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Involvement of catalase in the protective benefits of metformin in mice with oxidative liver injury

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ABSTRACT

Metformin is a commonly used anti-diabetic drug with AMP-activated protein kinase (AMPK)-dependent hypoglycemic activities. Recent studies have revealed its anti-inflammatory and anti-oxidative properties. In the present study, the anti-oxidative potential of metformin and its potential mechanisms were investigated in a mouse model with carbon tetrachloride (CCl_4)-induced severe oxidative liver injury. Our results showed that treatment with metformin significantly attenuated CCl_4-induced elevation of serum aminotransferases and hepatic histological abnormalities. The alleviated liver injury was associated with decreased hepatic contents of oxidized glutathione (GSSG) and malondialdehyde (MDA). In addition, metformin treatment dose-dependently enhanced the activities of catalase (CAT) and decreased CCl_4-induced elevation of hepatic H_2O_2 levels, but it had no obvious effects on the protein level of CAT. We also found that metformin increased the level of phosphorylated AMP-activated protein kinase (AMPK), but treatment with AMPK activator AICAR had no obvious effects on CAT activity. A molecular docking analysis indicated that metformin might interact with CAT via hydrogen bonds. These data suggested that metformin effectively alleviated CCl_4-induced oxidative liver injury in mice and these hepatoprotective effects might be associated with CAT.

1. Introduction

Metformin, also known as dimethylbiguanide, is a commonly used anti-diabetic drug [1]. Because metformin therapy appears to decrease the risk of diabetes-related endpoints and is associated with few adverse effects, it is recommended as the first-line pharmacological therapy of choice in type 2 diabetes [2]. Although the precise pharmacological mechanisms of metformin have not been fully elucidated, there are cumulative evidence suggesting that the activation of AMP-activated protein kinase (AMPK) is associated with the hypoglycemic actions of metformin [3]. AMPK is a conserved serine/threonine protein kinase viewed as a fuel gauge monitoring cellular energy status. AMPK is a heterotrimeric protein consisting of a catalytic α-subunit and two regulatory subunits, β and γ. AMPK activation requires phosphorylation on Thr172 within the activation loop of the catalytic α-subunit [4]. Activated AMPK switches cells from an anabolic to a catabolic state, shutting down the ATP-consuming synthetic pathways and restoring energy balance [5].

In addition to its hypoglycemic activities, a body of evidence has also revealed the anti-inflammatory properties of metformin [6–7]. The mechanisms underling the anti-inflammatory effects of metformin involve both AMPK-dependent and AMPK-independent pathways [8–10]. Recent studies have suggested that metformin might have anti-oxidative effects both in vivo and in vitro [11–13]. For example, treatment with metformin dose-dependently restored the depletion of glutathione (GSH) and the induction of reactive oxygen species (ROS) levels in rats with streptozotocin-induced diabetic nephropathy, which might contribute to the nephroprotective effect of metformin in diabetes mellitus [11]. In human aortic endothelial cells, palmitic acid-induced increases of intracellular ROS levels were reduced by metformin

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in a dose-dependent manner [12]. In addition, treatment of human peripheral blood lymphocytes with metformin significantly decreased cumene hydroperoxide-induced lipid peroxidation [13].

Free radicals generated under physiological conditions play an important role as regulatory mediators in signaling processes at moderate concentrations [14]. However, at high concentrations, free radicals are hazardous for living organisms and damage all major cellular constituents, which is termed oxidative stress and is involved in various pathological processes [15]. The catalytic removal of free radicals by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) represents the major defensive mechanisms against free radical-induced oxidative damage [16]. However, oxidative stress results in toxicity in mice in which the generating ROS exceed the capacity for their removal [15].

Our recent studies have found that metformin attenuated endotoxin-induced fulminant hepatitis in mice [17], suggesting metformin might have hepatoprotective effects via suppression of endotoxin-induced inflammation. In addition to endotoxin, carbon tetrachloride (CCl_4) is another commonly used hepatotoxin in laboratory researches. CCl_4 is metabolized by P450 in the hepatocytes to produce the highly reactive free radicals, which lead to severe oxidative liver injury in many species rat, mice, non-human primates and human [18]. CCl_4-induced liver injury in mice is widely used in investigating the mechanisms of clinical hepatitis and our group have previously used mouse model to investigate the pathogenesis of CCl_4-induced liver injury [19–20]. In the present studies, the hepatoprotective benefits of metformin were further investigated in a mouse model with CCl_4-induced liver injury.

