European Journal of Pharmacology 797 (2017) 94–105

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Full length article

Ilexgenin A, a novel pentacyclic triterpenoid extracted from Aquifoliaceae shows reduction of LPS-induced peritonitis in mice

Weidong Sun[a,b,c,1], Chang Liu[a,d,1], Yaqi Zhang[a], Xia Qiu[a,e], Li Zhang[d], Hongxia Zhao[a], Yi Rong[c], Yun Sun[a,*]

[a] Medical and Pharmaceutical Institute, Yangzhou University, Yangzhou 225001, Jiangsu, PR China
[b] Chinese Medicine Hospital of Yangzhou City, Yangzhou 225009, Jiangsu, PR China
[c] Nanjing University of Chinese Medicine, Nanjing 210009, PR China
[d] Pharmacy of School, University of Missouri-Kansas City, MO 64108, United States
[e] Jiangsu Hengrui Medicine Co.,ltd, Kunshan 222047, Jiangsu, PR China

ARTICLE INFO

Keywords:
Pentacyclic triterpenoid
Ilexgenin A
Aquifoliaceae
Anti-inflammation
Lipopolysaccaride
Peritonitis

ABSTRACT

Ilexgenin A (IA) is a novel pentacyclic triterpenoid, which extracted from leaves of *Ilex hainanensis Merr*. In the present study, we aim to explore anti-inflammatory activity of IA on LPS-induced peritonitis and its underlying molecular mechanism. The results determined that IA was capable of suppressing peritonitis in mice induced by intraperitoneal (i.p.) injection of lipopolysaccaride (LPS). Furthermore, the results showed that IA dramatically inhibited levels of inflammatory cells infiltration in peritoneal cavity and serum in LPS-induced mice peritonitis model. Besides, IA could dramatically inhibit levels of inflammatory cytokines (IL-1β, IL-6 and TNF-α) in peritoneal cavity in LPS-induced mice peritonitis model. *In vitro* study, the results showed that IA inhibited production of IL-1β, IL-6 and TNF-α at transcriptional and translational levels in RAW 264.7 cells induced by LPS. Furthermore, IA could suppress the LPS-induced activation of Akt and downstream degradation and phosphorylation of kappa B-α (IκB-α). Moreover, IA could significantly inhibit ERK 1/2 phosphorylation in RAW 264.7 cells induced by LPS. These results were concurrent with molecular docking which revealed ERK1/2 inhibition. These results demonstrated that IA might as an anti-inflammatory agent candidate for inflammatory disease therapy.

1. Introduction

Ilex is the only living genus of almost 600 species in the Aquifoliaceae family (Bracesco et al., 2011). In south China, its roots are used as Chinese herbal medicine for hypercholestaemia and coronary artery diseases (CADs) therapy (Zhou et al., 2014). Modern pharmacological studies demonstrated that *Ilex* extracts exhibited a series of bioactivities, i.e., to enlarge blood vessels, ease the blood pressure, improve microcirculation, inhibit platelet aggregation, prevent thrombosis as well as reduce cardiac ischemia (Bracesco et al., 2011; Hao et al., 2013). Shan-Lv-Cha, leaves of *Ilex hainanensis Merr*. (Aquifoliaceae), is widely distributed in south China (Chen et al., 2011). It is the most popular herbal tea in China, which can be used for the pleasure of drinking, or for ameliorating some physiological problems (Chen et al., 2011; Liu et al., 2013). As a traditional medicine, it is used for amelioration of hypertensive, hyperlipidemia, and some inflammatory diseases (Li et al., 2013).

