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ORIGINAL PAPER

Involvement of JNK regulation in oxidative stress-mediated murine liver injury by microcystin-LR

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Abstract Microcystin-LR (MC-LR) produced by cyanobacteria in diverse water systems is a potent specific hepatotoxin and has been documented to induce various liver diseases via oxidative stress. However, the underlying mechanisms are largely unknown. In the current study, we investigated the molecular events involved in the oxidative liver injury by MC-LR. Our results demonstrated that MC-LR induced liver injury in mice through a series of steps that began with the production of reactive oxygen species (ROS), which stimulated the sustained activation of JNK and its downstream targets, AP-1 and Bid. Furthermore, the mitochondrial proteomic analysis indicated that JNK activation affected some crucial enzymes of energy metabolism, led to mitochondria dysfunction, which contributed to hepatocyte apoptosis and oxidative liver injury by MC-LR. Our results reveal significant insights into the mechanisms of liver injury induced by microcystins, and serve as a framework for deciphering the role of JNK in oxidative stress-associated liver diseases.

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Introduction

Microcystin-LR (MC-LR), a potent environmental hepatotoxin produced by blue-green algae in eutrophic surface waters, has received increasing worldwide attention in the recent decades [1–3]. There is considerable evidence that the presence of microcystins (MCs) in drinking water is related to the pathogenesis of various liver diseases, including primary liver cancer [4], hepatitis [5], and liver injury [6]. Our previous studies [7, 8], as well as earlier studies by Ding et al. [9], find that MC-LR can produce a large amount of reactive oxygen species (ROS) in primary hepatocytes and liver tissue, suggesting a critical role of oxidative stress in MC-LR-induced hepatotoxicity. The underlying mechanisms, however, are largely uncertain. The involvement of oxidative stress has also been suggested in the pathophysiology of many liver diseases, ranging from viral hepatitis [10, 11], alcoholic-induced liver disease (ALD) [12], and nonalcoholic fatty liver disease [13] to cholestasis [14] and liver injury induced by chemical toxins [15].

It is well-established that oxidative stress can activate the c-Jun N-terminal protein kinase (JNK) pathway in the context of various stimuli or diseases [16, 17]. JNK has also been reported to be involved in liver damage, such as cold ischemia/warm reperfusion injury in liver transplantation, bile acid-induced hepatocyte death, nonalcoholic fatty liver disease, etc. [18]. The combined reports suggest that JNK may mediate the oxidative liver injury induced by different stimulus. As an important regulator in the cellular response to a variety of exogenous and endogenous stresses, JNK acts on many downstream targets. JNK may also

mediate some molecular events through various substrates, separately or synergistically. Moreover, there is considerable evidence that JNK activation may act through downstream substrates, such as AP-1 [18, 19] and Bcl-2 family molecules [20, 21], to induce apoptosis, especially via the mitochondria-dependent apoptotic pathway [22].

In the present study, we investigated in detail the molecular events mediating liver injury induced by MC-LR exposure. Our data showed that MC-LR leads to hepatocyte apoptosis and liver injury through a series of steps that began with the generation of an oxidative stress state, which prompted the sustained activation of JNK. Activated JNK, in turn, activated transcription factor AP-1 and the Bcl-2 family member Bid, which was subsequently accompanied by mitochondria dysfunction, as revealed by the proteomic analysis. Pretreatment with JNK inhibitor, sp600125, effectively attenuated the hepatocyte apoptosis and liver injury by inhibiting JNK activation. These findings shed some light on the mechanisms of the oxidative liver injury induced by MC-LR, thereby contributing to a better understanding of the hepatotoxicity of MCs and the potential mechanisms of oxidative stress-associated liver diseases.

Materials and methods

Reagents

MC-LR Standard was purchased from Alexis Inc. (Carlsbad, CA). Unless indicated otherwise, other reagents were obtained from Sigma Chemical Inc. (St. Louis, MO).

Animals and treatments

Male 6-week-old ICR mice were obtained from The Animal Center of Nanjing Medical University, China. On arrival, all mice had free access to food and water with 12-h light cycle. After 5-day quarantine, mice were randomized into groups. Some were injected intraperitoneally with MC-LR (55 μg/kg of body weight) or saline (control). After MC-LR treatment, mice were sacrificed at 0.5, 2, 6 and 12 h, respectively. Some received drinking water that contained 15 mg/ml *N*-acetyl-cysteine (NAC) for 48 h prior to MC-LR exposure and sacrificed 2, 6 or 12 h later. Some were pretreated with sp600125 (30 mg/kg of body weight, i.p.) 1.5 h prior to MC-LR exposure. Animal welfare and experimental procedures were carried out in strict accordance with the ''Guide for the Care and Use of Laboratory Animals'' (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university.

Serum enzyme activities

Serum activities of alanine transaminase (ALT) and aspatate transaminase (AST) were measured using a test kit (Catachem, Bridgeport, CT).

DNA fragmentation assay

DNA was extracted from approximately 80 mg of fresh liver tissue by Ting Chen's method [7] and then analyzed electrophoretically on 1% agarose gels containing 0.1 mg/ml ethidium bromide. Gels were visualized under UV illumination.

Histology and immunohistochemistry

Liver tissues were fixed in 4% phosphate-buffered formalin and later embedded in paraffin. For histological analysis, sections were stained with hematoxylin–eosin (H&E). For immunohistochemistry, dewaxed sections were boiled in 10 mM citrate (pH 6.0) in a pressure cooker to retrieve antigen. After cooling, sections were incubated in 3% H_2O_2 followed by goat serum to block nonspecific binding, and incubated with anti-pJNK (1:50, Santa Cruz) at 4°C overnight. After rinsing with TBST (Tris-Buffered Saline with 0.1% Tween-20), the sections were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories Inc.) for 15 min at room temperature. Finally, the sections were developed by diaminobenzidine (DAB), counterstained with hematoxylin.

MDA analysis

Lipid peroxidation of liver tissue was determined using the malondialdehyde (MDA) assay kit (Catachem, Bridgeport, CT), according to the kit protocol. Lipid peroxidation was calculated as nanomoles of MDA per milligram of protein.

