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(7*R*,8*S*)-9-Acetyl-dehydrodiconiferyl alcohol inhibits inflammation and migration in lipopolysaccharide-stimulated macrophages

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ABSTRACT

Background: (7*R*, 8*S*)-9-Acetyl-dehydrodiconiferyl alcohol (ADDA), a novel lignan compound isolated from *Clematis armandii* Franch (Ranunculaceae) stems, has been found to exert potential anti-inflammatory activities *in vitro*.

Purpose: To investigate the pharmacological effects and molecular mechanisms of ADDA on lipopolysaccharide (LPS)-induced activation and migration of macrophages.

Study design/methods: Macrophages were stimulated with LPS in the presence or absence of ADDA. Expression of inflammatory mediators, including cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and nitric oxide (NO) were measured by Western blot and commercial NO detection kit. Cellular viability and chemotactic properties of macrophages were investigated using MTT and transwell migration assays. The activation and expression of mitogen activated protein kinases, nuclear factor-κB (NF-κB), protein kinase B (Akt), Src, and focal adhesion kinase (FAK) were analyzed by Western blot.

Results: Non-toxic concentrations (12.5–50 μM) of ADDA concentration-dependently inhibited expression/release of inflammatory mediators (COX-2, iNOS, and NO), suppressed Akt and c-jun *N*-terminal kinase 1/2 (JNK) phosphorylation, and NF-κB activation in LPS-stimulated macrophages. In addition, ADDA blocked LPS-mediated macrophage migration and this was associated with inhibition of LPS-induced Src and FAK phosphorylation as well as Src expression in a concentration dependent manner. Notably, the inhibitory effects of ADDA on iNOS, NO, and Src could be mimicked by a Src inhibitor PP2 or an iNOS inhibitor L-NMMA.

Conclusion: Our results suggested that ADDA attenuated LPS-induced inflammatory responses in macrophages and cell migration, at least in part, through inhibition of NF-κB activation and modulation of iNOS/Src/FAK axis.

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Introduction

Macrophages, present in almost all tissues constitute the first line of innate immune defense to pathogens and diverse external stimuli. Under physiological conditions, as a part of host defense response to infection, macrophages sense, move to the site of infection or injury, and engulf microorganisms, foreign particles, and apoptotic bodies (Friedl 2004; Gordon 2003). Under pathological stimuli, macrophages activate their effector functions and excessive macrophage activation is associated with the expression/release of several inflammatory mediators, importantly, the proinflammatory enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), nitric oxide (NO), prostaglandins (PGs) (Jiang et al. 2014). Furthermore, overproduction of NO and cytokines/chemokines by activated macrophages contribute to recruitment and activation of inflammatory cells (Maa et al. 2008), which results in a variety of pathological disorders such as sepsis and atherosclerosis. Lipopolysaccharide (LPS), a major constituent of the Gram-negative bacterial endotoxin, plays a pivotal role in the initiation of inflammation *via* toll-like receptor 4 (Dalpke and Heeg 2002). LPS stimulates toll-like receptor 4 to ignite common downstream signaling pathways, i.e. activation of mitogen activated

Abbreviations: ADDA, (7R,8S)-9-acetyl-dehydrodiconiferyl alcohol; COX-2, cyclooxygenase-2; FAK, focal adhesion kinase; iNOS, inducible nitric oxide synthase; L-NMMA, N^G-monomethyl-l-arginine; LPS, lipopolysaccharide; JNK, c-jun *N*-terminal kinase 1/2; MAPK, mitogen activated protein kinases; NF-κB, nuclear factor-κB; NO, nitric oxide; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; SFKs, Src family kinases.

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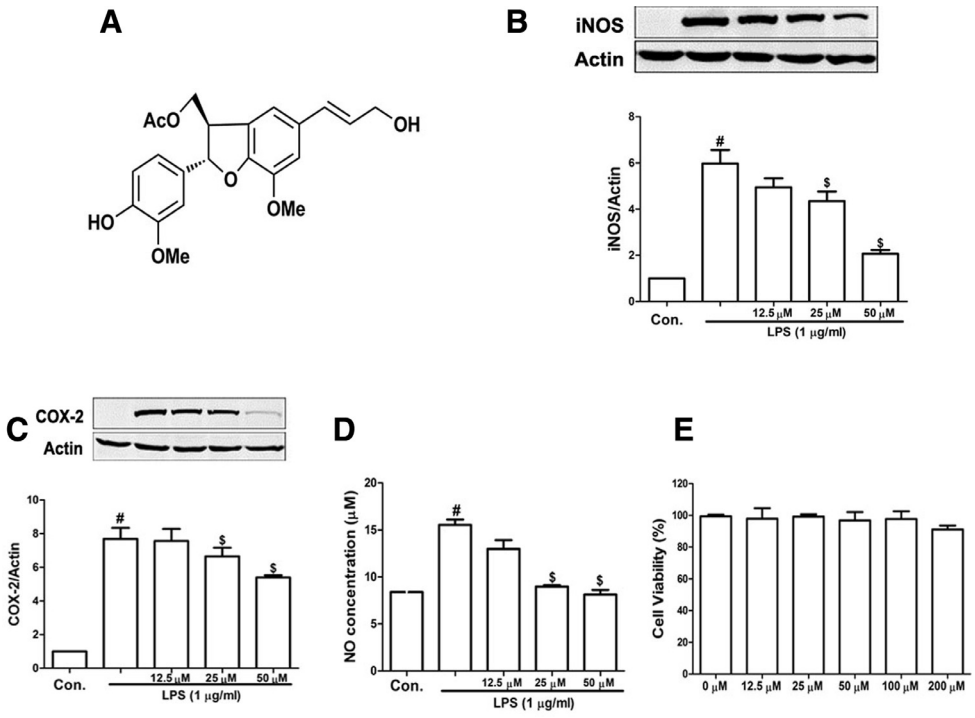

