induced liver injury by modulating JNK activity.⁽⁸⁾ RIP1 expression and JNK phosphorylation were substantially elevated in the livers of WT and *PUMA* KO mice treated with 250 mg/kg of APAP (Fig. 5A and Supporting Fig. S7A), and also in APAP-treated primary human hepatocytes (Supporting Fig. S7B). Pre-treating mice with the RIP1 inhibitor Nec-1 or the JNK inhibitor SP600125 suppressed the liver changes induced by 250 mg/kg of APAP, including serum ALT/AST activities (Fig. 5B), centrilobular necrosis (Supporting Fig. S7C), TUNEL staining (Supporting Fig. S7D), as well as cytoplasmic HMGB1 translocation (Supporting Fig. S7E). The induction of PUMA protein and mRNA was abrogated by RIP1 or JNK inhibition (Fig. 5C, D). The effects of RIP1 inhibition were verified by pre-treating mice with Nec-1s (Supporting Fig. S8A-C), a more specific RIP1 inhibitor than Nec-1.⁽¹⁵⁾ Using chromatin immunoprecipitation (ChIP), we detected markedly enhanced binding of c-Jun, a key component of the AP-1 complex, to the *PUMA* promoter in the livers of APAP-treated mice, which was abolished by Nec-1 or SP600125 pre-treatment (Fig. 5E). RIP1 or JNK inhibition suppressed PUMA induction in APAP-treated mouse primary hepatocytes (Fig. 5F). In line with RIP1-mediated JNK activation,⁽⁸⁾ Nec-1 pre-treatment suppressed APAP-induced JNK phosphorylation in mouse livers and primary hepatocytes (Fig. 5C, F).

Consistent with the results of APAP at 250 mg/kg, treating mice with 500 mg/kg of APAP induced RIP1, PUMA and JNK phosphorylation in the livers as early as 2 hr (Supporting Fig. S9A). RIP1 or JNK inhibition also suppressed liver injury, as well as PUMA induction, in response to 500 mg/kg of APAP treatment at 6 hr (Supporting Fig. S9B-D). Furthermore, adenovirus-mediated siRNA knockdown of RIP1 and JNK confirmed that PUMA induction by APAP requires RIP1 and JNK in the livers (Supporting Fig. S9E-G), and in primary mouse hepatocytes (Supporting Fig. S9H). Together, these results suggest a critical role of the RIP1/JNK/PUMA axis in APAP-induced liver injury.

A small-molecule PUMA inhibitor suppressed APAP-induced liver injury

RIP1 and JNK regulate complex signaling in cell death and survival in different cell types. We therefore explored PUMA inhibition for ameliorating APAP-induced liver injury using a recently described small-molecule PUMA inhibitor (PUMAi) (Fig. 6A).^(14, 29) The level of APAP-protein adducts in mouse liver was shown to peak at 2 hr after APAP injection,⁽³⁰⁾ and unaffected by *PUMA* KO (Supporting Fig. S3C). We therefore tested if PUMAi administered at this time point protects mice from liver injury. Similar to *PUMA* KO,

treatment with PUMAi attenuated APAP-induced serum ALT/AST activities (Fig. 6B), centrilobular necrosis (Fig. 6C), TUNEL staining (Fig. 6D), and HMGB1 cytoplasmic translocation (Fig. 6E). These results suggest that pharmacological inhibition of PUMA may be beneficial for treating liver injury after APAP overdose (Fig. 6F).

Discussion

We show for the first time that PUMA functions as a critical mediator of APAP-induced liver necrosis and tissue injury. PUMA protein and mRNA were continuously accumulated in the course of APAP treatment, parallel to APAP-induced pathological changes including increased ALT/AST activities and centrilobular necrosis (Fig. 1). Deletion of PUMA strongly suppressed these changes induced by APAP at 250 or 500 mg/kg (Fig. 2 and Supporting Fig. S3E and F). Our results demonstrate that hepatocyte necrosis mediated by RIP1/JNK-dependent PUMA induction is largely responsible for APAP-induced acute liver injury (Fig. 6F). Consistent with the observations from the liver injury model, PUMA and RIP1 were shown to mediate cardiac ischemia-reperfusion and cardiomyocyte necrosis in mice.^(31, 32) Our recent study revealed that PUMA mediates TNF-induced necroptosis by enhancing RIP3 and MLKL phosphorylation in a feedback amplification loop.⁽¹³⁾ Together, these findings uncover a novel functional role of PUMA in regulating non-apoptotic cell death and tissue injury.

