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Betulinic acid attenuates dexamethasone-induced oxidative damage through the JNK-P38 MAPK signaling pathway in mice

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ABSTRACT

Dexamethasone (Dex), a potent anti-inflammatory/immunosuppressive agent, has been shown to induce oxidative stress. Betulinic acid (BA) is a pentacyclic lupane triterpene with a potent antioxidant activity. The aim of this study was to investigate the ameliorative effect and underlying mechanisms of BA on Dex-induced oxidative damage. Mice were pretreated with BA orally (0, 0.25, 0.5, and 1.0 mg/kg) daily for 14 days, and then a single dose of Dex (25 mg/kg body weight) was administered intraperitoneally 8 h after the last administration of BA to induce oxidative stress. BA pretreatment significantly alleviated Dex-induced changes of blood biochemical indices, increased the total antioxidant capacity (T-AOC), the activity of superoxide dismutase (SOD), and the ability of inhibiting hydroxyl radical (AIHR), reduced the level of malondialdehyde (MDA) in serum. Moreover, BA pretreatment enhanced the T-AOC, AIHR and the activity of peroxidase (POD) in liver, spleen and thymus. Concomitant with these biochemical parameters, BA pretreatment significantly reduced gene and protein expressions of apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK) and P38 mitogen-activated protein kinase (P38 MAPK) in the lymphatic organs of Dex-treated mice. BA was found to effectively attenuate Dex-induced oxidative damage. These protective effects may be mediated in part through the JNK-P38 MAPK signaling transduction pathway and BA may be a potential therapeutic agent due to its anti-oxidative properties.

1. Introduction

Dexamethasone (Dex), a long-acting glucocorticoid (GC), is widely used as an anti-inflammatory agent. However, prolonged administration and/or overdose of Dex may result in serious side effects, including osteoporosis and immunosuppression [1,2]. In previous experiments, it has been shown that Dex disrupted the redox balance in poultry [3,4]. Increasing evidence has demonstrated that prolonged exposure to a high dosage of Dex may lead to an increase in reactive oxygen species (ROS) production that directly resulted in mitochondrial dysfunction, decreased cellular energy yield, elevated cytosolic calcium and increased mitochondrial permeability. These effects deepen oxidative stress culminating in apoptosis as shown in a number of cell types [5–7]. Oxidative stress disrupts intracellular redox homeostasis, causes irreversible oxidative modifications of lipid, protein, or DNA leads to various pathologies [8]. On the other hand, it was found that some antioxidants from natural sources such as plant phenols, terpene and chitosan can effectively scavenge free radicals and ROS, thus reducing or eliminating oxidative stress.

Betulinic acid (BA), a naturally occurring pentacyclic triterpene, is a compound found throughout the plant kingdom. Accumulating experimental evidence has revealed that BA has a variety of biological activities including anti-inflammatory, antitumor, anti-HIV, antibacterial, anti-fibrotic, antimalarial, antiangiogenic, anti-ulcer and antioxidant properties [9–12]. Moreover, due to low toxicity and high safety index, BA has been considered to be a promising candidate for clinical application as a therapeutic agent [13]. Some experiments have shown that BA has renoprotective effects by regulating c-Jun N-terminal kinase (JNK), P38 and extracellular signal-regulated kinase (ERK) in mitogen-activated protein kinase (MAPK) signaling transduction pathway [14–16]. Previous studies reported that BA possessed protective properties against Dex-induced cell apoptosis by reducing oxidative

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stress through the regulation of the mitochondrial mediated signaling pathway. These findings suggest a potential mechanism for BA-elicited immunomodulation [2]. JNK-P38 MAPK signaling pathway was found to play an important role in mediating many of the oxidative effects associated with apoptosis. However, it was not clear whether BA's protective effect against Dex-induced oxidative stress involved JNK-P38 MAPK. Thus, the aim of the current study was to explore the mechanism of BA on oxidative damage induced by Dex through the signaling transduction pathway of JNK-P38 MAPK.

2. Materials and methods

2.1. Reagents and chemicals

Dex was purchased from Puyang City Huiyuan Pharmaceutical Co., Ltd. (Puyang, Henan, China). Alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate transaminase (AST), total protein (TP), albumin (ALB), total cholesterols (TC), triacylglycerides (TG), total bilirubin (T-Bil) and calcium ions (Ca^{2+}) assay kits were purchased from Shenzhen Mindray Bio-Medical Electronics Co., Ltd (Shenzhen, China). The total antioxidant capacity (T-AOC), ability of inhibiting hydroxyl radical (AIHR), superoxide dismutase (SOD), malondialdehyde (MDA) and peroxidase (POD) assay kits were purchased from Nanjing Jiancheng Biotech (Nanjing, Jiangsu, China). Trizol was purchased from Life Technologies (Grand Island, NY, USA), while the primescript RTreagent Kit and SYBR Green I fluorescent dyes were purchased from Takara (Shiga, Japan). Primary antibodies for ASK1, JNK, P38, β-actin and secondary antibody were purchased from Cell Signaling Technology, Inc (Danvers, MA, USA). Enhanced chemiluminescence (ECL) reagent was purchased from Jiangsu Keygen Biotech Corp., Ltd (Beyotime, Jiangsu, China). BA was semi-synthesized as previously described with a purity of 96.5% as confirmed by high performance liquid chromatography–mass spectrometry (HPLC–MS) [17,18].

