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Role of JNK Translocation to Mitochondria Leading to Inhibition of Mitochondria Bioenergetics in Acetaminophen-induced Liver Injury*[S]

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Previously, we demonstrated JNK plays a central role in acetaminophen (APAP)-induced liver injury (Gunawan, B. K., Liu, Z. X., Han, D., Hanawa, N., Gaarde, W. A., and Kaplowitz, N. (2006) *Gastroenterology* 131, 165–178). In this study, we examine the mechanism involved in activating JNK and explore the downstream targets of JNK important in promoting APAP-induced liver injury *in vivo*. JNK inhibitor (SP600125) was observed to significantly protect against APAP-induced liver injury. Increased mitochondria-derived reactive oxygen species were implicated in APAP-induced JNK activation based on the following: 1) mitochondrial GSH depletion (maximal at 2 h) caused increased H_2O_2 release from mitochondria, which preceded JNK activation (maximal at 4 h); 2) treatment of isolated hepatocytes with H_2O_2 or inhibitors (*e.g.* antimycin) that cause increased H_2O_2 release from mitochondria-activated JNK. An important downstream target of JNK following activation was mitochondria based on the following: 1) JNK translocated to mitochondria following activation; 2) JNK inhibitor treatment partially protected against a decline in mitochondria respiration caused by APAP treatment; and 3) addition of purified active JNK to mitochondria isolated from mice treated with APAP plus JNK inhibitor (mitochondria with severe GSH depletion, covalent binding) directly inhibited respiration. Cyclosporin A blocked the inhibitory effect of JNK on mitochondria respiration, suggesting JNK was directly inducing mitochondrial permeability transition in isolated mitochondria from mice treated with APAP plus JNK inhibitor. Addition of JNK to mitochondria isolated from control mice did not affect respiration. Our results suggests that APAP-induced liver injury involves JNK activation, due to increased reactive oxygen species generated by GSH-depleted mitochondria, and translocation of activated JNK to mitochondria where JNK induces mitochondrial permeability transition and inhibits mitochondria bioenergetics.

c-Jun N-terminal protein kinases (JNK)[3] are a family of serine/threonine kinases important in responding to environmental stresses as well as growth factors and cytokines (1, 2). Important activators of JNK in many cell lines are reactive oxygen species (ROS) such as H_2O_2 generated from mitochondria and other sources (3, 4). JNK was first identified by its ability to phosphorylate the N terminus in the regulator site of the transcription factor c-Jun. Subsequent studies have demonstrated JNK can phosphorylate other transcription factors (*e.g.* p-53, Elk-1, and ATF-2) as well as many non-nuclear proteins (1, 2). Recent interest in JNK has focused on the ability of JNK to directly or indirectly regulate members of the Bcl2 family (*e.g.* bax and bcl-xl) and consequently influence apoptosis (5, 6). JNK has been shown to promote apoptosis by promoting bax translocation to mitochondria by directly phosphorylating bax (7) or by phosphorylation of 14-3-3, which anchors bax in cytoplasm (8). In addition, JNK has been shown to translocate to mitochondria and phosphorylate bcl-xl (inactivate) in mitochondrial membranes (5, 9). Similarly, JNK translocation to mitochondria can induce cytochrome *c* and SMAC release from the intermembrane space leading to apoptosis (10, 11).

JNK activation is an important component of stress response in cells, but when JNK activation is sustained, it is believed to promote cell injury and death (12, 13). In primary hepatocytes sensitized to TNF by NF-κB inhibition sustained JNK activation is essential in promoting TNF-induced apoptosis (12, 14). Sustained JNK activation has been suggested to play a pathologic role in many disorders, including liver injury from hepatic ischemia reperfusion and liver transplantation (15, 16). Recently, we demonstrated that JNK plays a central role in acetaminophen (APAP)-induced liver injury (17). Mice injected with JNK inhibitor (SP600125) or lacking JNK (antisense treatment, knock-out mice) were significantly protected against APAP-induced liver injury. Protection against APAP by JNK inhibitors was subsequently confirmed by two other laboratories (18, 19). Liver injury caused by APAP and hepatic ischemia is mainly a result of necrotic rather than apoptotic cell death in hepatocytes (20). This suggests that JNK may mediate necrotic

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[3] The abbreviations used are: JNK, c-Jun N-terminal kinase; AMAP, *N*-acetyl-*m*-aminophenol; MPT, mitochondrial permeability transition; ROS, reactive oxygen species; TNF, tumor necrosis factor; APAP, acetaminophen; ASO, antisense oligonucleotides; NAPQI, *N*-acetyl-*p*-benzoquinoneimine; PBS, phosphate-buffered saline; ALT, alanine transaminase; HPLC, high-performance liquid chromatography; TNF-R1, TNF-receptor 1; RCR, respiratory control ratio (state III/state IV).

signaling pathways as well as apoptotic signaling pathways in hepatocytes.

The signaling pathways regulated by JNK that are important in mediating hepatocyte injury following APAP treatment are not well understood. The hepatotoxicity of APAP is attributed to the formation of the reactive metabolite *N*-acetyl-*p*-benzoquinoneimine (NAPQI), generated from APAP metabolism by cytochrome P450 (CYP2E1) in hepatocytes (21, 22). Although a minor metabolite, NAPQI is highly reactive causing glutathione depletion (GSH) and forming covalent bonds with protein and non-protein thiols (23, 24). Our work involving JNK, along with other studies, suggests that NAPQI-mediated damage alone is insufficient to cause hepatocyte death and that the activation of specific signal transduction pathways involving JNK are necessary for hepatocyte death to occur with APAP treatment (22). This suggests APAP-induced hepatotoxicity entails two hits: 1) NAPQI-induced injury, through GSH depletion and covalent binding (23, 25); 2) activation of JNK and other signals that mediate cell death, through activation or inhibition of signaling pathways involved in cell survival, defense, and death (17, 22, 26). Previously, we observed that JNK activation induced bax translocation to mitochondria following APAP, which was inhibited by JNK inhibitor (17). However, recent studies with bax knock-out mice showed a similar susceptibility to APAP as wild-type mice (27), suggesting that JNK-induced bax translocation alone cannot account for APAP toxicity. The downstream targets of JNK that mediate APAP-induced injury still remain elusive, but recent studies suggest mitochondrial proteins may be important downstream targets of JNK (4). JNK translocation to mitochondria has been shown in some contexts to affect mitochondria bioenergetics through cytochrome *c* release (10) and inhibition of proteins such as pyruvate dehydrogenase (4). In the present study, we examined the possible role of JNK in directly impairing mitochondria function during APAP-induced liver injury.

EXPERIMENTAL PROCEDURES

Materials—JNK inhibitor (SP600125) was purchased from Calbiochem. Antisense oligonucleotides (ASOs) targeting mouse JNK1 (Isis 104492), JNK2 (Isis 101759), and a control oligonucleotide (Isis 141923) were synthesized as 20-nucleotide, uniform phosphorothioate chimeric oligonucleotides and purified as previously described (17). The oligonucleotides used in these studies were chimeric oligonucleotides containing five nuclease-resistant 2′-*O*-methoxyethylribose-modified phosphorothioate residues on the 5′- and 3′-ends, flanking a 2′-deoxyribonucleotide/phosphorothioate region that supports RNase H-based cleavage of the targeted mRNA. The sequences of the mouse JNK1 ASO and JNK2 ASO were 5′-TGTTGTCACGTTTACTTCTG-3′ and 5′-GCTCAGTGGACATGGATGAG-3′, respectively. The chemistry control oligonucleotide sequence was 5′-CCTTCCCTGAAGGTTCCTCC-3′. Anti-phospho-JNK, JNK, actin, and cytochrome oxidase IV antisera were purchased from Cell Signaling (Beverly, MA). Anti-Bax, bim, and cytochrome *c* antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bid and tBid levels were measured in whole cell extracts using anti-Bid antibody (R&D Antibody, Benicia, CA). Purified JNK1 and JNK2 were obtained from Millipore (Billerica, MA). *N*-Acetyl-*m*-aminophenol (AMAP) was a gift from Sid Nelson (School of Pharmacy, University of Washington).

Animals—Male C57 BL/6 (6–8 weeks of age) were obtained from Harlan Bioproducts for Science Inc. (Indianapolis, IN). Tumor necrosis factor (TNF)-R1 knock-out and wild-type (C57 BL/6) mice (4–6 weeks of age) were obtained from Jackson Laboratories (Bar Harbor, MN). The animals were housed in a temperature-controlled room and were allowed to acclimatize for a minimum of 3 days prior to use in experiments. They were maintained on a commercial pellet diet *ad libitum*. The animals were fasted overnight for food, but not water, prior to experiments.

All the treatments were administered intraperitoneally. APAP (Sigma) was dissolved in warm phosphate-buffered saline (PBS, 55 °C) and cooled to 37 °C before injection of mice. Previously, we demonstrated the protective effects of JNK inhibitor against APAP-induced liver injury using APAP dissolved in 10% DMSO in PBS (17). The presence of DMSO has however been shown to reduce the severity of APAP-induced liver injury (28–30), and consequently in this work we investigated whether JNK inhibition protects against APAP dissolved only in PBS. However JNK inhibitor (SP600125) required some DMSO (8.3%) in PBS (1 mg in 125 μl of DMSO diluted with 1375 μl of PBS) for solubility. JNK inhibitor, unless noted, was injected 1 h prior to APAP injection. Control mice received similar amounts of DMSO in PBS 1 h prior to APAP injection. Mice receiving the JNK inhibitor or vehicle received 1.24 μl/g body weight of DMSO. For ASO experiments, the animals were given 50 mg/kg in sterile saline intraperitoneal (7 injections, every other day) prior to APAP administration. The last dose of ASO was given 1 day prior to APAP administration. Blood was obtained after mice were anesthetized at the indicated time periods and serum alanine transaminase (ALT) was measured at the USC Pathology Reference Laboratory. All animals received care according to methods approved under institutional guidelines for the care and use of laboratory animals in research.

