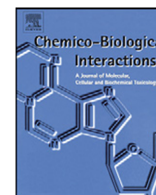
Chemico-Biological Interactions 188 (2010) 526–534

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint



Baicalein inhibits nuclear factor-κB and apoptosis via c-FLIP and MAPK in D-GalN/LPS induced acute liver failure in murine models

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ARTICLE INFO

Article history:
Received 2 July 2010
Received in revised form 7 September 2010
Accepted 7 September 2010
Available online 17 September 2010

Keywords:
Baicalein
D-Galactosamine/lipopolysaccharides
bcl-2
MAPK
c-FLIP

ABSTRACT

The hepatoprotective effects and molecular mechanisms of baicalein on acute liver failure induced by D-galactosamine (D-GalN)/lipopolysaccharides (LPS) were investigated *in vivo*. Mice were administered with different doses of baicalein (50, 100 or 150 mg/kg, p.o.) 1 h before injection of D-GalN (700 mg/kg)/LPS (10 μg/kg) and then sacrificed 6 h after treatment with D-GalN/LPS. Pretreatment with baicalein prevented D-GalN/LPS-induced liver damage by preventing associated increases of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and by reducing serum tumor necrosis factor α (TNF-α), nitric oxide (NO) or inducible nitric oxide synthase (iNOS) expressions. The molecular mechanisms involved in baicalein-induced inhibition of D-GalN/LPS-caused apoptosis were associated with the protection of mitochondria, increasing the Bcl-2/Bax ratio, blocking the release of cytochrome c, and suppressing the phosphorylation of IκBα, ERK and JNK. Moreover, baicalein activated c-FLIP$_L$, XIAP and cIAP2 proteins, potentially blocking the recruitment of NF-κB signaling molecules. The results support the investigation of baicalein as a therapeutic candidate for acute liver apoptosis or injury and indicate that baicalein might inhibit liver apoptosis by mediating one or more of these pathways.

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1. Introduction

D-GalN/LPS-induced hepatic injury is an animal model widely used in hepatoprotective drug screening [1]. The advantage of this model is that D-GalN can potentiate the toxic effects of LPS and produce fulminate hepatitis within a few hours. D-GalN is an amino sugar selectively metabolized by hepatocytes, inducing depletion of the uridine triphosphate pool and subsequent inhibition of mRNA and protein synthesis. These phenomena may lead to cellular damage and inflammation, which results in a histological and biochemical picture closely resembling viral hepatitis. Upon stimulation with LPS, liver macrophages secrete various pro-inflammatory cytokines, leading to hepatic necrosis, decreased levels of antioxidant enzymes and the scavenging of free radicals [2,3]. Thus, this model provides a practical tool for the evaluation of drugs or compounds that interfere with hepatic apoptosis as well as inflammatory liver injury.

Baicalein (5,6,7-trihydroxyflavone, BAE, $C_{15}H_{10}O_5$), is a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, a plant traditionally used in Oriental medicine [4]. Previous investigations have shown that baicalein protects against neuronal cell injuries induced by β-amyloid [5], oxidative stress [6], glutamate and glucose deprivation [7], and brain microglia death caused by lipopolysaccharide [8]. On the other hand, baicalein has been reported to induce apoptosis in human hepatoblastoma G2 cells (HepG2) [9], human breast cancer cells [10], human lung fibroblasts and peripheral lymphocytes [11], and human leukemia HL-60 cells [12,13]. Moreover, while apoptosis in human breast cancer cells was enhanced by combined treatment with baicalin and baicalein via the ERK/p38 MAPK pathway [14], baicalein could protect neurons against ischemic injury via the PI3K/Akt and PTEN pathway [15]. These results indicate that baicalein performs functions mediating MAPK or PI3K/Akt pathways, depending on the cell type, prompting us to study whether baicalein would mediate MAPK or PI3K/Akt in D-GalN/LPS-induced hepatic injuries.

All of the aforementioned investigations focused on baicalein-induced apoptosis of cancer cells. D-GalN/LPS induced acute liver failure in mice due to apoptosis (and even hepatic necrosis) caused, at least in part, by the secretion of various proinflammatory cytokines. Our prophase research shows that baicalein could pre-

Abbreviations: BAE, baicalein; D-GalN, D-galactosamine; LPS, lipopolysaccharides; AST, serum aspartate aminotransferase; ALT, alanine aminotransferase; TNF-α, tumor necrosis factor α; NO, nitric oxide; iNOS, inducible nitric oxide synthase; Bcl-2, B-cell CLL/lymphoma 2; MAPK, mitogen activated protein kinase; JNK, c-jun NH_2-terminal protein kinase; ERK, extracellular signal-regulated kinase; NF-κB, nuclear factor-κB; XIAP, X linked inhibitor of apoptosis; c-FLIP, cellular FLICE inhibitory protein; cIAP2, cellular inhibitor of apoptosis 2.

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doi:10.1016/j.cbi.2010.09.008

vent hepatic damage induced by D-GalN/LPS. It also indicates that baicalein could inhibit apoptosis of hepatocytes *in vivo* and selectively induce apoptosis of cancer cells *in vitro*, which is consistent with previous research [14,15].

Silymarin, a unique flavonoid complex containing silybin, silydianin and silychrisin, is a hepatoprotective agent with anti-inflammatory and anti-carcinogenic effects [16]. In this paper, we evaluated the hepatoprotective effect of baicalein compared with silymarin and further investigated the protection provided by baicalein against D-GalN/LPS-induced hepatotoxicity. The molecular mechanisms behind this protection were also investigated.

