Role of C-Jun N terminal kinase (JNK) signaling pathway in acetaminophen hepatotoxicity

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Abstract

Acetaminophen (APAP) is a commonly used analgesics and antipyretic agent. The therapeutic or recommended dose of APAP is not associated with adverse effects. However, intentional or unintentional overdose of APAP causes acute liver injury or acute liver failure if treatment is delayed. Currently, APAP-induced liver injury is one of the major causes of acute liver injury in the United States and other western countries. C-Jun N terminal kinase (JNK) implicated in stress-related signaling pathway plays an indispensable role in the mechanism of APAP hepatotoxicity. JNK mediates depletion of mitochondrial glutathione in the metabolic phase and enhances oxidative stress to aggravate liver injury. In addition, JNK plays an important role in APAP-induced apoptosis, necrosis or other forms of cell death. Furthermore, JNK plays a role in regulation of endogenous immune system and aseptic inflammatory responses induced by APAP. However, JNK may promote cell regeneration after APAP-induced cell death. The present review therefore highlights the functions of JNK in APAP-induced liver injury.

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Key words : Acetaminophen; JNK; hepatotoxicity; signaling pathway;

Acetaminophen (APAP), also known as paracetamol or N-acetyl para-aminophenol, is a commonly used analgesic and antipyretic agent. The recommended dose of APAP is usually less than 4g/day and this dose is considered to be safe [1, 2]. However, excessive intake of APAP can cause acute liver injury which may

progress to Acute Liver Failure (ALF). Notably, a previous study reported that a single dose of APAP > 125mg/kg can cause liver damage [3]. Currently, APAP-induced liver injury is a common cause of acute liver injury in the United States and most western countries.

C-Jun N terminal kinase (JNK) pathway is one of the 3 branches of the MAPK signaling pathway [4]. JNK is activated when cells are exposed to various forms of stress (osmotic stress, oxidative stress and radiation) or when they are treated with cytokines such as TNF and IL-1[4-6]. Therefore, JNK is considered to be a kinase activated by stress. JNK protein kinases are encoded by three genes, namely; JNK1, JNK2 and JNK3. JNK1 and JNK2 genes are expressed ubiquitously unlike JNK3 which has a more limited pattern of expression and is mainly expressed in the brain, heart and testis[4]. The aim of the present review was to explore the role of JNK in APAP-induced liver injury mainly focusing on JNK1 and JNK2 functions.

Acetyl-L-cysteine (NAC), the only FDA approved remedy for APAP-induced toxicity is a precursor for GSH that has a therapeutic effect against APAP overdose. NAC is only effective when administered during early phases of APAP toxicity, due to its narrow therapeutic window. JNK signaling pathway plays a significant role in APAP-induced liver injury. Therefore, JNK is a promising target for treatment of APAP hepatotoxicity. Therefore, this review summarizes the role of JNK in APAP-induced toxicity.

JNK in Metabolism of APAP in the liver.

APAP is mainly conjugated with glucuronic acid by enzymes in the UDP-glucuronosyltransferase (UGT) 1A subfamily and sulfated by sulfotransferase enzymes at therapeutic doses, to form hydrophilic compounds in the liver after which are then excreted through bile and urine. In addition, only a small portion of APAP is metabolized by phase I enzymes such as cytochrome P450 enzymes, to form the active metabolite N-acetyl-p-benzoquinone imine (NAPQI). NAPQI is a toxic metabolite that is detoxified by reduced Glutathione (GSH) to prevent it from binding to proteins containing sulfhydryl groups, reduce oxidative stress and prevent liver injury. On the other hand, excess NAPQI generated from an overdose of APAP results in depletion of GSH in the cytoplasm and mitochondria [7, 8]. NAPQI then covalently binds with protein sulfhydryl groups and forms NAPQI-protein adducts. Previous studies report that APAP-protein adducts are the main causes of APAP-induced hepatocyte injury [9]. However, formation of NAPQI-protein adducts does not solely cause severe liver injury because 3'-hydroxyacetanilide (AMAP), a non-toxic regioisomer of APAP in mice, also produces the same amount of covalent conjugates as APAP [10]. The difference between APAP and AMAP is that GSH in the cytoplasm and Endoplasmic Reticulum (ER) and not the mitochondria, is consumed when AMAP is used. As a result, there is no activation of various complex pathways associated with JNK and no liver damage occurs when AMAP is administered[11]. Previous studies report that depletion of mitochondrial GSH induces release of Reactive Oxygen Species (ROS) [12, 13] and activates the JNK signaling pathway[14]. Notably, AMAP is toxic to human liver cells as it forms mitochondrial protein adducts and causes mitochondrial dysfunction [15, 16]. Therefore, formation of NAPQI and protein adducts may initiate liver injury although they may not be the only factors implicated in progression of liver injury. Notably, other downstream events after formation of NAPQI-protein adducts play roles in APAP-induced liver injury.

The process of liver damage caused by APAP is therefore divided into two stages; the metabolism phase which comprises formation of NAPQI, depletion of GSH and formation of adducts and the oxidative phase that comprises oxidative stress, loss of mitochondrial membrane potential and Mitochondrial Membrane Permeability (MPT)[17, 18]. Notably, APAP or NAPQI are not required for the oxidative phase and studies report that adding them into experimental subjects at this phase does not exacerbate toxicity [18]. Therefore, oxidative stress amplification through JNK signaling pathway is the central mechanism in liver damage induced by APAP.

