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Succinate dehydrogenase inhibitor dimethyl malonate alleviates LPS/D-galactosamine-induced acute hepatic damage in mice

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Abstract

In addition to its energy-supplying function, increasing evidence suggests that mitochondria also play crucial roles in the regulation of inflammation. Succinate dehydrogenase is also known as mitochondrial complex II, and inhibition of succinate dehydrogenase by dimethyl malonate has been reported to suppress the production of pro-inflammatory cytokines. In the present study, the potential anti-inflammatory benefits of dimethyl malonate were investigated in a mouse model with LPS/D-galactosamine-induced acute hepatic damage. Male BALB/c mice were injected i.p. with LPS and D-galactosamine to cause liver injury. The degree of liver injury, inflammatory response and oxidative stress and the survival of the experimental animals were determined. The results indicated dimethyl malonate decreased the level of aminotransferases in plasma, alleviated histological abnormalities in liver, inhibited the induction of TNF-α and IL-6 in plasma, suppressed hepatocyte apoptosis and improved the survival of LPS/D-galactosamine-exposed mice. Therefore, inhibition of succinate dehydrogenase by dimethyl malonate significantly alleviated LPS/D-galactosamine-induced hepatic damage, which suggests that succinate dehydrogenase might become a novel target for the intervention of inflammation-based hepatic disorders.

Keywords

Dimethyl malonate, succinate dehydrogenase, hepatic injury, lipopolysaccharide, apoptosis

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Introduction

Acute hepatic damage is a serious clinical syndrome with a high mortality rate, which is usually induced by infection, alcohol, drugs and other harmful factors.[1] The pathogenesis of acute hepatic damage is complicated, and the uncontrolled inflammatory response plays a central role in mediating hepatic injury.[2] LPS, also known as endotoxin, is a major pro-inflammatory stimulator which is responsible for various inflammatory disorders.[3] LPS administered in combination with D-galactosamine (D-GalN) induces severe acute hepatic damage in mice,[4] which is attributed to the quick activation of inflammatory cells and the excessive production of pro-inflammatory cytokines.[5] The LPS/D-GalN model has been widely used to study the mechanisms of acute hepatic damage and to develop novel protective reagents.[6–8]

Mitochondria are key organelles for energy production, which might result from the complex metabolic processes in mitochondria, including the tricarboxylic

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acid cycle (TCA) and oxidative phosphorylation.[9,10] In addition to their energy-supplying function, increasing evidence suggests that mitochondria also play crucial roles in the regulation of inflammatory process.[11,12] Succinate dehydrogenase (SDH), anchored to the inner membrane of mitochondria, catalyses the oxidation of succinate to fumarate in the TCA cycle. Meanwhile, SDH, also known as mitochondrial complex II, is a critical integral component of the electron transport chain. SDH is a unique enzyme that plays essential roles in both the TCA cycle and the electron transport chain.[13] Cumulative evidence revealed that SDH plays key roles in regulating the inflammatory response,[14,15] which implies that the mitochondrial SDH might become a novel target for the intervention of inflammatory disorders.

Recently, a competitive inhibitor for SDH, dimethyl malonate (DMM), has been developed.[14] It has been reported that inhibition of SDH by DMM suppressed IL-1β production in LPS-stimulated bone marrow–derived macrophages.[15] In addition, treatment with DMM decreased the level of IL-1β in mice infected with bacteria.[16] Therefore, the SDH inhibitor DMM might have profound value for controlling inflammatory injury. In the current study, the SDH inhibitor DMM was administered in mice with LPS/D-GalN-induced acute hepatic injury, and the potential effects of DMM on histological abnormalities, inflammatory response, hepatocyte apoptosis and animal survival were determined.

Materials and methods

Reagents

LPS (*Escherichia coli*, 055: B5), D-GalN and DMM were from Sigma–Aldrich (St Louis, MO). The assay kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, PR China). The assay kits for detecting the activities of caspase-3, caspase-8, caspase-9 and the level of malondialdehyde (MDA) were from the Beyotime Institute of Biotechnology (Jiangsu, PR China). The ELISA kits for detecting mouse IL-6 and TNF-α were the products of the NeoBioscience Technology Company (Shenzhen, PR China). The In Situ Cell Death Detection Kit was provided by Roche (Indianapolis, IN). The rabbit anti-mouse cleaved caspase-3 and GAPDH were provided by Cell Signaling Technology (Danvers, MA). The BCA protein assay kit, the HRP-conjugated goat anti-rabbit Ab and the enhanced chemiluminescence (ECL) reagents were provided by Pierce Biotechnology (Rockford, IL).

