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**Caspase-12 mediates carbon tetrachloride induced hepatocyte apoptosis in mice**

Liu H *et al.* Caspase-12 mediated hepatocyte apoptosis

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**Abstract**

**AIM**: To investigate the role of caspase-12 and its downstream targets in carbon tetrachloride (CCl4)-induced hepatocyte apoptosis.

**METHODS**: The role of caspase-12 was determined by using caspase-12 knock-out mice. CCl4 (300 uL/kg BW) or vehicle (corn oil) was administered to wild-type or caspase-12 knock-out mice as a single intraperitoneal injection. The animals were sacrificed at 24 h after the CCl4 treatment. Blood was collected to evaluate liver function by the measurement of the activity of alanine aminotransferase. Liver samples were used for the measurements of reactive oxygen species by plasma malondialdehyde as biomarker, hepatocyte apoptosis by terminal transferase–mediated dUTP nick-end labeling and controlled by morphological study, cytochrome C release and caspase activations by western blot.

**RESULTS**: Administration of CCl4 at low dose resulted in hepatocyte apoptosis and acute liver injury in wild-type mice. CCl4 also induced the generation of reactive oxygen species and induction of endoplasmic reticulum stress in the liver followed by activations of caspases 12, 9 and 3 as well as release of small amounts of cytochrome C. However, in the CCl4-treated caspase-12 knock-out mice, activation of caspases 9 and 3 were significantly attenuated; no effect was seen in the cytochrome C release. CCl4-induced apoptosis and liver damage was markedly reduced in the knockout mice compared to wild-type mice (*P* < 0.05). The active form of caspase-8 was not detected neither in wild-type nor caspase-12 knock-out mice. There was no significant different in the formation of reactive oxygen species in the livers of wild-type and caspase-12 knock-out mice treated with CCl4.

**CONCLUSION:** These data indicate that caspase-12 plays a pivotal role in CCl4-induced hepatic apoptosis through the activation of the effector caspase-3 downstream directly and/or indirectly *via* capase-9 activation.

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**Key words:** Apoptosis; Caspases; Hepatocyte; Endoplasmic reticulum; Carbon tetrachloride; Reactive oxygen species

**Core tip**: Sublethal dose of carbon tetrachloride (CCl4) induced significant hepatocyte apoptosis and acute liver injury in wild-type mice. CCl4 also induced reactive oxygen species generation in the liver followed by activations of caspases 12, 9 and 3. *Caspase-12-/-* attenuated CCl4-induced activations of caspase-9 and 3, significantly reduced apoptosis, and preserved liver function. The active form of caspase-8 was not detected indicating that it does not play a significant role in this model of hepatocyte apoptosis. Together, our data indicate that caspase-12 plays a pivotal role in CCl4-induced hepatocyte apoptosis through the downstream activation of the effector caspase-3 directly and/or indirectly *via* caspase-9 activation.

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**INTRODUCTION**

Toxic liver damage may result in acute liver failure, hepatic fibrosis and carcinogenesis[1]. Hepatotoxicity remains a major reason for drug withdrawal from pharmaceutical development and clinical use[1]. Hepatocyte apoptosis is an important contributing factor to acute liver injury in a variety of liver diseases including toxic effects of drugs, alcohol, viral infection, non-alcoholic steatosis and cholestasis[2-4]. From the studies over last decade, hepatocyte apoptosis appears to be the first cellular response to toxic damage and is thought to be the main mode of cell death in liver diseases[4,5].

Apoptosis is executed through the activation of caspase cascades, *via* the apoptotic pathways. The pathways start with activation of an initiator caspase by different stimuli, caspase-8 in membrane-mediated, caspase-9 in mitochondrial-mediated, and caspase-12 in endoplasmic reticulum (ER) stress-mediated pathways[6-8]. The active initiator caspases then activate effector caspases-3, 6 or 7 which cleave key substrates required for normal cellular functions leading to apoptosis[6,7,9-12].

Carbon tetrachloride (CCl4) induced hepatic injury has been widely used to study the mechanisms of hepatotoxic injury and repair. Treatment of sublethal dose of CCl4 results in massive apoptotic damage in liver[13,14]. Previous studies have shown the activations of caspases 3and 9 in the liver of CCl4-treated mice or rat[15,16]. However, the role of caspase-12 and its down-stream targets in CCl4-induced hepatocyte apoptosis has not been defined.

Procaspase-12 is predominantly located on the cytoplasmic side of the ER and expressed at high levels in muscle, liver and kidney[8] and is activated by ER stress[17,18]. The initial event in the liver of CCl4 treated animals is generation of reactive oxygen species (ROS) within ER resulting from the interaction of CCl4 and cytochrome P450 (CYP)[19,20]. ER is highly sensitive to environmental insults such as oxidative stress which leads to ER stress[21,22]. Yuan *et al*[8] have demonstrated that ER-stress inducer tunicamycin-induced apoptosis in embryonic fibroblast and renal tubular epithelial cells was significantly attenuated in caspase-12 knockout mice (*caspase-12-/-*). In the same study, treatment of thymocytes isolated from wild-type and *caspase-12-/-* mice with anti-Fas antibody (activating the membrane-dependent pathway) or dexamethasone (activating mitochondria-dependent pathway through cytochrome C release) developed similar amounts of apoptosis[8]. The authors suggest that caspase-12 is involved in ER stress-induced apoptosis independent of membrane-mediated and mitochondrial pathways. In a cisplatin model of renal tubular apoptosis, we demonstrated that activation of caspase-12 prior to the activations of caspase-3 and 9 and transfection of anti-caspase-12 antibody into renal tubular epithelial cells prevented the activation of procaspase-12 and significantly attenuated cisplatin-induced renal tubular apoptosis[23]. The direct role of caspase-12 in hepatocyte apoptosis was not explored previously. Hence, the current study was to examine if caspase-12 plays essential role and its downstream targets in CCl4-induced hepatocyte apoptosis using *caspase12-/-* mice.

