Postprint: Mechanistic Study of Betulinic Acid Against Dexamethasone-Induced Oxidative Stress in Mice

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Abstract

This study aimed to investigate the protective effects and mechanisms of betulinic acid (BA) on dexamethasone (Dex)-induced oxidative stress in mice. Forty healthy male Kunming mice were randomly divided into five groups: control (NC) group, Dex group, 0.25 mg/kg BA group, 0.50 mg/kg BA group, and 1.00 mg/kg BA group. Mice in the NC and Dex groups were intragastrically administered 1% soluble starch solution, while the remaining groups received BA at different doses. After 14 consecutive days, except for the control group which was injected with normal saline, the other four groups were intraperitoneally injected with Dex (25 mg/kg) to induce an oxidative stress model. The total antioxidant capacity (T-AOC), hydroxyl radical scavenging capacity, and peroxidase (POD) activity in the liver, spleen, and thymus of mice in each group were measured. Reverse transcription (RT)-PCR was used to detect the mRNA expression levels of apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and P38 in the mitogen-activated protein kinase (MAPK) signaling pathway in the spleen and thymus, while Western blotting was employed to determine the protein expression levels of ASK1, JNK, and P38 in the spleen MAPK signaling pathway. The results showed: 1) Compared with the NC group, the liver T-AOC, hydroxyl radical scavenging capacity, spleen POD activity, thymus T-AOC, and hydroxyl radical scavenging capacity in the Dex group were extremely significantly decreased ($P<0.01$). Compared with the Dex group, the liver T-AOC, hydroxyl radical scavenging capacity, and POD activity in the 0.50 and 1.00 mg/kg BA groups were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$). The spleen T-AOC in the 0.50 mg/kg BA group, the spleen hydroxyl radical scavenging capacity in the 0.50 and 1.00 mg/kg BA groups, and the spleen POD activity in the 0.25 and 1.00 mg/kg BA groups were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$). The thymus T-AOC, hydroxyl radical scavenging capacity, and POD

activity in the 0.50 and 1.00 mg/kg BA groups were significantly or extremely significantly increased ($P<0.05$ or $P<0.01$). 2) Compared with the NC group, the mRNA expression levels of ASK1, JNK, and P38 in the spleen and thymus of the Dex group were extremely significantly increased ($P<0.01$). Compared with the Dex group, the mRNA expression levels of ASK1, JNK, and P38 in the spleen and thymus of the 0.50 and 1.00 mg/kg BA groups were significantly or extremely significantly decreased ($P<0.05$ or $P<0.01$). 3) Compared with the NC group, the protein expression levels of JNK and P38 in the spleen of the Dex group were extremely significantly increased ($P<0.01$). Compared with the Dex group, the protein expression level of ASK1 in the spleen of the 0.50 mg/kg BA group was significantly decreased ($P<0.05$), while the protein expression levels of JNK and P38 in the spleen of the 0.25, 0.50, and 1.00 mg/kg BA groups were significantly or extremely significantly decreased ($P<0.05$ or $P<0.01$). These results demonstrate that BA pretreatment enhanced the T-AOC, hydroxyl radical scavenging capacity, and POD activity in the liver, spleen, and thymus of Dex-stressed mice, and decreased the mRNA expression levels of ASK1, JNK, and P38 in the MAPK signaling pathway of the spleen and thymus, as well as the protein expression levels of ASK1, JNK, and P38 in the spleen. BA exhibits a preventive protective effect against oxidative damage caused by Dex, and this protective effect is associated with the JNK-P38 MAPK signaling pathway.

Full Text

Mechanism of Betulinic Acid on Dexamethasone-Induced Oxidative Stress in Mice

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Abstract

This study investigated the protective effects and underlying mechanism of betulinic acid (BA) against dexamethasone (Dex)-induced oxidative stress in mice. Forty healthy male Kunming mice were randomly divided into five groups: control (NC), Dex, 0.25 mg/kg BA, 0.50 mg/kg BA, and 1.00 mg/kg BA groups. The NC and Dex groups were administered 1% soluble starch solution orally, while the other groups received different doses of BA orally for 14 days. On day 14, all groups except the NC group were intraperitoneally injected with Dex (25 mg/kg) to induce oxidative stress, while the NC group received saline. Total antioxidant capacity (T-AOC), hydroxyl radical scavenging capacity, and

peroxidase (POD) activity were measured in liver, spleen, and thymus tissues. Reverse transcription (RT)-PCR was used to quantify mRNA expression of apoptosis signal-regulating kinase 1 (ASK1), c-Jun N-terminal kinase (JNK), and P38 in the MAPK signaling pathway of spleen and thymus, while Western blot was employed to detect protein expression of ASK1, JNK, and P38 in spleen.