In addition to PUMA, other proapoptotic Bcl-2 members including Bax and Bim have also been implicated in APAP-induced liver injury.^(33, 34) We observed the transient induction of Bax and Bim after APAP treatment (Supporting Fig. S1C), which did not correlate with markedly increased serum ALT/AST activities at 12–24 hr (Fig. 1A). *PUMA* KO did not affect the induction and mitochondrial translocation of JNK, Bax, Bim, and tBid (Fig. 3E), which may be responsible for the intact, low level of apoptosis observed in *PUMA* KO mice (Supporting Fig. S4A–C). Unlike *PUMA* KO, which blocked necrosis and liver injury throughout the treatment (Fig. 2 and Supporting Fig. S3), *Bax* or *Bim* KO primarily affected apoptosis at early time points, and only transiently protected mice from APAP-induced liver injury.^(33, 34) In contrast to the major role of PUMA-mediated necrosis, Bax- and Bim-dependent apoptosis appears to play a relatively minor role in APAP-induced liver injury by promoting early cell loss.

APAP-induced necrosis and liver injury are mediated by mitochondrial dysfunction. However, the mechanism by which APAP promotes mitochondrial damage is incompletely understood.^(3, 5, 33) We found PUMA was accumulated in the mitochondria, and *PUMA* KO suppressed APAP-induced mitochondrial fission and damage (Fig. 3C–E). APAP-induced mitochondrial fission was shown to be mediated by Drp1,⁽⁸⁾ which is induced and translocates to the mitochondria following APAP overdose.⁽⁹⁾ *PUMA* deficiency blocked APAP-induced Drp1 mitochondrial translocation and the release of Endo G and AIF (Fig. 3E), suggesting that PUMA promotes mitochondrial dysfunction via Drp1, which was also observed in PUMA-mediated apoptosis.⁽³⁵⁾ Biochemical and genetic studies indicate that PUMA induces cell death by binding to anti-apoptotic Bcl-2 family members such as Bcl-X_L,⁽¹¹⁾ which can directly interact with Drp1 and inhibit its GTPase activity,⁽³⁶⁾ and suppress APAP-induced hepatocyte necrosis.⁽³⁷⁾ We found Drp1 was dissociated from Bcl-

X_L in a PUMA-dependent manner in the livers of APAP-treated mice (Fig. 3F), suggesting that PUMA promotes hepatocyte necrosis by relieving Drp1 from Bcl-X_L, resulting in Drp1-mediated mitochondrial dysfunction. Despite the known interaction of PUMA and Bcl-X_L,⁽³⁸⁾ we could not detect such an interaction in the livers by immunoprecipitation, which might be limited by the specificity of the antibody used.

PUMA is primarily regulated at the transcriptional level in response to different stress conditions. In addition to p53, several transcription factors, including p73, FoxO3a, and p65, have been also shown to directly bind to *PUMA* promoter to activate its transcription.⁽¹¹⁾ PUMA is induced through a p53-independent, but RIP1/JNK-dependent mechanism in APAP-induced liver injury (Fig. 4, 5). *PUMA* is a direct transcriptional target of c-Jun (Fig. 5E), a subunit of the AP-1 complex, upon JNK-mediated c-Jun phosphorylation. JNK has been implicated in liver injury by causing cell death through transcription-dependent and -independent mechanisms.⁽³⁹⁾ Although other Bcl-2 family members are also regulated by JNK,⁽¹⁰⁾ our results suggest a major role of PUMA induction in JNK-mediated hepatocyte necrosis in APAP-induced liver injury. JNK/c-Jun-mediated PUMA induction is also involved in DEN-induced liver damage,⁽¹²⁾ and in hepatocyte lipoapoptosis induced by free fatty acids.⁽⁴⁰⁾ Therefore, PUMA induction may contribute to different forms of hepatocyte death and liver injury that are linked to JNK activation, and represent a useful target for drug development and therapeutic intervention.