**MATERIALS AND METHODS**

***Caspase-12 knockout mice***

*Caspase-12* knockout mice (developed on C57BL/6J mice) were purchased from Mutant Mouse Regional Resource Center (MMRRC, Chapel Hill, NC). The general *caspase-12* gene knockout mice were developed as described[8]. The genotype of the litter resuscitated from cryo-archive was examined by genotyping. The breeding was carried out by monogamous mating. A pair of male and female homozygous *caspase-12-/-* were kept in the same cage for mating. Pups were weaned at an age of 3 wk and separated according to gender. Animals were maintained under 12 h light/dark cycles with unlimited access to food and water. Male mice at eight-week age, weighing 25 to 30 g, were used for the experiments. All experimental procedures were conducted in accordance with our institutional guidelines and approved by the Institutional Animal Care and Use Committee at the University of Mississippi Medical Center (Permit Number: 1107B).

#### CC14-induced hepatic apoptosis

Our previous study indicated that a large dose of CCl4 (1000 uL/kg BW, equivalent to 1.59 g/kg BW; intraperitoneal, ip) induced severe liver damage and massive necrosis. Therefore, a pilot study was performed to determine the appropriate dose and time points for significant induction of hepatocyte apoptosis in littermate *capsase-12 +/+* mice. CCl4 (Sigma, St. Louis, MO) was administered as a single intraperitoneal dose with different volumes (100, 300 and 500 uL/kg BW, equivalent to 159, 477 and 795 ug/kg BW; dissolved in corn oil) or vehicle (corn oil). The animals were sacrificed at 6, 12, 24 and 48 h after CCl4 injection. Blood and liver samples were collected for the measurements of liver injury and apoptosis.

#### Measurement of alanine aminotransferase

Alanine aminotransferase (ALT) activity was measured on a Roche-cobas® c501 analyzer (Roche Diagnostics, Indianapolis, IN) using Roche-cobas® ALTL reagents. The assay is linear from 5-700 U/L and exhibits a slope of 1.003 (*r* = 0.996).

#### Detection of apoptotic cells

Detection of apoptotic cells by terminal transferase–mediated dUTP nick-end labeling (TUNEL) is extensively used for the quantification of apoptotic cells in tissue or cell culture. However, there have been concerns about its sensitivity and specificity. In the current study, apoptotic cells were quantified by a modified TUNEL protocol, and were controlled by morphological study. TUNEL stain on mouse liver sections was performed by use of ApoAlert DNA Fragmentation Assay Kit (Clontech, Palo Alto, CA) following the manufacture’s protocol and with modifications as described by Tamura *et al*[24] and Labat-Moleur *et al*[25]. Apoptosis was quantified as TUNEL-positive cells/mm2. In morphological examination, the following findings were considered to represent apoptosis: condensation of chromatin and cytoplasm, margination of nuclei, cell blebbing, and apoptotic bodies[26].

#### Determination of lipid peroxidation

Malondialdehyde (MDA), an intermediate of lipid peroxidation, was determined as a measure of lipid peroxidation following the protocol described by Wenger *et al*[27]. Briefly, liver tissue was rapidly frozen at the time of harvest and stored in liquid nitrogen. The tissue was homogenized in an ice cold buffer containing 20 mmol/L PBS pH 7.4, 140 mmol/L NaCl, 0.01% butylated hydroxyanisole, 0.25 mol/L DMSO, and then centrifuged at 900 g for 10 min. 20 µL of liver homogenate was incubated at 90 °C for 60 min in 0.8 mL reaction mixture of 1.35 mol/L acetic acid, 0.15 mol/L sodium dodecyl sulphate, 20 mol/L 1-thiobarbituric acid (pH 3.5). After cooling the reaction tubes in ice water, 0.2 mL distilled water and 1 mL n-butanol/pyridine mixture (15/1) were added. This mixture was mixed by vortexing and then centrifuged. The organic layer was taken for fluorometric measurements of the thiobarbituric acid reactive substances (TBARS) fluorescence (excitation, 515 nm; emission, 553 nm). The TBARS concentration was calculated using 1.1.3.3-tetraethoxypropane dissolved in acetic acid as standard.

#### Cell fraction preparation

Cell fractions were prepared as described previously[8]. Liver tissue was homogenized in an extraction buffer and cell fractions were obtained by differential centrifugations.

#### Western blot

Western blot was performed by the chemiluminescence method as described elsewhere[28]. The antibodies used were as follows: rabbit anti–caspase-12 (AB3612, Lot 22101182, Chemicon, Temecula, CA) and mouse anti-actin (MAB1501, Chemicon); rabbit anti–caspase-3 (sc-7148, Santa Cruz Biotechnology, Santa Cruz, CA), caspase-8 (sc-7890), caspase-9 (sc-8355), and rabbit anti–cytochrome C (sc-7159); anti-GRP78 (NBP1-06274, Novus Biologicals, Littleton, CO); anti-CHOP (#2895, Cell Signaling Technology, Danvers, MA); anti-IRE-1 (ab37073, Abcam, Cambridge, MA).

#### Genotyping

Genomic DNA was isolated from a small section of mouse tail by using a GentraPure gene Mouse Tail Kit (Qiagen, Valencia, CA). PCR was performed by a GoTaq Flexi DNA Polymerase kit (Promega, Madison, WI) and using three primers: GS (E), GCCAGGAGGACACATGAAAGAGATC; GS (E, T), AGCTGTTCCTGGGAAT TGGCAATG; Neo, GGGTGGGATTAGATAAATGCCTGCTCT. For routine genotyping, a PCR protocol employing a mixture of the three primers was used to detect wild allele (246 bp) and mutant allele (414 bp). PCR products were visualized in 2% agarose gel with ethidium bromide. Caspase-12 expression in mice was further confirmed by RT-PCR and western blot.