The results demonstrated: (1) Compared with the NC group, the Dex group showed extremely significant decreases in hepatic T-AOC and hydroxyl radical scavenging capacity ($P<0.01$), splenic POD activity ($P<0.01$), and thymic T-AOC and hydroxyl radical scavenging capacity ($P<0.01$). Compared with the Dex group, the 0.50 and 1.00 mg/kg BA groups exhibited significant or extremely significant increases in hepatic T-AOC, hydroxyl radical scavenging capacity, and POD activity ($P<0.05$ or $P<0.01$). The 0.50 mg/kg BA group showed significantly increased splenic T-AOC ($P<0.01$), while the 0.50 and 1.00 mg/kg BA groups demonstrated significantly enhanced splenic hydroxyl radical scavenging capacity ($P<0.05$). Splenic POD activity was significantly elevated in the 0.25 and 1.00 mg/kg BA groups ($P<0.05$). Additionally, the 0.50 and 1.00 mg/kg BA groups displayed significant or extremely significant increases in thymic T-AOC, hydroxyl radical scavenging capacity, and POD activity ($P<0.05$ or $P<0.01$).

(2) Compared with the NC group, the Dex group exhibited extremely significant upregulation of ASK1, JNK, and P38 mRNA expression in both spleen and thymus ($P<0.01$). In contrast, the 0.50 and 1.00 mg/kg BA groups showed significant or extremely significant downregulation of ASK1, JNK, and P38 mRNA expression in these organs ($P<0.05$ or $P<0.01$).

(3) Compared with the NC group, the Dex group displayed extremely significant increases in splenic JNK and P38 protein expression ($P<0.01$). Compared with the Dex group, the 0.50 mg/kg BA group showed significantly reduced ASK1 protein expression ($P<0.05$), while the 0.25, 0.50, and 1.00 mg/kg BA groups exhibited significant or extremely significant decreases in JNK and P38 protein expression ($P<0.05$ or $P<0.01$).

In summary, BA pretreatment enhanced T-AOC, hydroxyl radical scavenging capacity, and POD activity in liver, spleen, and thymus of Dex-challenged mice, while reducing mRNA expression of ASK1, JNK, and P38 in spleen and thymus and protein expression of these markers in spleen. These findings indicate that BA provides preventive protection against Dex-induced oxidative damage through modulation of the JNK-P38 MAPK signaling pathway.

Keywords: betulinic acid; dexamethasone; oxidative stress; MAPK

Introduction

Oxidative stress refers to a series of adaptive responses resulting from the imbalance between oxidant and antioxidant systems in the body. In livestock production, oxidative stress adversely affects animal health by reducing product quality, impairing reproductive performance in breeding animals, decreasing survival rates and increasing morbidity in young animals, and causing neurotoxicity [1-3]. Research has shown that certain antioxidants can scavenge free radicals, mitigate free radical-induced damage, improve production and reproductive performance, enhance immunity, increase survival rates of young animals, and improve product quality [3]. Natural antioxidants have become a research focus due to their safety and low toxicity.

Betulinic acid (BA) is a plant-derived pentacyclic triterpenoid with diverse biological activities including antioxidant, immunomodulatory, antitumor, anti-inflammatory, antimicrobial, and antiparasitic effects [4-6]. Our previous studies demonstrated that BA provides preventive protection against Dex-induced oxidative stress by enhancing superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, inhibiting free radical and lipid peroxide production, and thereby improving antioxidant capacity [7-10]. Further research revealed that BA blocks reactive oxygen species (ROS)-induced changes in mitochondrial membrane permeability, effectively scavenges ROS-mediated cellular damage, reduces lymphocyte apoptosis through mitochondrial signaling pathways, and protects lymphocytes via intracellular endogenous pathways [7-10]. The mitogen-activated protein kinase (MAPK) signaling pathway is intimately associated with oxidative stress. Building upon our previous findings [10], this study employed a Dex-induced oxidative stress model to investigate whether BA could alleviate oxidative stress by modulating the c-Jun N-terminal kinase (JNK)-P38 MAPK signaling pathway, thereby elucidating the molecular mechanisms underlying BA' s antioxidant effects.

