3-methyadenine attenuates chloroform-induced hepatotoxicity via autophagy activation

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ABSTRACT

Chloroform is a common contaminant in the drinking water. Exposure of human to chloroform leads to severe hepatotoxicity. In the present study, chloroform-induced acute liver injury was investigated in mice using 3-methyadenine (3-MA), a common autophagy inhibitor. At 24 h after intraperitoneal injection of 0.5 mL/kg chloroform, serum alanine aminotransferase (ALT) levels were increased significantly; extensive necrosis and inflammation occurred as identified by histological examinations. Moreover, chloroform induced an increase in lipid peroxidation as demonstrated by increased formation of malondialdehyde (MDA) in the liver tissues. Hepatic antioxidants including glutathione (GSH) and superoxide dismutase (SOD) were decreased by chloroform treatment. All these changes were significantly inhibited by 3-MA treatment. Further mechanistic insights demonstrated that chloroform up-regulated pro-inflammatory cytokine, IL-1β, in the livers and blood, which was suppressed by 3-MA. Surprisingly, Western blots results showed that after 24-hours of chloroform treatment 3-MA activated autophagy as indicated by decreased levels of LC3B II and p62 protein. Co-treatment of chloroquine with 3-MA to inhibit autophagy would abrogate the hepatoprotection of 3-MA in chloroform hepatotoxicity. Taken together, findings in the present study suggested that a widely-used autophagy inhibitor, 3-MA, significantly reduced chloroform hepatotoxicity in mice via autophagy activation. Findings in this study also suggested that caution should be exercised when using 3-MA to modulate autophagy in vivo.

Chloroform (trichloromethane, CHCL3) is a clear, colorless and volatile liquid and has been widely used as an intermediate and a general solvent in many chemical and pharmaceutical manufacturers.

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hepatotoxicity are needed and would be beneficial to the development of therapeutics.

Autophagy is a cellular catabolic process to degrade long-lived proteins and cellular organelles including misfolded molecules and dysfunctional organelles by lysosome (3, 26, 31). Autophagy is tightly-regulated and highly inducible (16, 31) and may be involved in the pathogenesis of a number of human diseases (17, 25). Recently, several reports demonstrate that autophagy is involved in drug/chemicals-induced liver injuries (7, 30). For example, activation of autophagy protects from ethanol-induced (5, 10) and acetaminophen-induced (26, 27) liver injuries. However, at present it is still unknown how autophagy influences chloroform-induced liver injury. In this study, a commonly used autophagy inhibitor, 3-methyadenine (3-MA), was used to investigate chloroform-induced liver injury in mice.

MATERIALS AND METHODS

Reagents. 3-methyladenine (3-MA) was bought from MedChem Express (Shanghai, China). Chloroform was purchased from Beijing Chemical Reagents Corp. (Beijing, China). Rabbit polyclonal anti-LC3B antibody (Cat. No. 18725-1-AP) and rabbit polyclonal anti-p62/SQSTM1 (Cat. No. 18420-1-AP) antibody were purchased from Proteintech company (Wuhan, China). Rabbit polyclonal anti-IL-1β antibody (Cat. No. ab9722) was bought from Abcam (Shanghai, China). Horseradish peroxidase (HRP)-conjugated (Cat. No. 111-035-003) and Cyanine-3-conjugated (Cat. No. 111-165-003) goat anti-rabbit secondary antibody were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

Animals and treatment. Male Balb/c mice (six weeks old, weighing ~20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The mice were housed under a specific-pathogen-free (SPF) environment with a 12 hour light/dark cycle and free access to food and water. All the mice were acclimated to the environment for a week before experiments were performed. All the mice received human care and all experiments were done according to the guidelines of the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals from the National Institutes of Health. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Tianjin Medical University and in accordance with Helsinki Declaration and with implications for replacement, refinement or reduction (the 3Rs) principle.

Twenty mice were used in this study and divided into four groups (five mice each group). Acute liver injury was induced by a single intraperitoneal injection of chloroform (0.5 mL/kg) in mice. To modulate autophagy in vivo, 3-methyadenine (3-MA, 30 mg/kg) was intraperitoneally administrated one hour before chloroform injection. Twenty-four hours later, mice were sacrificed and blood and livers were collected for further analyses.

Biochemical assays. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatotoxic malondialdehyde (MDA), hepatic superoxide dismutase (SOD) and serum glutathione (GSH) levels were determined using commercially available kits from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China) according to the manufacturer’s manual.