Current treatment options for APAP-induced hepatotoxicity are limited to N-acetylcysteine and liver transplantation.⁽⁴¹⁾ Novel and more effective agents for treating drug-induced acute liver injury are in demand. Previous studies showed that APAP-induced hepatotoxicity could be suppressed by inhibiting or silencing upstream kinases such as JNK and RIP1.^(8, 42) However, these kinases have numerous substrates and pleotropic effects, including those that are critical for normal physiology. On the other hand, PUMA mediates pathological cell death associated with inflammation and different types of tissue injury, such as APAP-induced liver injury, ischemia/reperfusion, and ulcerative colitis.^(9, 43, 44) Targeting PUMA, a selective cell death effector, may represent a more specific approach for reducing pathological cell death and tissue injury. This hypothesis is supported by our recent study showing that *PUMA* deletion is superior to *p53* deletion in protecting mice from radiation- or chemotherapy-induced stem cell loss and tissue injury.^(14, 45)

We previously used a structure-based pharmacophore model to perform *in silico* screen and identified PUMAi.⁽²⁹⁾ The cellular activity of PUMAi was demonstrated by disruption of the interaction of PUMA and Bcl-X_L; the on-target specificity was indicated by suppression of radiation-induced and PUMA-dependent apoptosis.⁽²⁹⁾ Our recent study showed that PUMAi had *in vivo* efficacy and protected mice against chemotherapy-induced and PUMA-dependent gastrointestinal injury and cell death.⁽¹⁴⁾ It is encouraging that PUMAi administered 2 hr after APAP injection had a comparable protective effect as RIP1 or JNK inhibitor pretreatment (Fig. 6), providing a proof-of-principle for using such inhibitors to suppress pathological cell death and liver injury due to APAP overdose. However, the effects of PUMAi remain to be further characterized, and its chemical structure and pharmacological properties need to be improved by structure activity relationship analysis and further modifications, which will be the focus of our future efforts.

In conclusion, we identified RIP1/JNK-dependent PUMA induction as a critical mediator of APAP-induced liver injury and hepatocyte necrosis. Pharmacological inhibition of PUMA may be useful for ameliorating tissue injury caused by APAP overdose (Fig. 6F).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIF	apoptosis inducing factor
ALT	alanine aminotransferase
APAP	acetaminophen
AST	aspartate aminotransferase
ChIP	chromatin immunoprecipitation
CYP2E1	cytochrome P-450 2E1
DAMP	damage associated molecular pattern
DEN	diethylnitrosamine
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DNPH	2,4-dinitrophenylhydrazine
Drp1	dynammin-related protein 1
Endo G	endonuclease G
GSH	glutathione
H&E	hematoxylin and eosin
HMGB1	high-mobility group box 1
IP	intraperitoneally
IV	intravenously

JNK	c-Jun N-terminal kinase
KO	knockout
MLKL	mixed lineage kinase domain-like protein
MOMP	mitochondrial outer membrane permeabilization
NAPQI	N-acetyl-p-benzoquinone imine
Nec-1	necrostatin 1
Nec-1s	7-Cl-O-Nec1
PUMA	p53 up-regulated modulator of apoptosis
PUMAi	PUMA inhibitor
RIP1	receptor interacting protein kinase 1
ROS	reactive oxygen species
RT PCR	reverse-transcription Polymerase Chain Reaction
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
WT	wild-type
z-VAD	z-VAD-fmk

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Figure 1. PUMA is induced in the livers of APAP-treated mice.