Animals

Male BALB/c mice (weighing 18–22 g and 6–8 wk old) were provided by Chongqing Medical University (Chongqing, PR China). The mice were kept in a controlled environment (20–25°C, 45–55% humidity and 12 h light/dark cycle) and allowed to feed and drink freely. All mice adapted to the environment for 7 d before use. All experiments related to animals were confirmed by the Animal Care and Use Committee of Chongqing Medical University.

Liver injury

BALB/c mice were injected i.p. with LPS (10 μg/kg) and D-GalN (700 mg/kg) to establish a hepatic damage model. To investigate the roles of DMM in hepatic damage, 32 mice were randomly divided into four groups (n=8/group). Animals in the LPS/D-GalN group were only treated with LPS and D-GalN. The DMM+LPS/D-GalN group indicated that mice were pre-treated with DMM (300 mg/kg, dissolved in normal saline (NS)) at 0.5 h before LPS/D-GalN exposure. The dose of DMM was used based on our pre-experiments. The control group and the DMM group were treated with the same amount of NS and DMM, respectively. Six h after LPS/D-GalN exposure, the mice were executed, and the blood and liver specimens were harvested for detection of aminotransferases, morphological evaluation and other examination. To evaluate the roles of DMM on inflammation, another set of mice (n=8/group) were executed 1.5 h after LPS/D-GalN injection. The blood was harvested, and the plasma TNF-α was detected. To investigate the roles of DMM on mortality, a third set of mice (n=20/group) was prepared, and mortality was monitored every 6 h for 1 wk after LPS/D-GalN exposure.

Histological analysis

Liver tissues were collected and fixed in paraformaldehyde. Then, the fixed tissues were embedded in paraffin and cut into sections (4 μm thick). Finally, through staining with hematoxylin and eosin, the histological changes of the livers were evaluated using light microscopy.

Measurement of aminotransferases

Hepatic damage was evaluated by measuring the concentration of plasma ALT and AST using the assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's instructions.

Analysis of IL-6 and TNF-α

Plasma IL-6 and TNF-α levels were measured using ELISA kits (IL-6 ELISA kit: catalogue number

EMC004; TNF-α ELISA kit: catalogue number EMC102a; NeoBioscience Technology Company) according to the manufacturer's instructions. IL-6 and TNF-α were measured separately at 6 and 1.5 h after LPS/D-GalN injection.

Measurement of MDA

The liver-tissue extracts were prepared in order for the level of MDA to be analysed using an assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Assay of caspase activities

The liver-tissue extracts were prepared in order to analyse the protease activities of caspase-3, caspase-8 and caspase-9 separately using assay kits (caspase-3: catalogue number C1116; caspase-8: catalogue number C1152; caspase-9: catalogue number C1158; Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Western blot analysis

Protein from the liver tissues was extracted using a protein extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology). The protein concentration was measured with a BCA protein assay kit (Pierce Biotechnology). The proteins were detached by SDS-PAGE and transferred to nitrocellulose membrane. The primary Ab, such as cleaved capase-3 and GAPDH, were used to incubate the membrane overnight at 4°C. Then, the membranes were incubated with the second Ab. The protein bands were visualised using an ECL chemiluminescence system. GAPDH was used as the internal control.

