

## Full-length article

## Apoptosis initiated by carbon tetrachloride in mitochondria of rat primary cultured hepatocytes

Yan CAI<sup>1,2</sup>, Li-kun GONG<sup>1,2</sup>, Xin-ming QI<sup>1,2</sup>, Xiang-hong LI<sup>1</sup>, Jin REN<sup>1,3</sup><sup>1</sup>State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 201203, China; <sup>2</sup>Graduate School of the Chinese Academy of Sciences, Shanghai 201203, China

### Key words

carbon tetrachloride; hepatocytes; mitochondria; caspase 3; cytochrome c

<sup>3</sup> Correspondence to Prof Jin REN.  
Phn/Fax 86-21-5080-6031.  
E-mail jren@mail.shcnc.ac.cn

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### Abstract

**Aim:** To investigate the mitochondria-initiated apoptosis pathway involved in Carbon tetrachloride (CCl<sub>4</sub>) hepatotoxicity *in vitro*. **Methods:** Several cytotoxicity endpoints, including WST-8 metabolism, lactate dehydrogenase leakage and morphological changes, were examined. The 5,5'-dithio-bis(2-nitrobenzoic acid) reaction was used to measure reduced glutathione level, and the malondialdehyde level was determined using the thiobarbituric acid assay. The release of cytochrome c and Bcl-X<sub>L</sub> was detected by Western blot. Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC. DNA fragmentation was used to evaluate cell apoptosis. **Results:** A time- and dose-dependent decrease in cellular glutathione content was observed, along with a concomitant increase in malondialdehyde levels following the application of CCl<sub>4</sub>. Caspase 3 activity was stimulated at all doses of CCl<sub>4</sub>, with the most significant activation at 3 mmol/L. Cytochrome c was released obviously after CCl<sub>4</sub> treatment. A time-dependent decrease in Bcl-X<sub>L</sub> expression was observed. DNA fragmentation results revealed apoptosis and necrosis following CCl<sub>4</sub> treatment. **Conclusion:** Oxidative damage is one of the essential mechanisms of CCl<sub>4</sub> hepatotoxicity, which triggers apoptosis via the mitochondria-initiated pathway.

### Introduction

Apoptosis is a genetically encoded form of cell suicide central to the development and homeostasis of multicellular organisms<sup>[1–3]</sup>. Once researchers assumed that the activation of endonucleases and specific proteases (such as caspases) reflect the key mechanism of apoptosis<sup>[4,5]</sup>. However, a number of studies disclose that mitochondria play a key role in apoptosis<sup>[1,4,6]</sup>. The mitochondrial pathway is partly dependent on the release of cytochrome c. After release from mitochondria to the cytosol, cytochrome c binds to apoptosis-activating factor-1 (Apaf-1), ATP (or dATP), and possibly a cytosolic protein (Apaf-3), and activates caspase 9, which in turn stimulates caspase 3 activity. Caspase 3 cleaves and activates DNA fragmentation factor (DFF), resulting in DNA degradation<sup>[2,4,7]</sup>.

Bcl-2 proteins act on mitochondria to regulate apoptosis. The Bcl-2 family consists of both cell death promoters and

preventers, including the anti-apoptotic proteins Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, A1/Bfl-1 and Bcl-W, and the pro-apoptotic members Bax, Bcl-X<sub>s</sub>, Bak, Bad, Bik, Bim, Bid, Hrk and Bok<sup>[8]</sup>. Bcl-2 and Bcl-X<sub>L</sub> prevent cytochrome c from entering the cytosol, either by blocking release or binding to the cytochrome in a direct or indirect fashion, and consequently inhibiting activation of the downstream caspase cascade<sup>[4]</sup>.

Reactive oxygen species (ROS), which induce the onset of the mitochondrial permeability transition (MPT), play an important role in mitochondrial apoptosis. Activation of MPT is a major controlling mechanism in some apoptotic systems, and also contributes to the release of cytochrome c and other apoptogenic proteins<sup>[4,9]</sup>. However, the mechanisms of ROS generation and its relationship with the well-documented caspase activation remain to be elucidated. In the present investigation, the levels of reduced glutathione (GSH) and malondialdehyde (MDA) are measured as reliable markers of oxidative stress.