In vivo studies report that depletion of GSH reached a peak after 2 hours of APAP injection, and similar findings are reported for covalent binding of NAPQI [11]. However, the metabolism phase is important because the metabolism of APAP to NAPQI which is mediated by cytochrome P450 enzymes, is an initial effector of liver injury. Notably, inhibition of CYP2E1 and CYP1A2 or increase in GSH synthesis pro-

tects the liver against APAP-induced toxicity [19]. Mitochondrial bioenergetic is inhibited 2 hours after APAP injection [20] which can be attributed to JNK-independent mechanism including depletion of GSH or covalent binding of APAP[21]. Moreover, NAPQI-mitochondrial protein adducts are formed in human hepatocytes and liver non-parenchymal cells during APAP-induced liver injury [22]. Previous studies report that mitochondrial proteins are important sources of oxidative stress and NAPQI indirectly (by binding with mitochondrial respiratory compounds or the electron transfer chain) or directly (by destroying redox cycling through GSH depletion or its redox reaction) leads to release of ROS [23]. In addition, a low-dose (75mg/kg) APAP that dose not form mitochondrial adduct does not cause JNK activation, therefore no amplification of oxidative stress [24]. Furthermore, only the depletion of mitochondrial GSH can induce sustained JNK activation [13] and only sustained and not transient JNK activation is significant. Moreover, transient JNK activation and reversible mitochondrial dysfunction occur when 150mg/kg APAP is injected. However, irreversible mitochondrial injury occurs at a dose of 300mg/kg [25]. Notably, transient activation of JNK does not cause mitochondrial injury. For instance, a previous study reported that pretreatment with DAVA (an extract containing Polyphenols) changed sustained activation of JNK into transient activation, subsequently improving liver injury [26].Therefore, further studies should explore the effect of mitochondrial GSH depletion and formation of protein adducts.

Activation of JNK starts 2 hours after injection of APAP and reaches a peak after 4 hours [13]. Liver damage gradually occurs after 4-6 hours [11]. However, the duration of JNK activation is still controversial. For instance, a previous study reported that JNK activation starts at about 2-4 hours after APAP injection, reaches a peak at 6 hours and remains high for 8 hours [27]. On the contrary a different study reported that activation of JNK starts 1 hour after APAP injection [28]. Moreover, other studies report that activation of JNK starts 0.5-6 hours after APAP injection [26]. A previous study reports that JNK induces changes in the quantity and expression of glutathione S-transferase A1, to promote liver injury, implying that activated JNK affects GSH metabolism [29]. In addition, activation of JNK mediates formation of peroxynitrite and nitrotyrosine, to induce oxidative and nitrosative stress [29]. Therefore, JNK-mitochondrial signaling loop plays a key role in liver injury.

In summary, JNK signaling pathway is a downstream pathway involved in depletion of mitochondrial GSH and formation of protein adducts. Therefore, JNK signaling pathway plays a key role in induction of acute liver injury by enhancing oxidative stress in APAP-induced liver injury.

Activated JNK signaling pathway exacerbates oxidative stress.

Depletion of GSH and formation of protein adducts in mitochondria damages the electron transport chain(ETC) participating in ATP synthesis. The damaged ETC results in production of more ROS[30]. Furthermore, depletion of GSH and formation of protein adducts lead to damage of the mitochondrial antioxidant defense system and inhibits elimination of ROS [30].In addition, NAPQI binds to mitochondrial respiratory compounds thus inhibiting electron transfer in the mitochondrial respiratory chain [31]. Moreover, JNK signaling cascade is activated to amplify oxidative stress [32].

JNK signaling pathway is an important part of the MAPK cascade where MAP Kinase Kinase Kinase (MAPKKK) is first activated to form activated ASK-1, MLK3 and GSK3β[33-36]. Τηις ις φολλοωεδ βψ μοδιφισατιον τηρουγη πηοσπηορψλατιον τηυς αστιατινγ ΜΑΠ Κινασε Κινασε (ΜΑΠΚΚ) ωηιςη υλτιματελψ αστιατες ΘΝΚ [37]. Πηοσπηορψλατεδ ΘΝΚ ανδ αστιατεδ ΓΣΚ-3β then translocate to the mitochondria [34, 36]. ASK-1, is inhibited through binding to thioredoxin in the cytoplasm and mitochondria [38], is activated by dissociating from thioredoxin due to oxidation of sulfhydryl groups in thioredoxin by ROS [39]. MLK3 induces JNK phosphorylation within 1 hour of APAP injection [28]. A previous study reports that ULK1/2 activates JNK through MKK4/7 and phosphorylation of Ser403 site of MKK7 by ULK1/2 is necessary for phosphorylation of Ser271/Thr275 site of MKK7 by MAP3K in APAP-induced liver injury. Therefore, ULK1/2-dependent phosphorylation of MKK7 is a crucial step in APAP-induced JNK activation [40]. Furthermore, MKK4 and MKK7 exhibit a synergistic effect in JNK activation [40]. Notably, activity of mTORC1 is inhibited when after administration of an overdose APAP [41], consequently inhibits phosphorylation of ULK1/2. This enhances activity of ULK1/2 and activation of JNK [40]. A previous study divided JNK phosphorylation

into the early and late stages [42]. The study reported that phosphorylated-GSK-3β/JNK axis is a major source of APAP-induced liver injury during the early phase whereas the ASK-1/JNK axis is implicated in liver injury during the late stages [42]. Therefore, inhibition of these upstream molecules of JNK pathway attenuates JNK activation and protects the liver against APAP-induced toxicity.

Mitochondria act as the upstream organelle for JNK activation and a downstream target for activated JNK. Therefore, inhibition of JNK prevents mitochondrial injury in APAP-induced liver injury [43]. Moreover, downstream targets for p-JNK are mitochondria under oxidative stress and not healthy mitochondria [11].

In addition, phosphorylated JNK translocates to the mitochondria and binds to the anchor protein, Sab, located in the outer mitochondrial membrane [44]. The effect of p-JNK is gradually amplified after translocation and binding to Sab. Proteins in the intermembrane space of mitochondria are released into the cytoplasm following translocation of p-JNK and binding to Sab. Moreover, protein tyrosine phosphatase nonreceptor type 6 (SHP1) is released into the cytoplasm from the mitochondrial Sab channel [45]. Notably, Sab is required for sustained activation of JNK and silencing Sab protects against APAP [46]. Moreover, JNK-mitochondrial signaling loop is a vicious circle that continuously amplifies APAP-induced liver damage [23].