Detection of ROS generation

The production of ROS was determined by detecting the fluorescent intensity of oxidant-sensitive probe dihydrorhodamine 123 (DHR, Molecular Probes) as previously described [8].

Western blot analysis

Tissue samples were homogenized in ice-cold lysis buffer (Beyotime, China) with 1% PMSF (Phenylmethylsulfonyl fluoride). Samples were centrifuged at 10,000*g* for 20 min at 4°C and the protein concentration of the resulting supernatant was determined by the BCA (bicinchoninic acid) protein assay kit (Beyotime, China). Proteins (30 μg)

were separated by SDS-PAGE electrophoresis and subsequently transferred to PVDF membranes. Membranes were blocked with 5% nonfat dry milk in incubation buffer and incubated with antibodies against mouse JNK, ERK, pERK, pP38 (Kangchen, China), Bid (BD Biosciences Pharmingen), p38, caspase 3, caspase 9 (Cell Signaling Technology, Beverly, MA), caspase 8 p20 and pJNK (Santa Cruz). Bound antibody was detected with peroxidase-linked secondary antibody and a chemiluminescence detection system. Loading accuracy was evaluated by membrane rehybridization with monoclonal antibodies against GAPDH (Kangchen, China).

Electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts was carried out as previously described [23]. Two double-stranded oligonucleotide probes containing a consensus binding sequence for AP-1 (5′-CGC TTG ATG AGT CAG CCG GAA-3′) were 3′ end-labeled with biotin. Binding activity of AP-1 to the probe was determined using a chemiluminescent EMSA kit (Beyotime, China), according to the manufacturer's protocol. Specificity of the DNA-protein complex was confirmed by competition with a 100-fold molar excess of unlabeled double stranded oligonucleotide added to the mixture.

Identification of mitochondrial proteins by mass spectrometry

Liver mitochondria were prepared according to the method previously described [24, 25]. The purity of the isolated mitochondrial proteins was analyzed by western blot using the β-actin antibody (Kangchen, China), which results verified the mitochondrial proteins were not contaminated by the cytosolic proteins (data not shown). Then the isolated mitochondrial proteins were resolved by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), stained with Coomassie Brilliant Blue R-250, and scanned on a Sharp JX-330 color image scanner. Spot detection, quantification, and matching were performed with PDQuest software (Bio-Rad). Then the protein gel spots were in-gel tryptic digested and analyzed using the Ultraflex TOF/TOF mass spectrometer system (Bruker) as previously described [7]. Finally, monoisotopic masses of peptides were bioinformatically analyzed using the Mascot search engine.

Statistical analysis

Data were expressed as mean ± SD. The data were statistically evaluated by a Student's t test when only two value sets were compared, and by a two-way ANOVA followed by Dunnett's t-test when the data involved three or more groups. Significance was declared when $P < 0.05$.

Results

ROS production in murine liver treated by MC-LR

To examine the kinetics of ROS generation induced by MC-LR in murine liver, we performed a time-course study. After injected a single intraperitoneal dose of MC-LR (55 μg/kg of body weight), the mice were sacrificed at 0.5, 2, 6 and 12 h, respectively. ROS generation in liver tissues was detected through the ROS-sensitive probe dihydrorhodamine 123 (DHR 123). As shown in Fig. 1a, the intensity of rhodamine 123 fluorescence was significantly enhanced from 0.5 to 12 h. MC-LR treatment initiated ROS production at 0.5 h ($P < 0.05$) and the ROS continued to accumulated until at least 12 h ($P < 0.01$) in a time-dependent manner. At the meantime, the level of MDA was assayed to detect the lipid peroxidation level in the liver tissue. Similarly, the results of MDA assay (Fig. 1b) showed that the MDA level significantly increased 2 h after the injection of MC-LR ($P < 0.05$). Moreover, the formation of MDA continued to increase for the duration of the study (12 h). In aggregate, these data strongly indicated that ROS production by MC-LR in murine liver rose promptly upon administration of the toxic and accumulated thereafter.

Sustained JNK activation induced by MC-LR

Recent evidence indicates that ROS is a key mediator in activating the members of the MAPK pathways, which contribute to the progression of various oxidative liver diseases. We therefore examined the impact of MC-LR on JNK, extracellular signal-regulated kinase (ERK) and p38. Mice were treated with MC-LR (55 μg/kg of body weight, i.p.) for the indicated times and liver protein extracts were subjected to Western blot analysis (Fig. 2a–c). JNK phosphorylation increased 2 h after exposure to MC-LR (Fig. 2a, top), and this phosphorylation was sustained for at least 12 h. The levels of ERK and p38 phosphorylation, however, remained unchanged (Fig. 2b and c). All blots were stripped and reprobed with the respective Abs recognizing JNK, ERK and p38 proteins, demonstrating no changes in total MAPK expression.

We performed immunohistochemistry analysis to confirm the result obtained from Western blot. As shown in Fig. 2d, JNK was activated by 2 h after MC-LR exposure, and this activation continued increasing for at least 12 h. The corresponding results from immunohistochemistry study and Western blot strengthen the conclusion that