Carbon tetrachloride (CCl<sub>4</sub>) is a typical poison that induces severe oxidative stress followed by activation of caspase 3 in rat liver<sup>[10]</sup>. Moreover, GSH depletion and MDA induction by CCl<sub>4</sub> are evident in rat primary hepatocytes and HepG2, a human hepatoma cell line<sup>[11]</sup>. However, it is currently unclear whether both oxidative stress and apoptosis occur in rat primary hepatocytes induced by CCl<sub>4</sub>.

Mitochondrial damage by CCl<sub>4</sub> is involved in the apoptotic process *in vivo*, during which caspase 3 is activated<sup>[10]</sup>. A histological study suggests that apoptosis is additionally induced by CCl<sub>4</sub> in liver<sup>[12]</sup>. However, the pathway by which CCl<sub>4</sub> mediates apoptosis in rat primary hepatocytes is currently unknown.

In the present report, CCl<sub>4</sub> mediation of the expression and activation of different proteins involved in apoptotic cell death is investigated. Moreover, the relationship between peroxidative damage and apoptosis induced by CCl<sub>4</sub> in rat primary hepatocytes is explored.

## Materials and methods

**Materials and culture of hepatocytes** CCl<sub>4</sub> and Me<sub>2</sub>SO were purchased from Shanghai Chemical Reagent Company (Shanghai, China). CCl<sub>4</sub> was dissolved in 10% Me<sub>2</sub>SO HBS solution (in mmol/L: HEPES 33, NaCl 160.8 mmol/L, KCl 3.15 mmol/L, Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 0.7 mmol/L) and diluted to final concentration of 0.1 mmol/L, 0.3 mmol/L, 1 mmol/L, 3 mmol/L, and 9 mmol/L, respectively.

Hepatocytes were isolated from Sprague-Dawley male rats (180 g–220 g) by 2-step collagenase perfusion, as described previously<sup>[13–15]</sup>, with some modifications. Collagenase IV was purchased from Sigma Chemical Company (St Louis, USA). Hepatocytes were seeded on collagen-coated plastic dishes at a density of 3×10<sup>5</sup> viable cells/mL, and cultured in Ham's F-12/Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) (1:1) medium supplemented with 15% fetal bovine serum (PAA Laboratories, Linz, Austria), 0.1 g/L penicillin (Shanghai Fourth Pharmaceuticals, Shanghai, China), 0.07 g/L streptomycin (Shanghai Fourth Pharmaceuticals, Shanghai, China) and 0.2% bovine serum albumin (Roche, NJ, USA). After 3 h, the medium was altered. Cells were incubated in medium containing different concentrations of CCl<sub>4</sub> solution.

**Cell viability and lactate dehydrogenase leakage assay** Rat primary hepatocytes were treated with CCl<sub>4</sub> for 20 h, and the general viability of cultured cells was assayed at 450 nm with the Cell Counting Kit-8 (Dojindo Laboratories, Tokyo, Japan).

Hepatocytes were seeded on collagen-coated 96-well

microtiter plates at a density of 3×10<sup>4</sup> cells/well. After treatment with increasing concentrations of CCl<sub>4</sub> for 20 h, lactate dehydrogenase (LDH) released into the culture supernatants was measured in a 3-min coupled enzymatic assay that results in the conversion of a tetrazolium salt (NBT) into a formazan product<sup>[16]</sup>. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data at 490 nm were collected using a standard 96-well plate reader (SOFTmax<sup>®</sup> PRO, Molecular Devices, Sunnyvale, CA, USA).

**General morphology of cultured cells** Hepatocytes were cultured with CCl<sub>4</sub> for 20 h, and cell morphology was examined using inverted microscopy.

**Intracellular glutathione content assay** Hepatocytes were treated separately with CCl<sub>4</sub> for 4 h, 8 h, 12 h, 16 h, or 24 h. Cells in monolayers were gently washed 3 times with phosphate-buffered saline (PBS) (pH 7.4), scraped in ice-cold PBS, and sonicated intermittently 3 times. Cell homogenates were added to solution containing 10% trichloroacetic acid and 20 mmol/L EDTA-Na<sub>2</sub>. The mixture was centrifuged for 5 min at 3000×g. The suspension was mixed with buffer (0.4 mmol/L Tris-HCl, 20 mmol/L EDTA-Na<sub>2</sub>) and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and measured at a wavelength of 412 nm within 5 min. Reduced pure GSH was used to obtain a standard curve.