Isolation of Liver Mitochondria and Cytoplasm—For most of the experiments liver mitochondria were isolated from mice by differential centrifugation (31). Livers from mice were excised, washed with 0.25 M sucrose, and homogenized in an H-medium (210 mM mannitol, 70 M sucrose, 2 mM HEPES, 0.05% bovine serum albumin, plus protease and phosphatase inhibitors). The homogenate was centrifuged at 800 × *g* for 10 min, the pellet was removed, and the centrifugation process was repeated. The resulting supernatant was centrifuged at 8500 × *g* for 15 min. The supernatant (cytoplasmic fraction) was collected and saved at −80 °C for future analysis. The pellet which represents the mitochondria fraction was washed with H-medium and the centrifugation repeated. The mitochondria were resuspended in H-medium before oxygen electrode and Western blot analysis. For measurement of H_2O_2 release from mitochondria discontinuous Percoll centrifugation was used as previously described (4).

Measurements of Respiration in Isolated Mitochondria—Respiration was measured in freshly isolated mitochondria by monitoring oxygen consumption polarographically with a

Clark-type electrode (Hanstech, UK) in respiration buffer containing 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4 (31). For isolated mitochondria, 0.75 mg of mitochondria protein was added to 1 ml of respiration buffer, and oxygen consumption was monitored in the presence of mitochondrial substrates.

Addition of Active JNK1 or JNK2 to Isolated Mitochondria—In some experiments isolated mitochondria from control and APAP plus JNK inhibitor-treated mice (following 4-h APAP treatment) were incubated with purified active JNK1 or JNK 2 (Millipore, Billerica, MA) in incubation buffer (230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EDTA, $MgCl_2$ 10 mM, ATP 500 μM, pH 7.4) for 10 min at room temperature (4). 1 mg of mitochondria was incubated with 0.35 μg of purified active JNK1 or JNK2. Controls represent isolated mitochondria from control and APAP plus JNK inhibitor-treated mice incubated in incubation buffer only for 10 min at room temperature. Following 10 min of incubation, mitochondria were spun (8500 $\times$ *g* for 15 min), washed with H-medium, centrifuged again, and redissolved in H-medium. Respiration measurements were immediately performed in the presence or absence of cyclosporine A (2 μM).

Western Blot for JNK—Aliquots of cytoplasmic or mitochondria extracts were fractionated by electrophoresis on 12% SDS-polyacrylamide gel (Bio-Rad). Subsequently, proteins were transferred to nitrocellulose membrane, and blots were blocked in 5% nonfat milk dissolved in Tris-buffered saline with Tween 20. The blots were then incubated with the desired primary and secondary antibodies. Finally, the proteins were detected by Luminol ECL reagent (Santa Cruz Biotechnology, Santa Cruz, CA). All gels shown are representative samples from three experiments.

GSH and ATP Measurements—GSH was detected using HPLC electrochemical detection (32). Total liver homogenate or mitochondria were mixed with metaphosphoric acid to obtain a 5% metaphosphoric acid solution to prevent GSH autoxidation. Samples were centrifuged (12,000 $\times$ *g* for 5 min), and the supernatant was injected into the HPLC. ATP was measured using HPLC with a UV detector set at 254 nm using the method developed by Folley *et al.* as described before (33).

Hydrogen Peroxide Measurements—H_2O_2 production by isolated liver mitochondria was measured by monitoring fluorescence of *p*-hydroxyphenyl acetate oxidation in the presence of horseradish peroxidase (34, 35). Fluorescence measurements (λ_{ex} = 320 nm; λ_{em} = 400 nm) were performed with a PerkinElmer Life Sciences LS-5 spectrofluorometer equipped with a thermal-controlled and magnetic stirring sample compartment. For all measurements mitochondria were incubated in 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EDTA, pH 7.4, at 25 °C.

Cell Isolation and Culture—Primary cultured hepatocytes were isolated as previously described (36). The liver was perfused with collagenase, and isolated hepatocytes were suspended in Dulbecco's modified Eagle's medium/F-12 containing 10% heat-inactive fetal bovine serum, 1 nM bovine insulin, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 50 nM hydrocortisone, 0.15 mg/ml methionine. 1.2 $\times$ 10^6 cells in 4 ml were plated in individual 60-mm diameter LUX culture dishes coated with 0.03% rat tail collagen and cultured in a 5% CO_2 atmosphere at 37 °C. The viability of the isolated hepatocytes was >90% as judged by trypan blue exclusion. After 3 h, the culture medium was changed to serum-free medium containing 100 units/ml penicillin and 0.1 mg/ml streptomycin. At this time, antimycin, rotenone, and H_2O_2 were added to hepatocytes. Hepatocytes were subsequently scraped (30 min following H_2O_2 treatment, 1 h after antimycin and rotenone treatment) and sonicated, and levels of phospho-JNK were analyzed by immunoblotting. In some samples mitochondria were isolated using differential centrifugation, using H-medium outlined above, and phospho-JNK levels in mitochondria were assessed by immunoblotting.

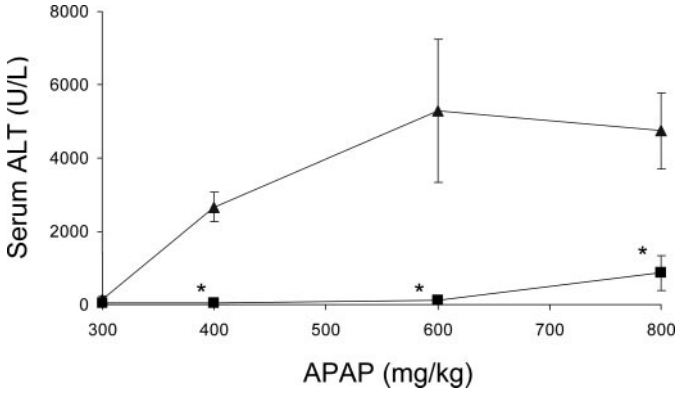

FIGURE 1. **Protective effects of JNK inhibitor (SP600125) on APAP-induced toxicity *in vivo* in C57BL/6 and TNF-R1 knock-out mice.** Protective effects of JNK inhibitor against APAP dissolved in warm PBS in C57BL/6 mice. ▲, acetaminophen; ■, APAP plus JNK inhibitor. APAP (600 mg/kg) was dissolved in warm PBS prior to injection of mice. In all experiments mice were pretreated with JNK inhibitor (10 mg/kg in DMSO (8.3%) and PBS) or equivalent amounts of DMSO in PBS as vehicle control, 1 h prior to APAP treatment. Serum ALT levels were measured 24 h after APAP treatment. n = 4–12 mice per group. Results are mean ± S.D. *, $p < 0.05$ *versus* APAP treatment alone.

Statistical Analysis—Statistical analyses were performed using the Student's *t* test for unpaired data or analysis of variance. A *p* value of <0.05 was considered significant.

RESULTS

Protective Effects of JNK Inhibitor against APAP-induced Liver Injury—The pretreatment of mice with JNK inhibitor protected mice from liver injury caused by various doses of APAP dissolved in hot PBS (Fig. 1), confirming our previous findings with APAP dissolved in DMSO (17). The high dose of APAP (800 mg/kg) dissolved in warm PBS induced 75% mortality, with lower doses having little effect on mortality. JNK inhibitor pretreatment completely protected against APAP-induced mortality (data not shown) and significantly reduced APAP-induced liver injury as shown by decreases in ALT levels. JNK inhibitor also protected against APAP-induced liver injury in TNF receptor 1 (TNF-R1) knock-out mice. TNF-R1 knock-out mice had similar ALT levels as wild-type mice (ALT-TNF-R1 KO: 5851 ± 1890, WT: 5988 ± 2530), and JNK inhibitor pretreatment protected both TNF-R1 knock-out and wild-type mice (ALT-TNF-R1 KO: 259 ± 197, WT: 315 ± 215). These data suggest that TNF does not play a major role in APAP-induced liver injury, that sustained JNK activation in the APAP model is unlikely to be due to TNF, and that the protec-