#### RT-PCR

RNA was isolated from the liver tissue using TRIzol Reagent (GibcoBRL, Gaithersburg, MD). Reverse transcription of 0.5 ug of total RNA and PCR were carried out by one step RT-PCR protocol using AccessQuick RT-PCR system (Promega, Madison, WI). Caspase-12 primers are: 5’-GAAGGAATCTGTGGGGTGAA (F), 5’-AGGCCTGCATGATGAG AATC (R) (product size about 133 bp). GAPDH primers include: 5’-AAGATGGTGAAGGTC GGTGT (F), 5’-TTGATGGCAACAATGTCCACT (R) (product size about 98 bp). The PCR products were visualized on a 2% agarose gel using ethidium bromide and ultraviolet trans-illumination.

#### Statistical analysis

Values are expressed as the mean ± SE. Statistical analysis was performed using an unpaired *t* test (for two groups only) and analysis of variance (for more than two groups). Statistical significance was considered at *P* < 0.05. Calculations were made with SigmaStat 3.5 (SPSS Science, Chicago, IL)

**RESULTS**

***CC14-induced acute liver damage and apoptosis***

We did a pilot study to determine the appropriate dose and time points for significant induction of apoptosis in the liver of wild-type mice. CCl4-induced hepatic injury as measured by ALT was noted starting at 12 h and this damage became more severe at 24 and 48 h (Figure 1A). The increases of ALT in 300 and 500 uL/kg BW treated groups were similar, and they were significantly higher than that of 100 uL/kg group. TUNEL staining demonstrated that there was virtually no positive apoptotic cell in the liver of control mice. Very few apoptotic cells (1-5) per mm2 were observed in the liver section of the mice 6 h after the CCl4 injection. Significant amount of apoptotic cells occurred after 12 h and increased to about 50 TUNEL-positive cells per mm2 at 24 and 48 h following the CCl4 treatment (Figure 1B). The TUNEL stain result was controlled by morphological examination which showed that about 85% TUNEL-positive cells exhibited the morphological features of apoptotic cell. It was determined that CCl4 administered as a single dose of 300 uL/kg (equivalent to 477 ug/kg BW) *i.p.* induced hepatocyte apoptosis at about 50 TUNEL-positive cells/mm2 at 24 h after the injection and this was the dose used for the rest of the animal experiments.

***CC14-induced ROS generation***

MDA was determined as a measure of lipid peroxidation. Lipid peroxidation started to increase 6 h after CCl4 treatment in *caspase-12+/+* and *12-/-* mice. It was significantly increased at 12 h, continued to rise at 24 h, and maintained until 48 h following the admission of CCl4 (Figure 1C). There was no significant difference in MDA generation between *caspase-12+/+* and *12-/-* mice.

***CC14-induced ER stress***

To examine the ER stress response induced by CCl4, we analyzed several key ER stress markers in CCl4-treated mice by western blot. As expected, glucose-regulated protein 78 (GRP78), CCAAT/-enhancer-binding protein homologous protein (CHOP) and serine/threonine-protein kinase/endoribonuclease (IRE-1) were up-regulated in the liver tissue from both *caspase-12+/+ and 12-/-* mice treated with CCl4 (Figure 2). *Caspase-12* gene knockout has no effect on the inductions of these proteins suggesting that activation of caspase-12 is a downstream event of ER stress.

***Subcelluar localization of procaspase-12 and activation of caspase-12 induced by CCl4***

Procaspase-12 was identified and localized in microsomal fraction of wild-type mouse liver homogenate, but not in the nuclear, mitochondria and soluble cell factions (Figure 3A). As shown in Figure 3B, procaspase-12 (55 kDa) was cleaved to the active form (36 kDa). CCl4 activated caspase-12 in the liver of *caspase-12+/+* mice 12 and 24 h after the treatment (Figure 3B).

***CC14-induced apoptosis and liver injury is caspase-12 dependent***

Initially, we confirmed the *caspase-12* knockout mice by genotyping, RT-PCR and western blot using the tissue from a small section of the tail. Genotyping results indicated the absence of wild allele and presence of mutant allele in the *caspase-12-/-* mice (Figure 4A). The absence caspase-12 in the knockout mice was confirmed by RT-PCR for mRNA expression (Figure 4B), and by Western blot for its protein expression (Figure 4C).

To examine the role of caspase-12 in CCl4-induced liver apoptosis, wild-type or *caspase-12-/-* mice were injected with single dose of CCl4 (300 uL/kg BW, *i.p.*), and the animals were sacrificed at 24 h following the injection. CCl4-induced marked hepatic apoptosis in wild-type mice, and this was significantly attenuated in the *caspase-12-/-* mice (Figure 5A and B). CCl4 injection resulted in marked liver damage in *caspase-12+/+* mice as measured by serum ALT, and this injury was significantly reduced in *caspase-12-/-* mice (Figure 5C).

***Effects of CC14 administration on the key enzymes in the different apoptotic pathways***

Western blot analysis was performed to examine activations of key caspases and cytochrome C release in response to the CCl4 treatment. As shown in Figure 6, caspase-12 was activated (presence of cleaved form of caspase-12) in the CCl4-treated wild-type mice (Figure 6A). CCl4 treatment also resulted in the activations of caspases 3and 9 in the liver of wild-type mice; activations of these caspases were significantly inhibited in*caspase-12-/-* mice (Figure 6B, D and G). The mitochondria-mediated apoptotic pathway involves release of mitochondrial protein cytochrome C, resulting in activation of caspase-9. In a western blot analysis using soluble cell factions, CCl4 treatment induced small amount of cytochrome C release from mitochondria, with no significant difference in the amount of cytochrome C released between wild-type and *caspase-12-/-* mice (Figure 6C and G).