The main molecular targets for p-JNK are members of the Bcl-2 family. Translocation of Bax to the mitochondria is a downstream response for JNK activation after APAP overdose [47] which has been reported several models [48]. P-JNK promotes translocation of Bax to the mitochondria by directly phosphorylating Bax or by phosphorylating 14-3-3 anchoring Bax in the cytoplasm [48, 49] thus inducing mitochondrial Membrane Permeability Transition (MPT). MPT is a common and important mechanism for different forms of hepatotoxicity. The inner mitochondrial membrane is permeable to small molecular solutes and the proton gradient of oxidative phosphorylation is disrupted when the mitochondria membrane is depolarized [50]. Loss of mitochondrial membrane potential results to uncoupling of oxidative phosphorylation [20] and swelling of the mitochondria [18, 51, 52]. Bax is necessary for MPT [2] implying that JNK and Bax have a synergistic effect. A previous study reports that mitochondria are still polarized 3.5 hours post APAP injection but were depolarized after 4.5 hours [47]. This observation implies that MPT is a downstream step in JNK activation. Previous studies divided MPT into two open modes, namely, regulatory MPT, which was inhibited by the MPT inhibitor, CsA and occurred following exposure to a low dose of APAP or a short time and non-regulatory MPT, which occurred when a high dose of APAP was administered [53]. These modes explain different outcomes from injection of the above-mentioned high or low doses of APAP, partially or completely. Release of ROS including superoxide from the mitochondria, results in oxidation of sulfhydryl groups in MPT pores which is a key factor for MPT. This is concept was proven through reduction of disulfides by dithiothreitol reduced thus preventing occurrence of MPT [18, 54]. However, studies report contradicting findings on the roles of Bid and Bcl-xL,the downstream members of Bcl-2 family of p-JNK. Studies report that the levels of Bax and Bid in the mitochondria increase after treatment with APAP. However, truncated Bid (tBid) may not have significant effect on APAP-induced liver injury. In addition, effect of Bax is blocked by JNK inhibitors although Bid and Bcl-xL are not affected by these inhibitors [11]. A different study reports that Bax and Bid translocate to the mitochondria after an APAP overdose and inhibition of caspases prevents cleavage of Bid [55]. Furthermore, a previous study reports that translocation of Bax to the mitochondria inactivates Bcl-xL, which is an anti-apoptotic member of the Bcl-2 family [56]. Moreover, loss of mitochondrial membrane potential reduces levels of Bcl-2 [31]. This implies that anti-apoptotic members of the Bcl-2 family in the mitochondria are inhibited despite their role in blocking MPT and levels of members of Bcl-2 family are affected by APA administration.

Bax, a downstream molecule of JNK activation forms channels by oligomerization or binding to MPT pores to promote release of cytochrome C [23]. Therefore, after the continuously activated JNK translocates to the mitochondria, mitochondrial respiration is severely inhibited and production of ROS increases [7, 57]. Translocation of phosphorylated JNK and subsequent loss of mitochondrial membrane potential have been reported in human liver cells [58] and in a model of UV-induced oxidative stress [59]. In addition to cytochrome C, other intermembrane proteins such as endonuclease G and the Apoptosis-inducing Factor

(AIF) are released which then translocate to the nucleus, leading to fragmentation of nuclear DNA [54, 60]. The blockage of release or translocation to the nucleus of these mitochondrial proteins, downstream molecules of the p-JNK has protective effect [61]. Moreover, ROS are further released thus reacting with the NO produced in this process to form peroxynitrite and nitrotyrosine. Peroxynitrite is a strong oxidant that is released into the cytoplasm through passive diffusion or VDAC anion channel [62].

Protein Kinase Cα(PKCα) is an important target for p-JNK[63]. Its activity increases after APAP overdose and is inhibited by the conventional JNK inhibitor, SP600125 [64]. A previous study reports that after APAP overdose, the levels of PKC-α increase then PKC-α is translocated to the mitochondria. In the mitochondria it also phosphorylates mitochondrial proteins and promotes phosphorylation and translocation of JNK to the mitochondria, subsequently promoting liver damage [63]. Moreover, JNK and PKC-α act synergistically to regulate mitochondrial respiration and mitochondrial-mediated necrotic and apoptotic cell death [21, 65]. Therefore, PKC-α and JNK interact with each other synergistically to participate in feed forward regulation of APAP-induced liver injury [63].

Grb2-associated binder 1 (Gab1) adaptor protein plays an important role in controlling the balance between death and compensatory proliferation of hepatocytes during APAP-induced liver injury[66]. Notably, silencing Gab1 induces activation of p-JNK and increases translocation of p-JNK to the mitochondria. Moreover, silencing Gab1 promotes release of mitochondrial enzymes into the cytoplasm and induces DNA fragmentation [66].

Previous studies report that the Dynamin related protein 1(Drp1), a downstream molecule of p-JNK, is translocated to the mitochondria, thus mediating mitochondrial division [67, 68]. RIPK3, an upstream molecule of JNK mediates production of ROS in mitochondria [69]. Silencing RIPK3 inhibits mitochondrial translocation of Drp1, and prevents release of mitochondrial AIF and DNA fragmentation [67]. In addition, RIPK1 plays a crucial role in sustained activation of JNK[70]. Notably, inhibition of RIPK1 prevents translocation of Drp1 to the mitochondria [68]. Furthermore, inhibition, knockout and/or silencing of ASK1, MLK3, GSK3b, PKCα, JNK, Sab and cyclophilin D (CypD) a mitochondrial permeability transition pore regulator[71], have protective effects against APAP [68]. P-AMPK signaling pathway which induces autophagy inhibits downstream events of p-JNK although it is inhibited after APAP administration. Upregulation of p-AMPK induced by a PKC inhibitor, exhibits protective effects against APAP toxicity despite sustained JNK activation [63].This is similar to blocking the release and translocation of endonuclease G and the Apoptosis-inducing Factor (AIF) to the nucleus (downstream events of p-JNK), which exhibit a protective effect.

Nrf2 and its Antidote Response Element (ARE) are important antioxidants in the body. Downstream target genes for Nrf2, including HO-1, NQO1 and GSH protects the body from oxidative stress [72, 73], implying that Nrf2 alleviates APAP hepatotoxicity. A previous study reports that upregulation of Nrf2 and its ARE inhibits JNK activation and protects against APAP-induced oxidative stress [74]. Furthermore, some compounds alleviates oxidative stress through AMPK/Akt/Nrf2 in APAP-induced liver injury [31, 75, 76]. However, p-JNK can target Nrf2 to promote its degradation [31, 75]. Biochemical analysis showed that p-JNK directly interacts with the Nrf2-ECH homology (Neh) 1 domain of Nrf2 and phosphorylates the serine-aspartate-serine motif 1 (SDS1) region in the Neh6 domain of Nrf2 [77]. Conversely, a recent report indicated that the protection of flagellin-induced Nrf2 against APAP was dependent upon the activation of TLR5-JNK/p38 pathways [78].