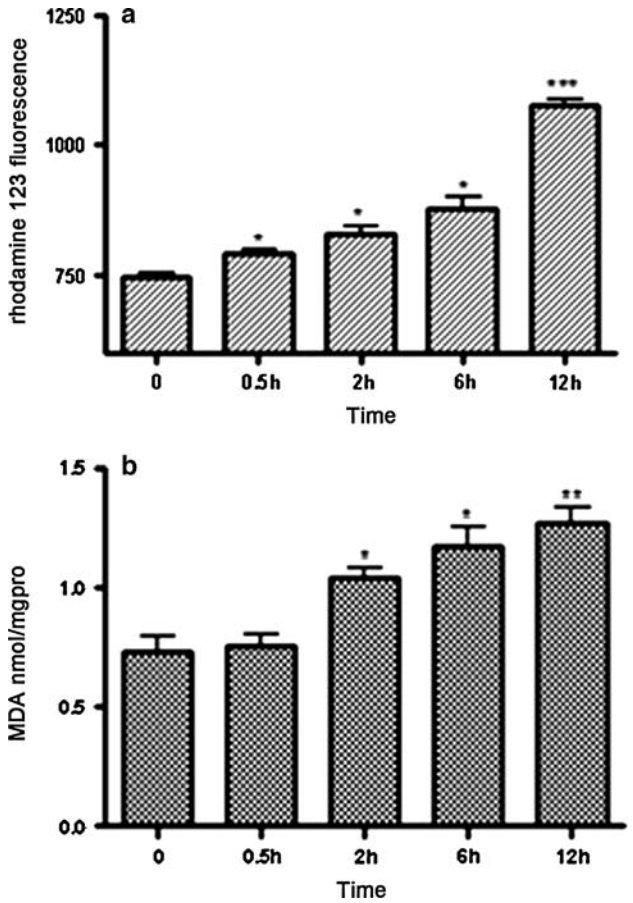

Fig. 1 Microcystin-LR (MC-LR) induced a time-dependent increase in ROS production and lipid peroxidation. Mice were treated with MC-LR (55 μg/kg of body weight, i.p.) for the indicated time. (**a**) The ROS production in liver tissue was determined by measuring the changes in the fluorescence using dihydrorhodamine 123 (DHR 123). MC-LR treatment initiated ROS production at 0.5 h and the ROS continued to accumulate until at least 12 h in a time-dependent manner. (**b**) The lipid peroxidation level of liver tissue was measured by MDA assay, which showed that the MDA level significantly increased 2 h after the injection of MC-LR and the formation of MDA continued to increase for the duration of the study (12 h), and TBARS are expressed in nmol/mg protein. Data were shown as mean ± SEM of three independent experiments. $*P < 0.05$ versus control group, $**P < 0.01$ versus control group, $***P < 0.01$ versus control group (Student's *t* test)

MC-LR induced a prolonged activation of JNK, which might be involved in the oxidative liver injury.

JNK activation by MC-LR is sensitive to antioxidant

To further evaluate whether there was a relationship between ROS production and JNK activation in MC-LR toxicity, we pretreated the mice with the NAC, an ROS inhibitor. Treatment of mice with NAC (in the presence of 15 mg/ml NAC in the drinking water) for 48 h followed by MC-LR injection resulted in the reduction of both ROS generation and MDA levels as compared to mice treated only with MC-LR (Fig. 3a and b). Western blot analysis revealed that phosphorylation of JNK was significantly reduced by pretreatment with NAC (Fig. 3c), suggesting that JNK activation by MC-LR was sensitive to cellular oxidative status.

Accordingly, we used an immunohistochemical method to detect the expression of phosphorylated JNK in the liver of mice treated with MC-LR with or without NAC (Figs. 2d and 3d). Showed in this analysis, liver tissue pretreated with NAC displayed attenuated p-JNK positive staining, indicating that the addition of ROS inhibitor almost completely reversed the activation of JNK brought about by MC-LR, thereby confirming the Western blot results. Together, these observations demonstrated that MC-LR mediated activation of JNK machinery via ROS production.

JNK plays a key role in oxidative liver injury induced by MC-LR

Several models of toxic liver injury, including those for TNF-α, GalN/LPS, CCl_4, have demonstrated the activation of JNK [26–28]. To investigate whether JNK activation was also required for the progression of MC-LR-induced oxidative liver injury, we treated mice with the specific JNK inhibitor sp600125 [29] 1.5 h before MC-LR injection. The results from the Western blot and immunohistochemistry analysis all showed that sp600125 pretreatment indeed inhibits JNK activation by MC-LR (Fig. 3c and d). Histological examination of the liver tissues (Fig. 4a) showed that liver from the mice sacrificed 6 and 12 h after receiving MC-LR injection exhibited severe damage, intrahepatic hemorrhage and destruction of hepatic structure. In marked contrast, the liver tissue from mice pretreated with NAC or sp600125 revealed a grossly intact architecture with minimal areas of tissue damage (Fig. 4b). Consistent with this observation, the serum ALT values of the group pretreated with sp600125 were reduced by 46% as compared with the group treated by MC-LR alone. Moreover, AST levels were reduced by 41% in the sp600125 group, suggesting that pretreatment with sp600125 significantly inhibits the increase of ALT and AST induced by MC-LR (Fig. 4c). Collectively, the significant cytoprotective effect of sp600125 in vivo demonstrated that JNK activation was of great importance to the oxidative liver injury induced by MC-LR.

AP-1 and Bid downstream of JNK activation

We assessed AP-1 DNA-binding activity after treatment with MC-LR. Nuclear extracts from the mice liver were used for EMSA. Results showed that MC-LR alone induced a time-dependent increase in AP-1 binding

