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Caloric restriction mimetic 2-deoxyglucose alleviated lethal liver injury induced by lipopolysaccharide/D-galactosamine in mice

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ABSTRACT

The glycolytic inhibitor 2-deoxyglucose (2-DG) is a calorie restriction (CR) mimetic produces CR-like beneficial effects in both acute and chronic pathological processes, but whether 2-DG is also helpful in critical and life-threatening situation is not known. In the present study, the potential benefits of 2-DG in lipopolysaccharide/D-galactosamine (LPS/D-Gal)-induced lethal liver injury were investigated. The results indicated that treatment with 2-DG suppressed the elevation of plasma aminotransferases, alleviated the histopathological abnormalities and improved the survival rate of LPS/D-Gal-exposed mice. Treatment with 2-DG also suppressed the production of pro-apoptotic cytokine TNF-α, the phosphorylation of JNK, the activation of caspase cascade and the count of TUNEL-positive apoptotic hepatocytes. These data suggested that the CR mimetic 2-DG could also provide beneficial effects in lethal pathological process such as LPS/D-Gal-induced fulminant liver injury.

1. Introduction

Calorie restriction (CR), which refers to restricting the intake of calories, has emerged as an active research area because it is a reliable intervention can effectively prolong lifespan and increase healthspan [1]. However, long-term CR would be highly problematic because of compliance challenge and other unpleasant side effects [2]. Therefore, the concept of a CR mimetic has been proposed. CR mimetic can mimic metabolic, hormonal, physiological effects of CR and produces CR-like beneficial effects on longevity and health without significantly reducing food intake [3]. The identification of CR mimetic provides novel promising approaches for the maintenance of health [2].

The glycolytic inhibitor 2-deoxyglucose (2-DG) was proposed as the first CR mimetic [4]. 2-DG blocks glycolysis primarily by competitively inhibiting phosphoglucose isomerase (PGI) because 2-DG is phosphorylated by hexokinase (HK) to form 2-DG-6-phosphate (2-DG-6-P) and 2-DG-6-P can compete with the fructose-6-phosphate (F-6-P) for PGI [5]. There is accumulative evidence indicating that treatment with 2-DG would be beneficial in chronic pathological processes such as cancer, Alzheimer's disease and Parkinson's disease [6–8]. In addition, several studies also found that administration of 2-DG protected against acute ischemia injury in rat [9,10]. Therefore, the CR mimetic 2-DG might also be helpful in acute pathological processes.

To further expand the investigation of CR mimetic, we questioned whether 2-DG could also provide beneficial effects in more serious and life-threatening situation. Recent studies have found that 2-DG suppressed lipopolysaccharid (LPS)-induced cytokine production in monocytes and inhibited the phagocytosis capacity of LPS-stimulated macrophages [11,12]. LPS, the major virulence factor of Gram-negative bacteria, is one of the representative pathogenic causers of lethal tissue injury [13,14]. Administration of LPS in D-galactosamine (D-Gal)-sensitized mice could selectively induce lethal liver injury that closely resembles clinical hepatitis in human [15]. In the present study, the potential effects of 2-DG on the degree of liver injury and the mortality of mice with LPS/D-Gal-induced lethal liver injury was investigated.

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2. Materials and methods

2.1. Materials

2-DG, LPS (from *Escherichia coli*, 055:B5) and D-Gal were purchased from Sigma (St. Louis, MO, USA). The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits were produced by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kit for detecting mouse TNF-α was the products of NeoBioscience Technology Company (Shenzhen, China). The total protein extract kit and caspase-3, -8, -9 colorimetric assay kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). In Situ Cell Death Detection Kit was purchased from Roche (Indianapolis, USA). The rabbit anti-mouse c-jun-N-terminal kinase (JNK), phosphorylated JNK (p-JNK), cleaved caspase-3 and β-actin antibodies were purchased from Cell Signaling Technology (Danvers, MA, United States). The BCA protein assay kit, horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA).

2.2. Animals

Male BALB/c mice weighing 20–25 g were obtained from the Experimental Animal Center of Chongqing Medical University. The animals were housed in a specific pathogen-free room at a temperature of 20–25 °C and 50 ± 5% relative humidity under a 12-h dark/light cycle. All animals were fed with a standard laboratory diet and water ad libitum. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Chongqing Medical University.