In addition, ER stress is an important mechanism for APAP-induced hepatotoxicity. JNK is an ER stress factor and two key proteins implicated in ER stress including CHOP and Bim are downstream molecules of JNK. 4-PBA, an inhibitor of ER stress effectively prevents JNK activation when administered before APAP injection [6]. c-Jun, a p-JNK downstream transcription factor, binds to the 5'regulatory region of CHOP gene [79, 80] and mediates transcription of the gene. Parkin is a post-translational regulation factor of CHOP that induces degeneration of CHOP through one of the branches of UPR, the PERK/eIF2α/ATF4 pathway. Notably, c-Jun can competitively bind to the binding site of ATF4 in the Parkin gene promoter[74]. Therefore, JNK/c-Jun can inhibit expression of Parkin [81].

Inhibition of JNK-dependent p53 up-regulated Modulator of Apoptosis (PUMA), a downstream molecule of JNK, significantly improves hepatocyte necrosis [82]. NF-ϰB response gene products inhibit JNK [83] but the process is blocked by depletion of GSH [17].

Some studies report that only JNK2 is involved in amplifying the effect of oxidative stress by translocating to the mitochondria whereas JNK1 has not significant effects on oxidative stress [84]. However, other studies report that both JNK1 and JNK2 translocate to the mitochondria, play a role in amplifying oxidative stress and participate in activation of downstream events. In addition, JNK1 has a greater effect on mitochondrial bioenergetics compared with JNK2 [11]. Therefore, the specific role of the two regioisomers of JNK should be explored further.

In summary, after depletion of mitochondrial GSH by NAPQI and formation of covalent adducts with mitochondrial proteins, oxidative stress, (amplified by the JNK signaling pathway and JNK-related signal transduction pathways) exerts a "second hit"[26] on damage of liver cells. Therefore, this process is a synergistic event that leads to decreased mitochondrial respiration and bioenergetics, mitochondrial dysfunction and eventually severe liver damage.

JNK mediates APPA-induced necrosis, apoptosis and other forms of cell death

In vivo and *in vitro* studies report that depletion of mitochondrial GSH results in necrosis [85, 86]. Depletion of mitochondrial GSH by DEM results in a significant increase in necrosis [4, 13]. Notably, DEM depletes cytoplasmic and mitochondrial GSH, leading to 100% necrosis instead of apoptosis despite exposure to TNF-α [13]. Administration of low concentration of DEM or APAP only depletes cytoplasmic GSH in hepatocytes, therefore, they become sensitive to TNF-α-induced apoptosis [13, 87]. Moreover, using GSH-EE to restore depleted GSH can reverse sensitivity to apoptosis, induced by TNF-α [87]. Furthermore, antioxidants can restore the levels of GSH and reduce levels of p-JNK [88, 89]. Previous studies report that JNK regulates apoptosis and necrosis [4, 45, 81].

Apoptosis:

JNK promotes cytokine receptor apoptosis signaling pathway. Previous studies report that JNK activation promoted the of release of Fas-L in an autocrine or paracrine manner to increase toxicity in adjacent liver cells [4, 90] and induce the cytokine receptor death signaling pathway. JNK is activated during APAP-induced oxidative stress in hepatocytes and non-parenchymal cells such as sinusoidal endothelial cells. On the other hand, inflammatory cells release pro-inflammatory cytokines such as TNF-α and INF-γ, thus promoting inflammatory reactions and aggravating liver damage under inflammatory conditions [91, 92]. The pro-apoptotic factors, p53 and c-Myc, are phosphorylated by activated JNK in cells exposed to stress factors [4]. Therefore, sustained activation of JNK mediates TNF-α [93] or FasL-induced apoptosis. Although endotoxin-induced production of TNF-α does not increase APAP-induced liver damage [94], Astaxanthin (ASX) reduces apoptosis by inhibiting TNF-α-mediated JNK signaling pathway [95]. Furthermore, TNFα-induced apoptosis following pretreatment with APAP, is inhibited by SNAP (producing NO). Furthermore, SNAP inhibits 50% of caspase3 activity, which is increased by combined treatment with APAP and TNF-α [13]. In addition, silencing TNF-α or TNFR1 exhibits protective effects against APAP-induced liver damage [96]. A previous study reported that JNK inhibitors partially block TNF-α-induced apoptosis in cells treated with APAP, without affecting GSH consumption [13]. Activation of TNF receptor induces activation of caspase 8, which in turn truncates Bid into tBid then tBid is translocated to the mitochondria. Bax is also translocated to the mitochondria where it forms MPT pores with Bak and Bad. These changes induce release of cytochrome C and AIF from mitochondria and translocation to the nucleus. As a result, apoptotic bodies are formed, caspase9 and caspase3 are activated in presence of sufficient ATP. Notably, the TNF superfamily activates death receptors including TNFα, (Fas ligand)FasL and TRAIL. FasL is the most harmful to hepatocytes. Moreover, sensitivity of cells to FasL and toxicity to adjacent liver cells increases with release of cell contents [97]. TRAIL which is a homologue of TNF-α, induces apoptosis in hepatocytes, a process which is independent of direct activation of caspases but is dependent on the activation of JNK and Bim. Therefore, inhibition of JNK and Bim protects hepatocytes against death induced by

FasL or TNF-α [97-99]. Furthermore, p-JNK phosphorylates Bim [100], Bax and Bak whereas Bcl-2 and Bcl-xL are inhibited during APAP-induced hepatotoxicity. Therefore, these findings imply that TRAIL induces apoptosis in hepatocytes through the JNK-Bim axis. Previous studies report that extramitochondrial depletion of GSH alters the thiol-disulfide redox state, leading to inhibition of transactivation of NF-kB and sustained activation of JNK, which ultimately induce sensitivity to TNF-α-induced apoptosis [13]. The JNK pro-apoptosis pathway, P38 and NF-kB pro-survival signaling pathways are simultaneously activated [101]. Sustained activation of JNK plays an essential role in primary hepatocytes sensitive to TNFα-induced apoptosis by inhibiting NF-kB [93]. Therefore, JNK plays an important role in promoting apoptosis in liver cells, induced by death receptor signaling during APAP hepatotoxicity. Additionally, sensitivity to TNFα-induced apoptosis in liver cells treated with APAP may promote APAP-induced liver damage. This explains why the dose of APAP required to induce acute liver injury in patients with basic liver diseases such as chronic hepatitis B, alcoholic liver and non-alcoholic fatty liver, is lower compared with that of patients without underlying diseases.

