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Inhibitor of apoptosis signal-regulating kinase 1 protects against acetaminophen-induced liver injury

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ABSTRACT

Metabolic activation and oxidant stress are key events in the pathophysiology of acetaminophen (APAP) hepatotoxicity. The initial mitochondrial oxidative stress triggered by protein adduct formation is amplified by c-jun-*N*-terminal kinase (JNK), resulting in mitochondrial dysfunction and ultimately cell necrosis. Apoptosis signal-regulating kinase 1 (ASK1) is considered the link between oxidant stress and JNK activation. The objective of the current study was to assess the efficacy and mechanism of action of the small-molecule ASK1 inhibitor GS-459679 in a murine model of APAP hepatotoxicity. APAP (300 mg/kg) caused extensive glutathione depletion, JNK activation and translocation to the mitochondria, oxidant stress and liver injury as indicated by plasma ALT activities and area of necrosis over a 24 h observation period. Pretreatment with 30 mg/kg of GS-459679 almost completely prevented JNK activation, oxidant stress and injury without affecting the metabolic activation of APAP. To evaluate the therapeutic potential of GS-459679, mice were treated with APAP and then with the inhibitor. Given 1.5 h after APAP, GS-459679 was still protective, which was paralleled by reduced JNK activation and p-JNK translocation to mitochondria. However, GS-459679 treatment was not more effective than *N*-acetylcysteine, and the combination of GS-459679 and *N*-acetylcysteine exhibited similar efficacy as *N*-acetylcysteine monotherapy, suggesting that GS-459769 and *N*-acetylcysteine affect the same pathway. Importantly, inhibition of ASK1 did not impair liver regeneration as indicated by PCNA staining. In conclusion, the ASK1 inhibitor GS-459679 protected against APAP toxicity by attenuating JNK activation and oxidant stress in mice and may have therapeutic potential for APAP overdose patients.

Introduction

Acetaminophen (APAP) is a widely used analgesic and antipyretic. A therapeutic dose of APAP is safe and effective while an overdose can cause severe liver injury in animals and humans (Larson, 2007; McGill et al., 2012). Hepatotoxicity is initiated by formation of a reactive metabolite, *N*-acetyl-*p*-benzoquinone imine, which is detoxified by glutathione but also binds to cellular proteins (Cohen et al., 1997). Although formation of protein adducts, especially in mitochondria, is a critical event in the pathophysiology (Tirmenstein and Nelson, 1989), it is not sufficient to cause cell death (Jaeschke and Bajt, 2006). The current hypothesis is that protein binding induces mitochondrial dysfunction including inhibition of mitochondrial respiration (Meyers et al., 1988) and formation of reactive oxygen species (ROS) (Jaeschke, 1990) and peroxynitrite (Hinson et al., 1998; Cover et al., 2005). This oxidant stress together with lysosomal iron taken up into the mitochondria by the Ca^{2+} uniporter (Kon et al., 2010), leads to the opening of the mitochondrial membrane permeability transition (MPT) pore and collapse of the membrane potential (Kon et al., 2004; Reid et al., 2005; Ramachandran et al., 2011a; LoGuidice and Boelsterli, 2011). The massive loss of mitochondrial function triggers necrotic cell death (Gujral et al., 2002). A number of therapeutic interventions that accelerated the recovery of mitochondrial glutathione and improved the detoxification of mitochondrial ROS and peroxynitrite provided clear evidence for the critical role of the mitochondrial oxidant stress in the mechanism of cell death (Knight et al., 2002; James et al., 2003; Ramachandran et al., 2011b; Saito et al., 2010b). However, it remained unclear how this extensive mitochondrial oxidant stress ultimately can be induced.