2. Materials and methods

2.1. Chemicals

Baicalein (purity >98%, 465119), D-GalN (≥99.0%, G0264), LPS (≥98.0% HPLC, L2880), and silymarin (254924) were purchased from Sigma–Aldrich Biotechnology (St. Louis, MO, USA). Detection kits for NO and NOS were purchased from Nanjing Jiancheng Bioengineering Institute. The mouse TNF ELISA kit was purchased from BD Biosciences (San Diego, CA, USA). Caspase-3 (SC-1226), PARP (CST-9542), caspase-8 (SC-7890), Bax (SC-493), Bcl-2 (SC-7382), β-actin (SC-7210), NF-κB p65 (SC-33039), p-p38 (SC-17852-R), cytochrome c (SC-7159) and c-IAP2 (SC-7944) monoclonal antibodies were purchased from Santa Cruz Biotechnology (CA, USA). XIAP (CST-2042), c-FLIP (CST-3210), JNK (CST-9252), p-JNK (CST-9251), ERK (CST-9102), p-ERK (CST-9106) and p38 (CST-9212) monoclonal antibodies were purchased from Cell Signaling Biotechnology. Peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies were obtained from iNtRON Biotechnology (Seougnam, Korea). ALT and AST reagent strips were purchased from Arkray Incorporated (Kyoto, Japan). All other chemicals were analytical grade.

2.2. Animals and treatment

Six- to eight-week-old C57BL/6 male mice (18–23 g) were obtained from Norman Bethune College of Medicine, Jilin University Animal Department. All mice received care according to the Guiding Principles in the Use of Animals in Toxicology adopted by the Society of Toxicology (USA) in July 1989 and revised in March 1999. The animal care committee of the local institution approved the study. All animals were acclimatized to the laboratory environment for 1 week before experiments were performed. Mice were allowed free access to drinking water and food and were maintained at a constant room temperature of 22 ± 2 °C and 50–60% relative humidity conditions with an automatic 12 h light and 12 h dark cycle.

Mice were starved overnight and were randomly divided into six groups. Normal groups were given the same volume injection of sterile saline only. Other groups were simultaneously injected intraperitoneally with 700 mg/kg D-GalN and 10 μg/kg LPS in sterile phosphate buffered saline (pH 7.4). BAE groups and silymarin groups were administered BAE (150, 100, or 50 mg/kg) or silymarin (100 mg/kg) 1 h before D-GalN/LPS injection. The dosages were selected based on preceding publications and a preliminary study. Blood was collected 2 h after D-GalN/LPS injection to measure serum TNF-α levels, and blood and liver tissue samples were collected 6 h after D-GalN/LPS injection to measure serum ALT, AST, and iNOS activities and NO levels. Blood samples were separated by centrifugation at 4 °C, 3000 rpm for 30 min. Serum and liver tissues were kept at −80 °C for subsequent analysis.

2.3. Measurements of serum ALT or AST, TNF-α, NO, and iNOS levels

Serum ALT and AST activities were measured using an Autodry chemistry analyzer (Spotchem SP4430, Arkray, and Kyoto, Japan). TNF-α levels were determined 2 h after D-GalN/LPS injection using a TNF-α ELISA kit (BD Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. NO and iNOS were assayed by colorimetric assay kits (Nanjing Jiancheng Biotechnology Institute, China) according to the manufacturer's protocols. In brief, NO is chemically active and is quickly converted to NO^{2-} and NO^{3-} *in vivo*, and NO^{2-} is converted to NO^{3-}. In the NO kits, nitrate reductase was used to reduce NO^{3-} to NO^{2-}, and the contents were then determined by colorimetry. NOS kits were used to quantify the activities of iNOS by colorimetry.

2.4. Liver homogenate preparation and isolation of nuclear extracts

Liver homogenates and nuclear extracts were isolated as previously described [17]. Liver tissues were ground in liquid nitrogen and then homogenized in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 2 mM ethylenediamine tetraacetic acid on ice. After centrifugation at 16,000 × *g* for 30 min at 4 °C, supernatants were collected. Another section of liver tissue was washed with phosphate-buffered saline and homogenized, then centrifuged at 1000 × *g* for 10 min at 4 °C, and supernatant was centrifuged at 7000 × *g* for 10 min. The pellet was washed and resuspended in the homogenization buffer. Liver homogenates and nuclear extracts were stored at −80 °C for further analysis.

2.5. Analysis of DNA fragmentation electrophoresis

Genomic DNA was extracted from liver tissue using a Wizard® genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Approximately 50 μg DNA samples were loaded into each well, and 1.5% agarose gel electrophoresis was carried out at 100 V in a TBE buffer for 30 min. After electrophoresis, DNA was visualized by soaking the gel in the TBE buffer containing 0.1 g/ml ethidium bromide. The gel was inspected using UV light and photographed.

2.6. Histopathology

For light microscopic investigations, liver samples were placed in 15% neutral buffered formalin and processed by embedding in paraffin. Consecutive 4-μm sections were prepared from frozen sections for hematoxylin and eosin (H&E) staining, after which the 4-μm slices were examined under an Olympus BH2 photomicroscope.

2.7. Western blotting analysis

For the NF-κB p65 assays, liver homogenates or nuclear extracts were separated by 10% or 12% denaturing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to PVDF membranes (Millipore) and blocked with 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween 20. Blots were incubated with the desired primary antibodies, then washed and incubated with an HRP-conjugate secondary antibody. Finally, the proteins were detected with ECL and exposed to X-ray film. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. Quantification of the immunoreactive bands was obtained with Bio-Rad Quantity One software (Bio-Rad Laboratories, Version 4.6.2).

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