The role of MAP2 kinases and p38 kinase in acute murine liver injury models

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c-Jun N-terminal kinase (JNK) mediates hepatotoxicity through interaction of its phospho-activated form with a mitochondrial outer membrane protein, Sh3bp5 or Sab, leading to dephosphorylation of intermembrane Src and consequent impaired mitochondrial respiration and enhanced ROS release. ROS production from mitochondria activates MAP3 kinases, such as MLK3 and ASK1, which continue to activate a pathway to sustain JNK activation, and amplifies the toxic effect of acetaminophen (APAP) and TNF/galactosamine (TNF/GalN). Downstream of MAP3K, in various contexts MKK4 activates both JNK and p38 kinases and MKK7 activates only JNK. The relative role of MKK4 versus 7 in liver injury is largely unexplored, as is the potential role of p38 kinase, which might be a key mediator of toxicity in addition to JNK. Antisense oligonucleotides (ASO) to MKK4, MKK7 and p38 (versus scrambled control) were used for in vivo knockdown, and in some experiments PMH were used after in vivo knockdown. Mice were treated with APAP or TNF/GalN and injury assessed. MKK4 and MKK7 were expressed in liver and each was efficiently knocked down with two different ASOs. Massive liver injury and ALT elevation were abrogated by MKK4 but not MKK7 ASO pretreatment in both injury models. The protection was confirmed in PMH. Knockdown of MKK4 completely inhibited basal P-p38 in both cytoplasm and mitochondria. However, ALT levels and histologic injury in APAP-treated mice were not altered with p38 knockdown versus scrambled control. p38 knockdown significantly increased P-JNK levels in cytoplasm but not mitochondria.

In the APAP model, many approaches to modulating JNK activity have supported the role of JNK activation in liver injury. However, a recent study using liver-specific deletion of JNK1 in global JNK2 knockout mice has suggested the opposite, that is a protective role. Therefore, the current studies were conducted to gain further insight concerning this controversy by addressing the role of MAP2K to determine whether silencing the expression of either of the two MAP2K upstream of JNK would protect against acute liver injury. As we found that the MAP2K involved (MKK4) activates both JNK and p38, we also explored its possible role in liver injury.

Results
To address the role of MKK4 or 7 in APAP toxicity, we first examined the basal level of expression and efficacy of knockdown of hepatic MKK4 or 7 in vivo. MKK4 and 7 expression in liver were efficiently knocked down by both their respective ASO: as shown by immunoblot and by qPCR. (>90%, Figures 1a and b). Neither ASO affected the other MKK indicating no cross reactivity. Knockdown of MKK4 markedly protected against histological liver necrosis and serum ALT elevation (Figures 1c and d) after a sublethal toxic dose of APAP (300 mg/kg). In contrast, MKK7 knockdown did not protect against liver injury. The MKK4 ASO treatment did not affect APAP metabolism (adducts and GSH depletion; Figures 1e and f).

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The striking protection by MKK4 ASO, but not MKK7 ASO, was also seen in the TNF/galactosamine model of acute massive apoptosis, a model independent of drug metabolism and GSH detoxification. Massive hemorrhagic liver injury and ALT elevation were abrogated by MKK4 but not MKK7 ASO pretreatment (Figure 2). Furthermore, we used a second ASO targeting a different part of the mRNA sequences of MKK4 and MKK7 and observed similar efficient knockdown in
the liver with the appropriate ASO. In both the APAP and TNF/GalN models, the second MKK4 ASO was also markedly protective, whereas the second MKK7 ASO was not protective (Supplementary Figures 1 and 2), as reflected in histological injury and serum ALT levels. These results support the conclusion that ASO knockdown of MKK4 is not protecting by an off-target effect of a single ASO.