Terminal deoxynucleotidyl transferase dUTP nick end labelling analysis

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was used to analyse the level of

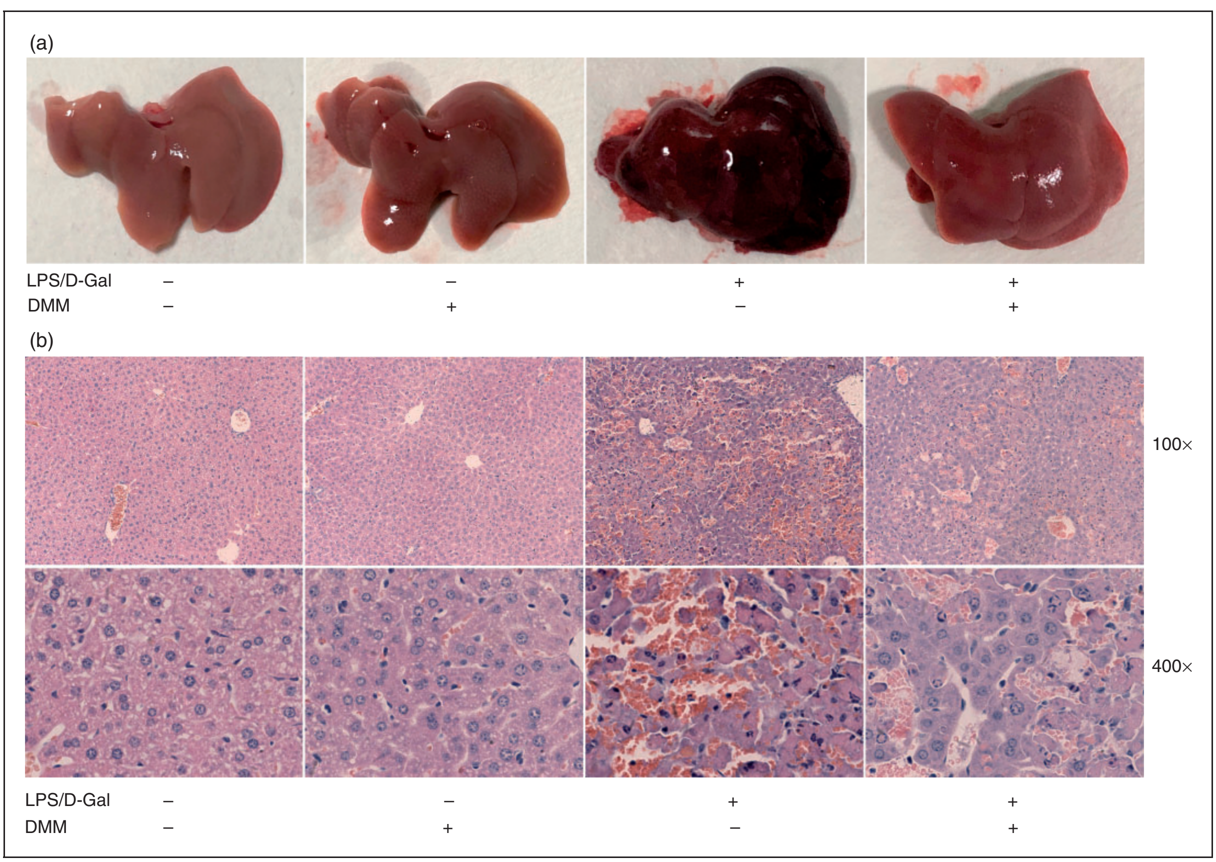

Figure 1. DMM mitigated LPS/D-galactosamine (D-GalN)-induced hepatic damage. Male BALB/c mice were injected i.p. with LPS (10 μg/kg) and D-GalN (700 mg/kg) to induce hepatic damage. Vehicle or DMM (300 mg/kg, dissolved in normal saline) were pre-treated in the absence or presence, respectively, of LPS/D-GalN injection. (a) The livers were observed by gross examination 6 h after LPS/D-GalN exposure. The representative livers of each group are shown. (b) Liver tissues were cut into sections and stained with hematoxylin and eosin for morphological evaluation. The representative sections of each group are shown (original magnification: ×100, ×400).

apoptosis in the liver tissues using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Dark-brown precipitate indicates apoptosis cells in the sections of the liver tissues.

Statistical analysis

All data are presented as the mean $\pm$ SD. The statistical significance was analysed by one-way ANOVAs with a *post hoc* test. The Kaplan–Meier curve and log-rank test were calculated to evaluate the survival rate. $P<0.05$ indicated statistical significance.