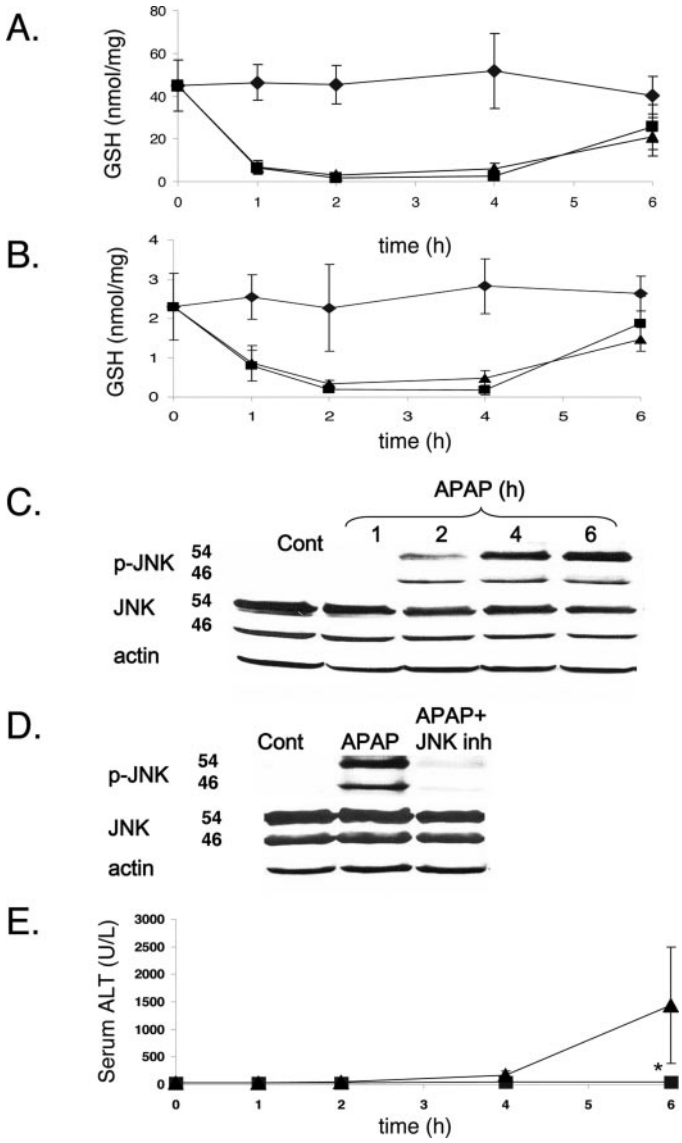

FIGURE 2. **Time course of GSH depletion, JNK activation, and liver injury following APAP treatment *in vivo*.** *A*, total liver homogenate GSH; *B*, mitochondrial GSH; *C*, JNK activation; *D*, JNK inhibitor prevents JNK activation (4 h following APAP treatment); *E*, early time course of serum ALT reflecting liver injury. C57BL/6 mice were treated with APAP (600 mg/kg) dissolved in warm PBS and pretreated with either JNK inhibitor (10 mg/kg in DMSO (8.3%) and PBS) or equivalent amounts of DMSO in PBS 1 h prior APAP treatment. At indicated times liver were taken, ALT measurements made in serum, GSH measured using HPLC with electrochemical detection, and Western blot analysis was performed using antisera against phospho-JNK, JNK, and actin as loading control. ♦, control; ▲, APAP; ■, APAP plus JNK inhibitor. Results are mean ± S.D. *, $p < 0.05$ *versus* APAP treatment alone. $n = 3$–5 mice.

tive effects of JNK inhibitor are mediated through other pathways than those involving TNF.

Time Course of GSH Depletion, JNK Activation, and Liver Injury following APAP Treatment—The depletion of GSH by NAPQI is an important component of APAP-induced liver injury (22). The results of GSH measurements demonstrate that the protective effects of JNK inhibitor against liver injury caused by APAP (delivered using warm PBS) was not due to its modulation of GSH (Fig. 2, *A* and *B*). Within 1 h of APAP, the bulk of hepatic GSH was depleted (~85%), and mitochondrial GSH (~65%) became depleted equivalently in both mice treated with APAP and APAP plus JNK inhibitor. The GSH depletion was maximal and profound at 2 h following APAP treatment, with later time points showing gradual recovery. These findings are in agreement with our previous findings using APAP dissolved in DMSO, indicating that JNK inhibitor does not modulate GSH to protect liver from APAP and that the production of the GSH-depleting metabolite, NAPQI, is not influenced by the JNK inhibitor.

An examination of the time course of JNK activation (phosphorylation) demonstrates that JNK activation does not begin until 2 h following APAP treatment, reaching a peak plateau at around 4 h (Fig. 2*C*). Comparison of the GSH and phospho-JNK time courses shows that cytoplasmic and mitochondrial GSH depletion precede JNK activation. Total JNK (p46 and p54) levels in liver were unaffected by APAP treatment. Pretreatment of mice with JNK inhibitor (SP600125) significantly decreased the levels of phospho-JNK at all times points (4 h is shown in Fig. 2*D*). Although SP600125 is a competitive inhibitor of ATP for the binding site of JNK, SP600125 has been previously shown to inhibit JNK phosphorylation, because maximal JNK activation involves autophosphorylation or a feed forward activation loop (37, 38). The activation of JNK by APAP treatment preceded liver injury, which started around 4–6 h (Fig. 2*E*). Thus taken together our data suggest that the sequence of events that take place following APAP treatment is cytoplasmic and mitochondrial GSH depletion, followed by JNK activation, which then somehow results in liver injury.

Effect of AMAP Treatment on Mitochondrial GSH and JNK Activation—AMAP is a regioisomer of APAP, which like APAP is metabolized by CYP2E1 to a reactive metabolite that depletes cytoplasmic GSH and covalently binds to proteins (39). However, unlike APAP, AMAP is not hepatotoxic, because the reactive intermediate of AMAP is hypothesized to be more reactive than NAPQI and consequently is mainly bound in endoplasmic reticulum and cytoplasm once cytoplasmic GSH is depleted. The metabolite is not sufficiently stable to reach mitochondria and thus does not significantly deplete mitochondria GSH or undergo covalent binding in mitochondria (39, 40). We therefore examined whether AMAP-induced extramitochondrial GSH depletion and covalent binding could induce JNK activation. Fig. 3 (*A* and *B*) demonstrates that AMAP treatment (600 mg/kg) caused significant GSH depletion in liver homogenate (~70%) but only a modest mitochondrial GSH depletion (~25%) 2 h following administration. Other doses of AMAP (400–800 mg/kg) resulted in similar mitochondria and liver homogenate GSH depletion (data not shown). Although AMAP caused significant GSH depletion in liver, it did not induce mitochondrial GSH depletion or JNK activation (Fig. 3*C*). The data therefore suggest that mitochondrial GSH depletion (and/or covalent binding) may be important in JNK activation following APAP. However, because AMAP caused less cytosolic GSH depletion (~70%) than APAP treatment (>90%), the direct involvement of severe cytosolic GSH depletion by APAP in activating JNK cannot be ruled out. Overall, AMAP does not significantly target mitochondria, does not cause JNK activation, and does not cause toxicity in contrast to the effects of APAP.

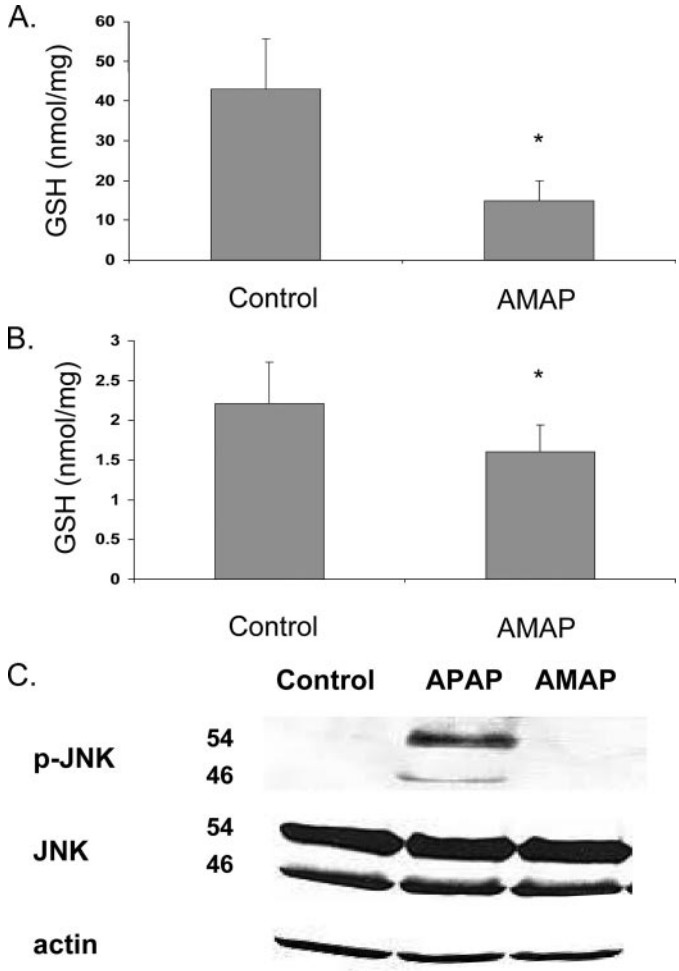

FIGURE 3. **Effect of AMAP on liver GSH levels and JNK activation.** *A*, total liver homogenate GSH; *B*, mitochondrial GSH; *C*, JNK activation. C57BL/6 mice were treated either APAP (400 mg/kg) or AMAP (600 mg/kg) dissolved in warm PBS. 2 h following APAP or AMAP treatment, liver mitochondria was isolated. Total liver homogenate and mitochondria GSH levels were measured using HPLC with electrochemical detection, and Western blot analysis was performed using antisera against phospho-JNK, JNK, and actin as loading control. $n = 4$ mice.

