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Antidiabetic drug metformin alleviates endotoxin-induced fulminant liver injury in mice

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ABSTRACT

Metformin is a first-line antidiabetic drug in type 2 diabetes for its hypoglycemic activity, but recently researches also revealed the anti-inflammatory properties of metformin. In the present study, the pharmacological efficiency of metformin in lipopolysaccharide (LPS)-induced hepatic injury in D-galactosamine (D-Gal)-sensitized mice was investigated. We found that pretreatment with metformin significantly decreased serum ALT and AST levels in LPS/D-Gal-exposed mice. These were accomplished with improved histological alterations in liver sections, decreased myeloperoxidase (MPO) activity, reduced malondialdehyde (MDA) content in liver homogenates and increased survival rate of experimental animals. Metformin also markedly reduced hepatic TNF-α mRNA content and blood TNF-α level. Additional experiment showed that metformin significantly attenuated LPS/D-Gal-induced hepatic apoptosis as evidenced by decreased caspase activities in liver tissues and reduced number of TUNEL-positive cells in liver sections. Furthermore, therapeutic administration of metformin after LPS/D-Gal challenge also improved the survival rate of experimental animal. These results indicated that the hypoglycemic reagent metformin could also provide therapeutic benefits in endotoxin-induced hepatic injury, suggesting its pharmacological potential in inflammation-base disorders.

1. Introduction

Lipopolysaccharide (LPS), also known as endotoxin, is the major toxic component of Gram-negative bacteria [1]. LPS is widely presented in the digestive tracts of humans and is regarded as a potent and predominant microbial mediator that activates the pro-inflammatory cascade and plays a central role in the progression of various inflammation-associated diseases [2,3]. The liver plays an important physiological role in detoxifying LPS but liver is also a pivotal target of LPS-induced inflammatory injury [4,5]. LPS-induced liver injury in D-Galactosamine (D-Gal)-sensitized mice is a well-established experimental animal model that closely resembles human hepatitis in both morphological and functional features [6]. The model has provided new insights in understanding the pathogenesis of clinical hepatitis and developing novel liver-protective reagents.

Metformin is a commonly used antidiabetic drug [7]. Since metformin therapy appears to decrease the risk of diabetes-related endpoints and be associated with few adverse effects, it is suggested as the first-line pharmacological therapy of choice in type 2 diabetes [8,9]. Besides its hypoglycemic activity, a body of evidence also revealed the anti-inflammatory properties of metformin. For example, metformin dose-dependently inhibited the production of pro-inflammatory cytokines in LPS-treated mouse macrophages and rat primary microglial cells [10,11]. Metformin also attenuated the expression of various cell adhesion molecules in tumor necrosis factor α (TNF-α)-activated endothelial cells [12]. Recently, experimental animal studies also revealed the therapeutic benefits of metformin in encephalomyelitis [13]. Since LPS-induced inflammatory injury represents a central mechanism of inflammation-associated disorders, we questioned whether metformin could provide protective efficiency in LPS/D-Gal-induced hepatitis.

In the present study, liver injury was induced by LPS/D-Gal in mice, the prophylactic and therapeutic effects of metformin on tissue injury and animal mortality were evaluated. Since LPS/D-Gal-induced liver injury predominantly dependent on TNF-α-induced hepatocyte apoptosis [6], the effects of metformin on TNF-α expression as well as the subsequent activation of caspases and apoptosis of hepatocytes was also investigated.

2. Materials and methods

2.1. Materials

Metformin, lipopolysaccharide (LPS, from *Escherichia coli*, 055: B5) and D-Galactosamine (D-Gal) were purchased from Sigma–

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Aldrich Chemical (St Louis, MO). Caspase-3, caspase-8 and caspase-9 colorimetric assay kits were the products of Enzo Life Sciences (Farmingdale, USA). In Situ Cell Death Detection Kit was purchased from Roche (Indianapolis, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA) and myeloperoxidase (MPO) detection kits were obtained from BioVision (California, USA). Mouse TNF-α Enzyme-Linked Immunosorbent Assays (ELISA) kit was the product of R&D (Shanghai, PRC).