There was no cleaved form of caspase-8 either in wild-type or *caspase-12-/-* mice following CCl4 treatment. To confirm this result, a positive control using liver tissue extract from lipopolysaccharide/galactosamine-treated rat was employed in western blot along with other samples. As shown in Figure 6E, active caspase-8 was generated following exposure to galactosamine/lipopolysaccharide, but not after administration of CCl4. The absence of active form of caspase-8 indicates the membrane-mediated pathway does not play a significant role in CCl4-induced hepatic apoptosis in the mice.

**DISCUSSION**

Apoptosis is the first cellular response to many toxic liver diseases[4]. CCl4 induced hepatic apoptosis as early as 6 h[14] far preceding the hepatic necrosis observed at 24 to 48 h following CCl4 treatment[19]. CCl4 is activated by CYP2E1, CYP2B1/CYP2B2 in the ER of hepatocytes to form the trichloromethyl radical and CCl3\*. This radical can react with oxygen leading to the formation of the trichloromethylperoxy radical (CCl3OO\*), a highly reactive species, which initiates a chain reaction of lipid peroxidation[29]. ROS are generated as early as 1 h after CCl4 administration and can serve as a cell death signal to induce apoptosis[30]. ROS formation in the ER leads to perturbation of Ca2+ homeostasis, altered glycosylation and the accumulation of misfolded proteins causing ER stress[31]. Caspase-12 is localized to the cytosolic interface of ER, making it vulnerable to the ER stress and, when activated, leading to further activation of the caspase cascade[8]. In fact, our current study demonstrated that CCl4 treatmentresulted insignificant ROS generation and up-regulations of ER stress response proteins GRP78, CHOP and IRE-1 in the liver tissue. Moreover, CCl4 induced similar degree of ROS generation and ER stress in the liver of both *caspase-12+/+ and 12-/-* mice. These results suggest that caspase-12 activation is a downstream event following ER stress.

In the current study, we confirmed that procaspase-12 is localized to the microsomal fraction of the hepatocytes in the wild-type mice but not in the mitochondrial, nuclear, and soluble fractions by western blot analysis. Sublethal dose of CCl4 induced ROS generation starting at 6 h followed by activation of caspase-12, hepatocyte apoptosis and liver injury at 12 h after CCl4 injection.

To explore the role of caspase-12 in CCl4-induced hepatocyte apoptosis, we conducted a study by using *caspase-12* knockout mice. A complete absence of caspase-12 in the knockout mice was confirmed by genotyping, western blot and RT-PCR. CCl4-induced apoptosis in the liver of *caspase-12 -/-* mice was significantly attenuated, and the liver damage (ALT level) was markedly reduced in the knockout mice as compared with wild-type mice. The nearly complete protection of *caspase-12-/-* mice against CCl4-induced hepatic apoptosis indicates that caspase-12 is a pivotal caspase in CCl4-induced apoptotic signaling.

Having demonstrated the role of caspase-12, we next examined its downstream targets in the apoptotic pathway. Caspase-9 was activated in the liver of wild-type mice treated with CCl4, and the activation of caspase-9 was significantly reduced in *caspase-12-/-*mice. This result implies that caspase-9 is a downstream target of the active caspase-12. It has been a common believe that cytochrome C-Apaf-1 complex namely apoptosome is required for the activation of caspase-9 during apoptosis. However, an in vitro study showed that ER stress induced activations of caspase-12, 9 and 3 in cytochrome C free cytosols[12]. *Apaf-1-/-*fibroblasts are known to be resistant to apoptotic insults that initiate the mitochondria pathway; however, they are susceptible to apoptosis induced by ER stress inducers[18]. In a study using cell-free system, addition of microsomes (isolated from ER-stress induced cells) to an *Apaf-1-/-* cell extract lacking mitochondria or cytochrome C resulted in activation of caspase-9[18]. These results indicate caspase-12 can activate caspase-9 in a cytochrome C and Apaf-1 independent fashion. Morishima *et al*[12] have shown that recombinant caspase-12 specifically cleaves and activates procaspase-9 in cytosolic extracts of a murine myoblast cell line C2C12.

Agents that induce ER stress can also result in release of cytochrome C from mitochondria due to its membrane damage[32,33]. Indeed, in the present study, a small amount of cytochrome C release was observed in the liver of both wild-type and *caspase-12-/-* mice treated with CCl4. There was no difference in the amount of cytochrome C release between wild-type and *caspase-12-/-* mice. However, *caspase-12-/-* significantly reduced caspase-9 activation and provided marked protection against CCl4-induced apoptosis. It appears that this release of cytochrome C does not have significant role in CCl4-induced apoptosis in the liver. As discussed earlier, cytochrome C is not required for caspase-9 activation under ER stress.

Caspase-3 is the key effector caspase that executes apoptosis through the cleavage of substrates required for normal cellular functions, such as the cytoskeletal proteins, nuclear proteins and DNA repairing enzymes[6]. It is well known that caspase-3 is activated by initiators such as caspases 8 and 9 through membrane- or mitochondrial-mediated pathway in response to different stimuli[6,9-12]. In the current study, caspase-8 was not activated in the CCl4-induced hepatocyte apoptosis. To confirm this result, a positive control using liver tissue extract from alactosamine/ lipopolysaccharide-treated rat was employed in western blot along with other samples. The active caspase-8 was presented in the liver of the animals exposed to lactosamine/lipopolysaccharide, but not in CCl4-treated mice. The absence of active form of caspase-8 indicates that the membrane-mediated pathway does not play a significant role in CCl4-induced hepatocyte apoptosis. The absence of caspase-8 activation in the apoptosis associated with ER-stress was also evidenced by our previous study[23] and by others[18] in different experimental animal models.