2.2. Animals and experiment designs

A total of 64 male Kunming mice were purchased from Hunan Silaikejingda Laboratory Animal Co., Ltd. (Changsha, Hunan, China). The animal experiments were complied with the Animal Care and Use Guidelines of China and approved by the Animal Care Committee of Hunan Agricultural University. The dosage of BA and duration of treatment were proposed based on previous studies, and the dosage of Dex was selected according to the preliminary experiment from our laboratory as well as the previous studies [17,19]. Mice were randomly divided into 8 groups: the control group, the low, medium and high dose of BA groups (BA at the dose of 0.25, 0.5, and 1.0 mg/kg body weight, respectively), the Dex group (25 mg/kg body weight), the low, medium and high dose of BA with Dex groups. BA was administered orally to mice with 1% starch jelly daily for 14 days, while mice in the control and Dex groups were given an equivalent amount of 1% starch jelly only. Mice were given a single dose of Dex intraperitoneally 8 h after the last administration of BA, while the control and the low, medium and high dose of BA groups received an equal volume of sterile saline.

Fifteen hours after Dex administration, blood samples were collected in tubes (Eppendorf, Germany) without anticoagulant by venous puncture with the mice under light anesthesia induced by diethyl ether (Sinopharm Chemical Reagent, Shanghai, China). Serum was collected by centrifugation (Z383K Universal High Speed Centrifuge, Hermle labortechnik, Germany) at 3000 × *g* for 10 min at 4 ℃ and frozen at −80 ℃ until analysis. The liver, spleen and thymus were quickly excised and weighed after all mice were sacrificed by cervical dislocation. The organs were washed in a cold 0.9% NaCl solution and minced thoroughly on ice. A 10% (w/v) homogenate (Tenbroeck tissue grinders; Wheaton, USA) was prepared in 10 mM phosphate buffer (pH 7.4) and centrifuged (Z383K Universal High Speed Centrifuge; Hermle labortechnik) at 2500 × *g* for 15 min at 4 ℃. The resulting supernatant was collected and stored at −80 ℃ until analysis.

2.3. Evaluation of blood biochemical parameters

The activities of ALP, ALT and AST as well as the contents of TP, ALB, TC, TG, T-Bil and Ca^{2+} in serum were measured using Mindray commercial kits and a Mindray BS-200 automatic biochemistry analyzer (Shenzhen Mindray Bio-Medical Electronics, China). The activities of ALT, ALP and AST were expressed as units per liter of serum (U/L). The contents of TC, TG and Ca^{2+} were expressed as mmol per liter of serum (mmol/L). The levels of TP and ALB were expressed as grams per liter of serum (g/L) while the content of T-Bil was expressed as μmol per liter of serum (μmol/L).

2.4. Evaluation of antioxidative capacity

T-AOC, AIHR and SOD activities were expressed as units per milliliter of serum (U/mL) while MDA content was expressed as nmol per milliliter of serum (nmol/mL). T-AOC, AIHR and POD levels in liver, spleen and thymus were expressed as units per milligram protein (U/mg).

2.5. Quantitative real-time polymerase chain reaction (RT-PCR)

The mRNA expression of apoptotic proteins in immune organs of mice were evaluated by RT-PCR. The spleen and thymus were grinded, and the total RNA was isolated with Trizol reagent. The RNA was reverse-transcribed into cDNA by using primescript RTreagent Kit. Real-time PCR was performed on a Rotor Gene 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Green I fluorescent dye kit. Quantitative measurements were determined using the $2^{-\triangle\triangle Ct}$ method, and the expression of β-actin was used as the internal control. Primer sequences were as follows: β-actin forward: 5′-CATCC GTAAAGACCTCTATGCC AAC-3′ and reverse: 5′-ATGGAGCCACCGATC CACA-3′; ASK1 forward: 5′-CCTGTG TGCCACCTGAACTCTC-3′ and reverse 5′-ACTAGCGTGTAATCCTCAGCCAGAA-3′; JNK forward: 5′-TCT CCAGCACCCATACATCAA-3′ and reverse 5′-CCCTCTCATCTA ACTGCT TGTCC-3′; P38 forward 5′-CGTTCTGAGCCAGGCAAGTG-3′ and reverse 5′-CAAACAGCTTGCTCCTGAAGTGA-3′.

2.6. Western blotting analysis

The frozen spleen were homogenized in lysis buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) at 4 ℃. After centrifugation for 5 min at 12,000 g and the supernatants were collected for Western blot. Samples were run in a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h at room temperature, washed in Tris-buffered saline with Tween 20 (TBST) and subsequently incubated with gentle shaking overnight at 4 ℃ with primary antibodies against ASK1, JNK, P38 and β-actin in diluent buffer (0.2% gelatin in TBST). After washing three times with TBST, membranes were incubated with secondary antibody for 1 h at room temperature and then washed three times in TBST buffer. The protein bands were detected by ECL reagent. The protein expression levels of ASK1, JNK, P38, and β-actin were determined by densitometry (Bio-Rad, Hercules, USA) and analyzed with an Image-Pro plus 6.0 software (Bio-Rad, Hercules, USA).