**Lipid peroxidation assay for malondialdehyde** Malondialdehyde levels were measured spectrophotometrically using a thiobarbituric acid (TBA) fluorescence assay. Cell homogenates were mixed with solution (20 mmol/L TBA/glacial acetic acid, 1:1), and heated for 60 min at 100 °C in a water bath. After cooling down, the mixture was extracted in methanol and centrifuged for 10 min at 5000×g. The suspension was measured in a fluorimeter with excitation at 515 nm and emission at 550 nm.

**Western blot analysis** Hepatocytes were scraped in ice-cold PBS obtained by centrifugation at 300×g for 5 min. Cells were gently lysed for 30 s in 75 μL ice-cold buffer containing 250 mmol/L sucrose, 1 mmol/L edetic acid, 0.05% digitonin, 25 mmol/L Tris, pH 6.8, 1 mmol/L dithiothreitol, and protease inhibitor (10<sup>-3</sup> g/L leupeptin and aprotinin, and 0.1 mmol/L phenylmethylsulfonyl fluoride). Lysates were then homogenized through a 26-gauge needle for 30 passages and centrifuged twice at 800×g for 20 min at 4 °C. The pooled supernatant was centrifuged at 10 000×g for 10 min at 4 °C to collect the mitochondria fractions. This supernatant was then transferred to fresh tubes and centrifuged at 16 000×g for 20 min at 4 °C to remove any residual mitochondria. The supernatant was stored as the cytosolic fraction of hepatocytes. Cytosolic and mitochondria fractions were

subjected to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The gel was transferred to a PVDF membrane. Monoclonal rat anti-cytochrome c antibody was purchased from Neomarkers (Lab Vision & NEOMARKERS, UK) and polyclonal rat anti-bcl-X<sub>L</sub> antibody was from Cell Signaling Technology (Beverly, MA, USA). Following incubation with horseradish peroxidase-conjugated secondary antibody (Rockland, Gilbertsville, PA, USA), the blot was developed using the enhancing chemiluminescence detection system.

**Caspase 3 activity assay** Following incubation of hepatocytes in the absence of CCl<sub>4</sub> for the indicated times, caspase 3 activation was measured with the Caspase 3 assay kit (BD Biosciences Pharmingen, San Diego, CA, USA). In brief, culture cells were washed with ice-cold PBS (pH 7.4), and lysed in buffer provided by the kit for 30 min on ice. Reaction mixtures containing Ac-DEVD-AMC and cell lysates in buffer were incubated for 1 h at 37 °C. AMC liberated from Ac-DEVD-AMC was measured in a fluorimeter with excitation at 380 nm and emission at 460 nm.

**DNA gel electrophoresis assay** Cultured cells were washed 3 times with ice-cold PBS, scraped in the same PBS, and collected by centrifugation for 5 min at 600×g. Cells were resuspended in 10 mmol/L EDTA and 50 mmol/L Tris-HCl (pH 8.0) containing 0.5% sodium lauryl sarcosinate and 0.5 g/L proteinase K, and incubated for 60 min at 50 °C. Next, 10 mmol/L EDTA containing 0.25% bromophenol blue and 40% sucrose was mixed with each DNA extract. Individual extracts were loaded into the wells of a 2% agarose gel containing 3×10<sup>-3</sup> g/L ethidium bromide. Electrophoresis was carried out in 40 mmol/L Tris-HCl containing 40 mmol/L acetic acid and 1 mmol/L EDTA.

**Statistical analysis** Data were entered into a database and analyzed using SPSS software. Group mean values and standard deviations were calculated. After homogenetic analysis, homogeneous data were analyzed with one-way analysis of variance and a *post hoc* test of least significant difference. Heterogeneous data were analyzed using the *t*-test. *P*<0.05 was considered statistically significant.

## Results

**Cytotoxicity induction by CCl<sub>4</sub> in rat primary hepatocytes** Cells were exposed to 0.1–9 mmol/L CCl<sub>4</sub> for 20 h, and viability was measured. Cell viability decreased in a dose-dependent manner (Table 1).

Intracellular LDH release as a result of plasma membrane breakdown and alteration of permeability was evaluated. A dose-dependent increase in LDH release in rat primary hepa-

**Table 1.** Cytotoxicity of CCl<sub>4</sub> in the rat primary hepatocytes. *n*=4. Mean±SD. <sup>c</sup>*P*<0.01 vs control group.