JNK plays a role in stress-induced apoptosis through the mitochondrial pathway [4]. Defect of JNK-deficient fibroblasts in stress-induced apoptosis is mainly located in the mitochondria [59]. Previous studies report that translocation of p-JNK to the mitochondria induces release of cytochrome C and SMAC from the mitochondrial intermembrane space, leading to apoptosis [65, 102]. Notably, release of cytochrome C from the mitochondria is inhibited by the absence of JNK [103]. Additionally, sustained activation of JNK promotes cell damage or death [93, 104]. The potential targets for p-JNK for regulation of release of cytochrome C are the apoptotic regulatory members of the Bcl-2 family[4]. In addition, p-JNK translocation to the mitochondria promotes translocation of Bax to the mitochondria thus activating caspase 9, leading to apoptosis through the intrinsic pathway. Moreover, the anti-apoptotic proteins Bcl-2 and Bcl-xL are phosphorylated by JNK [105, 106]. Therefore, apoptosis through the JNK-dependent but transcription-independent pathway, (the mitochondrial/caspase9 pathway), is implicated in APAP-induced cell death.

Notably, only sustained rather than transient activation of JNK is associated with apoptosis [107], which is consistent with the mechanism of APAP-induced liver injury. For instance, TNF-α causes transient JNK activation with no apoptotic response [13, 108]. Although several studies report that JNK-mediated apoptosis is involved in APAP-induced liver injury, the relationship between JNK-mediated apoptosis and APAP-induced liver injury has not been fully explored. Several studies report that apoptosis is not involved in APAP-induced liver injury because caspases, especially caspase 3, is not activated during APAP hepatotoxicity [109]. Furthermore, a previous study reported that the levels of activated caspase 3 did not increase in human hepatocytes after an APAP overdose [110]. Moreover, previous studies report that pan-caspase inhibitors do not alleviate APAP-induced liver damage [86, 111]. However, TRAIL induces apoptosis in hepatocytes and in a process that is not dependent on the direct activation of caspases but dependent on activation of JNK and Bim [97]. This observation partially explains why caspase 3 is not activated during APAP-induced liver injury as reported by some studies. However, recent studies report that caspase 3 is activated during APAP-induced liver damage [42, 112, 113]. In addition, hydrogen sulfide inhibits apoptosis through the JNK pathway, thus alleviating APAP-induced liver damage [114]. Several compounds protects liver cells against APAP-induced toxicity through anti-apoptotic mechanisms [115, 116]. Previous *in vitro* studies report a decrease in cell viability and a significant increase in levels of apoptotic cells after APAP administration [74]. Notably, treatment with caspase 3 inhibitors completely blocks APAP-induced apoptosis [47].

Necrosis:

Necrosis is the main form of liver-cell death during APAP-induced liver injury. Previous studies report that APAP-induced liver injury does not involve apoptosis [117], as mentioned above. Studies report that JNK inhibitors block APAP-induced necrotic cell death [13]. Notably, APAP-induced death of liver cells is not characterized by cell shrinkage, nuclear pyknosis and other common cytological features of apoptosis. On the contrary, cytological manifestations APAP-induced liver damage mainly include extensive mitochondrial dysfunction and nuclear lysis accompanied by swelling of cells and organelles and release of cellular con-

tents. These features show that APAP-induced cell death is a oncotic necrosis process [86].Mitochondrial respiration is inhibited after translocation of p-JNK to the mitochondria and ATP production is significantly reduced, leading to insufficient energy for activation of caspase and formation of apoptotic bodies. Previous studies report that when fructose and glycine are administered along with APAP, a significant increase in ATP production is observed and, cells are protected from necrosis although MPT was still normal and an increase in apoptosis was observed [47]. In addition, endonuclease G and the Apoptosis-inducing Factor (AIF) are released from the mitochondria, translocated to the nucleus, where they induce nuclear DNA fragmentation after JNK activation and ultimately initiate necrosis in cells [118]. In addition, apoptosis requires a reduced environment for caspase activation and the redox state of liver cells may affect apoptosis during APAP-induced liver injury [11, 119, 120].Furthermore, Damage-associated Molecular Pattern (DAMP) which induces inflammation, is released and interacts with pattern recognition receptors including toll-like receptors(TLR) on inflammatory cells. These events lead to release of pro-inflammatory cytokines (such as TNF$\alpha$and INF-γ) and induction of an inflammatory response [91, 92]. These are generally the characteristics of necrosis since apoptosis is not characterized by release of cellular contents. TRAIL-JNK-Bim axis is important in APAP-induced cell necrosis, and deletion of TRAIL or Bim protects hepatocytes and sinusoidal endothelial cells from necrosis [100, 121]. Therefore, ability of TRAIL-JNK-Bim axis in amplification of necrosis in liver cells is greater compared with the ability of the Bcl-2 family members to induce apoptosis through the mitochondrial pathway [97].

Previous studies also report that hepatocytes undergo both necrotic and apoptotic cell death in APAP-induced hepatotoxicity [47, 122]. In addition, upstream events such as JNK activation and translocation to the mitochondria, induction of pro-apoptotic Bcl-2 homologs and increase in MPT with subsequent release of cytochrome C and AIF, are indicators of apoptotic cell death. However, inhibition of mitochondrial respiration and ATP synthesis cannot solely induce apoptosis. Moreover, JNK and MPT mediate apoptosis and necrosis. Therefore, apoptosis is the main form of cell death in the early stages of APAP hepatotoxicity whereas necrosis mainly occurs in the late stages [113, 123]. A previous study reports presence of caspase-cleaved cytokeratin-18 in patients with an APAP overdose, indicating that apoptosis of hepatocytes occurred in the early stages of APAP-induced acute liver injury [124].