Previous studies demonstrated that activation of caspase-12 prior to the activation of executioner caspase-3 in other animal models of apoptosis associated ER-stress[23,34]. Treatment of human neuroblastoma cells with ER-stress inducers resulted in the activation of caspase-3 without the activations of caspases-8 and 9, and cells with stable expression of caspase-12 were more vulnerable to ER stress, concomitant with increased activation of caspase-3[35]. Our current study showed that caspase-3 activation was significantly attenuated in *caspase-12-/-* mice accompanied by a marked reduction of hepatocyte apoptosis. These findings suggest that caspase-12 activates caspase-3 downstream.

Taken together, our data establish the essential role of caspase-12 in CCl4-induced hepatocyte apoptosis. We postulate that the ER-associated caspase-12 services as an initiator caspase that activates caspase-3 downstream directly and/or indirectly through activation of caspase-9 resulting apoptosis. The primary effects of CCl4 in humans are on the liver, kidneys, and central nervous system. Human symptoms of acute (short-term) inhalation and oral exposures to CCl4 include headache, weakness, lethargy, nausea, and vomiting. Acute exposures to higher levels and chronic (long-term) inhalation or oral exposure to CCl4 produces liver and kidney damage in humans[36]. The United States Environmental Protection Agency has set a maximum allowable level of 0.005 mg/L for CCl4[36]. There are no known short-term or immediate illness symptoms reported in humans due to exposure at these levels[36]. However, exposure to CCl4 at the low level for longer timecould result in pathological changes in the liver of human. More clinical and animal study need to be done to investigate the long-term consequences of the minimal exposure.

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**COMMENTS**

***Background***

Carbon tetrachloride (CCl4) is a toxic agent that causes damages to liver, kidneys, and central nervous system in humans. Many studies have been done to investigate CCl4-induced acute liver failure and hepatic necrosis at lethal doses. At current time, CCl4 has been limited in use in many countries because of its toxicity. The United States Environmental Protection Agency has set a maximum allowable level of 0.005 mg/L for CCl4. There are no known short-term or immediate illness symptoms reported in humans due to exposure at this level. However, animal studies have shown that CCl4 induces hepatic apoptosis at sublethal doses. The mechanism involving CCl4-induced hepatocyte apoptosis is not well understood.

***Research frontiers***

Apoptosis is executed through the activation of caspase cascades, *via* the apoptotic pathways. The pathways start with activation of an initiator caspase by different stimuli, caspase-8 in membrane-mediated, caspase-9 in mitochondrial-mediated, and caspase-12 in endoplasmic reticulum (ER) stress-mediated pathways. The active initiator caspases then activate effector caspases-3, 6 or 7 which cleave key substrates required for normal cellular functions leading to apoptosis. The initial event in the liver of CCl4 treated animals is generation of reactive oxygen species within ER. ER is highly sensitive to environmental insults such as oxidative stress which leads to ER stress. Procaspase-12 is predominantly located on the cytoplasmic side of the ER, and it is activated by ER stress. The direct role of caspase-12 and its downstream targets in CCl4-induced hepatocyte apoptosis have not been examined previously.

***Innovations and breakthroughs***

This is believed to be the first study to investigate the direct role of caspase-12 and its downstream targets in CCl4-induced hepatocyte apoptosis using caspase-12 knockout animal. We have demonstrated that caspase-12 plays a pivotal role in CCl4-induced hepatocyte apoptosis through the downstream activation of the effector caspase-3 directly and/or indirectly.

***Applications***

Understanding the mechanism of CCl4-induced hepatocyte apoptosis is important for the development of potential therapeutic strategies, such as the uses of antioxidant, chemical ER chaperones and/or caspase-12 inhibitor for preventing/lessening CCl4-induced liver apoptosis and injury at the minimal exposure.

***Terminology***

ER is a major intracellular site for calcium storage and the production of secretory protein through protein synthesis, folding and modification. Disturbance in any of the functions will disrupt the proper protein folding and increase the load of unfolded or misfolded protein, the condition is called ER stress. The unfolded protein response (UPR) is a cellular response to ER stress. UPR has three aims: initially to restore normal cellular function by decreasing protein translation, degrading misfolded proteins and activating the signaling pathways that lead to increasing the production of molecular chaperones involved in protein folding. If these objectives are not achieved or the disruption is prolonged, the UPR aims towards apoptosis.

***Peer review***

The manuscript provides novel information. It is well written. The design, materials and methods are satisfactory. The results support the conclusions drawn.