CCl <sub>4</sub> /mmol·L <sup>-1</sup>	Cell viability of control group/%
0	100
0.1	82.5±6.0 <sup>c</sup>
0.3	72.4±5.3 <sup>c</sup>
1	68.2±4.4 <sup>c</sup>
3	61.8±4.4 <sup>c</sup>
9	47.3±2.9 <sup>c</sup>

**Table 2.** Intracellular lactate dehydrogenase release induced by CCl<sub>4</sub> after 20 h. *n*=5. Mean±SD. <sup>c</sup>*P*<0.01 vs control group.

CCl <sub>4</sub> /mmol·L <sup>-1</sup>	A <sub>490</sub>	Fold change in absorbance compared with control group
0	0.0047±0.0044	1
0.1	0.0025±0.0023	0.54±0.04
0.3	0.0098±0.0095	2.05±0.15 <sup>c</sup>
1	0.0321±0.0035	6.73±0.49 <sup>c</sup>
3	0.0869±0.0051	18.23±1.17 <sup>c</sup>
9	0.1499±0.0063	31.43±2.28 <sup>c</sup>

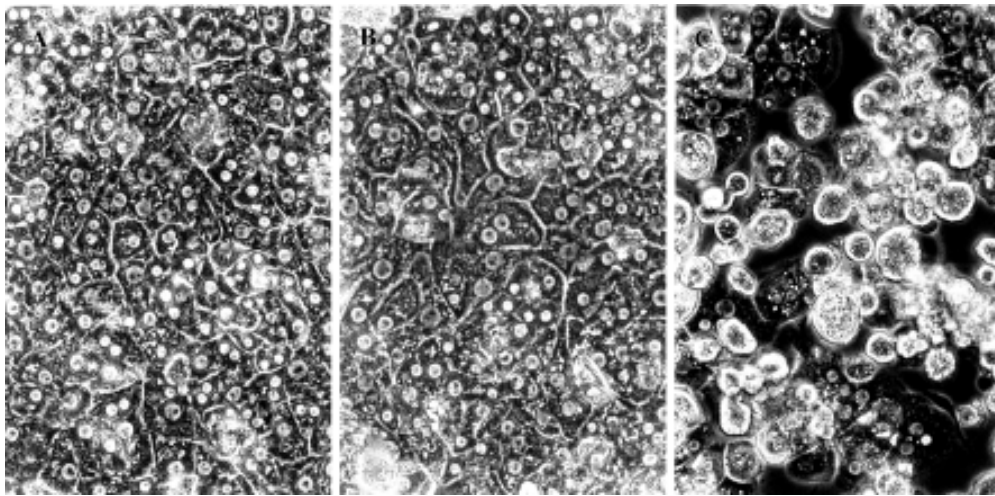
tocytes was observed in the presence of CCl<sub>4</sub> (Table 2).

**Hepatocyte morphology** Cultured cell morphology examination revealed that 9 mmol/L CCl<sub>4</sub> significantly inhibited the confluence of cultured hepatocytes (Figure 1).

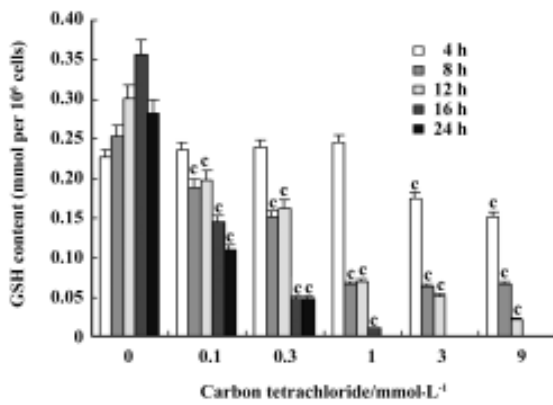
**Glutathione depletion** CCl<sub>4</sub> induced a dose- and time-dependent depletion of GSH in rat primary hepatocytes. The GSH level was significantly reduced after cell incubation with 1 mmol/L CCl<sub>4</sub> for 8 h, 12 h, 16 h, and 24 h, but not at 4 h. The exhaustion of GSH was observed initially with 3 mmol/L CCl<sub>4</sub> after a 16-h incubation (Figure 2).