(A) Serum ALT (*left*) and AST (*right*) levels in wild-type (WT) C57BL/6J mice treated with APAP (IP; 250 mg/kg) for 6, 12, or 24 hr (N = 12 for each time point). Each dot represents a different mouse. Means ± s.d. are indicated. (B) Western blotting of PUMA in the livers of 3 randomly selected mice treated as in (A). Each lane represents a different mouse. (C) Real-time RT-PCR analysis of *PUMA* mRNA expression in the livers from mice treated as in (A) (N = 3 for each group), with bars indicating means ± s.d. (D) Western blotting of PUMA in WT mouse hepatocytes treated with 10 mM APAP for 6 hr. (E) Western blotting of PUMA

in human hepatocytes treated with APAP at indicated concentrations for 6 hr. **(F)** Immunostaining of PUMA in the livers of WT and *PUMA* KO mice treated with APAP as in (A) for 6 or 24 hr. Representative images of necrotic centrilobular and non-necrotic secondary areas are shown (Scale bars: 20 μ m), with arrows indicating example areas with PUMA staining. DAPI was used for nuclear counter staining. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 2. *PUMA* deficiency suppresses APAP-induced liver injury and lethality.

(A) Western blotting of PUMA in the livers of 4 WT and 4 *PUMA* KO mice treated with APAP (IP; 250 mg/kg) for 24 hr. Each lane represents a different mouse. (B) Serum ALT (left) and AST (right) levels in WT and *PUMA* KO mice treated as in (A) (N = 12 for each genotype at each time point). Each symbol represents a different mouse. Means ± s.d. are indicated. (C) H&E staining of the liver sections from the mice treated as in (A) for 24 hr. Left, representative images with arrows indicating example necrotic centrilobular areas (Scale bars: 200 μm); right, quantification of necrotic areas by Image J software (N = 3 for

each group), with bars indicating means $\pm$ s.d. **(D)** Survival of WT and *PUMA* KO mice treated with APAP (IP; 500 mg/kg) for 72 hr (N = 18 for WT; N = 16 for *PUMA* KO). **, $P < 0.01$; ***, $P < 0.001$.

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Figure 3. PUMA mediates hepatocyte necrosis and mitochondrial dysfunction induced by APAP. (A), (B) WT and *PUMA* KO mice subjected to 2-hr pre-treatment with vehicle (1% DMSO) or the pan-caspase inhibitors z-VAD-fmk (IP; 10 mg/kg) were treated with APAP (IP; 250 mg/kg) for 24 hr. Liver sections were prepared and analyzed for cell death markers. (A) TUNEL staining of the liver sections. *Left*, representative images of necrotic centrilobular areas with arrows indicating example TUNEL+ cells (Scale bars: 20 μ m); *right*, quantification by counting TUNEL signals in at least 100 randomly selected individual cells (N = 3 for each group), with bars indicating means $\pm$ s.d. (B) HMGB1 (green) staining of the

liver sections. *Left*, representative images with arrows indicating example cells with cytoplasmic HMGB1 staining and hollow nuclei (Scale bars, 20 μ m); *right*, quantification of co-localization of HMGB1 and nuclei by Image J software in at least 20 randomly selected individual cells ($N = 3$ for each group), with bars indicating means $\pm$ s.d. **(C)-(F)** WT and *PUMA* KO mice were treated with APAP (IP; 250 mg/kg) for 24 hr. Liver sections and lysates were prepared and analyzed for mitochondria and protein-protein interactions. **(C)** Double staining of PUMA (green) and mitochondrial cytochrome oxidase subunit IV (COX IV; red), with arrows indicating example co-localization (yellow) signals (Scale bars: 5 μ m). **(D)** Representative TEM images of the liver sections with arrows indicating mitochondria (Scale bars: 500 nm). **(E)** Western blotting of indicated proteins in the mitochondrial and cytosolic fractions isolated from the liver lysates. Each lane represents liver samples from 4 mice in a group that were pooled and homogenized together as described in Materials and Methods. COX IV and tubulin, which are expressed in mitochondria and cytosol, respectively, were used as a control for loading and fractionation. **(F)** Analysis of the interaction of Bcl-X_L and Drp1 by immunoprecipitation, followed by western blotting of Drp1 and Bcl-X_L. IgG was used as a control. In (A)-(C), DAPI was used for nuclear counter staining. *NS*, $P > 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Figure 4. PUMA induction in APAP-induced liver injury is p53-independent.