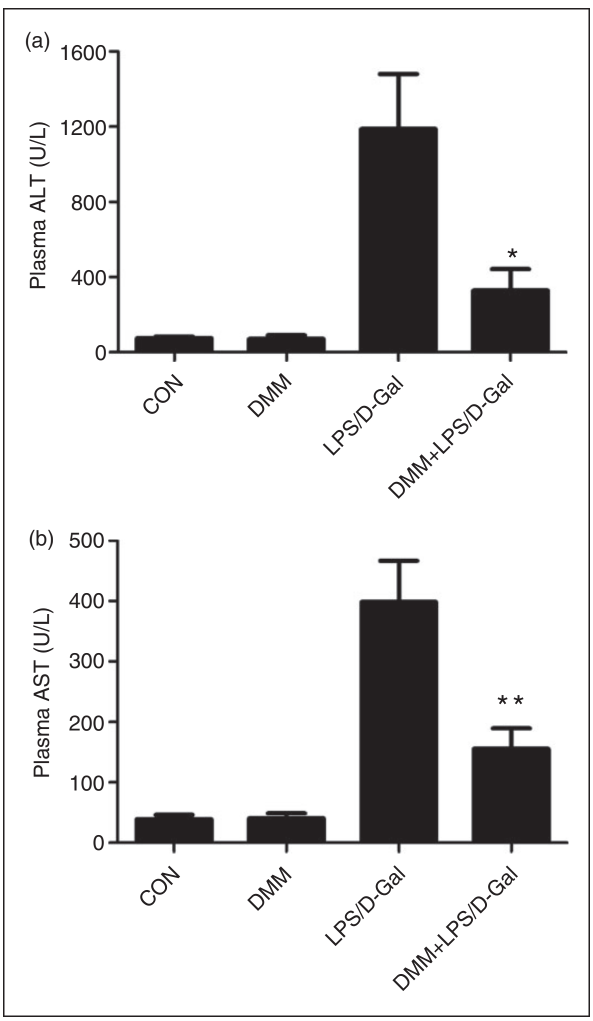

Figure 2. DMM ameliorated the level of plasma aminotransferases in mice with LPS/D-GalN-induced hepatic damage. The levels of plasma (a) alanine aminotransferase (ALT) and (b) aspartate aminotransferase (AST) were measured 6 h after LPS/D-GalN exposure. Data are presented as the mean $\pm$ *SD*, $n = 8$. *$P < 0.05$ and **$P < 0.01$ compared to the LPS/D-GalN group. CON: control.

Results

DMM mitigated LPS/D-GalN-induced hepatic damage

The liver in the LPS/D-GalN group showed severe haemorrhagic appearance upon gross examination, which was attenuated by pre-treatment with DMM (Figure 1a). Coincidentally, the histological changes induced by the LPS/D-GalN injection, such as hepatocyte necrosis, congestion and destruction of hepatic lobule, also ameliorated markedly in the DMM pre-treated mice (Figure 1b). The plasma levels of ALT and AST, as the biochemical indexes of hepatic damage,[17] up-regulated significantly at 6 h after LPS/D-GalN injection, while the up-regulation of ALT and AST were reversed by pre-treatment with DMM (Figure 2a and b). Survival analysis showed that pre-treatment with DMM markedly up-regulated the survival rate of mice with LPS/D-GalN-induced hepatic damage (Figure 3).

DMM ameliorated the level of IL-6 and TNF-α

TNF-α and IL-6 are key inflammatory mediators in LPS/D-GalN-induced hepatic damage.[18,19] In our study, the concentration of TNF-α and IL-6 in plasma increased significantly in the LPS/D-GalN group, which ameliorated markedly in the DMM+LPS/D-GalN group (Figure 4a and b). The results showed that DMM markedly alleviated the LPS/D-GalN-induced inflammatory response.