Histology. Liver tissues were fixed in 4% paraformaldehyde in PBS pH 7.4, embedded in paraffin and then cut into 4 μm-thick sections. Liver sections were subjected to standard hematoxylin-eosin (H.E.) staining and examined under a Nikon light microscope. The necrosis and inflammation were accessed and scored by an independent pathologist according to the Ishak scoring criteria (9, 11).

Immunohistochemistry. Four micrometer thick liver sections were deparaffinized and rehydrated and blocked with 3% H2O2 for 10 min to eliminate endogenous peroxidase activity. After antigen retrieval in a microwave oven for 15 min, the slides were blocked with 5% bovine serum albumin and then probed with primary antibodies against IL-1β (1 : 200) overnight in a humidified chamber at 4°C. Then the slides were washed twice with PBS. For immunohistochemistry, the liver sections were incubated with HRP-conjugated secondary antibody and stained with diaminobenzidine (DAB) chromogen for color development. At last, liver sections were lightly counter-stained with hematoxylin, mounted with mounting medium and examined under a light microscope.

Enzyme-linked immunosorbent assay (ELISA). Serum interleukin-1β (IL-1β) level was measured using a eBiosciences ELISA kit (San Diego, CA, USA) following the manufacturer’s manuals.

Western blot. Western blot analyses were performed as previously reported (19). Briefly, liver extracts were separated by sodium dodecyl sulfate polyacryl-
amid gel electrophoresis and transferred onto 0.2 μm polyvinylidene difluoride (PVDF) membrane (Millipore, Darmstadt, Germany). The membranes were blocked with 5% skimmed milk followed by incubation with certain primary antibody overnight at 4°C. Then the membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature and developed using Immunobilon enhanced chemiluminescence (ECL) reagents (Millipore, Darmstadt, Germany). Protein bands were visualized and imaged using a GelDoc XR System (Bio-Rad, Shanghai, China). Bands densities were semi-quantitatively analyzed using free software Image J (http://rsb.info.nih.gov/ij/).

Statistical analyses. Statistical analyses were performed using SPSS 21.0, version for Windows (SPSS Inc., Chicago, Illinois, USA). Differences between groups were compared by one way analysis of variance (ANOVA). P < 0.05 was considered as statistically significant.

RESULTS
3-MA reduces chloroform(CHCL3)-induced liver injury in mice
As shown in Fig. 1, intraperitoneal chloroform treatment induced significant increase in serum ALT and AST activities; while co-treatment with a common autophagy inhibitor, 3-methyadenine (3-MA), almost completely eliminated chloroform-induced serum ALT and AST increases. Histological examinations corroborated the serum ALT and AST changes. Chloroform treatment rendered extensive central necrosis in the hepatic lobules as illustrated by ballooning degeneration of hepatocytes, loss of liver sinus and nuclei in some hepatocytes (Fig. 2A). Chloroform treatment also induced inflammatory cells infiltration in the necrotic area (Fig. 2A). However, 3-MA treatment greatly reduced chloroform-induced central necrosis and inflammatory cells infiltration in the livers (Fig. 2A). Semi-quantitative analysis of the cell necrosis and inflammation by an independent pathologist further demonstrated 3-MA treatment could significantly reduce chloroform-induced liver injury (Fig. 2B).

3-MA reduces oxidative stress and inflammation
Fig. 3 shows the determinations of oxidative stress parameters. As indicated in that figure, chloroform treatment induced prominent lipid oxidation as indicated by increased formation of MDA (Fig. 3A). Hepatic antioxidants including GSH and SOD were also down-regulated by chloroform treatment (Fig. 3B and 3C). However, all these changes were significantly reduced by 3-MA treatment (Fig. 3).

IL-1β levels in liver tissues (Fig. 4A) and serum (Fig. 4B) were determined by immunohistochemistry and ELISA assays. Results demonstrated that chloroform administration significantly increased IL-1β levels in the liver tissues and serum; while 3-MA treatment almost completely suppressed chloroform-induced IL-1β production and secretion (Fig. 4A and 4B).

3-MA treatment increases autophagic flux in vivo in chloroform hepatotoxicity
Western blots were performed to determine LC3B and p62 levels in the livers to identify the autophagy status. As demonstrated in Fig. 5, accumulation of LC3B II and p62 proteins in the livers of chloroform-treated mice was reduced by 3-MA treatment,
Fig. 2  Liver damage and inflammation. (A) Histological changes examined by hematoxylin-eosin (H.E) staining. (B) Evaluation of the necrosis and inflammation by Ishak scoring system. *P<0.05 vs. Control group; **P<0.05 vs. CHCL3 group.