2.3. LPS/D-Gal-induced liver injury

LPS (10 μg/kg) and D-Gal (700 mg/kg) were injected intraperitoneally in mice to induce lethal hepatitis. The vehicle or a serial dose of 2-DG (125 mg/kg, 250 mg/kg, 500 mg/kg and 1000 mg/kg, dissolved in NS, i.p.) was administrated 0.5 h prior to LPS/D-Gal injection. Then, the animals were returned to their cages and allowed food and water ad libitum. To determine the degree of liver damage, mice were sacrificed at 6 h after LPS/D-Gal injection. The liver and plasma samples were harvested for morphological examination, aminotransferases determination and other biochemical analyses. To determine the degree of inflammation, another set of animals were sacrificed at 1.5 h after LPS/D-Gal injection. The plasma and liver samples were harvested for measuring the level of TNF-α. To determine the mortality, survival of the third set of mice (n = 20 per group) was assessed four times a day for at least 7 days and the cumulative survival curve was depicted using the Kaplan–Meier method.

2.4. Histological analysis

The liver tissues were fixed in formalin, embedded in paraffin and stained with hematoxylin & eosin for histopathological evaluation under light microscope (Olympus, Japan).

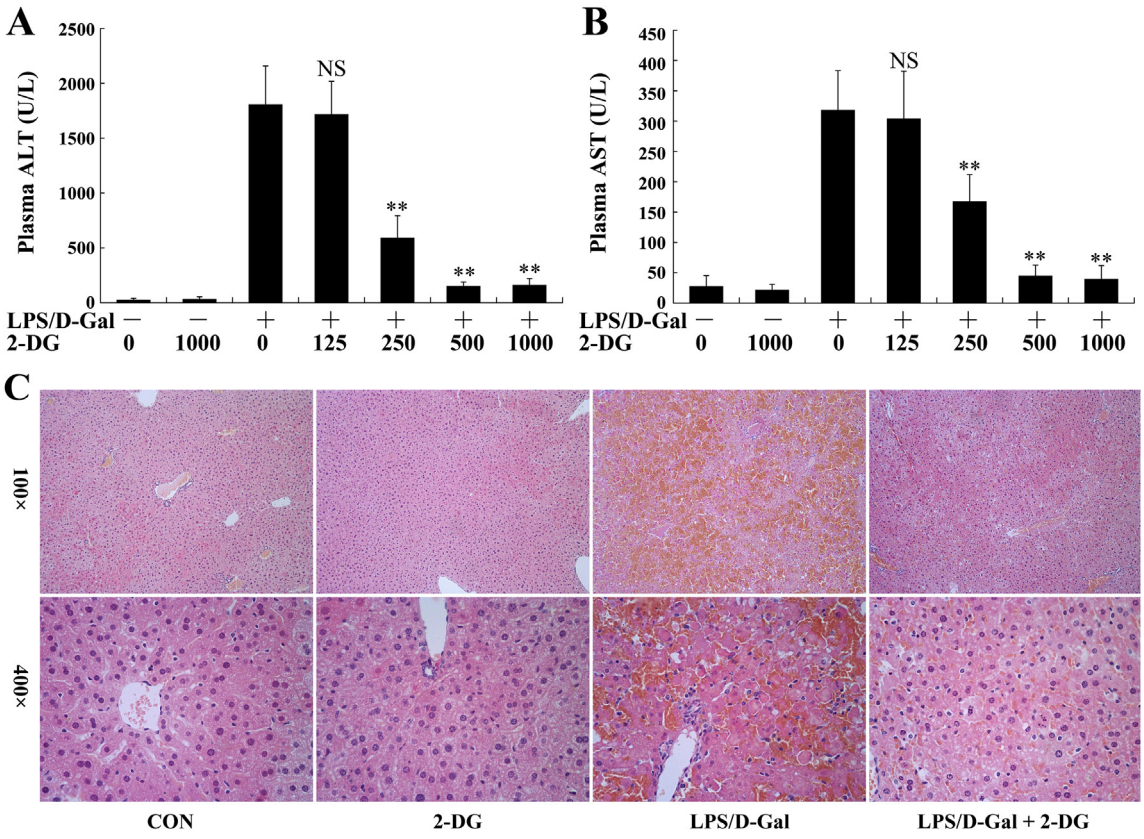

Fig. 1. 2-DG suppressed LPS/D-Gal-induced liver damage. Mice were treated with vehicle or various doses of 2-DG (125 mg/kg, 2500 mg/kg, 500 mg/kg and 1000 mg/kg) in the absence or presence of LPS/D-Gal challenge. The levels of (A) alanine aminotransferase (ALT) and (B) aspartate aminotransferase (AST) in plasma were determined at 6 h after LPS/D-Gal exposure. Data were expressed as mean ± SD, n = 8. NS $P > 0.05$, $^{**}P < 0.01$, compared with the LPS/D-Gal group (LPS/D-Gal +/2-DG 0). (C) Mice were treated with vehicle or 2-DG (500 mg/kg) in the absence or presence of LPS/D-Gal challenge. Liver samples were harvested at 6 h after LPS/D-Gal exposure and the liver sections were stained with hematoxylin-eosin for morphological evaluation. The representative liver sections of each group are shown (original magnification 100 and 400).

2.5. Determination of liver enzymes

The enzyme activities of ALT and AST in plasma were assessed according to the manufacturer's instructions (Nanjing Jiancheng, China).

2.6. TNF-α determination by ELISA

The protein levels of TNF-α in plasma were determined using ELISA kits according to the manufacturer's instructions (Neo-Bioscience, China).

2.7. Caspase activities determination