Shan-Lv-Cha contains different bioactive ingredients such as terpenes, flavonoids (Hao et al., 2013). Ilexgenin A (IA), a pentacyclic triterpene as showed in Fig. 1, is a primary component (~5%) in the leaves of *Ilex hainanensis Merr*. (Aquifoliaceae) (Chen et al., 2011). Recently, we demonstrated that IA has a series of pharmacological effects on hyperlipidemia (Liu et al., 2013), atherosclerosis (Liu et al., 2016), melanoma (Yang et al., 2015) and hepatocellular carcinoma (Yang et al., 2016a). Our studies found that IA significantly decreased total cholesterol (TC) and low-density lipoprotein-cholesterol (LDL-C) in hyperlipidemia mice (Liu et al., 2013). Our more recently study further demonstrated lipid-lowering effect and anti-inflammatory property of IA in Apo E deficient mice (Liu et al., 2016). We determined that treatment with IA attenuated atherosclerosis in high-fat diet-induced Apo E deficient mice via modulation of lipid parameters, decrease of atherosclerosis-related indexes, and inhibition of inflammatory cytokines secretion and pathological changes of liver and aortic root (Liu et al., 2016). One of our studies showed that IA remarkably

* Correspondence to: Medical and Pharmaceutical Institute, Yangzhou University, 11 Huaihai Road, Yangzhou, Jiangsu 225001, PR China.
E-mail address: ysun@yzu.edu.cn (Y. Sun).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejphar.2017.01.019
Received 6 September 2016; Received in revised form 6 January 2017; Accepted 16 January 2017
Available online 17 January 2017
0014-2999/

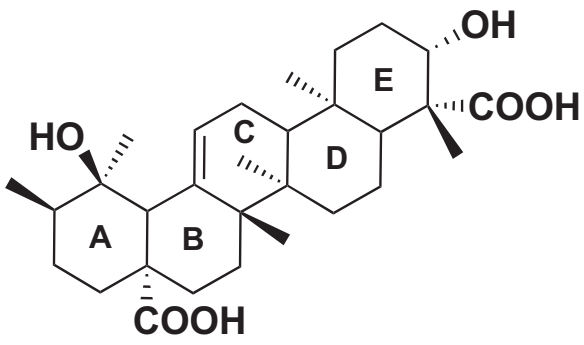

Fig. 1. The chemical structure of Ilexgenin A (IA).

inhibited serum inflammatory cytokines especially IL-6 in tumor-bearing mouse model (Yang et al., 2015). All of these findings imply that IA might be one of most important bioactive ingredients for multiply pharmacological effects of Shan-Lv-Cha.

Inflammation is a complex process, associated with a series of events such as vasodilatation, plasma extravasations, cell migration, and activation of coagulation cascades (Xiao, 2016). It is known that inflammation is a physiological response to invading pathogens and tissue injury (Henneke and Golenbock, 2004). However, excessive inflammatory reaction causes cancer and other chronic inflammatory diseases such as asthma, arthritis, multiple sclerosis and atherosclerosis (Kassi et al., 2015; Khatami, 2011; Skeoch and Bruce, 2015). Although non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are always used for amelioration of inflammation, a series of adverse effects cannot be avoided (Hynninen et al., 2000; Ikeda and Kohno, 2000). Therefore, effective anti-inflammatory agent candidates with lower toxicity are urgently needed. Plant-derived compounds may be potential candidates for drug discovery (Ni et al., 2016; Zhang et al., 2016; Zou, 2015). Among these compounds, triterpenoids have garnered significant interest as promising anti-inflammatory agents (Buus et al., 2011; Liu et al., 2014). IA belongs to pentacyclic triterpenoid family and is the taxonomic marker for Shan-Lv-Cha (Chen et al., 2011). According our previous studies, we found that IA suppressed chronic inflammation as well as inhibited melanoma proliferation (Liu et al., 2016; Yang et al., 2015). These findings implied that IA might be utilized for inflammatory diseases therapy.

In the present study, for the first time, we assessed the anti-inflammatory property of IA on LPS-induced peritonitis mice model. Furthermore, LPS-induced RAW264.7 cells were used to investigate underlying mechanism of IA on acute-inflammation.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with the guidelines of China Council on Animal Care and Use. All procedures performed on animals in this study were in accordance with the guidelines of the Yangzhou University Animal Care and Use Committee. All animals were kept and the experiments were performed in accordance with the European Community guidelines for the use of experimental animals (86/609/ EEC).