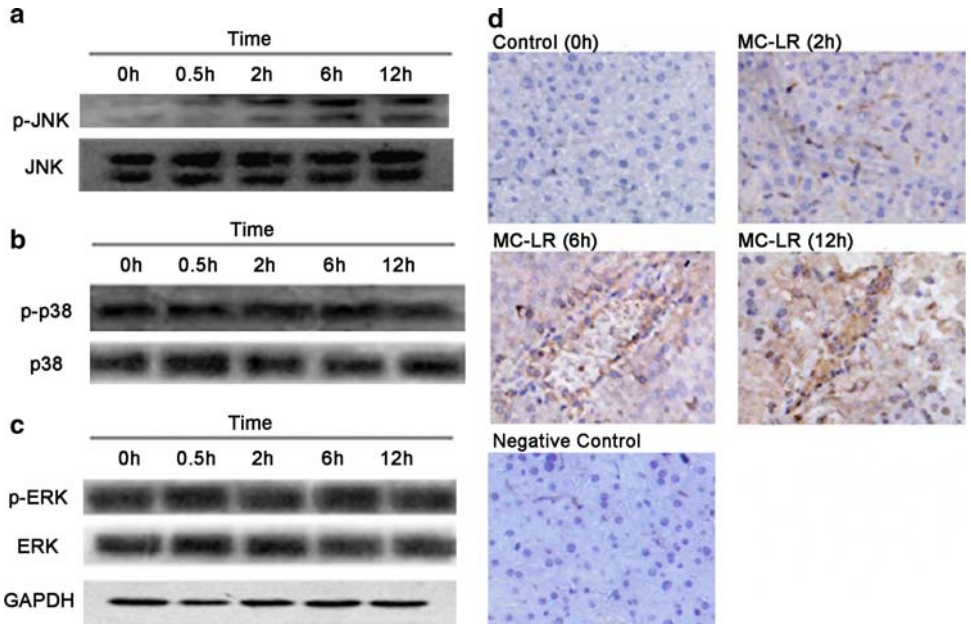

Fig. 2 Sustained JNK activation induced by MC-LR. The mice received MC-LR treatment (55 μg/kg of body weight, i.p.) for the number of hours indicated. Protein was isolated from liver tissue and aliquots of protein were immunoblotted with antibodies against (**a**) phosphorylated (p-JNK) or total c-Jun N-terminal protein kinase (JNK) or (**b**) phosphorylated (p-p38) or total p38 or (**c**) phosphorylated (p-ERK) or total extracellular signal-regulated kinase (ERK). MC-LR caused sustained activation of JNK, but had no effect on ERK and p38. (**d**) Detection of JNK activation in liver section of mice by immunohistochemistry using antibody against p-JNK. It was showed that JNK was activated by 2 h after MC-LR exposure, and this activation continued increasing for at least 12 h. The negative control was a control that showed the immunostaining with the non-immune IgG

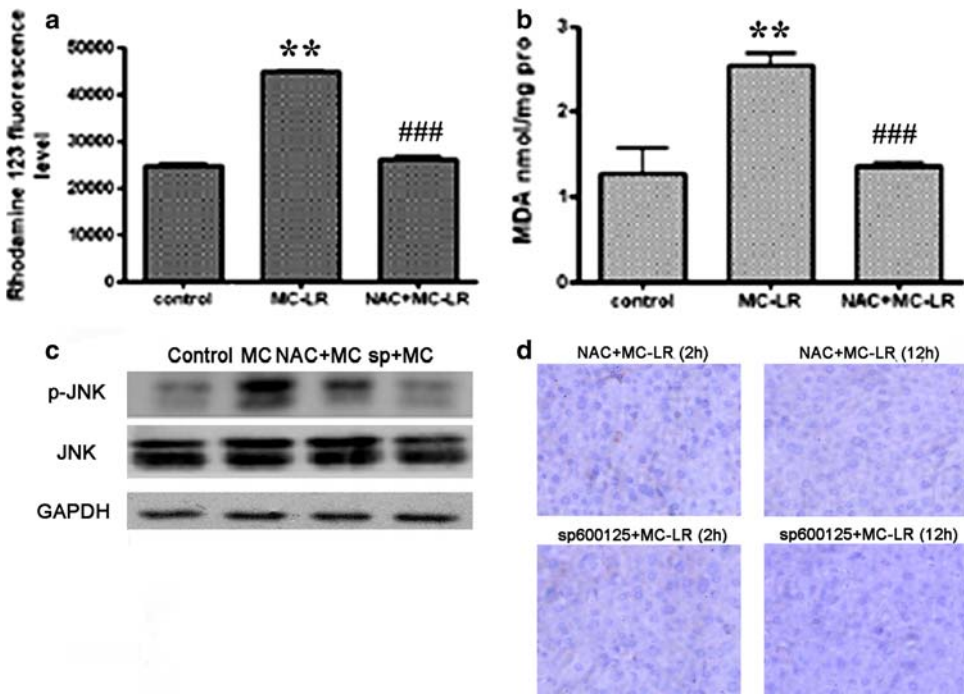

Fig. 3 JNK activation by MC-LR was blocked by the pretreatment with NAC or sp600125. (**a**) The ROS production by MC-LR was significantly decreased by *N*-acetyl cysteine (NAC) pretreatment. $^{***}P < 0.01$ versus control group, $^{\#\#\#}P < 0.01$ versus MC-LR treated only group. (**b**) The lipid peroxidation induced by MC-LR was attenuated by NAC pretreatment. $^{**}P < 0.01$ versus control group, $^{\#\#\#}P < 0.01$ versus MC-LR treated only group. The western blot (**c**) and immunohistochemistry study (**d**) were probed with anti-p-JNK antibodies. As shown, JNK activation by MC-LR was inhibited effectively by NAC or sp600125 pretreatment. Except the group of control, the mice were sacrificed after treated with MC-LR for 6 h (**a**–**c**), or 2 and 12 h (**d**)