The hepatic activities of caspase-3, -8 and -9 were determined using colorimetric assay kits according to the manufacturer's instructions. Briefly, the liver samples were homogenized in cell lysis buffer, the homogenates were centrifuged for 1 min at 10,000 g and the supernatant was incubated with Ac-DEVD-pNA, Ac-IETD-pNA and Ac-LEHD-pNA substrates for caspase-3, -8 and -9, respectively, for 90 min at 37 °C. The activities of caspases were assessed according to the absorbance measured at 405 nm and normalized by the total protein concentration of the same sample.

2.8. Terminal deoxynucleotidyl transferase-mediated nucleotide nick-end labeling (TUNEL) assay

The apoptotic hepatocytes were detected with an In Situ Cell Death Detection Kit according to the manufacturer's instructions. The terminal transferase reactions finally produced a dark-brown precipitate and then the sections were counterstained slightly with hematoxylin.

2.9. Western blot analysis

Total proteins from frozen liver samples were prepared according to the method described by the protein extract kit. The total protein concentration was determined using the BCA protein assay kit. Protein extracts were fractionated on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to nitrocellulose membrane. The membrane was blocked with 5% (w/v) nonfat milk in Tris-buffered saline containing 0.05% tween-20, and then the membrane was incubated with primary antibody overnight at 4 °C, followed by incubation with secondary antibody. Antibody binding was visualized with an ECL chemiluminescence systemand short exposure of the membrane to X-ray films.

2.10. Statistical analysis

All data from the experiments were expressed as a mean ± SD. Statistical significance was determined by the Student's t test for comparisons of two groups. Multigroup comparisons were performed using one-way ANOVA multiple comparisons among means, with the Turkey's post hoc test. The survival statistics were compared with a Kaplan–Meier curve and log-rank test. Results were considered statistically significant when $P < 0.05$.

3. Results

3.1. 2-DG attenuated LPS/D-Gal-induced liver injury and mortality

Injection of LPS/D-Gal induced marked increase in the plasma level ALT and AST, the elevation of ALT and AST was dose-dependently suppressed by 2-DG at doses less than 500 mg/kg (Fig. 1A and B). Therefore, we used 500 mg/kg of 2-DG in the

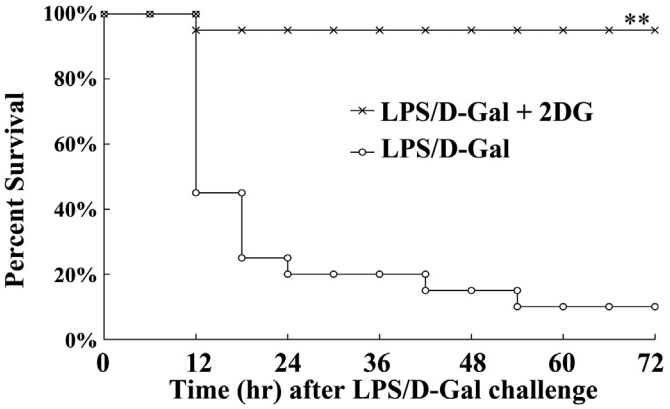

Fig. 2. 2-DG suppressed LPS/D-Gal-induced mortality. LPS/D-Gal-challenged mice were treated with vehicle or 2-DG (500 mg/kg). The mortality of the experimental animals was monitored and the percent survival rate was expressed as Kaplan–Meier survival curves (n = 20). $^{**}P < 0.01$, compared with the LPS/D-Gal group.