We examined the early signaling events in the liver before onset of APAP-induced hepatic necrosis. Consistent with protection from injury, the early activation of P-JNK and translocation to mitochondria was abrogated by MKK4 knockdown but not by MKK7 knockdown (Figure 3a). In addition the dephosphorylation of mitochondrial Src, which is critical in promoting ROS release and MAP3K activation, was prevented by MKK4 knockdown (Figure 3b). This result is an expected consequence of the interruption of the self-sustaining cycle of ROS $\rightarrow$ MAP3K $\rightarrow$ MAP2K $\rightarrow$ JNK $\rightarrow$ mitochondrial Sakt $\rightarrow$ P-Src $\rightarrow$ ↑ROS.

As MKK4 has the capacity to activate both JNK and p38, we examined p38 status. Active p38 was identified in cytoplasm under basal conditions and did not increase further after APAP, whereas P-p38 association with mitochondria did increase slightly but not significantly (Figure 3b). Knockdown of MKK4 completely inhibited P-p38 in both cytoplasm and mitochondria (Figure 3b), but we did not observe significant changes in p38 or P-p38 in control mice after APAP treatment. These findings alone do not fully exclude a role for p38 in liver and, therefore, we further addressed the possible contribution of p38 since MKK4 knockdown inhibited basal expression of P-p38 in the liver. These findings with p38 raise the possibility that the protection by MKK4 knockdown, and by inference the prior findings with MLK3 and ASK1 knockout, might be at least partially mediated by p38.
Although we previously showed that a small molecule p38 inhibitor did not protect, this was done with DMSO to dissolve the inhibitor, which complicates interpretation.\(^{13}\) Nakagawa et al.\(^{14}\) showed that p38α haplo-deficient mice were not protected against APAP. However, only 50% loss of p38α as well as possible consequences of developmental adaptations might have obscured the importance of p38. Therefore, we assessed the effect of highly efficient p38 knockdown versus scrambled control ASO treatment on basal hepatic p38 level and response to APAP or TNF/GalN. p38 ASO decreased expression by >90% (Figure 4a). The response of P-JNK at 1 and 2 h after APAP was not inhibited in cytoplasm or in mitochondrial translocation (Figure 4b). On the contrary, p38 knockdown significantly amplified the increase in sustained P-JNK in cytoplasm at 1 and 2 h after APAP, but did not significantly increase the association of P-JNK with mitochondria. Interestingly, a prior report indicated that liver-specific ablation of p38α resulted in excessive JNK activation in the liver after LPS challenge.\(^{14}\) Despite the increased P-JNK in p38 knockdown condition, the ALT levels and histological injury in both models were not altered (no protection) with p38 knockdown versus scrambled control (Figures 4c and d). A second p38α-targeted ASO verified the findings and did not affect the extent of liver injury from APAP or TNF/GalN (Supplementary Figures 1 and 2).

As we did observe P-p38 associated with the mitochondria under basal conditions and some small but not significant increase after APAP, we determined whether Sab was required for this association. Sab is the docking protein of JNK on mitochondria so it seemed possible that it might be responsible for binding of p38. We monitored P-p38 in mitochondrial fraction at 1 and 2 h after APAP in Tam-Sab f/f and Tam-SabLPC-KO KO mice dosing in both Tam-Sab f/f and Tam-SabLPC-KO KO mice (Figure 4e), whereas we previously showed that P-JNK was no longer associated.\(^{1}\) There was an insignificant transient increase in P-p38 in mitochondrial fraction at 1 h after APAP in Tam-SabLPC-KO versus Tam-SabLPC-KO littermates. Deletion of Sab did not eliminate the association of P-p38 with mitochondria (Figure 4e), whereas we previously showed that P-JNK was no longer associated.\(^{1}\) There was an insignificant transient increase in P-p38 in mitochondrial fraction at 1 h after APAP in Tam-Sab f/f and Tam-SabLPC-KO KO mice (Figure 4e). The physiological significance of the constitutive association P-p38 with mitochondria and its activation in the cytoplasm is unknown but does require MKK4.