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1. Materials and Methods

1.1 Experimental Reagents

Betulinic acid was prepared as previously described [10,11] with a purity of 96.53% as determined by high-performance liquid chromatography (HPLC). Dexamethasone sodium phosphate injection was purchased from Puyang Huiyuan Pharmaceutical Co., Ltd. Assay kits for total antioxidant capacity (T-AOC), hydroxyl radical scavenging capacity, and peroxidase (POD) activity were obtained from Nanjing Jiancheng Bioengineering Institute. Trizol reagent was from Life Technologies. Reverse transcription (RT)-PCR kits, quantitative real-time (qRT)-PCR primers, and SYBR Green were from Takara. Bicinchoninic acid (BCA) protein assay kits were from Lianke Biotechnology. Western and IP cell lysis buffer was from Beyotime Biotechnology. Antibodies against ASK1, JNK, and P38 were from Cell Signaling Technology.

Horseradish peroxidase-conjugated goat anti-rabbit IgG was from KPL (USA). ECL detection kits were from KeyGen Biotech. SDS-PAGE protein standards were from TransGen Biotech.

1.2 Experimental Animals and Diet

Forty healthy male Kunming mice (4 weeks old, SPF grade) weighing (20±2) g were used. The basal diet was a standard mouse growth formula provided by Hunan Slack Jingda Laboratory Animal Co., Ltd. The main ingredients included wheat, corn, soybean oil, bran, soybean meal, fish meal, maltodextrin, yeast, grass meal, and premix. The primary nutrient levels (air-dry basis) were: crude protein 20.50%, ether extract 4.62%, calcium 1.23%, phosphorus 0.91%, lysine 1.30%, and methionine + cysteine 0.68%.

1.3 Experimental Groups and Treatment

Mice were housed in an animal facility at 22-25°C with 50-70% relative humidity. After one week of acclimation, animals were randomly divided into five groups (n=8 each): normal control (NC), Dex, 0.25 mg/kg BA, 0.50 mg/kg BA, and 1.00 mg/kg BA. Different doses of BA were suspended in 1% soluble starch and administered orally once daily at 09:00 at a volume of 0.01 mL/g body weight. The NC and Dex groups received equivalent volumes of 1% soluble starch for 14 consecutive days. On day 14 at 17:00, all groups except the NC group were intraperitoneally injected with Dex (25 mg/kg) to induce oxidative stress, while the NC group received saline. After 15 hours of fasting (with free access to water), blood was collected via orbital bleeding, and mice were euthanized by cervical dislocation. Liver, spleen, and thymus were harvested, weighed, rinsed with saline, blotted dry, and homogenized in ice-cold saline at a 1:9 (w/v) ratio to prepare 10% tissue homogenates. Homogenates were centrifuged at 2,500 rpm for 10 minutes, and supernatants were collected for analysis.

1.4 Detection Methods

1.4.1 Detection of T-AOC, Hydroxyl Radical Scavenging Capacity, and POD Activity T-AOC, hydroxyl radical scavenging capacity, and POD activity in liver, spleen, and thymus were measured using commercial assay kits according to the manufacturer' s instructions. T-AOC was determined by colorimetric assay, hydroxyl radical scavenging capacity by Fenton reaction assay, and POD activity by colorimetric method.