However, several studies challenged the widely accepted conclusion that JNK mediates necrotic or apoptotic cell death in APAP-induced liver injury. For instance, knockout of Gst-pi, a negative regulator of JNK, protects mice from APAP-induced liver damage [125]. In addition, simultaneous activation of JNK1 and JNK2 in mice protected them against APAP-induced necrotic cell death by regulating oxidative stress response. Moreover, lack of JunD activation in hepatocytes with specifical knockout of JNK1 and JNK2 (Jnk[?]hepa), shows that JNK-JunD-dependent mechanism may be involved in protection of liver cells against APAP-induced liver damage [109]. Nevertheless, JNK signaling pathway plays a central role as it modulates necrotic or apoptotic cell death in APAP-induced liver injury probably through a mechanism that accelerates and amplifies oxidative damage.

In summary, JNK-mediated necrotic and apoptotic cell death are involved in APAP-induced liver injury and the specific form of death may depend on different conditions.

Necroptosis and other Forms of Cell Death

Several signaling pathways involved in stress initiation, amplification, expansion and ultimately cell death, have been identified in APAP-induced liver injury thus promoting the use of the term, programmed necrosis [126]. Previous studies report that RIPK1 and RIPK3 combine and translocate to the mitochondria to mediate necroptosis [67, 127]. In addition, RIPK3 and MLKL are up-regulated after APAP injection, in a time and dose-dependent manner similar to JNK [128]. Notably, JNK is a biomarker of UPR and ER stress. However, other studies report that necroptosis does not affect cell death in APAP-induced toxicity [68]. Moreover, additional studies report several forms of cell death during APAP hepatotoxicity including initial necrosis followed by pyroptosis, apoptosis and necroptosis [129].

In summary, JNK-mediated death of liver cells is necessary for APAP-induced liver toxicity and several

protective effects are exhibited through inhibition of JNK. For instance, the tumor suppressor, P53, protects liver cells against APAP-induced liver damage by inhibiting JNK activation [130]. Moreover, 4MP inhibits JNK activation and p-JNK translocation to the mitochondria, thus protecting liver cells against APAP-induced liver damage [131, 132]. Furthermore, the Gadd45β agonist inhibits phosphorylation of MKK4 and JNK [133] whereas Metformin inhibits JNK thus exhibiting protective effects against APAP through Gadd45β [134, 135]. Additionally, quercetin offers protection against APAP-induced liver injury by reducing JNK activation [136, 137]. Inhibition or inactivation of JNK can protect liver cells against APAP-induced liver injury [89, 138, 139].

Role of JNK in Liver Endogenous Immunity and Non-parenchymal Cells

JNK-mediated hepatocyte necrosis releases DAMP which interacts with pattern recognition receptors (such as toll-like receptors) on inflammatory cells, leading to release of pro-inflammatory cytokines (such as TNFαand INF-γ) thus promoting inflammatory responses. In addition, a previous study reports that phosphorylation of JNK was significantly inhibited after silencing TLR4 in the RAW264.7 immune cell line [140]. Therefore, TLR4 can activate JNK signaling pathway to induce an inflammatory response and infiltration of macrophages thus promoting APAP-induced liver injury [140]. Also, it has been reported that TLR3 can act on JNK to be injury-provoking in the model of APAP-induced liver injury [141]. Hematoxylin-eosin (HE) staining shows significant infiltration of inflammatory cells in areas with liver injury [142]. Therefore, the endogenous immune system, including liver macrophages, is induced during APAP hepatotoxicity, causing aseptic inflammation [143]. Moreover, previous studies report an increase in release of inflammatory factors such as TNF-α, IL-1β and IL-6 and immune cells such as neutrophils, macrophages, NK cells and NKT cells are recruited. These factors may exacerbate liver damage through the effect of NKT cells and effectors such as INF-γ[97]. Αδδιτιοναλλψ, νευτροπηιλ-μεδιατεδ δαμαγε, σεχονδαρψ το ηεπατοχψτε νεχροσις, μαψ προμοτε ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ [144]. Μανναν-βινδινγ Λεχτιν (ΜΒΛ), α μολεχυλε ιν τηε ιννατε ιμμυνε σψστεμ τηατ ις μαινλψ προδυχεδ βψ τηε λιερ, προμοτες ΘΝΚ αχτιατιον ανδ υπ-ρεγυλατες νυχλεαρ εξπρεσσιον οφ Σπεχιφιχιτψ Προτειν 1 (ΣΠ1) το αγγραατε λιερ δαμαγε [145]. Πρειους στυδιες ρεπορτ τηατ ΘΝΚ ρεγυλατες σεχρετιον οφ ΙΛ-2, προλιφερατιον οφ ΄Δ8+ Τ χελλς ανδ διφφερεντιατιον οφ ΄Δ4+ Τ χελλς ιντο ηελπερ Τ χελλς [146]. Φυρτηερμορε, κνοχκουτ οφ ΤΝΦ ορ ΤΝΦΡ1 προτεχτς λιερ χελλς αγαινστ ΑΠΑΠ-ινδυχεδ δαμαγε. Τηεσε φινδινγς σηοω τηατ ΘΝΚ ρεγυλατες ιμμυνε χελλς ορ τηε ιμμυνε φαχτορ ΤΝΦ ορ ΦασΛ-ινδυχεδ ινθυρψ οφ λιερ χελλς δυρινγ ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ. Ηοωεερ, τηεσε ιμμυνε ορ ινφλαμματορψ χελλς, ινχλυδινγ ΝΚ χελλς ανδ ΝΚΤ, χαν αλσο πλαψ προτεχτιε ρολες [147-149]. Φυρτηερμορε, μιχε δεοιδ οφ τηε ιντερφερον Φας ορ Φας λιγανδ διδ νοτ σηοω ΑΠΑΠ-ινδυχεδ λιερ δαμαγε ωηερεας τηοσε τηατ λαχκεδ ιντερλευκιν-10 ανδ ιντερλευκιν-6 ωερε προτεχτεδ αγαινστ λιερ δαμαγε [150].