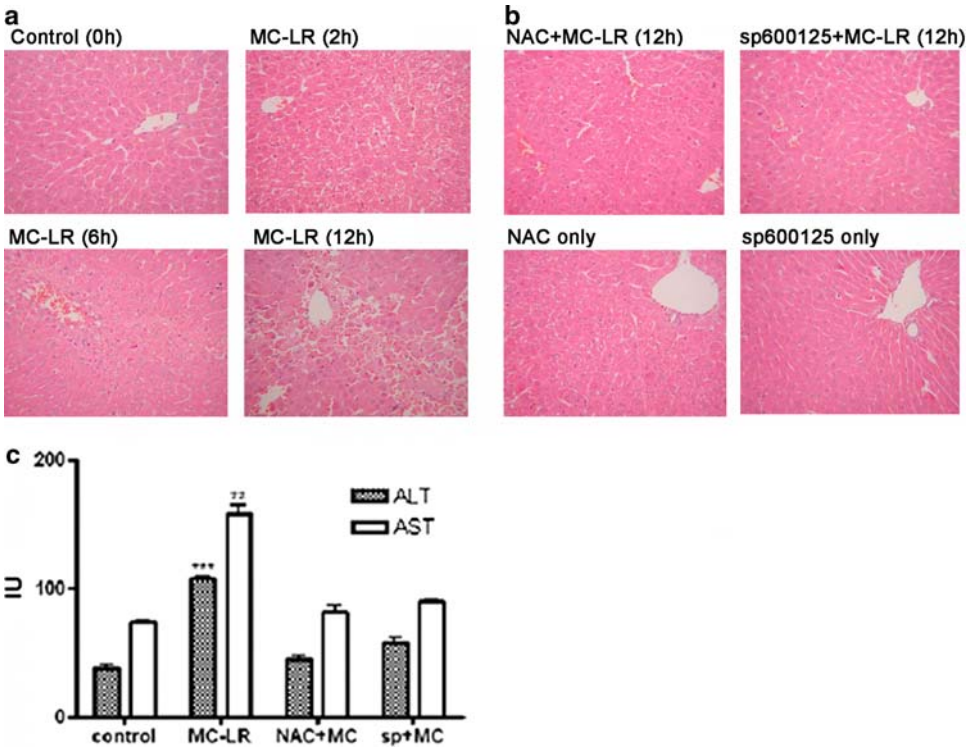

Fig. 4 The oxidative liver injury induced by MC-LR was detected. (**a**) Representative liver sections of mice treated with intraperitoneal MC-LR (55 μg/kg) only for 0, 0.5, 2, 6, 12 h. The liver from the mice sacrificed 6 and 12 h after receiving MC-LR exhibited severe liver damage, intrahepatic hemorrhage and destruction of liver structure. H&E staining, original magnification objective 200×. (**b**) NAC or sp600125 pretreatment revealed a grossly intact architecture with minimal areas of tissue damage. H&E staining, original magnification objective 200×. (**c**) The serum ALT and AST change in mice received the treatment as indicated. The serum ALT values of the group pretreated with sp600125 were reduced by 46% as compared with the group treated by MC-LR alone. Moreover, AST levels were reduced by 41% in the sp600125 group, suggesting that pretreatment with sp600125 significantly inhibits the increase of ALT and AST induced by MC-LR. Except the group of control and NAC or sp600125 only, the mice were sacrificed after treated with MC-LR for 12 h (**b** and **c**)

(Fig. 5a), and this increase initiated at 6 h after MC-LR injection (Fig. 5a, fifth lane). Pretreatment with sp600125 inhibited the binding activity of AP-1 induced by MC-LR (Fig. 5b). We examined Bid expression after MC-LR treatment using Western blot analysis and found that Bid was activated in mice subjected to MC-LR for 6 h in a time-dependent pattern (Fig. 5c). When JNK was inhibited by sp600125, activated Bid markedly decreased, as compared with the group treated with MC-LR alone (Fig. 5d). We also found that there was no clearage of caspase 8 (Fig. 6a), so we eliminate the effect of caspase 8 involved in the activation of Bid. Taken together, these data suggested that under the present conditions, activated JNK acted on AP-1 and Bid.

Activation of caspase 3 and caspase 9, hepatocyte apoptosis induced by MC-LR

Our previous work [7, 8] in conjunction with other studies [9], has characterized the role of hepatocyte apoptosis in MC-LR-induced liver injury. Recently, there is a confirmatory report showed MC-LR could induce cell apoptosis in engineered cell lines [30]. Other evidence has also been reported that sustained JNK activation leads to mitochondria-mediated apoptosis under various conditions [18, 31]. Therefore, it is possible that JNK activated by MC-LR leads to hepatocyte apoptosis mediated by mitochondria and eventually liver injury. To test this hypothesis, we first examined the expression of caspase 8, which clearage is the most important marker of death receptor pathway. It was confirmed that there was no cleaved caspase 8 (Fig. 6a). Then caspase 9 and caspase 3, two important regulatory caspases in the intrinsic apoptotic pathway, were assessed by Western blot. As shown in the illustrations, cleaved caspase 9 (Fig. 6b) and cleaved caspase 3 (Fig. 6c) were both markedly increased by MC-LR, both of which increases were inhibited by pretreatment with sp600125. We next employed a DNA fragmentation assay to detect apoptosis in murine liver. Figure 6d showed that a ladder pattern of internucleosomal fragmentation of DNA was apparent at 6 h after exposure to MC-LR, while the DNA from the mice which had received sp600125

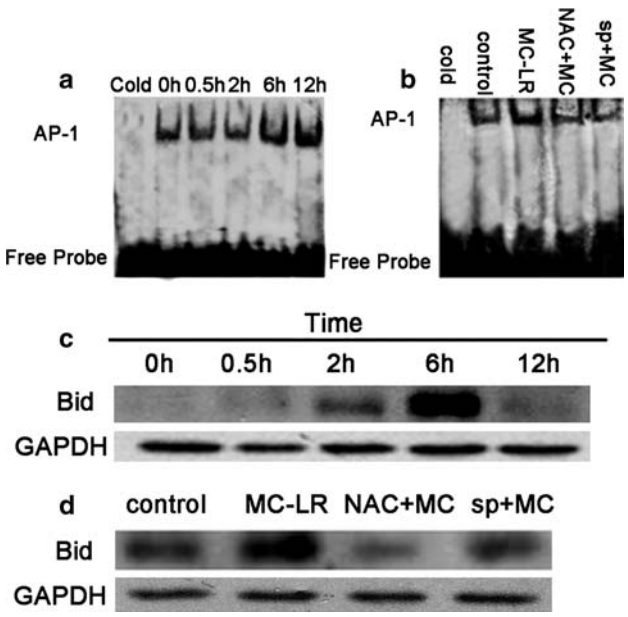

Fig. 5 Detection of the downstream targets of JNK activation. Detection of AP-1 DNA binding activity by electrophoretic mobility shift assay (EMSA). (**a**) MC-LR treatment (55 μg/kg of body weight, i.p.) enhanced AP-1 DNA binding in a time-dependent manner. (**b**) AP-1 DNA binding activity was decreased by pretreatment with sp600125. Except the group of control, the mice were sacrificed after treated with MC-LR for 6 h. Detection of the activation of Bid by Western blot. (**c**) MC-LR treatment (55 μg/kg of body weight, i.p.) enhanced Bid expression in a time-dependent manner. (**d**) The expression of Bid was decreased by pretreatment with NAC and sp600125. Except the group of control, the mice were sacrificed after treated with MC-LR for 6 h

pretreatment showed no sign of internucleosomal fragmentation. Altogether, these results indicated that JNK activation regulated the hepatocyte apoptosis by the intrinsic apoptotic pathway in MC-LR-induced liver injury.