1.4.2 qRT-PCR Detection of MAPK Signaling Pathway Genes Primer Design and Synthesis: Primers were designed based on GenBank sequences for ASK1 (NM_008580.4), JNK (NM_001310452), P38 (NM_001168508.1), and -actin (NM_007393). The primer sequences were: ASK1 forward 5 -CCTGTGTGCCACCTGAACTCTC-3 and reverse 5 -ACTAGCGTGTAATCCTCAGCCAGAA-3 ; JNK forward 5 -

TCTCCAGCACCCATACATCAA-3 and reverse 5 -CCCTCTCATCTAACTGCTTGTCC-3 ; P38 forward 5 -CGTTCTGAGCCAGGCAAGTG-3 and reverse 5 -CAAACAGCTTGCTCCTGAAGTGA-3 ; -actin forward 5 -CATCCGTAAAGACCTCTATGCCAAC-3 and reverse 5 -ATGGAGCCACCGATCCACA-3 . Primers were synthesized by Guangzhou Ruizhen Biotechnology Co., Ltd.

RNA Extraction and qRT-PCR: Total RNA was extracted from 0.05-0.10 g tissue samples using Trizol reagent. RNA concentration and purity were measured using a UV spectrophotometer, and integrity was verified by 0.8% agarose gel electrophoresis. cDNA was synthesized using a reverse transcription kit. qRT-PCR was performed using SYBR Green I and a Rotor-Gene 7300 Real-Time PCR System under the following conditions: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Melting and amplification curves were recorded for target and reference genes. Ct values for -actin and target genes were obtained, and relative quantification was performed using the 2^(-ΔΔCt) method.

1.4.3 Western Blot Detection of MAPK Signaling Pathway Proteins

Tissue samples were lysed in Western/IP lysis buffer, homogenized, and centrifuged at 12,000 rpm for 5 minutes at 4°C. Protein concentration was determined by BCA assay. Samples were denatured by boiling, separated by 10% SDS-PAGE, and transferred to PVDF membranes. Membranes were blocked with 0.2% gelatin in TBST for 1 hour, then incubated overnight at 4°C with primary antibodies against ASK1, JNK, P38, and -actin diluted in blocking buffer. After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 37°C for 1 hour. Protein bands were visualized using ECL reagent and imaged with a gel documentation system. Band intensities were quantified using ImageJ software, normalized to -actin, and expressed relative to the NC group (set as 1).

1.5 Statistical Analysis

Data were analyzed using SPSS 17.0 software. Comparisons among multiple groups were performed by one-way ANOVA followed by q-test. Results are expressed as mean ± standard deviation. $P<0.05$ was considered statistically significant.

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2. Results

2.1 Effects of BA on Liver T-AOC, Hydroxyl Radical Scavenging Capacity, and POD Activity

As shown in Table 1 , compared with the NC group, the Dex group exhibited extremely significant decreases in hepatic T-AOC and hydroxyl radical scavenging capacity ($P<0.01$), while hepatic POD activity decreased without statistical

significance (P>0.05). Compared with the Dex group, the 0.25, 0.50, and 1.00 mg/kg BA groups showed significant or extremely significant increases in hepatic T-AOC and hydroxyl radical scavenging capacity (P<0.05 or P<0.01). Hepatic POD activity was significantly elevated in the 0.50 and 1.00 mg/kg BA groups (P<0.05 and P<0.01, respectively).

Table 1 Effects of BA on T-AOC, hydroxyl radical scavenging capacity, and POD activity in mouse liver

Group	T-AOC (U/mg prot)	Hydroxyl radical scavenging capacity (U/mg prot)	POD (U/mg prot)
NC	0.35±0.04	29.78±4.97	1.12±0.09
Dex	0.22±0.08^{b}	19.31±5.00^{b}	1.06±0.10
0.25 mg/kg BA	0.45±0.10^{d}	30.45±4.63^{d}	1.08±0.07
0.50 mg/kg BA	0.65±0.07^{d}	29.08±5.98^{d}	1.19±0.13^{c}
1.00 mg/kg BA	0.48±0.10^{d}	31.28±3.09^{d}	1.21±0.05^{d}

^{a}P<0.05, ^{b}P<0.01 vs. control group; ^{c}P<0.05, ^{d}P<0.01 vs. Dex group. The same applies below.