Studies during the last decade aimed at elucidating intracellular signaling events have demonstrated a critical role of the mitogen-activated protein kinase (MAPK) c-jun-N-terminal kinase (JNK) in APAP-induced

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cell death in mice (Gunawan et al., 2006; Henderson et al., 2007; Latchoumycandane et al., 2007) and in human hepatocytes (Xie et al., 2014). Importantly, JNK was not only activated (phosphorylated) in the cytosol, p-JNK also translocated to the mitochondria (Hanawa et al., 2008) and amplified the mitochondrial oxidant stress (Saito et al., 2010a) through binding to an anchor protein in the outer mitochondrial membrane (Win et al., 2011). It was also shown that an early mitochondrial oxidant stress triggered JNK activation (Hanawa et al., 2008; Saito et al., 2010a). However, JNK is not a redox-sensitive kinase. In fact, the upstream kinases, apoptosis signal-regulating kinase 1 (ASK1) (Nakagawa et al., 2008) and mixed-lineage kinase 3 (MLK3) (Sharma et al., 2012) have been identified as critical upstream regulators of JNK activation. Oxidant stress can release ASK1 from its complex with reduced thioredoxin by oxidation of thioredoxin resulting in the liberation of the active kinase (Saitoh et al., 1998). Because ASK1 is thought to be mainly responsible for sustained activation of JNK, the substantial reduction in APAP-induced liver injury in ASK1-deficient mice demonstrated the critical role of ASK1 in the pathophysiology (Nakagawa et al., 2008). Recently a potent and selective small molecule inhibitor of ASK1 with properties amenable for in vivo efficacy testing in rodents was developed (Gerczuk et al., 2012; Toldo et al., 2012). This inhibitor enabled us to test if pharmacologic inhibition of ASK has therapeutic potential for treating APAP hepatotoxicity and to study the mechanisms of protection.

Materials and methods

Animals. 8 week old male C57Bl/6 mice were acquired from Jackson Laboratories for the experiments. Animals were housed in a controlled environment with a 12 hour light/dark cycle and free access to food and water. Animals were acclimatized for at least 3 days and fasted overnight before experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Kansas Medical Center.

Inhibitors. GS-459679 and GS-444217 are potent and selective ATP competitive inhibitors of ASK1 developed by Gilead Sciences, Inc., Foster City, CA (Breckenridge et al., manuscript in preparation). In a competitive, time-resolved fluorescence resonance energy transfer immunoassay, GS-459679 and GS-444217 directly inhibit ASK1 kinase activity with IC_{50} (concentration that inhibits ASK1 kinase by 50%) values of 6.1 and 2.9 nM, respectively. One micromolar GS-459679 results in 99% inhibition of the ASK1 kinase but does not inhibit 20 other kinases implicated in cellular stress signaling (Gerczuk et al., 2012).

Experimental design. For pre-treatment studies, animals were administered either the ASK1 inhibitor GS-459679 (ASK1i) (10 or 30 mg/kg), or vehicle (55% PEG in H_2O) 30 min prior to administration of APAP (300 mg/kg, i.p.). Animals were euthanized 0.5, 6 or 24 h after APAP. For post-treatment experiments, animals were administered APAP (300 mg/kg, i.p.) followed by ASK1i (30 mg/kg, i.p.), *N*-acetylcysteine (NAC) (500 mg/kg, i.p.), ASK1i + NAC or vehicle (saline) at 1.5 or 3 h after APAP. Animals were allowed access to regular food at 6 h after APAP treatment; the animals were euthanized at 24 h. For 48 h experiments evaluating liver regeneration, animals were given back food (control diet or diet containing 0.2% of the ASK1 inhibitor GS-444217) 6 h after APAP administration. At the end of the experiments, animals were anesthetized with isoflurane and blood was drawn from vena cava into heparinized syringes for determination of alanine aminotransferase (ALT) activity. The liver was excised and rinsed in saline before being divided for histology and subcellular fractionation, and the rest being snap frozen in liquid nitrogen and subsequently stored at −80 °C.

Histology. Formalin-fixed tissue samples were embedded in paraffin and 4 μm sections were cut. Replicate sections were stained with hematoxylin and eosin (H&E) to evaluate necrosis as described (Gujral et al., 2002).