Τηερεφορε, *ιν ιο* στυδιες προιδε ρελιαβλε φινδινγς χομπαρεδ ωιτη *ιν ιτρο* εξπεριμεντς ον τηε ρολε οφ τηε ενδογενους ιμμυνε σψστεμ ιν ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ (ρεγαρδλεσς οφ ωηετηερ ιτ αγγραατες δαμαγε ορ οφφερς προτεχτιον). Ιν αδδιτιον, σηουλδ βε χαρριεδ ουτ το εξπλορε τηε σπεχιφις ρολε οφ ΘΝΚ ιν ρεγυλατιον οφ ιμμυνε χελλς ιν ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ.

Α πρειους στυδψ ρεπορτς τηατ ΘΝΚ1 ις ηιγηλψ ινδυχεδ ανδ α σιγνιφιχαντ ινχρεασε ιν λεελς οφ π-ΘΝΚ ις οβσερεδ ιν μιχε ωιτη σπεχιφις κνοχκουτ οφ ΘΝΚ1 ανδ ΘΝΚ2 ιν τηειρ ηεπατοχψτες. Ιν αδδιτιον, τηε λεελς οφ ινφιλτρατιον ιν τηε λιερ εξπλαινς τηε πηενομενον οφ ΘΝΚ αχτιατιον αφτερ ΑΠΑΠ αδμινιστρατιον [109]. Νοταβλψ, α πρειους στυδψ υσινγ α ηεπατοχελλυλαρ χαρχινομα μοδελ ρεπορτεδ τηατ τηε εφφεστ οφ ΘΝΚ ιν προμοτινγ δεελοπμεντ οφ λιερ χανχερ μαινλψ ινολες νον-παρενχηψμαλ χελλς ανδ νοτ ηεπατοχψτες [151]. Φυρτηερμορε, σινυσοιδαλ ενδοτηελιαλ χελλς αρε μορε σενσιτιε χομπαρεδ ωιτη ηεπατοχψτες το ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ [152]. Τηερεφορε, στυδιες ον τηε ρολε οφ ΘΝΚ ιν ΑΠΑΠ-ινδυχεδ λιερ ινθυρψ σηουλδ αλσο φοχυς ον τηε εφφεστς ον νον-παρενχηψμαλ χελλς.

Ρολε οφ ΘΝΚ ιν Ρεστορατιον ανδ Ρεγενερατιον Πηασε

Προλιφερατιον ανδ ρεχοερψ οφ χελλς αρε εξτρεμελψ ιμπορταντ αφτερ ΑΠΑΠ-ινδυχεδ χελλ δεατη. Ινηιβιτιον οφ ΕΓΦΡ ιν λιερ χελλς, 12 ηουρς αφτερ ΑΠΑΠ ινθεστιον, λεαδς το ιμπαιρμεντ ιν προλιφερατιον, περσιστενχε οφ λιερ ινθυρψ ανδ αν ινχρεασε ιν μουσε μορταλιτψ [153]. ΘΝΚ πλαψς αν ιμπορταντ ρολε ιν χελλ προλιφερατιον

[59]. Τηερεφορε, κνοςκουτ οφ ΘΝΚ1 ινηιβιτς προγρεσσιον οφ λιερ ςανςερ ανδ προλιφερατιον οφ ηεπατοςψτες ις σιγνιφισαντλψ αφφεςτεδ. Ιν αδδιτιον, ΘΝΚ1 ις ινολεδ ιν τρανσφορματιον οφ ηεπατις στελλατε ςελλς ιντο φιβροβλαστς. Νοταβλψ, ινηιβιτιον οφ ΘΝΚ αττενυατες φιβροσις ιν μοδελς οφ βιλε δυςτ λιγατιον ορ ""λ4-ινδυςεδ λιερ ινθυρψ [154]. Τηερεφορε, ΘΝΚ μαψ προμοτε ρεγενερατιον οφ λιερ ςελλς δυρινγ τηε λατε σταγες οφ ΑΠΑΠ-ινδυςεδ λιερ ινθυρψ ανδ προτεςτ λιερ ςελλς αγαινστ ΑΠΑΠ ηεπατοτοξιςιτψ. Φυρτηερμορε, ρεςεντ στυδιες ρεπορτ τηατ ΘΝΚ μαψ βε ςριτιςαλ το ςελλ συριαλ [155] βεςαυσε ΘΝΚ προμοτες ρεςοερψ ανδ ρεγενερατιον οφ λιερ ςελλς αφτερ ΑΠΑΠ-ινδυςεδ δεατη οφ λιερ ςελλς. Αδδιτιοναλλψ, σιλενςινγ "ΗΟΠ, ωηιςη ις α δοωνστρεαμ γενε οφ ΘΝΚ, προμοτες προλιφερατιον οφ ηεπατοςψτες δυρινγ ΑΠΑΠ-ινδυςεδ λιερ ινθυρψ [117]. Ηοωεερ, φυρτηερ στυδιες σηουλδ εξπλορε τηε ρολε οφ ΘΝΚ ιν ρεςοερψ ανδ ρεγενερατιον οφ ςελλς φολλοωινγ ΑΠΑΠ-ινδυςεδ λιερ ινθυρψ.

Ὁνςλυσιον

Ιντεντιοναλ ορ υνιντεντιοναλ οερδοσε οφ ασεταμινοπηεν λεαδς το Ασεταμινοπηεν-ινδυςεδ λιερ ινθυρψ, ωηιςη ις τηε μοστ ςομμον ςαυσε οφ ασυτε λιερ ινθυρψ ιν τηε Υνιτεδ Στατες ανδ μοστ ωεστερν ςουντριες. "-Θυν Ν τερμιναλ κινασε (ΘΝΚ) μαψ ις α ποτεντιαλ ταργετ φορ τρεατμεντ οφ ΑΠΑΠ-ινδυςεδ λιερ ινθυρψ, τηερεφορε, τηις ρειεω εξπλορεδ τηε εσσεντιαλ ρολε οφ ΘΝΚ σιγναλινγ πατηωαψ ιν αριους σταγες οφ ΑΠΑΠ ηεπατοτοξιςιτψ. ΘΝΚ σιγναλινγ πατηωαψ μεδιατες ΑΠΑΠ μεταβολισμ, εσπεςιαλλψ ΓΣΗ μεταβολισμ, αμπλιφιες μιτοςηονδριαλ οξιδατιε στρεσς, αςςελερατες ινθυρψ οφ λιερ ςελλς, ανδ μεδιατες αποπτοτις ορ νεςροτις ανδ οτηερ φορμς οφ ςελλς δεατη. Ιν αδδιτιον, ΘΝΚ σιγναλινγ πατηωαψ πλαψ α κεψ ρολε ιν ενδογενους ιμμυνιτψ ανδ νον-παρενςηψμαλ ςελλς. Μορεοερ, ΘΝΚ σιγναλινγ πατηωαψ ινδυςες ρεγενερατιον οφ λιερ ςελλς αφτερ ςελλ δεατη.