2.2 Effects of BA on Spleen T-AOC, Hydroxyl Radical Scavenging Capacity, and POD Activity

As shown in Table 2 , compared with the NC group, the Dex group showed decreased splenic T-AOC and hydroxyl radical scavenging capacity without statistical significance (P>0.05), while splenic POD activity decreased extremely significantly (P<0.01). Compared with the Dex group, the 0.50 mg/kg BA group exhibited extremely significant elevation in splenic T-AOC (P<0.01). The 0.50 and 1.00 mg/kg BA groups showed significant increases in splenic hydroxyl radical scavenging capacity (P<0.05), while the 0.25 and 1.00 mg/kg BA groups demonstrated significant elevation in splenic POD activity (P<0.05).

Table 2 Effects of BA on T-AOC, hydroxyl radical scavenging capacity, and POD activity in mouse spleen

Group	T-AOC (U/mg prot)	Hydroxyl radical scavenging capacity (U/mg prot)	POD (U/mg prot)
NC	0.88±0.09	21.39±1.76	4.33±0.54
Dex	0.82±0.07	18.56±2.34	3.62±0.29^{b}

Group	T-AOC (U/mg prot)	Hydroxyl radical scavenging capacity (U/mg prot)	POD (U/mg prot)
0.25 mg/kg BA	0.94±0.18	21.37±1.77	4.20±0.46^{c}
0.50 mg/kg BA	1.03±0.14^{d}	22.10±1.92^{c}	4.07±0.64
1.00 mg/kg BA	0.89±0.23	21.77±1.76^{c}	4.21±0.22^{c}

2.3 Effects of BA on Thymus T-AOC, Hydroxyl Radical Scavenging Capacity, and POD Activity

As shown in Table 3 , compared with the NC group, the Dex group exhibited extremely significant decreases in thymic T-AOC and hydroxyl radical scavenging capacity ($P<0.01$), along with a significant reduction in thymic POD activity ($P<0.05$). Compared with the Dex group, the 0.50 and 1.00 mg/kg BA groups showed significant or extremely significant increases in thymic T-AOC, hydroxyl radical scavenging capacity, and POD activity ($P<0.05$ or $P<0.01$).

Table 3 Effects of BA on T-AOC, hydroxyl radical scavenging capacity, and POD activity in mouse thymus

Group	T-AOC (U/mg prot)	Hydroxyl radical scavenging capacity (U/mg prot)	POD (U/mg prot)
NC	0.60±0.07	19.77±3.08	1.72±0.30
Dex	0.50±0.06^{b}	12.08±1.61^{b}	1.37±0.11^{a}
0.25 mg/kg BA	0.51±0.04	13.79±3.70	1.57±0.16
0.50 mg/kg BA	0.58±0.10^{c}	15.76±2.65^{c}	2.03±0.15^{d}
1.00 mg/kg BA	0.64±0.06^{d}	16.52±2.66^{d}	2.42±0.24^{d}

2.4 Effects of BA on ASK1, JNK, and P38 mRNA Expression in Mouse Spleen

As shown in Figure 1 [Figure 1: see original paper], compared with the NC group, the Dex group exhibited extremely significant upregulation of ASK1,

JNK, and P38 mRNA expression in spleen ($P<0.01$). Compared with the Dex group, the 0.25 mg/kg BA group showed significantly decreased ASK1 mRNA expression ($P<0.05$), while the 0.50 and 1.00 mg/kg BA groups demonstrated extremely significant downregulation of ASK1, JNK, and P38 mRNA expression ($P<0.01$).

Figure 1 Effects of BA on mRNA expression of ASK1, JNK, and P38 in mouse spleen

2.5 Effects of BA on ASK1, JNK, and P38 mRNA Expression in Mouse Thymus

As shown in Figure 2 [Figure 2: see original paper], compared with the NC group, the Dex group exhibited extremely significant upregulation of ASK1, JNK, and P38 mRNA expression in thymus ($P<0.01$). Compared with the Dex group, the 0.25, 0.50, and 1.00 mg/kg BA groups showed significant or extremely significant decreases in thymic ASK1, JNK, and P38 mRNA expression ($P<0.05$ or $P<0.01$).