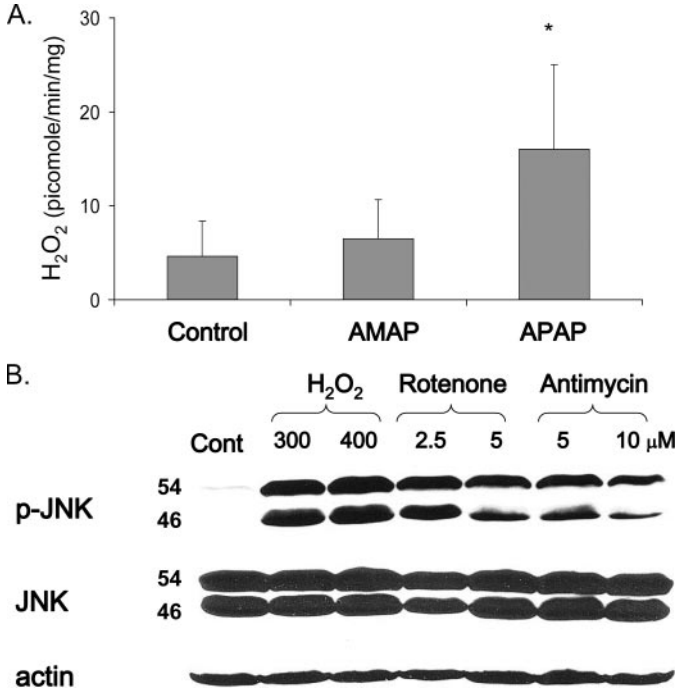

FIGURE 4. **Activation of JNK by mitochondrial ROS.** *A*, APAP treatment induces increased H_2O_2 generation from mitochondria. C57BL/6 mice were treated with either APAP (400 mg/kg) or AMAP (600 mg/kg) dissolved in warm PBS. 1 h following APAP treatment, livers were taken and mitochondria was isolated using discontinuous Percoll gradient centrifugation, and H_2O_2 release from mitochondria was measured by monitoring fluorescence of *p*-hydroxyphenyl acetate oxidation in the presence of horseradish peroxidase. H_2O_2 measurements were made with mitochondria treated with complex I substrates (glutamate/malate 7.5 mM). *B*, H_2O_2 and inhibitors that increase mitochondrial H_2O_2 generation induce JNK activation in isolated primary cultured hepatocytes. Primary cultured hepatocytes were treated with various doses of H_2O_2 (300 and 400 μM for 30 min) or rotenone (2.5 and 5 μM for 60 min) or antimycin (5 and 10 μM for 60 min). Western blot analysis was performed using antisera against phospho-JNK, JNK, and actin as loading control.

Mitochondrial ROS Activates JNK in Hepatocytes—Mitochondrial GSH is an essential substrate for GSH peroxidase to detoxify H_2O_2 in the mitochondrial matrix generated by the respiratory chain (41). We previously demonstrated that extensive mitochondrial GSH depletion (>40%) is associated with significant increases in H_2O_2 release from stressed mitochondria (35). In addition, we previously showed that diethylmaleate-induced GSH depletion caused sustained JNK activation only at doses that depleted mitochondrial GSH (36). In agreement with these findings, we observed increased H_2O_2 release from mitochondria isolated from mice treated with APAP (mitochondria with severe GSH depletion) compared with mitochondria isolated from control and AMAP-treated mice (Fig. 4*A*). AMAP treatment, which only caused a small decrease in mitochondrial GSH (Fig. 3), did not cause a significant increase in H_2O_2 release from mitochondria. These findings suggest that the extensive mitochondrial GSH depletion and consequent H_2O_2 generation caused by APAP treatment may be responsible for JNK activation. This notion is also supported by our observation that GSH depletion and H_2O_2 generation preceded JNK activation following APAP treatment (Figs. 3 and 4).

To confirm that mitochondrial H_2O_2 is likely to be responsible for activating JNK during APAP-induced liver injury, we investigated JNK activation by ROS in primary cultured hepatocytes treated with either H_2O_2 or mitochondria inhibitors (antimycin, complex III inhibitor, rotenone, and complex I inhibitor) that have been documented to increase H_2O_2 generation specifically from mitochondria (35, 42). Fig. 4*B* demonstrates that H_2O_2, rotenone, or antimycin all induced JNK activation in whole cell extracts. Taken together these observations strongly suggest that JNK activation during APAP-induced liver injury is a likely consequence of increase H_2O_2 release from mitochondria contributed by mitochondrial GSH depletion and other possible effect of APAP on mitochondria.

Impairment of Mitochondria Bioenergetics by JNK—We next examined the possibility that mitochondria were not only a source of ROS-induced JNK activation but also downstream targets of JNK during APAP-induced liver injury. Mitochondria bioenergetics has been reported to decline in liver mitochondria following APAP treatment (43, 44), suggesting the possibility that JNK may be mediating mitochondria dysfunction during APAP-induced live injury. Therefore, we examined the

effect JNK inhibitor treatment on mitochondria bioenergetics following APAP treatment. Fig. 5 demonstrates that APAP treatment caused a significant decline in state III respiration (using complex I substrate (glutamate/malate) plus ADP), a slight decline in state IV respiration (glutamate/malate only), and significant decline in the respiratory control ratio (RCR, state III/state IV). The decline in mitochondria respiration following APAP treatment started at 2 h, a time that coincides with JNK activation and translocation to mitochondria, and was maximal at 4 h, which corresponds to maximal JNK activation.

Pretreatment of mice with JNK inhibitor protected against a decline in mitochondria respiration and RCR caused by APAP treatment. The protection of JNK inhibitor was not complete, with mitochondria from mice treated with JNK inhibitor and APAP having a respiratory rate and RCR lower than control but significantly higher than mice treated with APAP alone. These results suggest that part of the decline in mitochondria respiration following APAP treatment involved a component that was JNK-independent (not affected by the JNK inhibitor treatment) and a component that was JNK-dependent (prevented by JNK inhibitor treatment). The JNK-independent component presumably involves NAPQI-induced mitochondrial GSH depletion and covalent binding, which are not prevented by inhibition of JNK (22, 23). However, this alone was insufficient to cause necrosis, so the additional JNK inhibitable effect on mitochondria function was necessary for necrosis to develop.

Not surprisingly, liver ATP levels paralleled changes in mitochondria bioenergetics caused by APAP and JNK inhibitor treatment (Fig. 5*D*). Liver from mice treated with APAP experienced a dramatic decline in ATP levels in liver (at 4 h ~88% decline in ATP levels). JNK inhibitor pretreatment partially prevented the decline in liver ATP levels caused by APAP, a finding that corresponds with mitochondria respiration results.

JNK Regulation of bcl2 Family Members and Cytochrome c in Mitochondria—JNK has also been shown to regulate members of the bcl2 family such as bax, which translocates to mitochondria, as well as to promote cytochrome *c* release from mitochondria (10, 11). Loss of cytochrome *c* from mitochondria could explain, in part, the decline in mitochondria respiration induced by APAP treatment. We consequently examined the effect of APAP and JNK inhibitor on various members of the bcl2 family and cytochrome *c* in cytoplasm and mitochondria. APAP had little effect on the cytoplasmic levels of members of the bcl2 family but did promote release of cytochrome *c* from mitochondria to cytoplasm (Fig. 6*A*). APAP treatment caused ~2-fold increase in cytoplasmic cytochrome *c*, which was prevented by JNK inhibitor pretreatment. On the other hand, APAP treatment caused an increase in bax and bid levels in mitochondria, as well as a slight decline in cytochrome *c* levels in mitochondria (Fig. 6*B*). Contrary to previous reports, we did not observe an increase in tBid following APAP treatment (tBid was observed in hepatocytes treated with actinomycin D plus TNF as positive control (data not shown)) (45). JNK inhibitor treatment inhibited bax translocation to mitochondria as we previously reported and prevented cytochrome *c* release from mitochondria. Bid levels, which were not affected by JNK inhibitor, increased in mitochondria. However, because intact bid is not considered to modulate mitochondria function and not found to be affected by JNK inhibitor, its importance can be questioned. Also, we observed a doublet of Bcl-X_L following APAP treatment, suggesting a phosphorylated and non-phosphorylated form, but these did not change with JNK inhibitor pretreatment. The amount of cytochrome *c* loss from mitochondria induced by APAP treatment was small (~23%) but

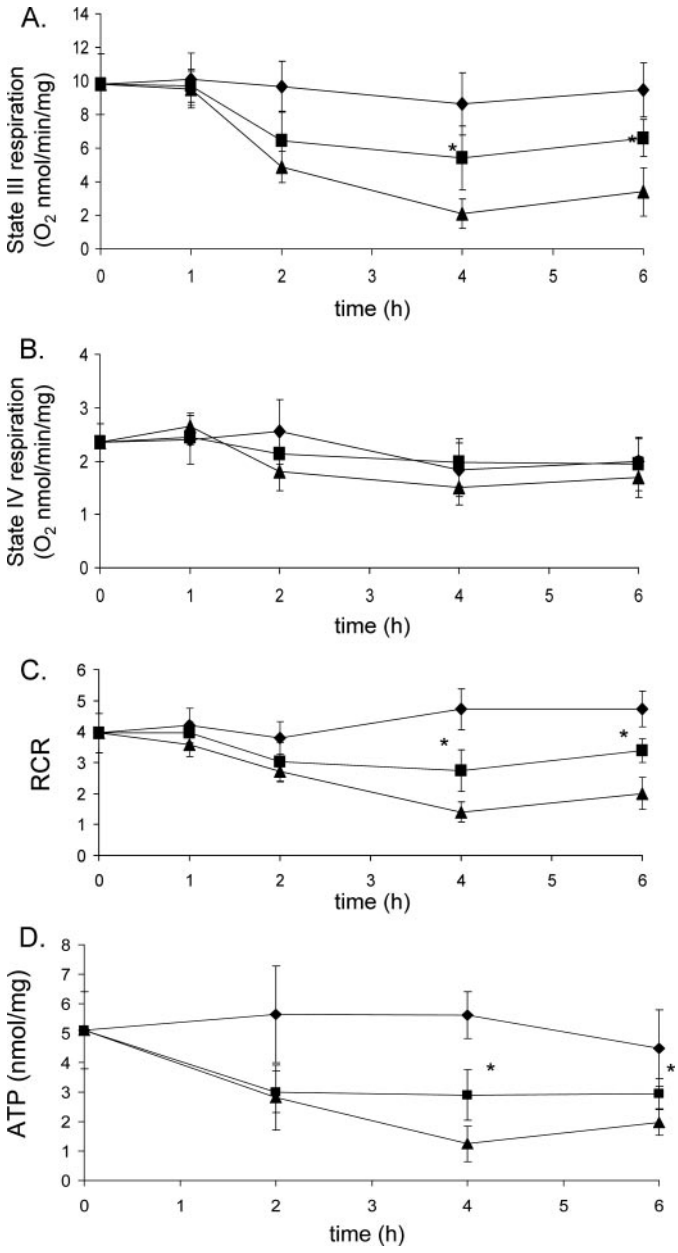

FIGURE 5. **JNK inhibitor protects against a decline in mitochondria bioenergetics caused by APAP *in vivo*.** *A*, state III respiration (glutamate/malate plus ADP) in isolated liver mitochondria. *B*, state IV respiration (glutamate/malate). *C*, RCR (state III/state IV). *D*, ATP levels in liver homogenate. C57BL/6 mice were treated with APAP (600 mg/kg) dissolved in warm PBS and pretreated with either JNK inhibitor (10 mg/kg in DMSO (8.3%) and PBS) or equivalent amounts of DMSO in PBS 1 h prior APAP treatment. At the indicated times livers were taken, mitochondria was isolated using differential centrifugation, and mitochondrial oxygen consumption was measured using a Clarke type electrode. ATP measurements were made using HPLC with UV detection as described under "Experimental Procedures." ◆, control; ▲, APAP; ■, APAP plus JNK inhibitor. Results are mean ± S.D. *, $p < 0.05$ *versus* APAP treatment alone. $n = 3$ mice per time point per group.