DMM alleviated the level of MDA in liver

As a result of lipid peroxidation, MDA is regarded as a hallmark of oxidative stress.[20] MDA was measured to

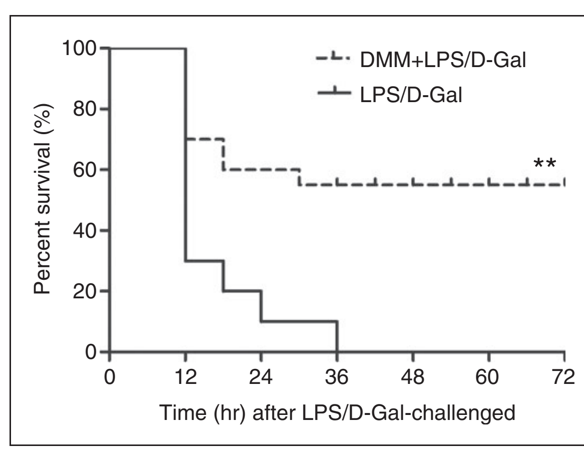

Figure 3. DMM up-regulated the survival rate of mice with LPS/D-GalN-induced hepatic damage. The survival rate of the mice was monitored every 6 h for 1 wk after LPS/D-GalN injection. The survival rate is shown as Kaplan–Meier curves, $n = 20$ per group. **$P < 0.01$ compared to the LPS/D-GalN group.

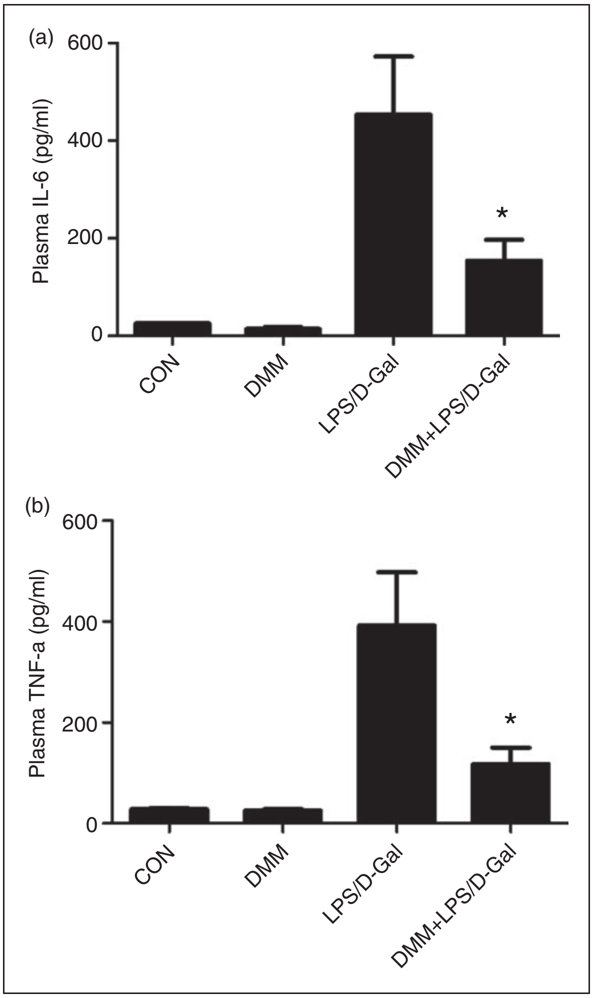

Figure 4. DMM decreased the level of TNF-α and IL-6 in plasma in LPS/D-GalN-challenged mice. (a) IL-6 and (b) TNF-α in plasma were detected at 6 h and 1.5 h after LPS/D-GalN exposure, respectively. Data are presented as the mean ± *SD*, $n = 8$. **P* < 0.05 compared to the LPS/D-GalN group.

evaluate the level of oxidative stress. The level of MDA in the LPS/D-GalN group increased markedly, and the increase of MDA in the LPS/D-GalN group was reversed by pre-treatment with DMM (Figure 5).

DMM ameliorated caspase activation and hepatocyte apoptosis

Apoptosis is a major characteristic in LPS/D-GalN-induced hepatic damage.[21] Exposure to LPS/D-GalN increased the protease activity of caspase-3, caspase-8 and caspase-9, while the activity of caspases ameliorated markedly after pre-treatment with DMM (Figure 6a–c). Consistently, the cleaved caspase-3 induced by LPS/D-GalN-exposure was also markedly suppressed by pre-treatment with DMM (Figure 7a and b). As expected, LPS/D-GalN-induced hepatocyte