**Malondialdehyde formation** We observed dose- and time-dependent induction of MDA formation in the presence of CCl<sub>4</sub> in rat primary hepatocytes. CCl<sub>4</sub> induced significant amounts of MDA at concentrations of 3 mmol/L and 9 mmol/L after a 12-h incubation and at 0.3–9 mmol/L after 20 h. There was no evident MDA induction after 4 h of CCl<sub>4</sub> treatment at all the doses tested (Table 3).

**Cytochrome c release** Cytochrome c was detected in the cytosolic fractions of hepatocytes. Hepatocytes were treated with 0.3, 1, 3 and 9 mmol/L CCl<sub>4</sub> up to 16 h. CCl<sub>4</sub> induced time-dependent cytochrome c release in rat primary hepatocytes. Evident dose-dependent release of cytochrome c was observed at 4 h and 8 h (Figure 3A). In contrast, a dose- and time-dependent decrease in mitochondria cyto-



**Figure 1.** Light micrographs of rat primary hepatocytes at 20 h after treatment with CCl<sub>4</sub>. (A) Control cells; (B) CCl<sub>4</sub> 3 mmol/L; (C) CCl<sub>4</sub> 9 mmol/L. ×20.



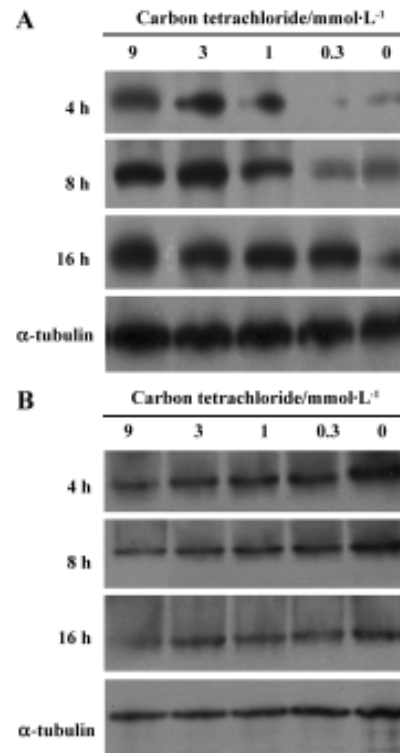
**Figure 2.** Levels of glutathione (GSH) measured in rat primary hepatocytes with increasing concentrations of CCl<sub>4</sub>. Results are expressed as GSH content (mmol) per 10<sup>6</sup> cells. *n*=4. Mean±SD. <sup>c</sup>*P*<0.01 vs control (CCl<sub>4</sub> 0 mmol/L).

**Table 3.** Time and dose course of malondialdehyde (MDA) content in rat primary hepatocytes treated with CCl<sub>4</sub>. *n*=4. Mean±SD. <sup>b</sup>*P*<0.05 vs 12 h control. <sup>c</sup>*P*<0.05 vs 20 h control.

CCl <sub>4</sub> /mmol·L <sup>-1</sup>	MDA/μmol·g <sup>-1</sup> protein		
	4 h	12 h	20 h
0	0.041±0.004	0.038±0.006	0.022±0.003
0.1	0.043±0.001	0.046±0.002	0.021±0.013
0.3	0.038±0.004	0.038±0.004	0.026±0.006 <sup>c</sup>
1	0.048±0.018	0.042±0.004	0.026±0.006 <sup>c</sup>
3	0.036±0.003	0.054±0.001 <sup>b</sup>	0.029±0.001 <sup>c</sup>
9	0.042±0.002	0.053±0.003 <sup>b</sup>	0.040±0.007 <sup>c</sup>

chrome c was observed (Figure 3B).