Figure 2 Effects of BA on mRNA expression of ASK1, JNK, and P38 in mouse thymus

2.6 Effects of BA on ASK1, JNK, and P38 Protein Expression in Mouse Spleen

As shown in Figure 3 [Figure 3: see original paper], compared with the NC group, the Dex group showed elevated ASK1 protein expression without statistical significance ($P>0.05$), while JNK and P38 protein expression increased extremely significantly ($P<0.01$). Compared with the Dex group, the 0.50 mg/kg BA group exhibited significantly reduced ASK1 protein expression ($P<0.05$), while the 0.25, 0.50, and 1.00 mg/kg BA groups showed significant or extremely significant decreases in JNK and P38 protein expression ($P<0.05$ or $P<0.01$).

Figure 3 Effects of BA on protein expression of ASK1, JNK, and P38 in mouse spleen detected by Western blot. (A) Representative Western blot bands for ASK1, JNK, and P38 protein expression in spleen. (B) Quantitative analysis of protein band densities normalized to -actin.

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3. Discussion

T-AOC, hydroxyl radical scavenging capacity, and POD activity serve as important indicators of oxidative damage severity. In this study, Dex-induced oxidative stress significantly reduced these parameters in liver, spleen, and thymus, confirming successful establishment of the oxidative damage model. BA, a plant-derived antioxidant and immunomodulator, has been shown in previous

in vivo studies to enhance SOD and GSH-Px activities while decreasing malondialdehyde content in mouse immune organs, thereby improving antioxidant capacity [12]. Additionally, BA has demonstrated preventive protective effects against alcohol-induced liver injury by improving hepatic redox status, enhancing antioxidant capacity, and reducing lipid peroxidation [13]. Our current findings demonstrate that BA pretreatment enhances T-AOC, hydroxyl radical scavenging capacity, and POD activity in liver and lymphoid organs, conferring protection against Dex-induced oxidative damage.

MAPK signaling pathways are evolutionarily conserved across eukaryotes and most prokaryotes, playing crucial roles in cellular signal transduction. Through a three-tiered kinase cascade, MAPKs regulate diverse physiological and pathological processes including cell growth, differentiation, stress adaptation, and inflammatory responses. The MAPK family comprises JNK, P38, and extracellular signal-regulated kinases (ERKs). ASK1, a member of the MAPK kinase kinase (MAPKKK) family, remains inactive when bound to thioredoxin (Trx) under physiological conditions. During oxidative stress, ROS either directly activate ASK1 or induce Trx dimerization and dissociation from ASK1, leading to ASK1 activation. Activated ASK1 phosphorylates MAPK kinases (MAPKK) including MKK4/MKK7-JNK and MKK3/MKK6-P38, causing JNK and P38 translocation from cytoplasm to nucleus. This promotes transcription of relevant genes and phosphorylation of additional MAPKs, PK2, and PK3, activates low-molecular-weight heat shock proteins, and ultimately triggers caspase cascades resulting in apoptosis [14-17]. Therefore, we investigated the molecular mechanisms of BA' s protective effects by examining JNK-P38 MAPK signaling pathway components.

Previous studies have reported that Dex induces oxidative stress in broiler chicken skeletal muscle satellite cells primarily through JNK and P38 MAPK pathways [18]. Consistent with these findings, our results showed Dex administration increased ASK1, JNK, and P38 mRNA and protein expression in mouse lymphoid organs, confirming that Dex induces lymphocyte oxidative stress via JNK and P38 signaling pathways. Zheng et al. [19] reported that BA protects against D-galactosamine/lipopolysaccharide (LPS)-induced liver injury by enhancing glutathione expression, inhibiting lipid peroxidation, promoting Bcl-2 expression, and suppressing JNK and ERK phosphorylation through mitochondrial-dependent mechanisms. Szuster-Ciesielska et al. [16] demonstrated that BA, as an antioxidant, inhibits ethanol-induced ROS production in hepatic stellate cells and reduces oxidative damage by downregulating the JNK pathway. In our study, BA pretreatment decreased mRNA and protein expression of ASK1, JNK, and P38 in lymphoid organs, indicating that BA alleviates Dex-induced oxidative damage in lymphocytes by inhibiting the JNK-P38 MAPK signaling pathway.

4. Conclusion

BA provides protective effects against Dex-induced oxidative damage in lymphocytes, and this protection is mediated through modulation of the JNK-P38 MAPK signaling pathway.

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Note: Figure translations are in progress. See original paper for figures.