Activated JNK by MC-LR affected mitochondria energy metabolism

Mitochondrial functions are dependent on the maintenance of mitochondrial membrane potential (MMP) [32]. Disruption of MMP always indicates mitochondrial dysfunction. We previously reported that MC-LR caused MMP loss, led to the occurrence of mitochondrial permeability transition (MPT) and mitochondrial dysfunction, and finally led to hepatocyte apoptosis [8]. Furthermore, both Bid and AP-1 have been reported to act as links between JNK activation and mitochondria-dependent apoptosis [21]. To investigate whether JNK activated by MC-LR initiated the apoptosis machinery by influencing mitochondrial function, we isolated and compared the liver mitochondrial proteins of the control, MC-LR-only, and sp600125/MC-LR-treated groups using 2-DE and mass spectrometry. Three gels per sample were processed simultaneously. Figure 7a shows three representative 2-D gel images of the three groups. Reproducible protein expression profiles were found on gels (pH 3–10) following Coomassie Blue R-250 staining. The matching analysis of paired gels was done in automatic mode and further manual editing was performed to correct the mismatched and unmatched spots. The protein spots which were found to be significantly changed, defined as having increased or decreased more than two times in either comparable group than the control, were then cut out of the 2-D gels, trypsin digested and measured using a MALDI-TOF mass spectrometer. The identified proteins included ATP synthase subunit beta (ATPB, the catalytic subunit of ATP synthase, which produces ATP from ADP in the presence of a proton gradient across the membrane); Malate dehydrogenase (MDH, an enzyme in the citric acid cycle that catalyzes the conversion of malate into oxaloacetate (using NAD+) and

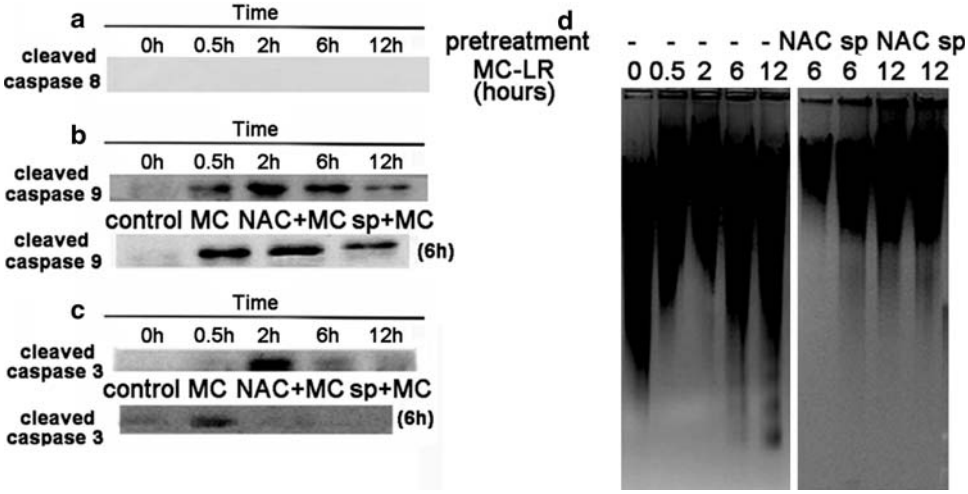

Fig. 6 MC-LR caused the mitochondria-dependent apoptosis of hepatocytes, and pretreatment with NAC, or sp600125 significantly attenuated. The cleaved fragments of caspase 8 (**a**), caspase 9 (**b**) and caspase 3 (**c**) were analyzed. There was no cleaved caspase 8, but cleaved caspase 9 and cleaved caspase 3 were both markedly increased by MC-LR, both of which increases were inhibited by pretreatment with sp600125. (**d**) Occurrence of hepatocyte apoptosis was also examined by DNA fragmentation assay.

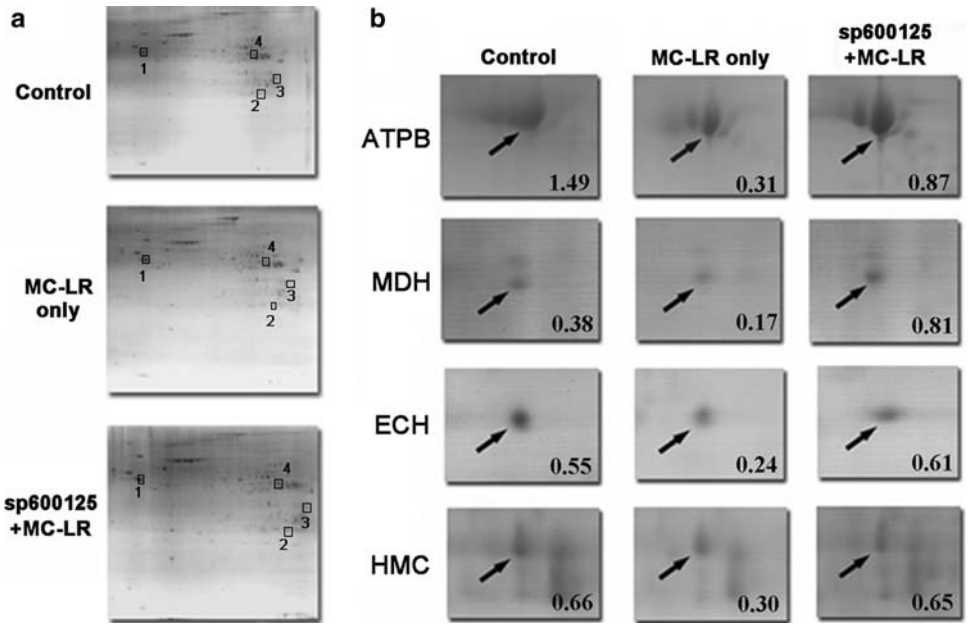

Fig. 7 Activated JNK by MC-LR interfered with mitochondrial energy metabolism. Images of two-dimensional (2-D) gel stained with Coomassie Blue R-250 are given. The liver mitochondria were isolated from three distinct groups: control (NS), MC-LR only (55 μg/kg of body weight, i.p., 6 h exposure), and sp600125 plus MC-LR (pretreatment with sp600125, 30 mg/kg of body weight, i.p., 1.5 h prior to the 6 h' exposure to MC-LR) and then lysed in 1%SDS buffer, assayed for protein concentration. 1 mg of total protein was prepared for two-dimensional gel separation. (**a**) 2-D SDS-PAGE gels (12.5% Tris–HCl) show the entire pI range 3–10 and molecular masses of 10–100 kDa. The boxes enclose are the positions of the four proteins of each group. (1) ATPB, (2) MDH, (3) ECH, (4) HMC. (**b**) Differential protein expression analysis with zoomed images of selected gel areas, where the value of gray scale analyzed by Imagemaster 2D Plantinum were indicated. The identified proteins including ATP synthase subunit beta (ATPB), Malate dehydrogenase (MDH), Enoyl-CoA hydratase (ECH), and Hydroxymethylglutaryl-CoA synthase (HMC) were down-regulated by MC-LR, but sp600125, significantly reversed the expression levels of these proteins