Finally, to insure that the protection by MKK4 knockdown was not mediated by an effect on nonparenchymal cells and innate immunity/inflammation, we examined the cell autonomous effect of MKK4 knockdown. Hepatocytes were isolated and cultured after in vivo treatment with MKK4 or control ASO. The PMH were then exposed to APAP or TNF/actinomycin D for up to 2 h to assess early signaling events. Both toxic treatments activated cellular JNK in controls but prior in vivo MKK4 knockdown prevented JNK activation with both treatments (Figures 5a and b). Subsequent cell death was markedly inhibited in both models (Figures 5c and d). Thus, MKK4 in hepatocytes is critical in mediating sustained JNK activation and cell death.
**Discussion**

The findings in the current work provide new insights into the mechanisms of hepatotoxicity in two models in which we have previously shown that the interaction of JNK and mitochondrial Sh3bp5 are critical in promoting oxidative stress and sustained JNK activation.\(^1,^2\) APAP toxicity involves its biotransformation to NAPQI, which directly impairs mitochondrial function, initiating a self-amplifying mitochondrial ROS cycle which ultimately leads to MPT and necrosis.\(^2,^4,^3,^5,^7,^15,^16\) In the TNF/GalN model, hepatocytes are sensitized to direct TNF-induced apoptosis; galactosamine inhibits antiapoptosis gene expression, particularly genes that dampen TNF-induced mitochondrial ROS production.\(^17,^18\) As a consequence, JNK leads to Bcl family-mediated mitochondrial outer membrane permeabilization and apoptosis.\(^3,^9,^19–^22\) In the APAP model, both ASK1 and MLK3 have been shown to participate in activating downstream JNK.\(^3,^4,^23–^25\) These two MAP3 kinases, activated by ROS, then activate MAP2 kinase.\(^26,^27\) There are two MAP2 kinases, which activate JNK: MKK4 and MKK7. MKK4 activates both JNK and p38, whereas MKK7 activates only JNK.\(^10,^11\) Therefore, we explored the contribution of MKK4 and MKK7 in mouse liver to the activation of JNK and the development of acute liver injury in both the APAP and TNF/GalN models. Furthermore, we examined the role of p38 in APAP toxicity.

We found that efficient ASO knockdown of MKK4, but not ASO knockdown of MKK7, protected against liver injury and sustained JNK activation in both models. As the ASO knockdowns are not hepatocyte specific, we assessed whether the effect of MKK4 knockdown was cell autonomous. PMH cultured after \textit{in vivo} knockdown of MKK4 were protected against APAP or TNF/actinomycin D and JNK activation before PMH cell death was markedly suppressed before the onset of cell death when MKK4 was silenced. To ensure that the protection observed was not due to off-target effects of one specific ASO, all results were confirmed with a second ASO targeting a different portion of the mRNA of MKK4 and MKK7.
Our findings need to be discussed in light of the recent findings of Cubero et al. suggesting that hepatocellular JNK1 and JNK2 dampen hepatotoxicity in liver injury from acute APAP and chronic CCl4. Although we cannot fully explain the apparent discrepancy, these studies involved the combined embryonic liver-specific deletion of JNK1 and global JNK2 deletion and the results were characterized by markedly heightened oxidative stress in response to APAP, which suggested that the embryonic deletions lead to a dampening oxidant defense. A large body of evidence supports the role of JNK in amplifying APAP toxicity, including a variety of approaches such as different small molecule JNK inhibitors, ASO knockdown of JNK1 and JNK2, JNK2 global knockout, ASK1 and MLK3 global knockout, highly specific ASK1 inhibitor, MKP-1 global knockout, Sab knockdown and liver-specific inducible knockout of