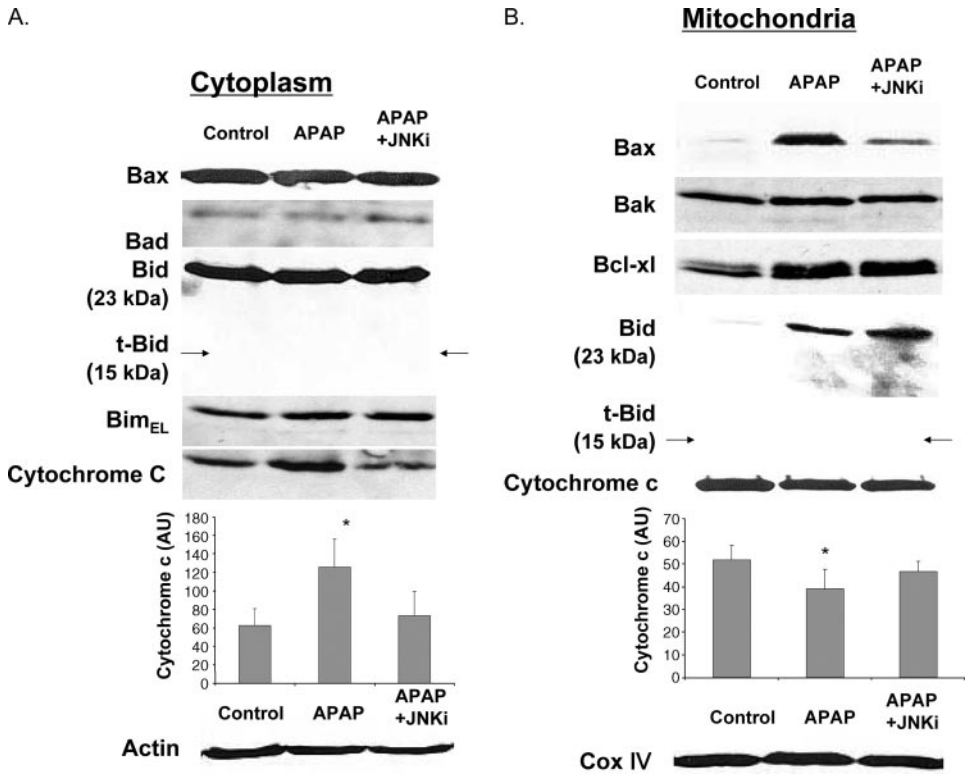

FIGURE 6. **Effect of APAP and JNK inhibitor on bcl2 family members and apoptotic factors in cytoplasm and mitochondria *in vivo*.** *A*, cytoplasm; *B*, mitochondria. Bax, Bad, Bak, Bid, tBid, Bcl_{xL}, Bim_{EL}, and cytochrome *c* were measured by Western blot analysis at 4 h after APAP (600 mg/kg) treatment pretreated with either JNK inhibitor or DMSO-PBS vehicle. A positive control for tBid was made by treating primary culture hepatocytes with actinomycin D (10 μM) plus TNF (20 ng) for 6 h (data not shown). Densitometry was performed using Scion. *AU* = arbitrary units. Results are mean ± S.D. *, $p < 0.05$ *versus* control.

significant. Bax is known to form channels that promote cytochrome *c* release from mitochondria, which may be responsible in part for the loss of cytochrome *c* from mitochondria following APAP treatment. The loss of cytochrome *c* from mitochondria may explain part of the decline in mitochondria respiration that occurs with APAP treatment. However, because significant amounts of cytochrome *c* must be lost for mitochondria respiration to be completely inhibited, the loss of cytochrome *c* from mitochondria alone probably cannot explain the significant decline in mitochondria respiration caused by APAP treatment, which suggests that other factors must be responsible for the decline in mitochondria bioenergetics caused by APAP treatment.

APAP Induces JNK Translocation to Mitochondria—To address whether the regulation of mitochondria function may be a direct effect of JNK on mitochondria, we examined whether APAP treatment induced JNK translocation to mitochondria following its activation in cytoplasm. Fig. 7*A* shows that phospho-JNK (p46 and p54) and total JNK translocated to mitochondria following APAP treatment. JNK translocation to mitochondria was maximal 4 h following APAP treatment, a time coinciding with maximal JNK activation and maximal inhibition of mitochondria bioenergetics (Fig. 5). The pretreatment of mice with JNK inhibitor, which prevented JNK phosphorylation (Fig. 2*D*), prevented the translocation of JNK to mitochondria (Fig. 7*B*). Similarly, primary cultured hepatocytes treated with agents that activated JNK (rotenone, antimycin, and H_2O_2, Fig. 4*B*) all induced JNK translocation to mitochondria (supplemental Fig. S1). Taken together, the data suggest that JNK translocation to mitochondria appears to be a consequence of JNK activation (phosphorylation) by ROS.

Because JNK1 is predominantly associated with the p46 JNK isoform, whereas JNK2 is associated with the p54 isoform of JNK (46), our data suggest both isoforms of JNK translocate to mitochondria following APAP treatment. This was further confirmed with experiments using antisense to knock down JNK 1 and/or JNK 2. Treatment of mice with JNK1 ASO or JNK2 ASO, caused reduction in protein levels of p46 (JNK1) or p54 (JNK2) in the cytoplasm (supplemental Fig. S2), with JNK1 ASO being more effective than JNK2 ASO in lowering the respective protein levels. The treatment of APAP in JNK isoform-specific ASO pretreated mice still resulted in translocation of the other JNK isoform to mitochondria, suggesting both isoforms of JNK translocate to mitochondria following APAP treatment. The treatment of mice with both JNK1 and JNK2 ASO similarly resulted in silencing of JNK1 and JNK2 (Fig. 8*A*). Consequently, very little JNK was observed to translocate to mitochondria following APAP treatment following JNK1 and -2 ASO treatment. This decrease in JNK translocation to mitochondria by silencing JNK1 and -2 was associated with less impairment of state III respiration (data not shown) and RCR (Fig. 8*B*) following APAP treatment. The protective effects on mitochondria respiration by silencing both JNK1 and -2 suggest that both activated JNK isoforms may be negatively regulating mitochondria bioenergetics.

The silencing of JNK1 or JNK2 alone was not effective in preventing liver injury caused by APAP delivered in warm PBS (Fig. 8*C*). This result differs from previous results in which we observed significant protective effects by silencing JNK2 against APAP delivered using DMSO as vehicle. However, we observed that silencing of both JNK1 and JNK2 protected against APAP dissolved in warm PBS, which is in agreement with results we previously observed with APAP dissolved in DMSO (17).

Addition of Purified Active JNK Inhibits Mitochondria Bioenergetics and Induces MPT in Redox-altered Mitochondria—We next examined whether JNK translocation was directly regulating mitochondria bioenergetics. To address this question, purified active JNK1 or JNK2 were added to mitochondria isolated from control and JNK inhibitor plus APAP-treated mice. The latter represent mitochondria that are redox-altered (GSH

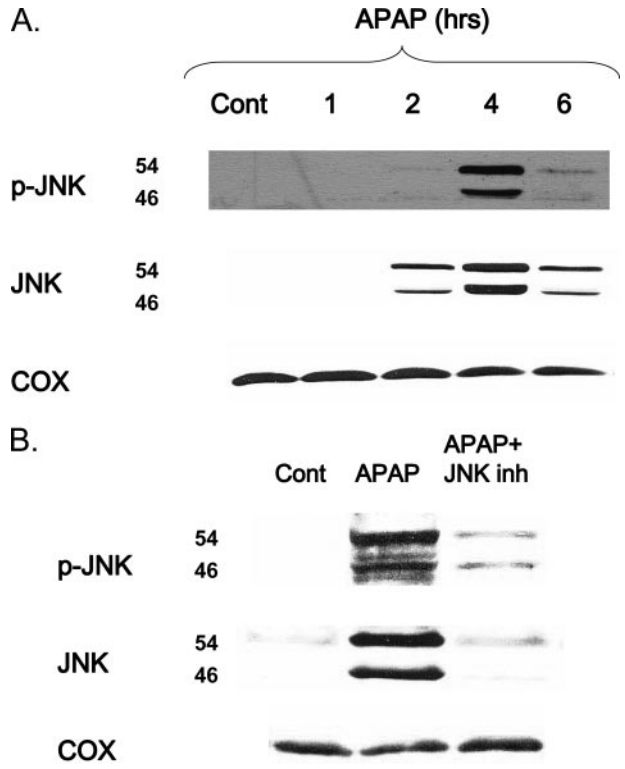

FIGURE 7. **Time course of JNK translocation to mitochondria following APAP treatment *in vivo*: modulation by JNK inhibitor.** *A*, time course of JNK translocation to mitochondria following APAP treatment. *B*, JNK inhibitor prevents JNK translocation to mitochondria (4 h following APAP treatment). C57BL/6 mice were treated APAP (600 mg/kg) dissolved in warm PBS and pretreated with either JNK inhibitor (10 mg/kg in DMSO (8.3%) and PBS) or equivalent amounts of DMSO in PBS 1 h prior APAP treatment. At indicated times livers were taken, and mitochondria were separated from cytoplasm by differential centrifugation. Western blot analysis was performed using antisera against phospho-JNK, JNK, and cytochrome oxidase as loading control.