2.2. Animals

Male Balb/c mice weighing 20–25 g were obtained from the Experimental Animal Center of Chongqing Medical University. All animals were fed with a standard laboratory diet and water ad libitum. They 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, and acclimatized for at least l week before use. 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

Acute liver injury was induced in Balb/c mice with intraperitoneal injection of LPS (10 μg/kg) combined with D-Gal (700 mg/kg). To evaluate the prophylactic effects of metformin, vehicle or various dose of metformin (100 mg/kg, 200 mg/kg or 400 mg/kg, dissolved in normal saline, i.p.) was administrated 0.5 h prior to LPS/D-Gal challenge. We chose these doses based on previous reports and these doses of metformin have no detectable toxicity to experimental animal [13–16]. Then, the animals were returned to their cages and allowed food and water ad libitum.

To determine the effect of metformin on mortality from LPS/D-Gal-challenge, survival of experimental animals ($n = 25$ 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. To evaluate the effects of metformin on hepatic lesions, a separated set of animals were allocated and mice were sacrificed by decapitation at 1.5 h ($n = 8$ per group) or 6 h ($n = 8$ per group) after LPS/D-Gal challenge, blood samples were harvested for measuring TNF-α levels or determining aminotransferases (AST and ALT) activities respectively. The right lobe of the liver was fixed in formalin for morphological analysis. Remaining liver tissues were thoroughly washed in cold physiological saline and stored at −80 °C until required.

To evaluate the therapeutic effects of metformin, metformin (400 mg/kg, i.p.) was administrated at 1 h, 3 h or 6 h after LPS/D-Gal challenge ($n = 25$ each group) and survival of experimental animals 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. Determination of liver enzymes

Liver injury was assessed 6 h after LPS/D-Gal administration by measuring plasma enzyme activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) using corresponding detection kits according to the manufacturer's instructions (BioVision, California, USA).

2.5. Measurement of MDA content

Liver tissues were prepared to make 1:10 (w/v) homogenates, and the homogenates were then centrifuged at 12,000 g (4 °C) for 20 min to collect supernatants for determination of MDA concentrations. MDA was evaluated by the thiobarbituric acid-reactive substances method (TBARS) using a MDA detection kit according to the manufacturer's instructions (BioVision, California, USA). All values were normalized by the total protein concentration of the same sample.

2.6. Measurement of MPO activity

Liver tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The enzyme activity was determined spectrophotometrically using a MPO detection kit according to the manufacturer's instructions (BioVision, California, USA). MPO acitivity was assessed according to the absorbance measured at 450 nm and normalized by the total protein concentration of the same sample.

2.7. Quantitative real-time PCR

Total RNA was isolated from tissue samples using Trizol reagent. First-strand complementary DNA (cDNA) was synthesized using oligo-dT primer and the M-MLV reverse transcriptase. Quantitative PCR was performed with SYBR green PCR Master Mix. The following primers were used to amplify TNF-α cDNA: sense, 5′-CGGGCAGGTCTACTTTGGAG-3′, and antisense, 5′- CAGGTCACTGTCCCAGCATC-3′; β-actin cDNA: sense, 5′-CTGAGAGGGAAATCGTGCGT-3′, and antisense, 5′-CCACAGGATTCCATACCCAAGA-3′. PCR was performed using the following PCR condition: denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and elongation at 72 °C for 45 s. The mRNA levels of TNF-α were normalized by that of β-actin.

2.8. Enzyme-Linked Immunosorbent Assays (ELISA)

Blood sample was harvested and the concentrations of TNF-α were determined using ELISA kits according to the manufacturer's instructions (R&D, Shanghai, PRC).

2.9. Histological analysis

Formalin-fixed specimens were embedded in paraffin and stained with hematoxylin & eosin routinely for conventional morphological evaluation under light microscope (Olympus, Tokyo, Japan).

2.10. Caspase activities determination

Caspase-3, caspase-8 and caspase-9 protease activities in liver tissue were measured using caspase-3, caspase-8 and caspase-9 colorimetric assay kits (Enzo Life Sciences, Farmingdale, USA), respectively, according to the manufacturer's instructions. Briefly, after homogenization of liver tissue in cell lysis buffer, homogenates were centrifuged for 1 min at 10,000 g. The supernatant (100 μg protein) was incubated with Ac-DEVD-pNA and Ac-IETD-pNA substrates for caspase-3, caspase-8 and caspase-9, respectively, and reaction buffer for 90 min at 37°C. The activity caspase was assessed according to the absorbance measured at 405 nm and normalized by the total protein concentration of the same sample.

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

The assessment of liver apoptosis was performed using In Situ Cell Death Detection Kit according to the manufacturer's instructions (Roche, Indianapolis, USA). The terminal transferase reactions finally produced a dark-brown precipitate and then the sections were counterstained slightly with hematoxylin.

2.12. Statistical analysis

All data except survival 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. Survival statistics were