**Figure 5** Effect of MKK4 knockdown on APAP and TNF-α-induced toxicity in cultured hepatocytes. Immunoblots assessing (a) APAP and (b) TNF-α/ActD-induced JNK activation in PMHs. After control ASO and MKK4 ASO injections, hepatocytes were isolated. Cultured hepatocytes from these mice were incubated with PBS or APAP (5 mM) or TNF-α (20 ng/ml)/ActD (0.5 μg/ml), and whole-cell extracts were examined by western blotting for p-JNK and JNK at the indicated times. GAPDH was used as a loading control. Bars represent densitometric analysis of immunoblots from three mice per group. (c) PMHs from control and MKK4 KD mice were incubated with APAP 16 h or (d) TNF-α/ActD for 6 h, and hepatocytes were stained with Hoechst 3325. Dead cells were counted as described in the ‘Materials and Methods’ section. *P < 0.05 versus control group (n = three experiments, three different primary culture from three mice)
Sab. The current study adds MKK4 knockdown to the evidence supporting the role of JNK in APAP toxicity while excluding a role for the other downstream substrate of MKK4, namely p38 in mediating toxicity. It appears that the sensitization to APAP toxicity in the combined embryonic JNK1/2 deletion may be accounted for by the observed enhanced mitochondrial oxidant stress observed with this model. However, further work assessing liver-specific inducible deletion of JNK1 and JNK2 may shed more light on this controversy.

We have previously observed by P-MKK4 translocation to mitochondrial with P-JNK. P-JNK association with mitochondria only occurs in the presence of Sab. P-MKK4 activation is not sustained in the absence of Sab making it difficult to determine whether P-MKK4 binds directly to Sab or binds to the P-JNK/Sab complex. We have previously shown that P-JNK (plus ATP) directly impairs mitochondrial respiration and increases ROS, which requires binding to Sab. Interestingly, some P-p38 was associated with mitochondria of APAP or TNF/GalN as reflected in histology and serum ALT.

*Materials and Methods*

**Animals.** All animal experiments followed procedures approved by the Institutional Animal Care and Use Committee of the University of Southern California. Male C57BL/6 Nhrsd mice (6–8 weeks of age) were obtained from ENVIGO Bioproducts Inc. (Madison, WI, USA). IoxP-flanked Sab mutant mice (Shbp5tm1aKOMP/Wtsi) were generated in C57BL/6N background by KOMP. UC Davis. Exon 5, 6 and 7 were flanked by IoxP. Primers for genotyping of Sab mutant mice are: for flaxed mice 5′-GAGATGGCGCAAGCATTAGTATG-3′ and 5′-TGTG TAGCAAGTAGACCCCTGATGC-3′, for post-Cre mice 5′-GCTTACATACCGTGAGG-3′ and 5′-TGTGTAACAGAGTGACCTCAGCTGC-3′. To generate tamoxifen-induced hepatocyte-specific Sab KO mice (Tam-Sab-/-), Sab floxed mice were crossed with AlbCreERT2 mice (AlbCreERT2tm1a(CAG-CAG-CAG-3′), kindly provided by Dr. Pierre CHAMBON, Institut de Génétique et de Biologie Moleculaire et Cellulaire (IGBMC), France. Sab−/AlbCreERT2−− were crossed with Sab−/− floxed mice at least 10 times, and then 6-week-old male mice were fed with tamoxifen (Tam) 1 g/kg of diet pellet for 7 days and the experiment was performed 5–7 days later after the last day of Tam diet.