depleted with protein covalent binding, Fig. 2) and somewhat impaired (decline in RCR, Fig. 5) but do not have any associated JNK. The mitochondria preparations were also washed free of JNK inhibitor. The incubation of JNK1 or JNK2 in buffer containing ATP had no effect on mitochondria bioenergetics in mitochondria isolated from control mice (Fig. 9, *A* and *B*). However, addition of JNK1 or JNK2 decreased state III respiration (complex I substrates (glutamate/malate) plus ADP) in mitochondria isolated from mice that had been treated with JNK inhibitor plus APAP (Fig. 9*A*). Mitochondria from control and JNK inhibitor plus APAP-treated mice incubated in buffer containing ATP for 10 min without JNK exhibited no change in mitochondria respiration (data not shown). State IV respiration (glutamate/malate only) was only slightly impaired in mitochondria from JNK inhibitor plus APAP-treated mice (data not shown), however because state III respiration was altered there was a dramatic decline in RCR (state III/IV) of mitochondria (Fig. 9*B*). These changes in mitochondria bioenergetics (*e.g.* drop in state III, RCR) induced by addition of active JNK to mitochondria isolated from APAP plus JNK inhibitor-treated mice caused respiration and RCR values similar to mitochondria from mice treated with APAP alone (Fig. 9). These results demonstrate that either JNK1 or JNK2 directly inhibits mitochondria bioenergetics in redox-altered and -impaired mitochondria but not in normal mitochondria. JNK1 was found to be slightly more effective than JNK2 in causing a decrease in mitochondria bioenergetics. JNK1 and JNK2 were found to associate equally with mitochondria, even following washing (Fig. 9*C*). Taken together our data support the hypothesis that part of the decline in mitochondria bioenergetics following APAP treatment is a direct result of JNK translocating to mitochondria with the APAP-induced covalent binding and/or GSH depletion sensitizing the mitochondria to the effects of JNK (dual hit).

The pretreatment of isolated mitochondria with cyclosporin A (2 μM) was observed to prevent the decline in mitochondria bioenergetics caused by treatment of mitochondria with purified active JNK. Because cyclosporin A is known to bind cyclophilin D and prevent MPT (47), our data suggest that active JNK was directly inducing MPT in isolated mitochondria. MPT, by increasing the permeability of the matrix to protons, is known to inhibit mitochondria respiration (47, 48). Taken together, the findings suggest that JNK translocates to mitochondria and inhibits mitochondria bioenergetics, at least in part, by triggering of MPT.

JNK Inhibitor Given after APAP Treatment Still Partially Protects against JNK-induced Mitochondria Dysfunction—JNK translocation to mitochondria was maximal at 4 h following APAP treatment. We consequently investigated the time course following APAP injection that JNK inhibitor would still be effective in protecting mice from APAP-induced liver injury. JNK inhibitor given 2 h after APAP treatment still significantly protected liver from APAP-induced injury, while JNK inhibitor given 4 h after APAP treatment (where JNK activation is maximal) was not effective in protecting mice from liver injury (Fig. 10*A*). Western blot analysis shows that JNK activation and translocation to mitochondria was partially inhibited by providing JNK treatment 2 h after APAP treatment (Fig. 10*B*). The decreased JNK translocation to mitochondria caused by providing JNK inhibitor 2 h after APAP treatment was associated with less impairment of mitochondria respiration (state III respiration on glutamate/malate plus ADP) than APAP alone (Fig. 10*C*). These observations support a strong correlation between JNK translocation to mitochondria and mitochondria respiration. Preventing JNK translocation to mitochondria even at later time points after maximal GSH depletion still prevented further decline in mitochondria bioenergetics.

DISCUSSION

Mitochondria have gained prominence in the field of cell death, because both apoptotic and necrotic cell death are believed to involve mitochondria (47–49). Mitochondria control cellular energy, apoptotic factors (*e.g.* cytochrome *c*, SMAC, and AIF) and are the major sources of ROS that can initiate or modulate apoptotic and necrotic signaling pathways in cells (32, 50). Recent studies suggest that cytoplasmic kinases such as JNK and protein kinase C can regulate mitochondria bioenergetics as well as mitochondria-driven apoptosis and necrosis (4, 10, 51). Consequently, it is perhaps not surprising that we found mitochondria are a major target of JNK signaling in APAP-induced liver injury. In the present study, we demonstrate in a clinically relevant liver injury model that a JNK-mitochondria signaling loop is important in mediating liver injury

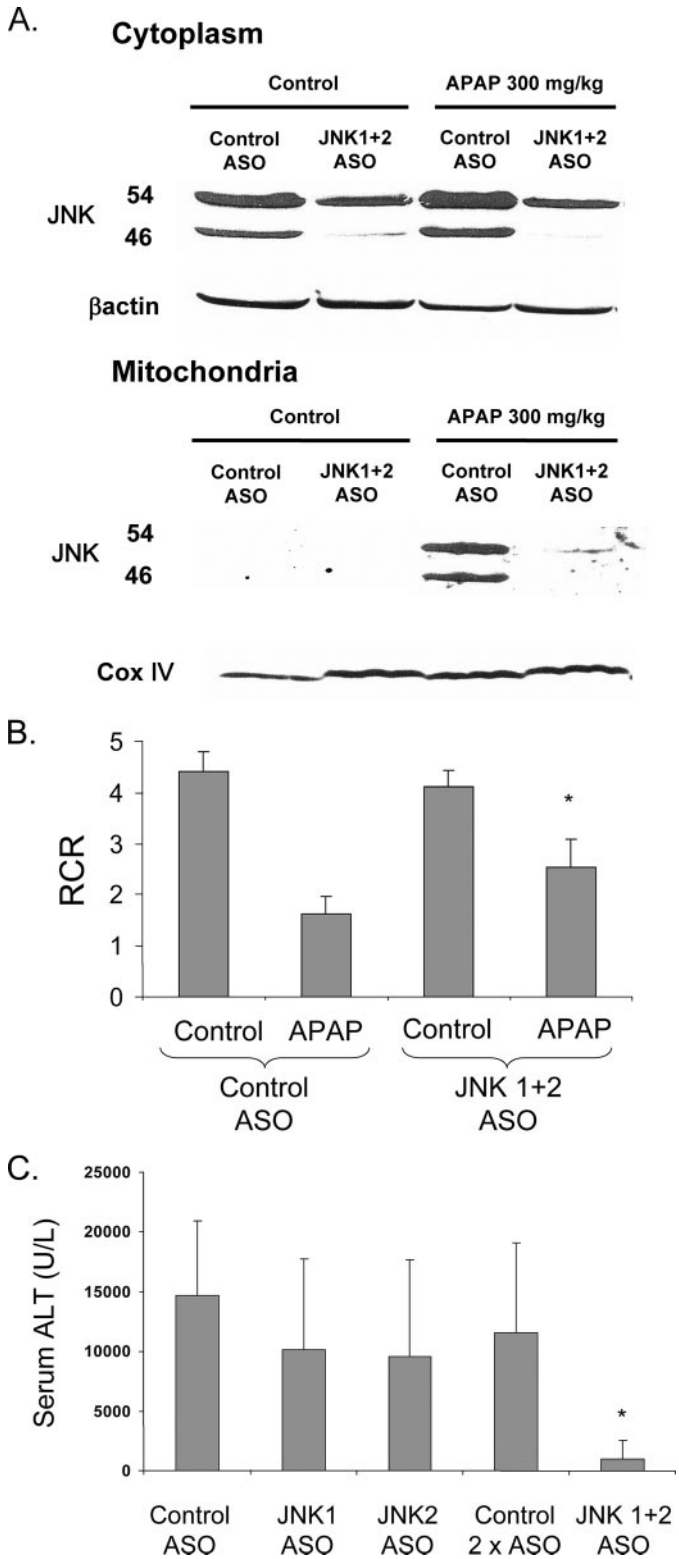

FIGURE 8. **Effect of knocking down JNK1 and/or -2 on JNK translocation to mitochondria, mitochondria bioenergetics, and liver injury.** *A*, effect of JNK1 and -2 ASO on JNK translocation to mitochondria induced by APAP treatment. *B*, effect of silencing JNK1 and -2 on mitochondria bioenergetics following APAP treatment *C*, effect of silencing JNK1, JNK2, or JNK1 and -2 on APAP-induced liver injury. One set of C56BL/6 mice were treated with control or JNK1 or JNK2 antisense (ASO) every other day for 2 weeks (7 doses). The other set of C56BL/6 mice were treated with control (double ASO dose) or JNK1 plus JNK2 antisense (ASO) every other day for 2 weeks (7 doses). Mice were subsequently treated with APAP (300 mg/kg) dissolved in warm PBS. For mitochondria measurements, livers were taken 2 h following APAP treatment, and mitochondria were separated from cytoplasm by differential centrifugation. Western blot analysis was performed using antisera against JNK with cytochrome oxidase and actin serving as loading controls, and mitochondria bioenergetics was measured as described under "Experimental Procedures." In other experiments, serum ALT levels were measured 24 h after APAP treatment. $n = 4–8$ mice. Results are mean ± S.D. *, $p < 0.05$ *versus* APAP treatment alone.