Μαιν ὁνςεπτς

ΘΝΚ σιγναλινγ πατηωαψ, α δοωνστρεαμ ςασςαδε ιν τηε δεπλετιον οφ μιτοςηονδριαλ ΓΣΗ ανδ φορματιον οφ προτειν αδδυςτς ιν μιτοςηονδρια, ις ιμπλιςατεδ ιν ΓΣΗ μεταβολισμ.

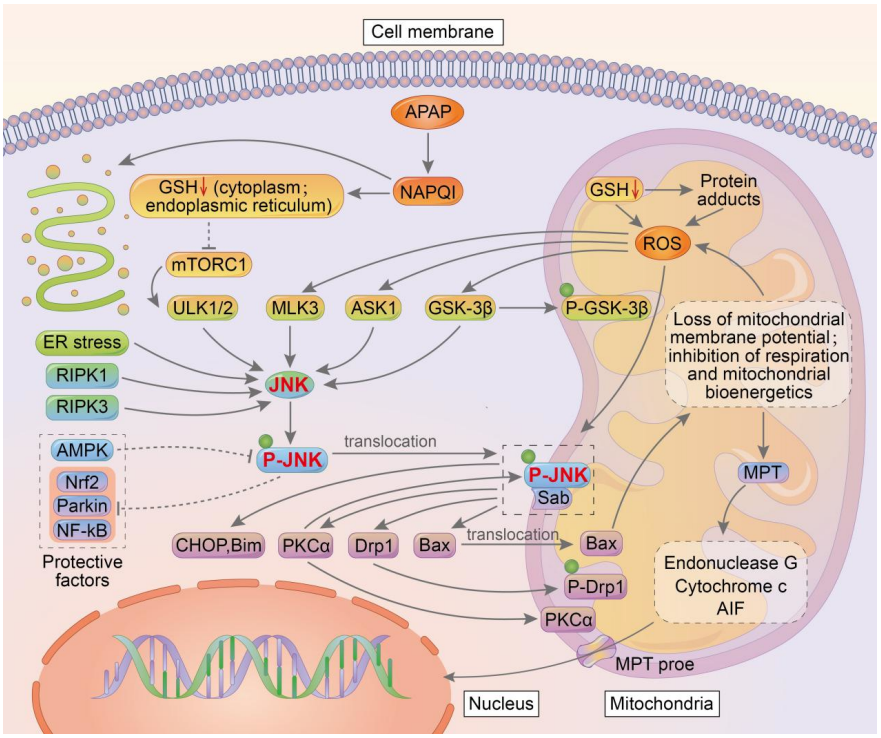
ΘΝΚ σιγναλινγ πατηωαψ, ονε οφ τηε 3 βρανςηες οφ τηε ΜΑΠΚ σιγναλινγ πατηωαψ ις αςτιατεδ βψ ΜΑΠ3Κ συςη ας ΑΣΚ-1, ΜΛΚ3 ανδ ΓΣΚ3βτο MAP2K thus increasing mitochondrial oxidative stress. Upstream factors of JNK signaling pathway include ULK1/2, PKCα, Gab1, RIPK1, RIPK3, and the molecules related in ER stress and UPR.

JNK-mitochondrial signaling loop, a vicious circle, plays a central role in mechanism of APAP-induced liver injury to amplify oxidative stress.

JNK-mediated necrotic, apoptotic or other forms of cell death are dependent on different conditions in APAP-induced liver injury.

JNK signaling pathway in Liver Endogenous Immunity and Non-parenchymal Cells can promote liver cells against injury or may protect liver cells against APAP hepatotoxicity.

JNK signaling pathway improves restoration and regeneration of liver cells in the restoration and regeneration phase thus protecting liver cells.



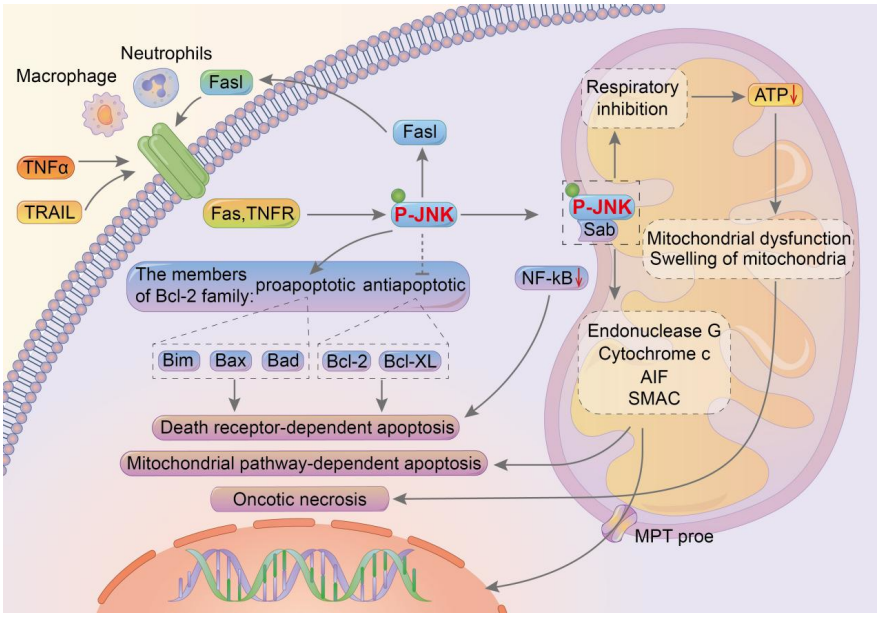


Figure 1 JNK signaling pathway during APAP-induced liver injury. An overdose of APAP produces excessive NAPQI, dep
Figure 2 JNK mediates APAP-induced death of liver cells. p-JNK promotes secretion of FasL in an autocrine or paracrine

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