Fig. 3  Changes of oxidative stress parameters. (A) Malondialdehyde (MDA), (B) Glutathione (GSH), (C) Superoxide dismutase (SOD). *P<0.05 vs. Control group; **P<0.05 vs. CHCL3 group.
which indicated the increased autophagic flux by 3-MA. Suppression of the autophagy flux by chloroquine abrogated the hepatoprotection of 3-MA in chloroform hepatotoxicity (Fig. 6).

**DISCUSSION**

In this study, chloroform-induced hepatotoxicity was investigated. Our results showed that chloroform treatment greatly damaged liver tissues. A common autophagy inhibitor, 3-MA, significantly reduced
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Fig. 6 Serum alanine aminotransferase (ALT) (A) and aspartate aminotransferase (AST) (B) activities. Co-treatment of chloroquine (CQ) abrogated the hepatoprotection of 3-MA. *P < 0.05 vs. Control group; **P < 0.05 vs. CHCL3 group.

chloroform-induced hepatotoxicity. What’s more, Western blot results suggested that 3-MA treatment significantly reduced chloroform-increased expressions of LC3B and p62/SQSTM1, two important proteins in autophagy pathway. Autophagy is an important cellular process and self-degrades cellular components under many conditions (3, 26, 31). LC3 and p62 are widely used as autophagy markers to determine autophagy flux (14). In the process of autophagy, LC3 is conjugated to the autophagosomal membranes and ultimately degraded in autolysosome (14). p62 is multifunctional protein and functions as a receptor in mammalian autophagy (6). What’s more, p62 can directly bind LC3B and this serves as a mechanism to transfer autophagic cargo for degradation by autophagy (14). Expression levels of p62 inversely correlate with autophagy activity (23). When autophagy flux is increased, p62 expression will be decreased; when autophagy flux is inhibited, p62 protein will accumulate (23). In this study, our results showed that 3-MA treatment reduced both p62 protein and LC3B levels, which indicated the increased autophagy flux by 3-MA (22).

A lot of studies have suggested that autophagy is involved in many human diseases (17, 25). The role of autophagy in cell survival or death is complex. Under some conditions autophagy exerts protective effects to promote cell survival, while under some other conditions autophagy is detrimental to the cell (24, 28, 33). Previous reports showed that in hepatotoxic models induced by acetaminophen and ethanol, induction of autophagy was protective and inhibition of autophagy was detrimental (5, 20, 26, 27). Consistent with these reports, in our study we found that autophagy enhancement by 3-MA protected against chloroform-induced hepatotoxicity in mice. Inhibition of autophagy with chloroquine abrogated the hepatoprotection of 3-MA in chloroform hepatotoxicity. However, at present it was still unknown why 3-MA enhances autophagy in vivo. Previous in vitro study demonstrated that long-term treatment of 3-MA could activate autophagy via persistent inhibition of class I phosphoinositide-3-kinase (PI3K)/Akt/mTOR pathway in cultured cells (34). So, we speculated that long-term (24 h) treatment of 3-MA in vivo might also activate autophagy through suppression of PI3K/Akt/mTOR pathway.

In the liver chloroform is mainly metabolized by cytochrome P450 (CYP2E1 and CYP2A6) into phosgene (CCl4O) and free radicals. These reactive metabolites deplete hepatocellular GSH, cause mitochondrial damage and hepatocyte death (8, 13, 21, 29). In this study, besides increases in serum ALT activity and histological changes after chloroform administration, we also observed the chloroform-induced decreases in GSH levels and SOD activity and increased formation of lipid peroxidation product, malondialdehyde (MDA), in the liver tissues. These changes were reduced by 3-MA, which suggested that autophagy activation was protective in chloroform hepatotoxicity.

IL-1β was an important effector cytokine in innate immune and inflammation (18). Blocking IL-1β signaling also alleviated liver injury (12, 32). In present study, IL-1β expression was upregulated in the liver tissues and blood by chloroform administration. 3-MA treatment significantly reduced chloroform-induced IL-1β expression. These results suggested that 3-MA exerted hepatic protection against chloroform toxicity through inhibiting inflammation and the
subsequent production of pro-inflammatory cytokine, IL-1β.

In summary, findings in our study suggested that chloroform-induced acute liver injury would be greatly reduced by 3-MA, a widely-used autophagy inhibitor. Surprisingly, protection of 3-MA against chloroform hepatotoxicity might attribute to the autophagy activation by 3-MA. Furthermore, results from present study also suggested that caution should be exercised when using 3-MA in modulating autophagy in vivo.

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CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

31. Ravikumar B, Sarkar S, Davies JE, Futter M, Garcia-Arencibia M, Green-Thompson ZW, Jimenez-Sanchez M, Korolechuk...