**REFERENCES**

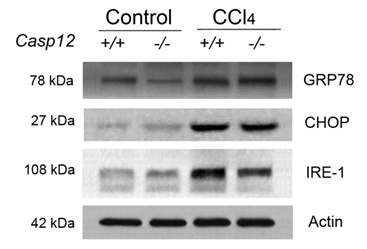
|  |
| --- |
| 1 **Jaeschke H**, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D, Lemasters JJ. Mechanisms of hepatotoxicity. *Toxicol Sci* 2002; **65**: 166-176 [PMID: 11812920 DOI: [10.1093/toxsci/65.2.166](http://dx.doi.org/10.1093/toxsci/65.2.166)]  2 **Higuchi H**, Gores GJ. Mechanisms of liver injury: an overview. *Curr Mol Med* 2003; **3**: 483-490 [PMID: 14527080 DOI: [10.2174/1566524033479528](http://dx.doi.org/10.2174/1566524033479528)]  3 **Wright MC**, Issa R, Smart DE, Trim N, Murray GI, Primrose JN, Arthur MJ, Iredale JP, Mann DA. Gliotoxin stimulates the apoptosis of human and rat hepatic stellate cells and enhances the resolution of liver fibrosis in rats. *Gastroenterology* 2001; **121**: 685-698 [PMID: 11522753 DOI: [10.1053/gast.2001.27188](http://dx.doi.org/10.1053/gast.2001.27188)]  4 **Canbay A**, Friedman S, Gores GJ. Apoptosis: the nexus of liver injury and fibrosis. *Hepatology* 2004; **39**: 273-278 [PMID: 14767974 DOI: [10.1002/Hep.20051](http://dx.doi.org/10.1002/hep.20051)]  5 **Bilodeau M**. Liver cell death: update on apoptosis. *Can J Gastroenterol* 2003; **17**: 501-506 [PMID: 12945012]  6 **Earnshaw WC**, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999; **68**: 383-424 [PMID: 10872455 DOI: [10.1146/annurev.biochem.68.1.383](http://dx.doi.org/10.1146/annurev.biochem.68.1.383)]  7 **Zou H**, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. *J Biol Chem* 1999; **274**: 11549-11556 [PMID: 10206961 DOI: [10.1074/jbc.274.17.11549](http://dx.doi.org/10.1074/jbc.274.17.11549)]  8 **Nakagawa T**, Zhu H, Morishima N, Li E, Xu J, Yankner BA, Yuan J. Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000; **403**: 98-103 [PMID: 10638761 DOI: [10.1038/47513](http://dx.doi.org/10.1038/47513)]  9 **Thornberry NA**, Lazebnik Y. Caspases: enemies within. *Science* 1998; **281**: 1312-1316 [PMID: 9721091 DOI: [10.1126/science.281.5381.1312](http://dx.doi.org/10.1126/science.281.5381.1312)]  10 **Fraser A**, Evan G. A license to kill. *Cell* 1996; **85**: 781-784 [PMID: 8681372 DOI: [10.1016/S0092-8674(00)81005-3](http://dx.doi.org/10.1016/S0092-8674(00)81005-3)]  11 **Chinnaiyan AM**, Dixit VM. The cell-death machine. *Curr Biol* 1996; **6**: 555-562 [PMID: 8805273 DOI: [10.1016/S0960-9822(02)00541-9](http://dx.doi.org/10.1016/S0960-9822(02)00541-9)]  12 **Morishima N**, Nakanishi K, Takenouchi H, Shibata T, Yasuhiko Y. An endoplasmic reticulum stress-specific caspase cascade in apoptosis. Cytochrome c-independent activation of caspase-9 by caspase-12. *J Biol Chem* 2002; **277**: 34287-34294 [PMID: 12097332 DOI: [10.1074/jbc.M204973200](http://dx.doi.org/10.1074/jbc.M204973200)]  13 **Zahedi K**, Barone SL, Xu J, Steinbergs N, Schuster R, Lentsch AB, Amlal H, Wang J, Casero RA, Soleimani M. Hepatocyte-specific ablation of spermine/spermidine-N1-acetyltransferase gene reduces the severity of CCl4-induced acute liver injury. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G546-G560 [PMID: 22723264 DOI: 10.1152/ajpgi.00431.2011]  14 **Shi J**, Aisaki K, Ikawa Y, Wake K. Evidence of hepatocyte apoptosis in rat liver after the administration of carbon tetrachloride. *Am J Pathol* 1998; **153**: 515-525 [PMID: 9708811 DOI: [10.1016/S0002-9440(10)65594-0](http://dx.doi.org/10.1016/S0002-9440(10)65594-0)]  15 **Schattenberg JM**, Nagel M, Kim YO, Kohl T, Wörns MA, Zimmermann T, Schad A, Longerich T, Schuppan D, He YW, Galle PR, Schuchmann M. Increased hepatic fibrosis and JNK2-dependent liver injury in mice exhibiting hepatocyte-specific deletion of cFLIP. *Am J Physiol Gastrointest Liver Physiol* 2012; **303**: G498-G506 [PMID: 22700824 DOI: 10.1152/ajpgi.00525.2011]  16 **Tien YC**, Liao JC, Chiu CS, Huang TH, Huang CY, Chang WT, Peng WH. Esculetin ameliorates carbon tetrachloride-mediated hepatic apoptosis in rats. *Int J Mol Sci* 2011; **12**: 4053-4067 [PMID: 21747724 DOI: 10.3390/ijms12064053]  17 **Szegezdi E**, Fitzgerald U, Samali A. Caspase-12 and ER-stress-mediated apoptosis: the story so far. *Ann N Y Acad Sci* 2003; **1010**: 186-194 [PMID: 15033718 DOI: [10.1196/annals.1299.032](http://dx.doi.org/10.1196/annals.1299.032)]  18 **Rao RV**, Castro-Obregon S, Frankowski H, Schuler M, Stoka V, del Rio G, Bredesen DE, Ellerby HM. Coupling endoplasmic reticulum stress to the cell death program. An Apaf-1-independent intrinsic pathway. *J Biol Chem* 2002; **277**: 21836-21842 [PMID: 11919205 DOI: [10.1074/jbc.M202726200](http://dx.doi.org/10.1074/jbc.M202726200)]  