**Preparation of antisense oligonucleotide (ASO) and in vivo knockdown.** MKK4 ASO (5′-TACCTGCTGCTTACG-3′), and its control ASO (5′-GGCAATACGCGGCTGA-3′); MK7 ASO (5′-ACTTTGTTGTCTTCTGTA-3′); MK7 ASO (5′-ACTTTGTTGTCTGATGGA-3′), or P38 ASO (5′-GCACCGCTCTGCATGTCG-3′) and its control ASO (5′-CCCTCTCTCTACAGGTCCTC-3′) were provided by Ionis Pharmaceuticals. Experiments with a second set of ASO and control ASO were conducted to verify the findings: MKK4 ASO (5′-GTTGTTT AGCTGTTCTC-3′) and its control ASO (5′-GGCAATACGCGGCTGA-3′); MK7 ASO (5′-CCGTCACAGTGTGTCCG-3′) or P38 ASO (5′-CGGCTGCTGACAGGAGCGA-3′). The ASO was applied for quantitative PCR in ABI 7900 HT Fast Real-Time PCR System using 500 ng of mRNA. A total 2.5 ng of cDNA was synthetized by QIAGEN OneStep RT-PCR Kit using 500 ng of mRNA. RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthetized by Qiagen OneStep RT-PCR Kit using 500 ng of mRNA. A total 2.5 ng of cDNA was applied for quantitative PCR in ABI 7900 HT Fast Real-Time PCR System using QuantiTect SYBR Green PCR Kit. Specific primer-amplified RNA amount was
determined from standard curve generated by serial dilution of pool mRNA of normal three male mice. MKK4 or MKK7 mRNA was normalized by housekeeping gene TBP (TATA box binding protein). Primers for MKK4 mRNA are 5′-AATCGACAGCAGGTCACTG-3′ and 5′-TGAAATCCGAGTTGTTGACAG-3′. Primers for MKK7 are 5′-GATTCGCGTCTGTGGTAT-3′ and 5′-ACTTTGGAGAAGGTGTTGGGAAG-3′. Primers for TBP are 5′-GAACACTTTGGACTGTGACAGCA-3′ and 5′-GGGAACCTCACTCAGACGCTC-3′.

**Isolation of liver mitochondria and cytoplasm.** Mitochondria were isolated from mouse liver by differential centrifugation as described before.1,2 Livers were homogenized in homogenizing buffer (250 mM sucrose, 10 mM Tris, 2 mM EDTA, pH 7.4 in ice) and centrifuged at 1000× g for 10 min to collect fibrous tissue debris and nuclei. Mitochondria were collected by centrifugation at 9000 × g for 10 min. The mitochondrial pellet was washed once. Mitochondrial protein was extracted in RIPA lysis buffer for Western blot analysis. The supernatant (cytoplasmic fraction) and mitochondrial lysate were stored in −80 °C.

**Antibodies and reagents.** Antisera to P-JNK (#4686), SEK1/MKK4 (SC10; #3346), Phospho-SEK1/MKK4 (Ser257; C38C11; #4514), P-Src(Tyr416; #6943), c-Src (2109), P-c-Src (#4511), total c-Src (#8690), MKK7 (#4172), PHB1 (#4226; Cell Signaling Technology, Danvers, MA, USA), total JNK (JNK1/2/3; sc571), Santa Cruz Biotechnology, Dallas, TX, USA), GAPDH (G2925; Sigma-Aldrich) were used. ALT was determined by reagents from Teco Diagnostics (Anahiem, CA, USA). Covalent binding to liver proteins was assessed using antisemur to NAPQI protein adducts provided by Dr. Laura James of University of Arkansas for Medical Sciences. GSH was determined as described before.36

**Western blot analysis.** Aliquots of cytoplasmic or mitochondrial extracts were fractionated by electrophoresis on 7.5, 10, or 12% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA). Subsequently, proteins were transferred to nitrocellulose membrane using iBlot Gel transfer device (Invitrogen, Grand Island, NY, USA), and blots were blocked with 5% (w/v) nonfat milk or 5% (w/v) BSA dissolved in Tris buffered saline with 0.05% Tween 20 (TBS-T). The blots were then incubated with the desired primary and secondary antibodies. Finally, the proteins were detected by ECL reagent (Thermo Scientific or GE Lifesciences, Waltham, MA, USA) using autoradiography film (Denville) or Amersham Hyperfilm ECL (GE Lifesciences). All gels shown are representative samples from at least three experiments.

**Conflict of Interest**

MA is an employee and shareholder of Ionis Pharmaceuticals. The remaining authors declare no conflict of interest.

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