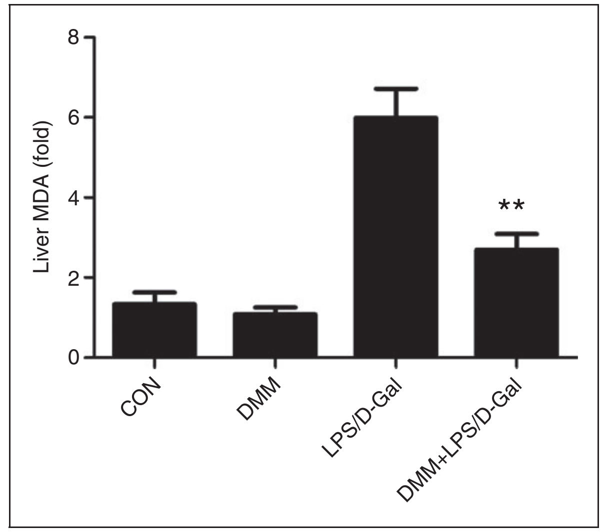

Figure 5. DMM ameliorated malondialdehyde (MDA) in the liver in LPS/D-GalN-challenged mice. The level of MDA in the liver was detected 6 h after LPS/D-GalN exposure. Data are presented as the mean ± *SD*, $n = 8$. ***P* < 0.01 compared to the LPS/D-GalN group.

apoptosis also decreased significantly after pre-treatment with DMM (Figure 8).

Discussion

In addition to its crucial roles in the TCA cycle and oxidative phosphorylation, recent studies have suggested that SDH might become a new checkpoint for controlling the inflammatory response.[14,15] Several experimental investigations have found that inhibition of SDH by its inhibitor DMM significantly suppressed the expression of pro-inflammatory cytokines both *in vitro* and *in vivo*.[14,15] In the present study, we found that treatment with DMM resulted in beneficial outcomes in mice with LPS/D-GalN-induced hepatic damage because DMM ameliorated the hepatic histological abnormalities, mitigated the elevation of aminotransferase and up-regulated the survival rate of LPS/D-GalN-exposed mice. These data suggest that DMM might play important roles in the progression of LPS/D-GalN-induced hepatic damage.

LPS-D-GalN-induced hepatic damage mainly depends on the quick induction of the pro-inflammatory mediators.[22] Consistent with beneficial effects on liver damage, treatment with DMM prominently decreased the level of TNF-α in LPS/D-GalN-challenged mice. TNF-α has been regarded as the most important detrimental factor during the development of LPS/D-GalN-induced hepatic damage. The ligation of TNF-α with its receptor activates the death receptor–dependent apoptotic pathway, which leads to the activation of the caspase cascade and the cleavage of