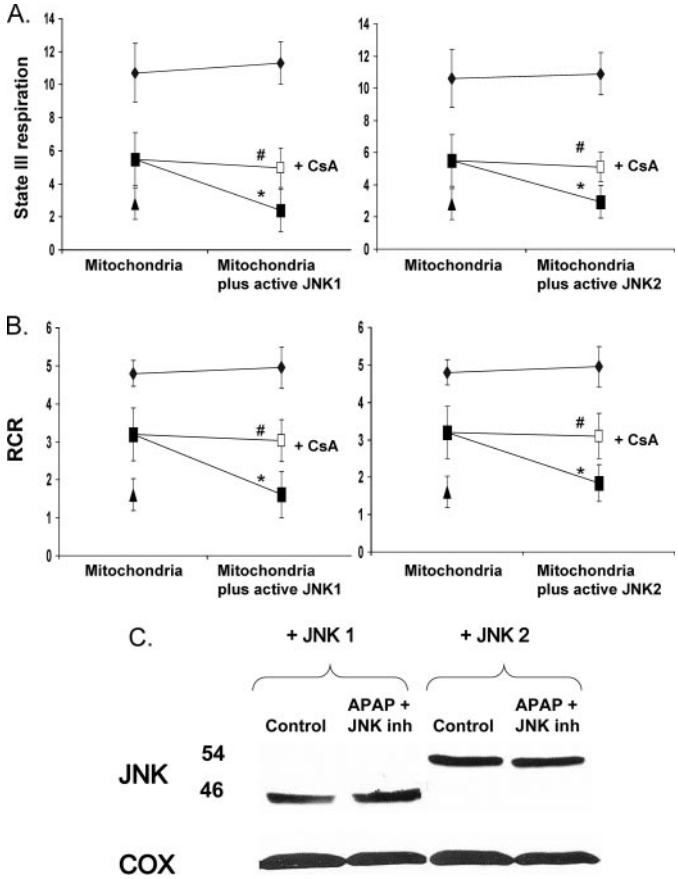

FIGURE 9. **Addition of purified active JNK1 or JNK2 inhibits mitochondria bioenergetics and induces MPT in redox altered mitochondria.** Isolated mitochondria from control and JNK inhibitor plus APAP treated (redox altered due to GSH depletion and covalent binding) mice were incubated with purified active JNK1 or JNK2. *A*, state III respiration; *B*, RCR; *C*, Western blot of JNK1 and JNK2 associated with mitochondria following incubation. ♦, control mitochondria; ▲, mitochondria from APAP-treated mice; ■, mitochondria from APAP plus JNK inhibitor-treated mice; □, mitochondria from APAP plus JNK inhibitor-treated mice treated with purified active JNK and cyclosporin A. Mitochondria were incubated with purified active JNK1 or JNK2 (0.35 μg/mg of mitochondria) or with buffer alone (control) for 10 min in 230 mM mannitol, 70 mM sucrose, 30 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EDTA, 10 mM $MgCl_2$, 600 μM ATP, pH 7.4. In some samples isolated mitochondria were pretreated with cyclosporin A (*CsA*, 2 μM) 5 min prior to JNK treatment. The mitochondria were centrifuged, washed, and centrifuged one more time. Mitochondria respiration was measured with a Clark type electrode using complex I substrates (glutamate/malate, 7.5 mM) and ADP. *, $p < 0.05$ *versus* untreated mitochondria; #, $p < 0.05$ *versus* APAP plus JNK inhibitor plus active JNK-treated mitochondria.

(Fig. 11). APAP treatment increases H_2O_2 generation from mitochondria due to NAPQI depletion of mitochondrial GSH and perhaps other effects on the electron transport chain. The increased H_2O_2 release from mitochondria activates JNK in cytoplasm and triggers its translocation to mitochondria. JNK associates with mitochondria and this can induce MPT, cytochrome *c* release, and possibly other changes that inhibit mito-

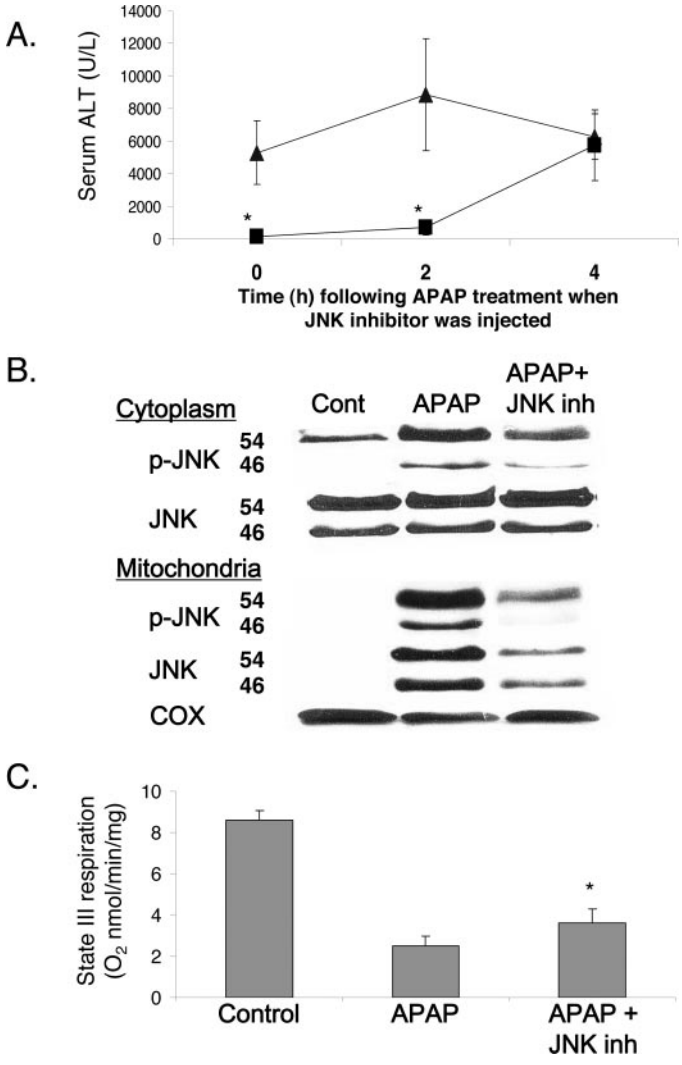

FIGURE 10. **JNK inhibitor given after APAP treatment still partially protects against JNK-induced mitochondria dysfunction.** *A*, protective effects of JNK inhibitor treatment following APAP administration in C57BL/6 mice. ▲, APAP; ■, APAP plus JNK inhibitor. Mice received either JNK inhibitor or vehicle (DMSO-PBS) at time points indicated following APAP treatments. Serum ALT levels were measured 24 h after APAP. *B*, effect of delaying JNK inhibitor (2 h following APAP) on JNK activation and translocation to mitochondria (at 4 h following APAP). *C*, effect of delaying JNK inhibitor (2 h following APAP) on mitochondria state III respiration (at 4 h following APAP treatment). Mitochondria respiration was measured using an oxygen electrode in the presence of complex I substrate (glutamate/malate) plus ADP. Results are mean ± S.D. *, $p < 0.05$ *versus* APAP treatment alone. $n = 4$ mice.

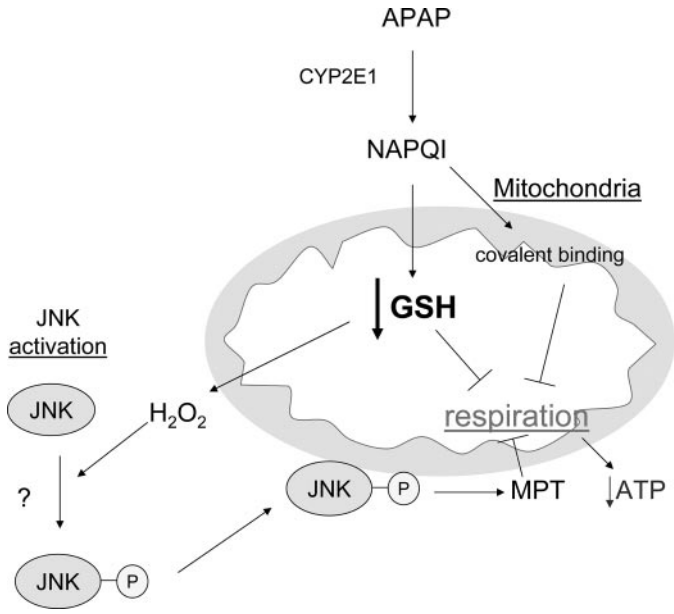

FIGURE 11. **Proposed model of the JNK-mitochondria signaling loop important in mediating liver injury.** APAP is metabolized to NAPQI by CYP2E1, which depletes mitochondria GSH and covalently binds to protein in mitochondria. Mitochondria GSH depletion and possible covalent binding cause a partial decrease in mitochondria respiration. Mitochondrial GSH levels are inadequate to detoxify H_2O_2 formed in mitochondria, causing an increase release of H_2O_2 from mitochondria. The H_2O_2 released from mitochondria activates JNK in cytoplasm and triggers its translocation to mitochondria. Activated JNK also promotes bax translocation to mitochondria (not shown) which may contribute to mitochondrial dysfunction in a redundant fashion. JNK associated with mitochondria induces MPT and promotes cytochrome *c* release, which inhibits mitochondria bioenergetics. The mechanism of MPT could be a direct effect of JNK on a pore constituent or possibly through an intermediary target, which then induces pore opening.

chondria bioenergetics in mitochondria rendered susceptible by APAP. The decline in mitochondria respiration causes a drop in ATP levels in liver and hepatocyte necrosis ensues. Thus, JNK translocation to mitochondria and inhibition of mitochondria bioenergetics through induction of MPT is a major component of APAP-induced liver injury.