19 **Zimmerman HJ**. Hepatotoxicity: The adverse effects of drugs and other chemicals on the liver, New York: Appleton-Century-Crofts, 1978: 349-369  20 **Recknagel RO**, Glende EA, Hruszwcyz AM. Free Radicals in Biology: Chemical mechanisms in carbon tetrachloride toxicity. New York: Academic Press, 1977: 97-132 [DOI: 10.1016/B978-0-12-566503-2.50010-0]  21 **Zhang K**, Kaufman RJ. The unfolded protein response: a stress signaling pathway critical for health and disease. *Neurology* 2006; **66**: S102-S109 [PMID: 16432136 DOI: [10.1212/01.wnl.0000192306.98198.ec](http://dx.doi.org/10.1212/01.wnl.0000192306.98198.ec)]  22 **Szegezdi E**, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Rep* 2006; **7**: 880-885 [PMID: 16953201 DOI: [10.1038/sj.embor.7400779](http://dx.doi.org/10.1038/sj.embor.7400779)]  23 **Liu H**, Baliga R. Endoplasmic reticulum stress-associated caspase 12 mediates cisplatin-induced LLC-PK1 cell apoptosis. *J Am Soc Nephrol* 2005; **16**: 1985-1992 [PMID: 15901768 DOI: [10.1681/ASN.2004090768](http://dx.doi.org/10.1681/ASN.2004090768)]  24 **Tamura T**, Said S, Lu W, Neufeld D. Specificity of TUNEL method depends on duration of fixation. *Biotech Histochem* 2000; **75**: 197-200 [PMID: 10999571 DOI: [10.3109/10520290009066501](http://dx.doi.org/10.3109/10520290009066501)]  25 **Labat-Moleur F**, Guillermet C, Lorimier P, Robert C, Lantuejoul S, Brambilla E, Negoescu A. TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement. *J Histochem Cytochem* 1998; **46**: 327-334 [PMID: 9487114 DOI: [10.1177/002215549804600306](http://dx.doi.org/10.1177/002215549804600306)]  26 **Gold R**, Schmied M, Giegerich G, Breitschopf H, Hartung HP, Toyka KV, Lassmann H. Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques. *Lab Invest* 1994; **71**: 219-225 [PMID: 8078301]  27 **Wenger FA**, Kilian M, Mautsch I, Jacobi CA, Steiert A, Peter FJ, Guski H, Schimke I, Müller JM. Influence of octreotide on liver metastasis and hepatic lipid peroxidation in BOP-induced pancreatic cancer in Syrian hamsters. *Pancreas* 2001; **23**: 266-272 [PMID: 11590322 DOI: [10.1097/00006676-200110000-00007](http://dx.doi.org/10.1097/00006676-200110000-00007)]  28 **Bradd SJ**, Dunn MJ. Analysis of membrane proteins by western blotting and enhanced chemiluminescence. *Methods Mol Biol* 1993; **19**: 211-218 [PMID: 7693221]  29 **Weber LW**, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: carbon tetrachloride as a toxicological model. *Crit Rev Toxicol* 2003; **33**: 105-136 [PMID: 12708612 DOI: [10.1080/713611034](http://dx.doi.org/10.1080/713611034)]  30 **Czaja MJ**. Induction and regulation of hepatocyte apoptosis by oxidative stress. *Antioxid Redox Signal* 2002; **4**: 759-767 [PMID: 12470503 DOI: [10.1089/152308602760598909](http://dx.doi.org/10.1089/152308602760598909)]  31 **Paschen W**, Frandsen A. Endoplasmic reticulum dysfunction--a common denominator for cell injury in acute and degenerative diseases of the brain? *J Neurochem* 2001; **79**: 719-725 [PMID: 11723164 DOI: [10.1046/j.1471-4159.2001.00623.x](http://dx.doi.org/10.1046/j.1471-4159.2001.00623.x)]  32 **Häcki J**, Egger L, Monney L, Conus S, Rossé T, Fellay I, Borner C. Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene* 2000; **19**: 2286-2295 [PMID: 10822379 DOI: [10.1038/sj.onc.1203592](http://dx.doi.org/10.1038/sj.onc.1203592)]  33 **Ito Y**, Pandey P, Mishra N, Kumar S, Narula N, Kharbanda S, Saxena S, Kufe D. Targeting of the c-Abl tyrosine kinase to mitochondria in endoplasmic reticulum stress-induced apoptosis. *Mol Cell Biol* 2001; **21**: 6233-6242 [PMID: 11509666 DOI: [10.1128/MCB.21.18.6233-6242.2001](http://dx.doi.org/10.1128/MCB.21.18.6233-6242.2001)]  34 **Jayanthi S**, Deng X, Noailles PA, Ladenheim B, Cadet JL. Methamphetamine induces neuronal apoptosis via cross-talks between endoplasmic reticulum and mitochondria-dependent death cascades. *FASEB J* 2004; **18**: 238-251 [PMID: 14769818 DOI: [10.1096/fj.03-0295com](http://dx.doi.org/10.1096/fj.03-0295com)]  35 **Hitomi J**, Katayama T, Taniguchi M, Honda A, Imaizumi K, Tohyama M. Apoptosis induced by endoplasmic reticulum stress depends on activation of caspase-3 via caspase-12. *Neurosci Lett* 2004; **357**: 127-130 [PMID: 15036591 DOI: [10.1016/j.neulet.2003.12.080](http://dx.doi.org/10.1016/j.neulet.2003.12.080)]  36 **Agency for Toxic Substances and Disease Registry** (ATSDR). Toxicological Profile for Carbon tetrachloride (Update). Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA. 1994 |