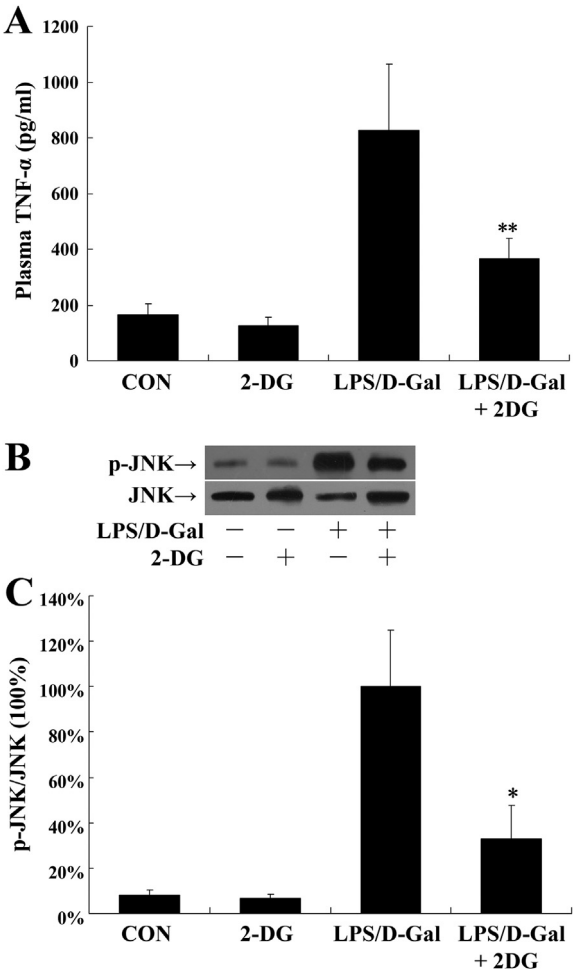

Fig. 3. 2-DG suppressed LPS/D-Gal-induced production of TNF-α and phosphorylation of JNK. Mice were treated with vehicle or 2-DG (500 mg/kg) in the absence or presence of LPS/D-Gal challenge. (A) The levels of TNF-α in plasma were determined by ELISA. Data were expressed as mean ± SD, n = 8. $^{**}P < 0.01$, compared with LPS/D-Gal group. (B) The levels of phosphrylated JNK (p-JNK) and total JNK (JNK) in liver were determined by immunoblot analysis. The bands of p-JNK and JNK were indicated by arrows. (C) The blots were scanned by densitometry and data presented as relative intensity units. Data were expressed as mean ± SD, n = 4. $^{*}P < 0.05$, compared with LPS/D-Gal group.

following experiments. Consistent with the suppressed elevation of liver enzymes, treatment with 2-DG at a dose of 500 mg/kg significantly alleviated LPS/D-Gal-induced congestion in the sinusoids and destruction of hepatic architecture (Fig. 1C). In the survival analysis, 90% of LPS/D-Gal-exposed mice died within 3 days, but the mortality rate dropped to 5% after 2-DG treatment (Fig. 2).

3.2. 2-DG suppressed LPS/D-Gal-induced pro-apoptotic signals

Liver injury induced by LPS/D-Gal largely depends on the upregulation of pro-apoptotic cytokine TNF-α [15]. Our resultant data indicated that the production of TNF-α in LPS/D-Gal-exposed mice was significantly suppressed by 2-DG (Fig. 3A). In addition, treatment with 2-DG also suppressed the phosphorylation of JNK (Fig. 3B and C), a pivotal pro-apoptotic signal in LPS/D-Gal model [16].

3.3. 2-DG suppressed LPS/D-Gal-induced activation of caspases and hepatocyte apoptosis

The activation of caspase cascade is crucial for the induction of apoptosis [17]. Our experiments found that 2-DG significantly suppressed the LPS/D-Gal-induced upregulation of caspase-3, -8 and -9 activities (Fig. 4A–C). Consistently, the increased level of cleaved caspase-3 in LPS/D-Gal-exposed mice was suppressed by 2-DG (Fig. 4D and E). The TUNEL assay indicated that the TUNEL-positive apoptotic hepatocytes presented in LPS/D-Gal-exposed mice decreased after 2-DG treatment (Fig. 4F).