2. Materials and methods

2.1. Animals

BALB/c mice weighing 18–22 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. The mice were maintained in a controlled environment at a temperature of 20–25 °Cand 50 ± 5% relative humidity under a 12-h dark/light cycle. The mice were allowed to acclimatize for at least 1 week prior to use. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Chongqing Medical University.

2.2. Reagents

Metformin, AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranosid (AICAR) and CAT inhibitor aminotriazole were produced by Sigma (St. Louis, MO, USA). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and myeloperoxidase (MPO) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx) and glutathione reductase (GR), hydrogen peroxide (H_2O_2), malondialdehyde (MDA) and glutathione (GSH) assay kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Enzyme-linked immunosorbent assay (ELISA) kits for detecting mouse tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were purchased from NeoBioscience Technology Company (Shenzhen, China). CCl_4 was purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). Rabbit anti-mouse CAT, AMPK, phosphorylated AMPK (p-AMPK) and β-actin antibody were purchased from Abcam (Cambridge, UK). The BCA protein assay kit, horseradish peroxidase-conjugated goat anti-rabbit antibody and enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL).

2.3. CCl_4-induced liver injury

Acute oxidative liver injury was induced in mice with an intraperitoneal injection of CCl_4 (50 μl/kg, i.p. dissolved in olive oil), vehicle or various doses of metformin (100 mg/kg, 200 mg/kg or 400 mg/kg, dissolved in normal saline, i.p.) was administered 0.5 h prior to the CCl_4 challenge ($n = 8$ per group). To investigate the potential mechanisms, AMPK activator AICAR (500 mg/kg, dissolved in normal saline, i.p.) or CAT inhibitor aminotriazole (500 mg/kg, dissolved in normal saline, i.p.) was administered 0.5 h prior to the vehicle or CCl_4 treatment. The dose of AICAR and aminotriazole was determined based on previous reports [21,22]. The mice were then returned to their cages and provided food and water ad libitum. Twenty-four hours after CCl_4 insult, massive hepatocyte necrosis is manifest [18,23], and the mice were anesthetized and sacrificed, and blood samples were obtained for measuring cytokine (TNF-α, IL-6) levels or to determine the aminotransferases (AST and ALT) activities, respectively. The right lobe of the liver was fixed in formalin for morphological analysis. The remaining liver tissues were thoroughly washed in cold physiological saline and stored at −80 °C for the MPO and antioxidant enzymes assays and to determine the MDA and H_2O_2 contents.

2.4. Determination of liver enzymes

The serum ALT and AST activities were determined by using ALT and AST detection kits according to the manufacturer's instructions to assess the extent of CCl_4-induced acute liver injury.

2.5. Histological analysis

Formalin-fixed specimens were embedded in paraffin and stained with hematoxylin & eosin routinely for conventional morphological evaluation under light microscopy.

2.6. MPO activity assay

Frozen liver tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. The enzymatic activity was determined using a MPO detection kit according to the manufacturer's instructions. MPO activity was assessed according to the absorbance measured at 450 nm and normalized by the total protein concentration of the same sample.

2.7. Measurement of MDA and GSH content

Liver tissues were prepared to obtain 1:10 (w/v) homogenates, and the homogenates were then centrifuged at 10,000×g (4 °C) for 10 min to collect supernatants to determine the MDA and oxidized glutathione (GSSG) concentrations using colorimetric assay kits according to the manufacturer's instructions. All values were normalized according to the total protein concentration of the same sample.

2.8. H_2O_2 content determination

The H_2O_2 content in liver tissue was determined using the H_2O_2 assay kit according to the manufacturer's instructions. H_2O_2 can oxidize Fe^{2+} to Fe^{3+}. Fe^{3+} can subsequently react with xylenol orange, resulting in a colorimetric reaction that can be further detected using a spectrometer. The concentration of H_2O_2 was calculated according to the standard concentration curve and normalized against the total protein concentration of the same sample.