Fig. 1. ADDA attenuated LPS-induced inflammatory mediators in RAW264.7 macrophages. (A) The chemical structure of ADDA. (B–D) Cells were pretreated with indicated concentration of ADDA for 4 h, and stimulated with or without LPS (1 μg/ml) for 24 h, iNOS expression (B), COX-2 (C), and NO production (D) were detected as described in Materials and Methods, respectively; β-actin was used as loading control. (E) Cells were pretreated with indicated concentration of ADDA for 24 h, then cell viability was analyzed as described in Materials and Methods. Data were shown means ± SEM; $^{\#}p < 0.05$, compared to unstimulated cells, $^{\$}p < 0.05$, compared with LPS-stimulated cells. Data were from at least three independent experiments, each performed in duplicate.

protein kinases (MAPK), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and nuclear factor-κB (NF-κB) (Kyriakis and Avruch 2012), leading to expression of proinflammatory mediators (Israf et al. 2010; Liu et al. 2013b) and the resultant inflammatory processes and tissue injury.

Cellular Src is the prototype of Src family kinases (SFKs) of highly conserved proteins, including Blk, Fgr, Fyn, Hck, Lck, Lyn, and Yes (Miguelez et al. 2014). SFKs have been implicated in a spectrum of signaling pathways and cellular events. In macrophages, the majority of the myeloid-specific SFK members (i.e. Fgr, Hck and Lyn) are constitutively expressed and almost unaltered in response to inflammatory stimuli such as LPS (Maa et al. 2008). In contrast, Src is barely detectable in resting macrophages and is greatly up-regulated by LPS, leading to focal adhesion kinase (FAK) activation and cell motility (Chen et al. 2012; Leu et al. 2006). FAK, one of the Src substrates, plays a critical role in macrophage adhesion and motility (Owen et al. 2007). Mounting evidence indicates that LPS induces Src expression and subsequently FAK auto-phosphorylation at Tyr 397, triggering macrophage migration, which is iNOS-dependent (Fernandez-Arche et al. 2010; Leu and Maa 2002). Hence, targeting Src/FAK axis may effectively prevent LPS-triggered macrophages locomotion.

Clematis armandii Franch. (Ranunculaceae) (*Caulis clematidis armandii,* or "Chuan-Mu-Tong" in Chinese), a flowering climbing plant, is frequently found in southwestern China, especially in Si-Chuan (Szechwan) Province (Xiong et al. 2014). *Clematis armandii* has long been used for the treatment of inflammation conditions, such as rheumatism and urinary tract infection (Chawla et al. 2012). Previously, we reported that (7*R*, 8*S*)-9-acetyldehydrodiconiferyl alcohol (ADDA, Fig. 1A), a novel lignan isolated and identified from the dried stems of *Clematis armandii*, had anti-inflammatory and cytoprotective effects in activated microglial cells *in vitro* (Xiong et al. 2014). The current study was conducted to investigate the pharmacological effects and underlying mechanism of ADDA on inflammatory responses in LPS-stimulated murine RAW264.7 macrophages.

Materials and methods

Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) medium and fetal bovine serum (FBS) were from GIBCO-BRL (USA). 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), N^G-monomethyl-L-arginine (L-NMMA), and LPS (*E. coli* 055:B5) were obtained from Sigma (St. Louis, MO, USA). Antibodies against β-actin, iNOS, Hck, and COX-2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Lamin A/C, NF-κB p65, FAK, and Src were from Epitomics (Burlingame, CA). Antibodies against total- and phosphor (p)- c-Jun N-terminal kinase 1/2 (JNK) (Thr^{183} and Tyr^{185}), total- and p- p38 (Thr^{180} and Tyr^{182}), total- and p- extracellular signal-regulated kinase 1/2 (ERK) (Thr^{202}/Tyr^{204}), total- and p- Akt, p-Src (Tyr^{416}), p-FAK (Tyr^{397}) and p-p65 (Ser^{563}) were purchased from Cell Signaling Technology (Danvers, MA).

Isolation and purity of ADDA

The dried stem of *Clematis armandii* (10 kg) was pulverized and extracted with 95% EtOH at room temperature to give a brown crude extract (500 g), which was suspended in H_2O and then extracted with CH_2Cl_2. After removal of the solvent under reduced pressure, the CH_2Cl_2 extract (200 g) was chromatographed over a silica gel column with a gradient elution of CH_2Cl_2–MeOH (90:1 to 0:1) to afford seven fractions (Fr. 1–Fr. 7). Fr. 4 (15.0 g) was subjected to silica gel CC with PE–acetone (3:1 to 0:1) to yield five subfractions (Fr. 4.1–Fr. 4.5). Fr. 4.2 and Fr. 4.3 were purified by semi-preparative HPLC [Waters e2695 system with a Waters

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