4. Discussion

2-DG is a representative CR mimetic induces a remarkable phenotype of CR without the need to reduce calorie intake [4]. It was reported that treatment with 2-DG suppressed antitumor drug doxorubicin-induced cardiomyocyte death and induced by and protects retinal ganglion cells against excitotoxicity [18,19]. In experimental animal models, administration of 2-DG has shown protective benefits in mice with Parkinson's disease and ischemic brain damage [8,9]. In the present study, we found that 2-DG significantly alleviated LPS/D-Gal-induced lethal liver injury. The protective benefits were evidenced by suppressed elevation of aminotransferases in plasma, improved histopathological abnormality in liver and increased survival rate of the experimental animals.

LPS/D-Gal exposure can induce massive apoptosis of hepatocytes [15]. There were evidence suggesting that blockage of glycolysis by 2-DG might induce apoptosis or enhance apoptosis induced by other pro-apoptotic reagents [20,21]. However, the present study found that LPS/D-Gal-induced activation of caspase cascade and elevation of TUNEL-positive hepatocytes were significantly suppressed by 2-DG treatment, indicating 2-DG exert anti-apoptotic effects in LPS/D-Gal-induced liver injury. In agree with our findings, several studies also observed that 2-DG suppressed apoptosis induced by hydrogen peroxide, hypoxia or antitumor topoisomerase II inhibitor etoposide [22–24]. More interestingly, 2-DG enhanced the pharmacological effects of pro-apoptotic drugs cisplatin and staurosporine but hindered the pro-apoptotic effects