**Caspase 3 activation by carbon tetrachloride** Caspase 3 activity was examined in cultured hepatocytes treated with



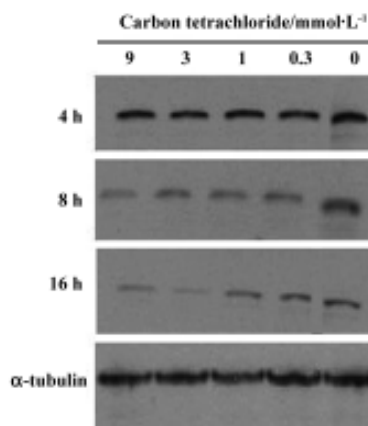
**Figure 3.** Effect of CCl<sub>4</sub> on cytochrome c release from mitochondria into the cytosol in rat primary hepatocytes. Cytochrome c was analyzed in the cytosolic (A) and mitochondrial (B) fractions of hepatocytes treated with increasing concentrations of CCl<sub>4</sub> for up to 16 h.

increasing concentrations of CCl<sub>4</sub> for 8 h. A dose-dependent increase in caspase 3 activity was observed in the presence of 0.3–3 mmol/L CCl<sub>4</sub>, which decreased slightly at 9 mmol/L CCl<sub>4</sub> (Table 4).

**Table 4.** Dose-course analysis of caspase 3 activation after 8 h treatment of CCl<sub>4</sub>. n=4. Mean±SD. <sup>c</sup>P<0.01 vs control group.

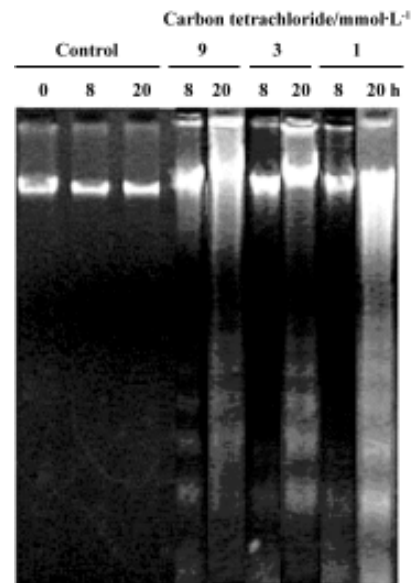
CCl <sub>4</sub> /mmol·L	Caspase 3 activity/fluorescence·h <sup>-1</sup> ·g <sup>-1</sup> protein
0	685±85
0.3	1040±44 <sup>c</sup>
1	1227±55 <sup>c</sup>
3	1442±30 <sup>c</sup>
9	1062±55 <sup>c</sup>

**Bcl-X<sub>L</sub> analysis** Bcl-X<sub>L</sub> protein levels were analyzed in cultured hepatocytes treated with different concentrations of CCl<sub>4</sub>. Hepatocytes were exposed to CCl<sub>4</sub> for 4 h, 8 h and 16 h (Figure 4). No significant dose-dependent change was observed, but a time-dependent decrease in Bcl-X<sub>L</sub> expression was observed.



**Figure 4.** Effect of CCl<sub>4</sub> on Bcl-X<sub>L</sub> expression in rat primary hepatocytes treated with 0.3 mmol/L, 1 mmol/L, 3 mmol/L, and 9 mmol/L CCl<sub>4</sub> for up to 16 h.

**DNA fragmentation** To confirm whether CCl<sub>4</sub> induces DNA fragmentation in rat primary hepatocytes, DNA was extracted from treated cells. No ladder was observed in control cells at 0 h, 8 h, or 20 h of CCl<sub>4</sub> treatment. Apoptosis was initially observed in the presence of 9 mmol/L CCl<sub>4</sub> after 8 h treatment, followed by 3 mmol/L and 1 mmol/L CCl<sub>4</sub> after 20 h treatment (Figure 5).



**Figure 5.** DNA fragmentation in rat primary hepatocytes treated with CCl<sub>4</sub> 1 mmol/L, 3 mmol/L and 9 mmol/L CCl<sub>4</sub> for 8 h and 20 h.

## Discussion

Carbon tetrachloride is a typical hepatotoxin used in liver injury research. Early studies showed that the damage induced by CCl<sub>4</sub> in liver is partly involved in the apoptosis pathway *in vivo*. At least 2 different apoptosis pathways – the mitochondrial pathway and the death-receptor pathway – lead to caspase activation<sup>[17]</sup>. Although past reports have disclosed caspase 3 activation and other histopathological changes in CCl<sub>4</sub>-induced apoptotic hepatocytes<sup>[10,12]</sup>, little is known about the precise molecular mechanisms of apoptosis induction. In this study, we investigated the molecular mechanism of CCl<sub>4</sub>-induced apoptosis in rat primary hepatocytes. Release of cytochrome c first occurred at 4 h and then up to 16 h (Figure 3A). On the other hand, cytochrome c remaining in the mitochondria was found to decrease in a dose- and time-dependent manner (Figure 3B). In addition, it was found that caspase 3 was significantly activated after 8 h at all doses (Table 4). As a result of caspases cascade activation, DNA fragmentation first appeared at 9 mmol/L after 8 h and formed at all concentrations after 20 h (Figure 5). It is well known that cytochrome c released from mitochondria into the cytosol triggers the activation of caspase 9 and caspase 3 in the mitochondrial pathway. In view of this, we propose the involvement of the mitochondrial pathway via cytochrome c release in CCl<sub>4</sub>-induced apoptosis.