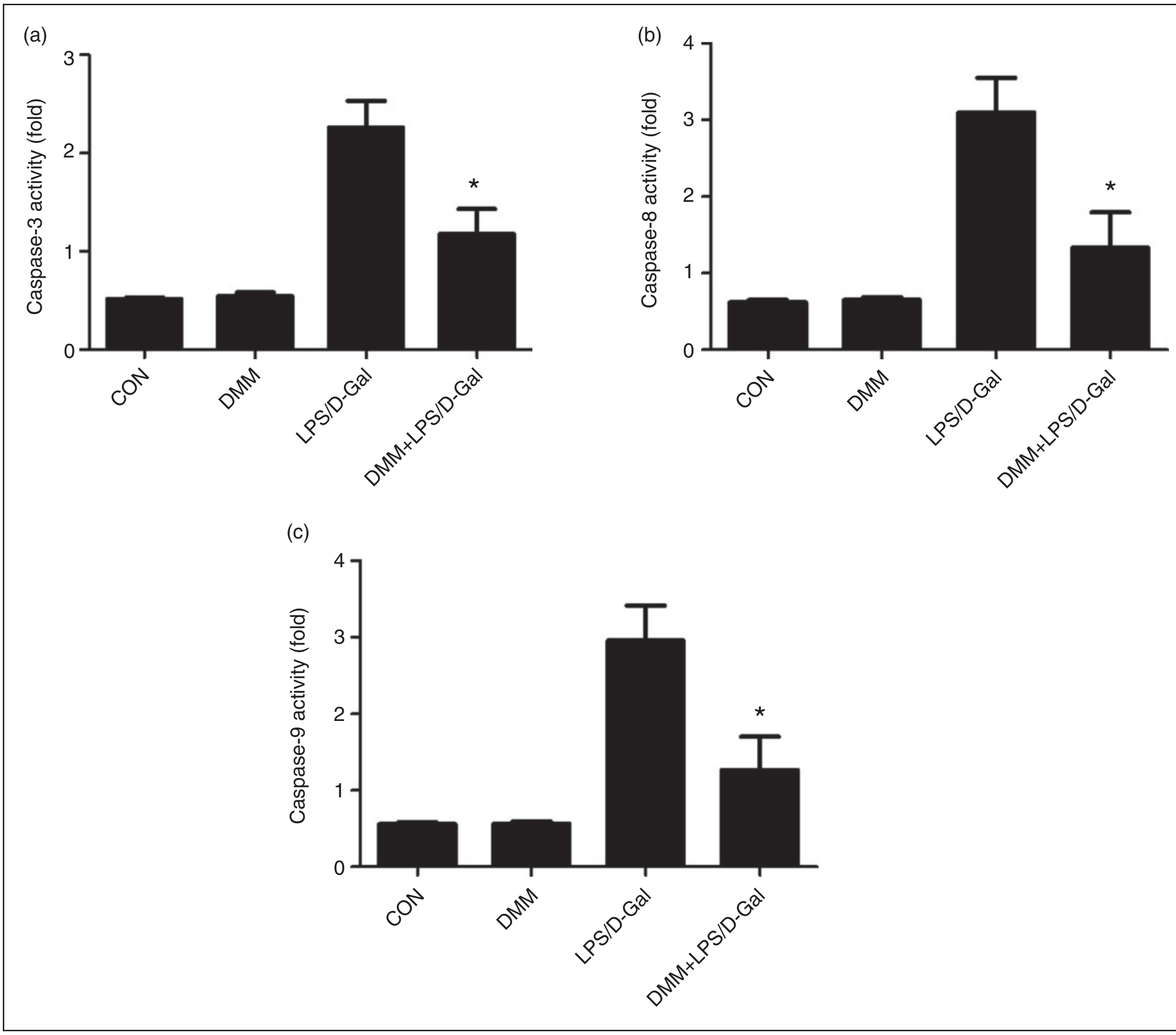

Figure 6. DMM suppressed caspase activation in LPS/D-GalN-challenged mice. The protease activities of (a) caspase-3, (b) caspase-8 and (c) caspase-9 in the liver tissue were detected 6 h after LPS/D-GalN exposure. Data are presented as the mean $\pm$ *SD*, $n = 8$. $^*P < 0.05$ compared to the LPS/D-GalN group.

structural proteins.[23,24] Therefore, the suppressive effect of DMM on TNF-α production might result in the suppressed activation of caspases, reduced TUNEL-positive cells and alleviated liver injury seen in the present study.

In addition, DMM also decreased the level of IL-6, another pro-inflammatory cytokine involved in liver injury induced by LPS/D-GalN. In agreement with our findings, a previous study reported that DMM can suppress the inflammatory response in bone marrow–derived macrophages by down-regulating the level of IL-1β.[15] In addition, it was also found that DMM was effective in an LPS-induced sepsis model by decreasing the production of IL-1β.[14] Therefore, the anti-inflammatory properties of DMM might be the basic mechanism responsible for its beneficial effects in the LPS/D-GalN model.

The detailed mechanisms underlying the inflammation/regulatory roles of SDH have not been fully identified. SDH, as mitochondrial complex II, plays a critical role in the electron transport chain.[13,25] The electron transport chain is important for the generation of ATP, but the deleterious reactive oxygen species (ROS) is also produced as a by-product, especially under pathological circumstances.[26,27] The oxidation of succinate via SDH might produce a burst of mitochondrial ROS, while the ROS production can be restrained by DMM.[28,29] In addition to inducing direct tissue injury, excessive ROS also plays crucial regulatory roles in inflammation and apoptosis by activating the