Measurement of GSH and GSSG. Total soluble GSH and GSSG were determined in the liver homogenate with a modified method of Tietze (Jaeschke and Mitchell, 1990). In brief, the frozen tissue was homogenized at 0 °C in 3% sulfosalicylic acid containing 0.1 mM EDTA. For measurement of GSSG, GSH was trapped with 10 mM N-ethylmaleimide. The sample was centrifuged after dilution with 0.01 N HCl and the supernatant was further diluted with 100 mM potassium phosphate buffer (KPP), pH 7.4. The samples were then assayed using dithionitrobenzoic acid. All data are expressed as GSH-equivalents.

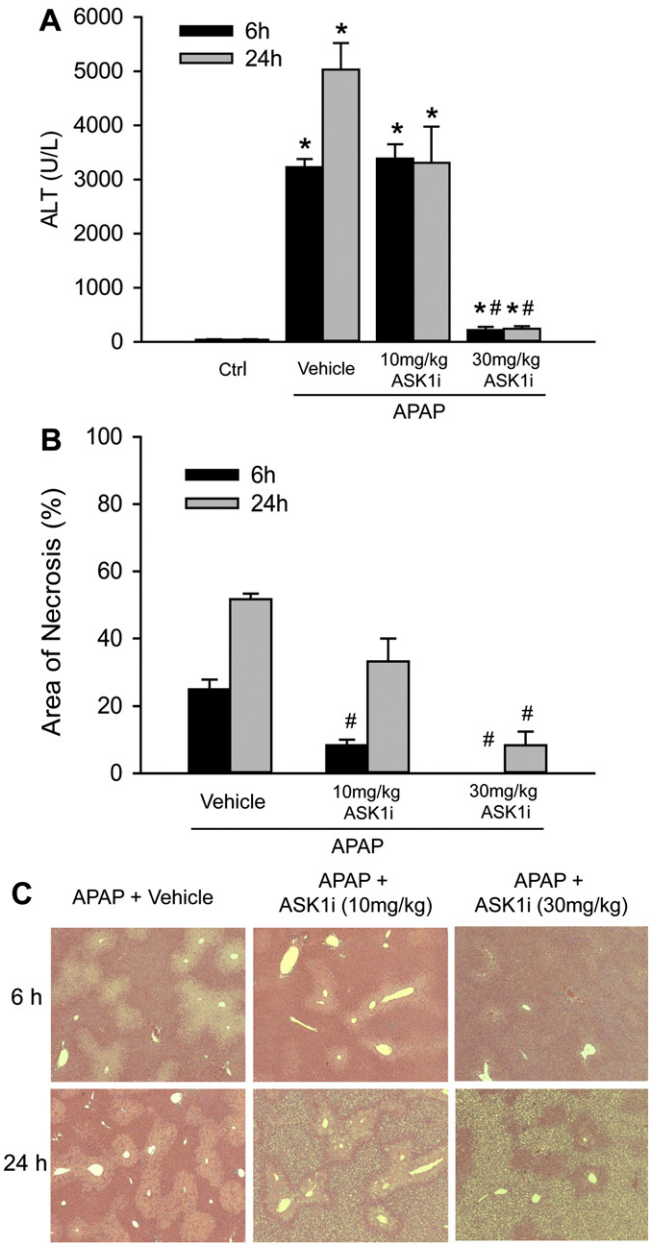

Fig. 1. Protective effect of the ASK1 inhibitor GS-459679 against APAP-induced liver injury. C57BL/6 mice were pretreated with either 10 or 30 mg/kg GS-459679 or an equivalent dose of the vehicle (55% PEG in water) 30 min before 300 mg/kg APAP administration. (A) Plasma alanine aminotransferase (ALT) activities measured at 6 and 24 h after APAP treatment. (B) Quantitation of the areas of necrosis in H&E-stained liver sections. (C) Representative sections from each group with H&E staining. Data represent means ± SE of n = 4–11 animals per group. *P < 0.05 compared with control (Ctrl) groups, #P < 0.05 compared with corresponding APAP + vehicle groups.