vice versa); Enoyl-CoA hydratase (ECH, the enzyme used to catalyze the second step of β-oxidation in Fatty acid metabolism); and Hydroxymethylglutaryl-CoA synthase (HMC, the enzyme condensed acetyl-CoA with acetoacetyl-CoA to form HMG-CoA) (Fig. 7b). All these proteins were down-regulated in response to exposure to MC-LR alone. Of note, these four identified proteins are all involved in the process of mitochondrial energy metabolism. However, pretreatment with the JNK inhibitor, sp600125, significantly reversed the expression levels of these proteins, as shown in Fig. 7b, suggesting that JNK activated by MC-LR at least partially mediated the molecular events which finally led to liver injury, by acting on mitochondria and interfering with mitochondrial energy metabolism.

Discussion

In recent years, a growing number of human poisonings and illnesses caused by MCs have been reported. In 1996, 60 patients in Brazil died because of the water used for their dialysis contaminated with MCs [33]. Epidemiological studies by Yu et al. reported that the high incidence of primary liver cancer in some regions of China was related to the presence of MCs in drinking water [34, 35]. Thus, the liver diseases induced by MCs have garnered increasing attention from the WHO as well as related scientific communities.

It was first proposed by Ding et al. that MC-LR can induce the production of large amount of ROS in primary hepatocytes [9]. And then they demonstrated that an early increase of mitochondrial Ca^{2+} by MC-LR could have led to the uncoupling of mitochondrial electron transport and may favor the mitochondrial ROS formation as the upstream events in MC-LR-induced hepatocytes apoptosis [36, 37]. As shown in Fig. 1, our results as exhibited by time-course study confirmed that MC-LR induced ROS generation and accumulation in murine liver after exposure. Moreover, it has also been reviewed that ROS formation may be both a cause and a result of onset of MPT, and a possible amplification loop may exist between ROS formation and MPT, in which ROS formation led to MPT and this further led to more ROS formation [38]. The accumulation of ROS in our model in the later periods was possibly according to this mechanism, as we had previously found the occurrence of MPT induced by MC-LR [8]. Consequently, the above results were consistent with

MC-LR's time-dependent destructive effect on murine liver tissue. The appearance of liver tissue examined in this study revealed swelling and congestion from 2 h until 12 h after exposure (data not shown). Moreover, the section of liver stained by H&E showed hepatic hemorrhage with a complete disruption of the lobular and sinusoidal liver architecture (Fig. 4a). ALT and AST assays also confirmed these results (Fig. 4c, second column). Taken together, these are strong evidences that MC-LR destroyed the redox balance by generating large amounts of ROS, resulting in severe liver insult.

Other studies have reported that JNK is involved in various oxidative stress-associated liver diseases [16, 18], and ROS generation could make the sustained JNK activation [39]. We therefore tested whether JNK was activated under the conditions of our study and whether it played a role in the oxidative liver injury caused by MC-LR. Both of the Western blot assay and immunohistochemistry analysis in situ indicated that JNK is significantly activated by 2 h after MC-LR treatment (Fig. 2), by which time there was already substantial ROS accumulation. In addition, when ROS production was diminished by pretreatment with the antioxidant NAC, JNK activation was reduced accordingly by a significant extent (Fig. 3). There was no significant change of the ROS generation when the activation of JNK was inhibited by sp600125 (data not shown). These findings indicated that the sustained activation of JNK was mediated by ROS under the conditions of our study.

We hypothesized that the sustained JNK activation might be involved in the MC-LR-induced liver injury. To test this hypothesis, we further utilized the specific JNK inhibitor sp600125 to inhibit the activation of JNK by MC-LR. The results from ALT/AST assays as well as the histochemistry analysis (Fig. 4) revealed that pretreatment with sp600125 effectively attenuated the liver injury induced by MC-LR, suggesting that JNK activation was indeed involved in MC-LR-induced liver damage. Taken together, these findings established a regulatory role for JNK in the pathology of MC-LR-induced oxidative liver injury.

Transcription factor AP-1 and the Bcl-2 family member Bid are two important downstream substrates of JNK. Activated JNK can phosphorylate the DNA binding protein c-Jun and increase AP-1 (the heterodimer of c-Jun and c-Fos) transcriptional activity. We first explored the effects of JNK activation by probing AP-1 DNA-binding activity. The results of EMSA indicated that treatment with MC-LR for 6 h increased AP-1 binding. Additionally, pretreatment with sp600125 inhibited the binding of AP-1 by a significant extent (Fig. 5a and b). In the other hand, sustained activated JNK can trigger Bid truncation and promote the Bid translocation to mitochondria and its activation [21]. However, there are some other evidences that both truncated Bid and full-length Bid have the capacity to translocate to mitochondria during apoptosis [40, 41]. In other words, if the cells or the stimulators are different, the activation of Bid would be different. For these reasons, we further analyzed the total expressions of full-length Bid. The results indicated that JNK activation was associated with a marked increase in Bid at 6 h (Fig. 5c and d). However, the expression of full-length Bid disappeared at 12 h after MC-LR exposure. It was possible that, in our model, JNK triggered the related translocation and activation of full-length Bid in early stage, and latter induced Bid truncation. The underlie mechanisms of the Bid activation by JNK were uncertain in our model, which we would make clear in our future works. Besides AP-1 and Bid, we also analyzed phosphorylation of Bcl-2 and Bcl-xL, which are the targets of JNK as reported and relate to mitochondria dependent apoptosis. Our results showed that there was no significant diversity after sp600125 was used in our model (data not show).