**P-Reviewer:** Breitkopf-Heinlein K, Jeschke MG **S-Editor:** Ma YJ

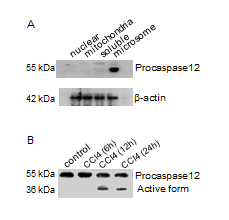
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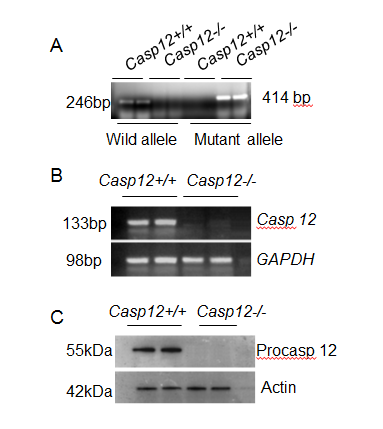
**Figure 1 Dose and time dependent liver damage, apoptosis and reactive oxygen species generation.** A: Carbon tetrachloride (CCl4)-induced hepatic injury as measured by alanine aminotransferase (ALT) was noted starting at 12 h and becoming more severe at 24 and 48 h. The increases in ALT in 300 and 500 uL/kg BW groups were similar and they are significantly higher than that of 100 uL/kg BW group. Values are means ± SE, *n* = 5, a*P* < 0.05 *vs* control, c*P* < 0.05 *vs* 12 h at the same dose; B: Hepatic apoptosis was examined by terminal transferase–mediated dUTP nick-end labeling (TUNEL) staining. There was virtually no positive apoptotic cell in the liver of control mice. Very few apoptotic cells (1-5) per mm2 were observed at 6 h, while significant amount of apoptotic cells appeared after 12 h and reached around 50 TUNEL-positive cells/mm2 at 24 and 48 h after CCl4 treatment at doses of 300 and 500 uL/kg BW. For each sample, five randomly selected fields at x 200 magnification were evaluated. Values are means ± SE, *n* = 5, e*P* < 0.05 *vs* 100 uL/kg BW group at the same time point; C: There was a significant generation of reactive oxygen species as measured by malondialdehyde (MDA) in both *caspase-12+/+ and 12-/-* mouse liver 12, 24 and 48 h following CCl4 treatment (300 uL/kg BW) as demonstrated by the increase in MDA. No significant different in MDA formation between *caspase-12+/+* and *12-/-* mice. Values are means ± SE, *n* = 8, g*P* < 0.05 *vs* controls.



**Figure 2 Carbon tetrachloride induced endoplasmic reticulum stress in the liver**. The representative western blots show that there was an increase in endoplasmic reticulum stress response in carbon tetrachloride (CCl4)-treated mice. Glucose-regulated protein 78, CCAAT/-enhancer-binding protein homologous protein and serine/threonine-protein kinase/endoribonuclease were up-regulated in the liver of both *caspase-12+/+* and *12-/-* mice treated with CCl4.



**Figure 3 Localization of procaspase-12 in the subcellular components of liver cells and the activation of caspase-12 by carbon tetrachloride**. A: Procaspase-12 was detected only in the microsome fraction but not in the nuclear, mitochondria and soluble fractions; B: Activation of caspase-12 was detected in the liver *of caspase-12+/+* mice at 12 and 24 h after carbon tetrachloridetreatment. Western blot was performed using an antibody recognizing both pro- and active forms of caspase-12. Procaspase-12, a about 55 kDa protein, was cleaved to the active form with a molecular weight about 36 kDa. The representative blot was from the results of 3 experiments.



**Figure 4 Genotyping and confirmation of the lack of caspase-12 in the *caspase-12-/-* mice.** A: Genotyping result indicates the absence of wild allele and presence of mutant allele in the *caspase-12-/-* (*Casp*) mice; B: RT-PCR confirmed the absence of caspase-12 mRNA in *caspase-12-/-*; C: Western blot showed the absence of caspase-12 protein in *caspase-12-/-* mice.

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**Figure 5 *Caspase-12-/-* mice attenuated carbon tetrachloride-induced hepatic apoptosis and liver injury**. A: Statistical analysis of terminal transferase–mediated dUTP nick-end labeling (TUNEL)-positive cells indicates a significant reduction of carbon tetrachloride (CCl4)-induced hepatocyte apoptosis in *caspase-12-/-* (*Casp*) mice. Five randomly selected fields at x 200 magnification were evaluated for each tissue section. The numbers of apoptotic cells were counted as TUNEL-positive cells/mm2. Values are means ± SE, *n* = 8, a*P* < 0.05 *vs* controls, c*P* < 0.05 *vs* *caspase12+/+*; B: The representative sections of TUNEL staining under fluorescence microscope (x 200). TUNEL-positive cells were identified by the nuclear fluorescence staining; C: *Caspase-12-/-* mice significantly attenuated CCl4-induced liver injury as measured by serum ALT. Values are means ± SE, *n* = 8, e*P* < 0.05 *vs* controls, g*P* < 0.05 *vs* *caspase-12+/+* mice.

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**Figure 6 Caspase activations and cytochrome C release in mice treated with carbon tetrachloride**. **Activations of caspases and cytochrome C release were examined by western blot.** A: Procaspase-12 was significantly reduced in carbon tetrachloride (CCl4)-treated *caspase-12+/+* mice associated with the presence of cleaved caspase-12, and both forms of caspase-12 were absence in *caspase-12-/-* mice; B: There was an induction of active form of caspase-3 in the CCl4-treated *caspase-12+/+* mice. The activation of caspase-3 was significant reduced in CCl4-treated *caspase-12-/-* mice and not noticeable in the controls; C: CCl4 treatment induced cytochrome C release from mitochondria both in wild-type and *caspase-12-/-* mice; D: Casapse-9 was activated in CCl4-treated *caspase-12+/+* mice, but this activation was significantly reduced in CCl4-treated *caspase-12-/-* mice; E: Administration of CCl4 did not activate caspase-8 neither in *caspase-12+/+* nor *caspase-12-/-* mice. The bands (asterisk), first line from right, are the positive control for the anti-caspase-8 antibody. Liver tissue extract from lipopolysaccharide/galactosamine-treated rat was used for the positive control; F: A representative blot for actin as control; G: Densitometric analyses of cytosolic cytochrome C and cleaved caspases-3 and 9 were performed by four independent experiments from the western blots. The values of density for caspases and cytochrome C were corrected by loading control actin for each individual sample. Values are means ± SE, a*P* < 0.05 *vs* CCl4-treated *caspase-12+/+*.