In the present study, we investigated the level of Bcl-X<sub>L</sub>, an anti-apoptotic protein, in CCl<sub>4</sub>-induced apoptosis in rat

primary hepatocytes. Bcl-X<sub>L</sub>, the only member of the Bcl-2 family present in hepatocytes, is one of the markers used to identify apoptosis in rat primary hepatocytes<sup>[18]</sup>. Bcl-X<sub>L</sub> exerts an anti-apoptosis function by interacting with cytochrome c, either directly or indirectly via Apaf-1, which binds to both Bcl-X<sub>L</sub> and cytochrome c<sup>[2,4,19,20]</sup>. Therefore, decreased Bcl-X<sub>L</sub> levels and increased cytochrome c release are specific biomarkers for the mitochondrial pathway of apoptosis<sup>[18]</sup>. In our experiments, cytochrome c levels increased markedly after a 8-h CCl<sub>4</sub> treatment with a concomitant decrease in Bcl-X<sub>L</sub> protein expression. Bcl-X<sub>L</sub> may not execute its anti-apoptotic function once large amounts of cytochrome c are released from the mitochondria when the cell is over-exposed to stimuli, for example, lipid peroxidation induced by CCl<sub>4</sub> in this system. These findings strongly suggest the involvement of cytochrome c release from mitochondria accompanied by Bcl-X<sub>L</sub> regulation in CCl<sub>4</sub>-induced apoptosis in rat primary hepatocytes.

Hepatic injury through CCl<sub>4</sub>-induced lipid peroxidation is used extensively in experimental models to elucidate the cellular mechanisms behind oxidative damage<sup>[11,21–23]</sup>. In the present study, we confirm time- and dose-dependent depletion in the intracellular GSH content after CCl<sub>4</sub> treatment. MDA, an important lipid peroxidation product, increased in a time- and dose-dependent manner in our system. The data suggest that oxidative damage is one of the essential mechanisms of hepatotoxicity induced by CCl<sub>4</sub> *in vitro*.

The relationship between ROS and apoptosis has been under investigation for a number of years<sup>[4,9]</sup>. ROS play important roles in apoptosis initiated in mitochondria<sup>[24,25]</sup>. It has been documented that GSH depletion and MDA increase, events observed frequently during oxidative damage, are inducers of mitochondrial permeability transition (MPT)<sup>[11,26]</sup>. Triggering of MPT leads to the release of several different factors relevant to apoptosis, such as cytochrome c, Apoptosis-inducing factor (AIF), and endonuclease G<sup>[2,8,9,27]</sup>. In this study, we demonstrated GSH depletion, MDA increase and apoptosis initiated from mitochondria of CCl<sub>4</sub>-treated rat primary hepatocytes. Our results collectively indicate that CCl<sub>4</sub> induces apoptosis partly via a mitochondria-dependent pathway, dependent on ROS production in rat primary hepatocytes.

To elucidate other possible mechanisms of CCl<sub>4</sub>-induced apoptosis, we analyzed the expression of endonuclease G, a caspase-independent apoptotic protein released from mitochondria that translocates to the nucleus during apoptosis<sup>[28,29]</sup>. Western blot analysis of endonuclease G revealed no evident release in rat primary hepatocytes treated with CCl<sub>4</sub> (data not shown). The finding implies that endonu-

lease G does not participate in apoptosis induced by CCl<sub>4</sub>.

In summary, our results confirm that oxidative damage is one of the essential mechanisms of hepatotoxicity induced by CCl<sub>4</sub>. Moreover, mitochondria-initiated apoptosis triggered by ROS plays an important role in this hepatotoxicity in rat primary hepatocytes.

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