Central to JNK activation is the increase in ROS generation by mitochondria. ROS have been suggested to be important in initiating free radical reactions (lipid peroxidation, protein oxidation, etc.) that damage hepatocytes and promote liver injury during APAP-induced hepatotoxicity (25, 52). However our work suggests that ROS are not only damaging molecules but also important signaling molecules that activate JNK. The increase in mitochondrial ROS generation is likely a consequence of mitochondrial GSH depletion by NAPQI based on the JNK activation time course (mitochondrial GSH depletion preceded JNK activation) and our previous findings that mitochondrial GSH depletion (>40%) increases H_2O_2 release from mitochondria (42) and diethyl maleate-induced mitochondrial GSH depletion activates JNK (36). However, other factors such as NAPQI redox cycling and covalent binding of NAPQI to respiratory complexes may play contributing roles. Several mitogen-activated protein kinase family members have been demonstrated to phosphorylate and activate JNK and may be responsible for JNK activation following APAP treatment. One intriguing pathway by which ROS may activate JNK involves oxidation of kinase inhibitors, which sequester JNK or upstream ASK1, such as thioredoxin (53) and GSH *S*-transferase subunits (54). Alternatively, sustained JNK activation may be a consequence of redox inactivation of JNK phosphatase by ROS (55). The exact mechanism(s) by which ROS promotes JNK activation during APAP-induced liver injury needs to be further elucidated. Regardless of the mechanism, our work demonstrates that ROS generated from mitochondria are plausible second messengers in activating JNK which is a key step in promoting APAP-induced liver injury.

APAP-induced liver injury is characterized by a dramatic decline in mitochondria bioenergetics, which leads to diminished ATP production and ultimately cell necrosis. The findings presented in this report suggest that the loss of mitochondria functions caused by APAP treatment is due to at least two

sequential steps: 1) a JNK-independent step, probably involving mitochondrial GSH depletion and covalent binding, and 2) a JNK-dependent step involving JNK association with mitochondria and induction of MPT and cytochrome *c* release. Even in the presence of JNK inhibitor, APAP treatment caused a significant decline in mitochondria respiration suggesting factors not affected by JNK (redox changes, mitochondrial GSH depletion, and covalent binding) can impair mitochondria functionality. Previous studies have established that redox changes by GSH depletion and NAPQI covalent binding can alter protein activity (23, 36). JNK inhibitor partially prevented the decline in mitochondria bioenergetics and cytochrome *c* loss from mitochondria caused by APAP treatment, suggesting JNK is responsible in part for the significant inhibition of mitochondria respiration following APAP treatment. JNK translocation to mitochondria can therefore be viewed as the final blow that diminished respiration in mitochondria, already impaired by redox alterations, below a critical threshold level needed for survival. Knocking down JNK1 or JNK2 alone did not protect mice against APAP, whereas knocking down both JNK1 and JNK2 protected against APAP-induced liver injury, suggesting that translocation of either JNK1 or JNK2 to mitochondria is sufficient to induce MPT and impair mitochondria bioenergetics.

Our work supports a growing body of literature demonstrating that JNK directly regulates mitochondria function. Previous work using various cell models demonstrated that JNK translocates to mitochondria to regulate mitochondrial PDH activity (4) and cytochrome *c* and SMAC release (10, 11). Similarly, Schroeter *et al.* demonstrated that purified JNK can directly induce MPT in isolated brain mitochondria (6). However, our work demonstrates that JNK affects mitochondria MPT and bioenergetics of liver mitochondria, which have been redox-altered after APAP treatment (mitochondria with GSH depleted and covalent binding), whereas JNK was observed to have little effect in control mitochondria. Redox changes to mitochondrial proteins may be causing conformation changes allowing greater access of mitochondrial proteins to JNK, and/or redox changes may be altering JNK signaling pathways in mitochondria. Alternatively, the effects of GSH depletion/covalent binding and JNK on mitochondria work in parallel or are additive. The mitochondrial targets of JNK remain to be elucidated; however, because JNK appears to regulate MPT, proteins that comprise the MPT pore (VDAC, ANT, cyclophilin, etc.) are possible candidates (47, 49). Most of the direct mitochondria targets of JNK are likely to be in the outer membrane, because it was shown by Zhou *et al.* that JNK associates with the outer membrane of mitochondria but does not enter the intermembrane space or matrix of mitochondria (4).

MPT is associated with increased permeability of the inner membrane to protons and other ions in cytoplasm that results in an inhibition of bioenergetics and swelling of mitochondria (47, 48). MPT has been suggested to promote cytochrome *c* release in some cases, however in other cases it has been shown not to trigger cytochrome *c* release (47, 49). The fact that cytochrome *c* release from mitochondria was observed following APAP treatment suggests that MPT induced by APAP was associated with some cytochrome *c* loss. The amount of cytochrome *c* loss from mitochondria (~23%) was, however, not sufficient to explain the almost complete loss of mitochondria bioenergetics following APAP treatment, suggesting that MPT without cytochrome *c* loss along with other factors (GSH depletion, covalent binding) may be responsible for the decline in mitochondria respiration following APAP treatment. Previously, we suggested that JNK regulation of bax, which forms channels that promote cytochrome *c* release, as an important mechanism by which JNK mediates APAP-induced liver injury (17). Work with bax knock-out mice, however, has demonstrated an equal susceptibility to APAP-induced liver injury, suggesting bax is not essential to APAP-induced liver injury (27). However, bax may still be one of many redundant factors that damage mitochondria and promote cytochrome *c* release in APAP-induced liver injury. Further work will be needed to see if JNK directly phosphorylates proteins associated with MPT or whether JNK modulates other proteins that regulate MPT.

An interesting and important caveat of our studies is the comparison of APAP delivered in warm saline *versus* DMSO. APAP delivered in DMSO was associated with less severe liver injury, confirming the work of others (28–30). Thus, LD_{50} is much higher and injury evolves more slowly with DMSO delivery of APAP. Our initial JNK work used the DMSO delivery and a preferential role of JNK2 in APAP-induced injury was identified using ASO silencing and knock-out mice. In addition, in relation to the slow evolution of injury JNK activation was not seen until 4 h and delayed treatment with the JNK inhibitor was effective in protective even up to 6 h after APAP treatment. In the present studies, we verified that small molecule JNK inhibitor (SP600125) and JNK ASO were markedly protective when APAP was given in warm saline despite more severe and rapidly progressive injury. However, some differences were revealed. First, the rapid progression was associated with a somewhat faster onset and peaking of JNK activation so that the protection afforded by delayed JNK inhibitor administration exhibited a more narrow window, and the difference between JNK1 and JNK2 was no longer observed, *i.e.* silencing of both JNK1 and JNK2 were required to protect against warm APAP. This finding is consistent with isolated mitochondria studies, which showed that either JNK1 or -2 could lead to inhibition of respiration. We currently do not understand why APAP delivery with DMSO unmasked a more selective role for JNK2 in APAP toxicity, but this has proven to be highly reproducible in our hands. It is however also possible in these studies that the expression of JNK2 alone was not sufficiently silenced to be effective in preventing APAP-induced liver injury. The key point is that, irrespective of the mode of APAP delivery, JNK plays a major role in the toxicity and inhibition of JNK protects against APAP-induced liver injury.

JNK activation is a major component of liver injury and is activated during hepatocyte injury caused by TNF, APAP, bile acids, concanavalin A, and ischemia-reperfusion injury (15, 16). APAP treatment mainly induces necrosis (20), whereas TNF-induced cell death is primarily apoptotic (32, 36), suggesting JNK regulates both necrotic and apoptotic signaling in hepatocytes. Although modest cytochrome *c* release is seen following APAP treatment, it may not be sufficient for apoptosome for-

mation. Most importantly, however, apoptosis is a process that requires energy, and APAP-induced liver injury is associated with a dramatic fall in ATP levels, due in part to JNK inhibition of mitochondria function. In addition, apoptosis requires a reduced intracellular environment for caspases to be fully functional (56, 57), and APAP induces an oxidized environment due to GSH depletion and increased ROS. Thus, although cytochrome *c* release is a part of APAP-induced liver injury, metabolic and redox status of hepatocytes probably prevent apoptosis from being a major component of APAP-induced liver injury.

In summary, our findings suggest that mitochondria damage caused by APAP is the result of two events, a JNK-independent event and a JNK-dependent event, both of which are required for necrosis to occur. Initially NAPQI causes significant cytoplasmic and mitochondrial GSH depletion and covalent binding to proteins, the functional consequences being an impairment of mitochondria bioenergetics and increase ROS generation that activates JNK. The second event is the translocation of activated JNK to mitochondria, which trigger MPT, cytochrome *c* release, and impairs mitochondria respiration, the functional consequences being a dramatic decrease in ATP production and necrosis. Taken together, our findings outline a mechanistic understanding of how sustained JNK activation promotes APAP-induced liver injury. However, more work will be needed to determine precisely how JNK inhibits mitochondrial function, which mitochondria protein(s) are direct substrates for JNK, and the interplay between MPT, cytochrome *c* release, and respiration.

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