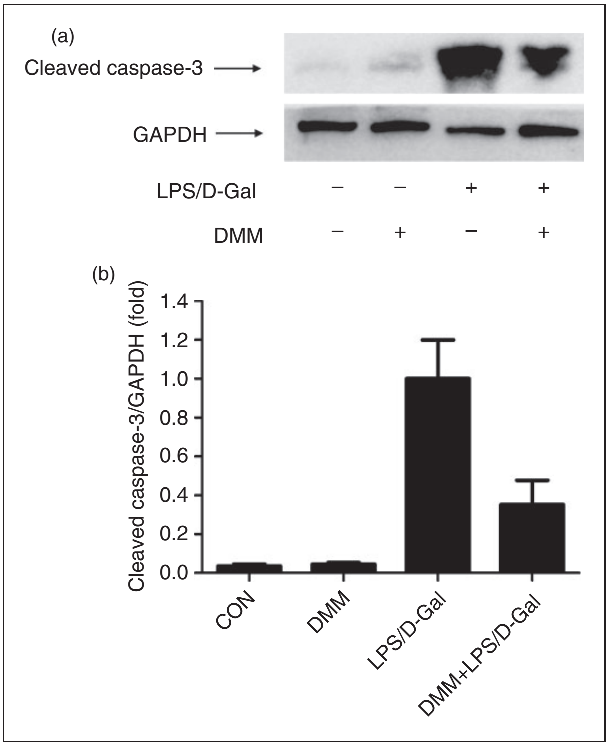

Figure 7. DMM decreased the level of cleaved caspase-3 induced by LPS/D-GalN. (a) The cleaved caspase-3 in liver was measured by Western blot analysis 6 h after LPS/D-GalN injection. GAPDH was used as the internal control. (b) The blots were scanned and semi-quantified. $n = 4$. $^*P < 0.05$ compared to the LPS/D-GalN group.

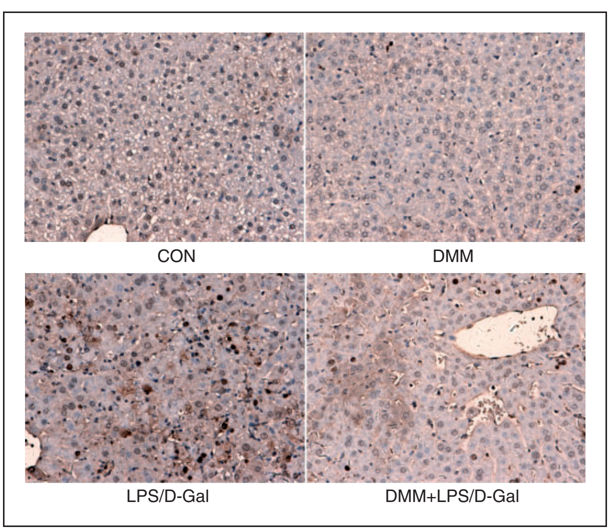

Figure 8. DMM suppressed LPS/D-GalN-induced hepatocyte apoptosis. Liver tissues were harvested and cut into sections 6 h after LPS/D-GalN injection. Hepatocyte apoptosis were detected by terminal deoxynucleotidyl transferase dUTP nick end labelling assay, and the dark-brown nucleus represents apoptotic cells in the sections. The representative sections of each group are shown (original magnification ×200).

transcription factors hypoxia-inducible factor-1, NF-κB and activator protein-1, as well as the NLRP3 inflammasome, and increasing the release of pro-inflammatory cytokines.[30–32] Actually, ROS has also been regarded as a crucial factor involved in the progress of liver damage.[33] In the current study, MDA, a molecular marker of oxidative injury, increased markedly in LPS/D-GalN-challenged mice, which was reversed by pretreatment with DMM. Therefore, the suppression of SDH-dependent oxidative stress might contribute to, at least partially, the anti-inflammatory benefits of DMM in LPS/D-GalN-induced hepatic damage.

Taken together, the current study found that treatment with the SDH inhibitor DMM significantly suppressed the production of the pro-inflammatory cytokines and ameliorated hepatic damage in LPS/D-GalN-exposed mice, and the beneficial effects of DMM in liver injury seem to be attributed to its capacity to inhibit oxidative stress. The study indicates that SDH inhibitors, including DMM, might have promising value in the intervention of inflammation-based hepatic disorders.

Declaration of conflicting interests

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