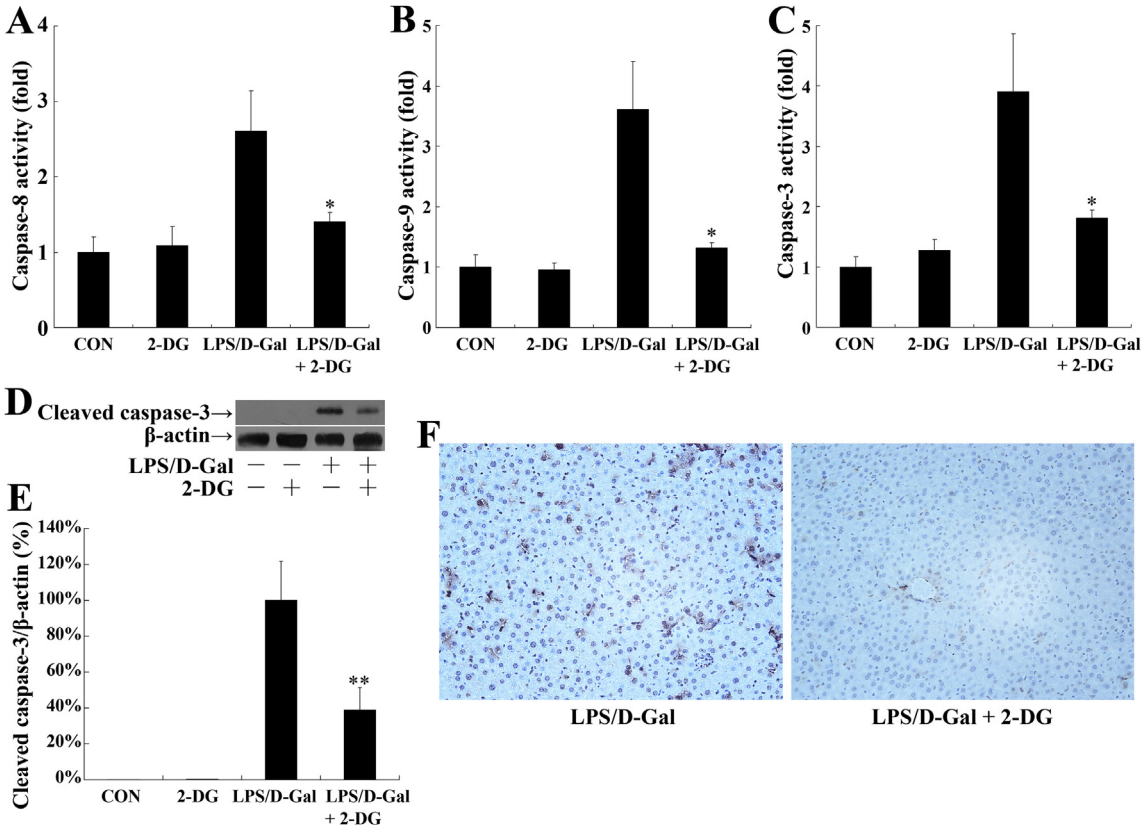

Fig. 4. 2-DG suppressed LPS/D-Gal-induced activation of caspase cascade and apoptosis of hepatocytes. Mice were treated with vehicle or 2-DG (500 mg/kg) in the absence or presence of LPS/D-Gal challenge. The activities of caspase-8 (A), caspase-9 (B) and caspase-3 (C) were determined. Data were expressed as mean ± SD, n = 8. *$P < 0.05$, compared with LPS/D-Gal group. (D) The levels of cleaved caspase-3 in liver were determined by immunoblot analysis. The bands of cleaved caspase-3 and β-actin were indicated by arrows. (E) The blots were scanned by densitometry and data presented as relative intensity units. Data were expressed as mean ± SD, n = 4. *$P < 0.05$, compared with LPS/D-Gal group. (F) The apoptotic cells were determined by TUNEL assay and the TUNEL-positive cells showed a dark-brown nucleus. Representative liver sections of each group are shown (original magnification 400).

of pyrimethamine, a pro-apoptotic antifolate drug [25]. Therefore, the modulatory effects of 2-DG on apoptosis seems context dependent.

The pro-inflammatory cytokine TNF-α is a major deleterious pro-apoptotic factor in LPS/D-Gal-induced liver damage because the TNF-α deficient mice and TNF-α receptor 1 (TNFR1) deficient mice were resistant to LPS/D-Gal-induced liver damage [26,27]. The binding of TNF-α to TNFR1 results in the recruitment of a series of intracellular proteins and the subsequent activation of caspase-8 [28]. The suppressive effect of 2-DG on TNF-α production has been found in LPS-stimulated monocytes [11]. The present study also found that treatment with 2-DG suppressed the induction of TNF-α in LPS/D-Gal-challenged mice. Consistently, the activity of caspase-8, the cleavage and activation of caspase-3 decreased after 2-DG treatment. In addition to the activation of extrinsic apoptosis pathway, TNFR1 induced apoptotic signal is closely interlinked with the intrinsic apoptosis pathway. The following activation of the mitochondria apoptosis pathway and the activation of caspase-9 greatly amplify the activation of caspase-3 and the ultimate induction of apoptosis [29]. Therefore, the present study also found that the activity of caspase-9 increased in LPS/D-Gal-challenged mice, but this alteration was suppressed by 2-DG.

JNK is a mitogen-activated protein kinase (MAPK) family member that is activated by diverse environmental stresses as well as inflammatory cytokines [30]. TNF-α signaling via TNFR1 is one of the best-studied pathways that lead to JNK activation [29]. Sustained activation of JNK pathway promotes activation of caspases and apoptosis of hepatocytes [31]. The pivotal roles of JNK signaling pathway in mediating LPS/D-Gal-induced liver injury have been confirmed in several studies based on the gene-knockout approaches or pharmacological reagents [16,32]. In the present study, LPS/D-Gal-induce phosphorylation of JNK was suppressed by 2-DG. In U937 cells, 2-DG also suppressed topoisomerase II inhibitor etoposide-induced apoptosis via inhibiting JNK signaling pathway [24]. Thus, inhibition of JNK-mediated pro-apoptotic signal might be associated with the anti-apoptosis effects of 2-DG.

Taken together, the present study has shown that the CR mimetic 2-DG effectively alleviated LPS/D-Gal-induced apoptotic liver damage and improved the survival rate of experimental animals, these effects were associated with reduced production of pro-apoptotic cytokine TNF-α and suppressed activation of JNK-mediated pro-apoptotic signal. Although the mechanisms underlying the protective benefits of 2-DG in LPS/D-Gal-induced lethal liver injury remain more intensive studies, the present experimental data suggested that the CR mimetic 2-DG could also provide beneficial effects in fulminant pathological process such as LPS/D-Gal-induced lethal liver injury.

Conflict of interest

The authors declare that they have no conflicts of interest concerning this article.

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