WT and *p53* KO mice were treated with APAP (IP; 250 mg/kg) for 24 hr (N = 12 for each group). **(A)** Western blotting of p53 and PUMA in the livers of 3 randomly selected mice from each group. **(B)** Real-time RT-PCR analysis of *PUMA* mRNA expression in the livers from the treated mice (N = 3 for each group), with bars indicating means $\pm$ s.d. **(C)** Serum ALT (*left*) and AST (*right*) levels. Each symbol represents a different mouse. Means $\pm$ s.d. are indicated. **(D)** H&E staining of the liver sections from the treated mice. *Left*, representative images with arrows indicating example necrotic centrilobular areas (Scale bars: 200 μ m); *right*, quantification of necrotic areas by Image J software (N = 3 for each group), with bars indicating means $\pm$ s.d. NS, $P > 0.05$; *, $P < 0.05$.

Figure 5. PUMA induction in the livers of APAP-treated mice is RIP1- and JNK-dependent.

(A) Western blotting of indicated proteins in the livers of 4 WT mice with or without APAP treatment (IP; 250 mg/kg) for 24 hr. Each lane represents a different mouse. (B)-(E) WT mice subjected to 2-hr pretreatment with vehicle (1% DMSO), the RIP1 inhibitor Nec-1 (IP; 25 mg/kg) or the JNK inhibitor SP600125 (IP; 20 mg/kg) were treated with APAP (IP; 250 mg/kg) for 24 hr (N = 12 for each group). (B) Serum ALT (upper) and AST (lower) levels. Each symbol represents a different mouse. Means $\pm$ s.d. are indicated. (C) Western blotting of PUMA, p-JNK, and total JNK in the livers of 3 randomly selected mice from each group. Each lane represents a different mouse. (D) Real-time RT-PCR analysis of *PUMA* mRNA expression in the livers (N = 3 for each group), with bars indicating means $\pm$ s.d. (E) Binding of c-Jun to the *PUMA* promoter in the livers was analyzed by chromatin

immunoprecipitation (ChIP) using anti-c-Jun antibody with IgG as control. **(F)** Western blotting of PUMA, p-JNK and total JNK in mouse primary hepatocytes treated with vehicle (1% DMSO) alone, or indicated combination of APAP (10 mM) with vehicle, Nec-1 (40 μ M), or SP600125 (10 μ M) simultaneously for 6 hr. *, $P<0.05$; ***, $P<0.001$.

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Figure 6. A PUMA inhibitor ameliorates APAP-induced liver injury.

(A) Structure of the small-molecule PUMA inhibitor (PUMAi).⁽¹⁴⁾ (B)-(E) WT mice were treated with APAP (IP; 250 mg/kg) for 2 hr, and then with vehicle (1% DMSO) or PUMAi (IP; 10 mg/kg) for 24 hr (N = 12 for each group). (B) Serum ALT (upper) and AST (lower) levels. Each symbol represents a different mouse. Means $\pm$ s.d. are indicated. (C) H&E staining of the liver sections from the treated mice. *Left*, representative images with arrows indicating example necrotic centrilobular areas (Scale bars: 200 μ m); *right*, quantification of necrotic areas by Image J software (N = 3 for each group), with bars indicating means $\pm$ s.d.

(D) TUNEL staining of the liver sections from the treated mice. *Left*, representative images of necrotic centrilobular areas with arrows indicating example TUNEL+ cells (Scale bars, 20 μ m); *right*, quantification by counting TUNEL signals in at least 100 randomly selected individual cells ($N = 3$ for each group), with bars indicating means $\pm$ s.d. **(E)** HMGB1 staining of the liver sections from the treated mice. *Left*, representative images with arrows indicating example cells with cytoplasmic HMGB1 staining and hollow nuclei (Scale bars: 20 μ m); *right*, quantification of co-localization of HMGB1 and nuclei by Image J software in at least 20 randomly selected individual cells ($N = 3$ for each group), with bars indicating means $\pm$ s.d. **(F)** A model of APAP-induced and PUMA-mediated liver necrosis and injury. TFs: transcription factors. In (D) and (E), DAPI was used for nuclear counter staining. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.