2.2. Animals and cell lines

Male ICR mice (6–8 weeks old) were purchased from Comparative Medicine Center of Yangzhou University, License No: SCXK (Su) 20120029. All mice were housed in a room under a 12 h light/dark cycle and fed under experimental conditions with a temperature of 22–24 °C and humidity of 50 ± 5%.

RAW 264.7 cells were purchased from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China) and maintained in RPMI 1640 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Wisent, Quebec, Canada) at 37 °C in the presence of 5% CO_2.

2.3. Antibodies and reagents

IA was prepared from the dried leaves of *Ilex hainanensis Merr.* (Guangxi, China) and was authenticated by Prof. Qiang Wang (China Pharmaceutical University, China). Purity of IA was determined to exceed 98% by using HPLC-ELSD (Chen et al., 2011). Voucher specimens were deposited at the Department of Chinese Material Medical Analysis (China Pharmaceutical University, Nanjing, PR China).

LPS, 3-[4, 5-dime-thylthylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Polyclonal antibodies against JNK/SAPK, phospho-JNK/SAPK (Thr183/Tyr185), p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), ERK 1/2, phospho-ERK 1/2 (Thr202/Tyr204), AKT, phospho-AKT (Ser473), IKKα, phospho-IKK α (Ser176/Ser180), NF-κB p65 and Histone 3 (H3) were purchased from Cell Signaling Technology (Bervely, MA, USA).

2.4. Induction of peritonitis and therapy

Acute peritonitis model was established according to previously described method (Borges et al., 2014). Peritonitis was induced in mice by an intraperitoneal (i.p.) injection of LPS (1 mg/kg) or sterile saline solution (0.9% NaCl, 10 ml/kg) as a control. Briefly, fifty mice were randomly divided into five groups (n=10). In the model group, mice were received 0.5% sodium carboxymethyl cellulose (CMC-Na) solution by intra-gastric gavage (i.g.) 60 min before LPS injection. In the control group, mice were received 0.5% CMC-Na solution by intra-gastric gavage (i.g.) 60 min before injection of sterile saline solution (0.9% NaCl, 10 ml/kg). In the IA group, mice were received IA at doses of 30 or 60 mg/kg by intra-gastric gavage (i.g.) 60 min before LPS injection. The doses of IA were chosen according to our previous study (Yang et al., 2015). IA was suspended in 0.5% CMC-Na solution according to our previous studies. In the positive control group, mice were received dexamethasone (0.5 mg/kg, i.p.) 60 min before LPS injection. The dose of dexamethasone was chosen according to the reference (Borges et al., 2014).

All animals were killed by CO_2 as phyxiation 4 h later after the induction of peritonitis, and their peritoneal cavity were opened and washed with 1 ml PBS containing heparin (25 UI/ml). The total numbers of inflammatory cells and the differential leukocyte cells in peritoneal fluid and serum were measured by using an automatic veterinary hematology analyzer (Hemavet 950, USA). In addition, the levels of IL-1β, TNF-α, and IL-6 in peritoneal cavity were determined by using enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA).

2.5. Cell culture and viability assay

RAW 264.7 cells were maintained in RPMI 1640 (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Wisent, Quebec, Canada) at 37 ℃ in the presence of 5% CO_2.

The cell viability was measured by a MTT assay according to our previously described method (Yang et al., 2015). Briefly, the RAW 264.7 cells were seeded into 96-well plates at 5×10^4 cells per well 24 h before treatment. After treatment with IA (10, 20, 40, 60, 80 and 100 μmol) for 18 h in the presence or absence of LPS (1 μg/ml), MTT solution (5 mg/ml) was added to each well and incubated for another 4 h at 37 °C. After incubation, media was removed and DMSO was added to dissolve purple precipitates. Then plates were read at 570 nm using an ELx 800 Universal Microplate Reader (Bio-tek, inc).