AP-1 is the principal substrate among the transcription factors downstream of JNK. Previous studies have suggested that JNK/AP-1 mediates a caspase-dependent apoptosis through the activation of the mitochondrial death pathway [19, 22]. An apoptosis defect was also observed in fibroblasts derived from mice with a mutation in the c-Jun gene [42]. The pro-apoptotic action of AP-1 may be correlated with the increased expression of some BH-3 only Bcl-2 family members, such as Bim and DP5/Hrk [43–45], which induces apoptosis, since it antagonizes the functions of both Bcl-2 and Bcl-XL and concomitantly causes activation of Bax and dissipation of $\Delta\psi_m$ [46]. In addition, it was reported that activated Bid translocate to mitochondria, leading to the induction of apoptosis [21, 40, 41]. Importantly, it has long been recognized that mitochondria, which play a critical role in apoptosis, are an important target for the damaging effects of oxidative stress. Combining these reports, it is conceivable that the activation of AP-1 and Bid might prompt oxidative liver injury by acting on mitochondria and leading to apoptosis. Therefore, an important question we considered was if activated JNK stimulates the induction of apoptosis. Thus, we examined whether there existed any relationship between apoptosis and sustained JNK activation in our model. The results demonstrated that MC-LR treatment activated caspase 9 and caspase 3, rather than caspase 8 (Fig. 6). Pretreatment with sp600125 decreased the activation of caspase 9 and caspase 3 by MC-LR, suggesting that activated JNK has a connection with the induction of apoptosis. The results from DNA fragmentation assay revealed that MC-LR treatment alone induced the apparent DNA fragmentation pattern, while the DNA extracted from sp600125-pretreated mice did not show any obvious DNA

fragmentation, confirming that apoptosis was regulated by activated JNK.

The JNK substrates AP-1 and Bid have been reported to be involved in apoptosis via effects on mitochondria, leading to the mitochondrial membrane loss, mitochondrial dysfunction, and eventually cell death [19, 21, 22]. Interestingly, we not only found that JNK activated AP-1 and Bid in our study, but our proteomic data (Fig. 7) also revealed that the activation of JNK changed the expression pattern of some crucial energy metabolism-related enzymes. The proteome studies revealed that four mitochondrial proteins, ATPB, MDH, ECH and HMC, were all down-regulated in the group treated with MC-LR alone. Among the four mitochondrial proteins, ATPB is a pivotal element in energy metabolism that catalyzes the synthesis of ATP using the proton force across the inner mitochondrial membrane [47]. MDH catalyses the conversation of malate and NAD+ to oxaloacetate and NADH, and the generated NADH is directly available to the respiratory chain [48]. And the release of MDH from the mitochondrial matrix is one of the biochemical manifestations of the MPT [49]. ECH is involved in the oxidation of fatty acids and mitochondrial fatty acid synthesis [50]. HMC is a key enzyme in steroid biosynthesis through catalyzing the synthesis of hydroxynmethylglutaryl-CoA from acyl-CoA and acetoacetyl-CoA [51]. All four of these proteins are involved in the electron transport chain and mitochondrial energy metabolism, suggesting that MC-LR disturbs mitochondrial energy metabolism, leading to mitochondrial dysfunction, which could also be validated by the loss of mitochondrial membrane potential as mentioned in our previous report [8]. Notably, these four proteins all returned to normal levels in the mitochondria from mice in the group treated with both sp600125 and MC-LR. Considering the protective effects of sp600125 on MC-LR-induced liver injury, these proteomic data suggested that activated JNK acted on mitochondria, inducing mitochondrial dysfunction, which has long been recognized to contribute to the apoptosis.

In conclusion (Fig. 8), the results presented in this work suggested that MC-LR triggered liver injury by initiating ROS accumulation and causing oxidative stress. The oxidative stress then stimulated sustained activation of JNK, which in turn induced apoptosis via AP-1 and Bid, two important substrates downstream of JNK. Of note, our proteomic analysis identified the mitochondria as a sensitive target of JNK, the activation of which led to the disturbance of mitochondrial energy metabolism and later mitochondria dysfunction, thereby contributing to the hepatocyte apoptosis and oxidative liver injury induced by MC-LR. These findings represented a novel characterization of the mechanisms regarding the liver injury and diseases induced by MC-LR, adding a new level of understanding to the pathological mechanisms of MCs, which may aid in clinical detoxification.

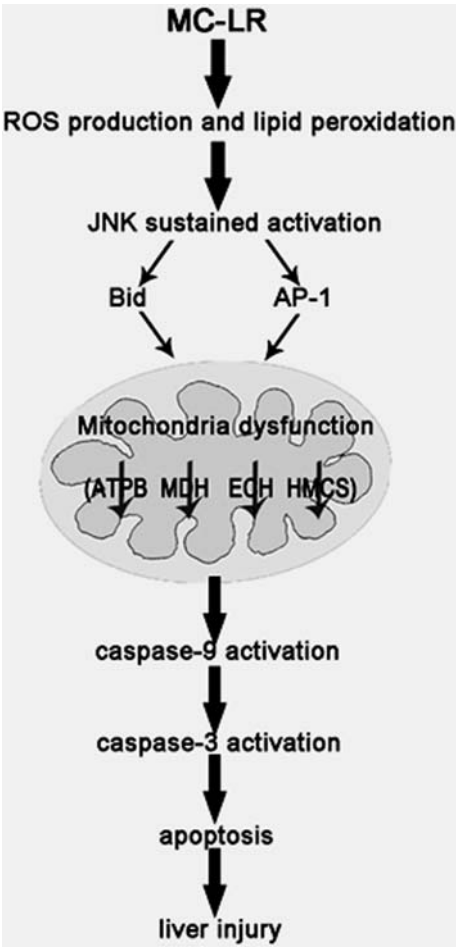

Fig. 8 Proposed pathways and mechanisms of the model in the present paper. MC-LR initiates ROS accumulation and causing oxidative stress. The oxidative stress then stimulates sustained activation of JNK, which in turn induces mitochondria-dependent apoptosis via AP-1 and Bid

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