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Short communication

Anti-inflammatory and antiviral activities of cynanversicoside A and cynanversicoside C isolated from *Cynanchun paniculatum* in influenza A virus-infected mice pulmonary microvascular endothelial cells

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ABSTRACT

Background: Outbreaks of the influenza A virus (IAV) often occur in various avian and mammalian species, including humans, causing serious respiratory injury worldwide. Therapeutic actions are limited to vaccines and a few antiviral drugs. Combination antiviral compounds and anti-inflammatory modulators to control the propagation of viruses would be more efficient therapeutic strategies for infectious diseases.

Purpose: This study was designed to isolate anti-inflammatory and antiviral compounds from *Cynanchun paniculatum* and elucidate their potential molecular mechanisms.

Methods/Study designs: Bioactivity-guided isolation (*via* in vitro anti-inflammatory assay) was performed on the ethanolic extract of *C. paniculatum*, the structures of active compounds were elucidated by comparing spectral data (ESI-MS, ^{1}H NMR and ^{13}C NMR) with literature values. The antiviral activity of active compounds against Influenza A virus (IAV) was determined using the cytopathic effect (CPE) inhibition assay. Inhibitory effects of active compounds on influenza A/FM1/1/47 (H1N1) virus infection were also determined by RT-PCR. Effect of active compounds on NF-kB and MAPK signaling pathways after virus infection was determined by ELISA.

Results: Two compounds that showed great anti-inflammatory activity were isolated from *C. paniculatum* and elucidated as cynanversicoside A and cynanversicoside C. Cytokine assay demonstrated that cynanversicoside A and cynanversicoside C can suppress the production of TNF-α, IL-6 and IL-1β in Mice Pulmonary Microvascular Endothelial Cells (MPMEC) after Influenza virus A/FM/1/47 infection ($p < .05$) and also decreased the expressions of p-p65 and p-IκBα in infected cells. Furthermore, the phosphorylation of p38, ERK and JNK was also significantly attenuated. Subsequently, cynanversicoside A and cynanversicoside C treatment resulted in decreased viral replication and viral mRNA synthesis.

Conclusions: These results indicate that cynanversicoside A isolated from *C. paniculatum* has potent anti-inflammatory and antiviral effects on IAV-infected MPMEC by the regulation of NF-κB and MAPK signaling pathways.

Introduction

Influenza A virus (IAV) causes a contagious respiratory disease in humans that is responsible for the annual epidemics with a high rate of morbidity and mortality worldwide. Oseltamivir, amantadine and rimantadine have been used for the control of IAV with varying levels of success (Hayden and de Jong, 2011). However, the threats of drug effectiveness or resistance, risk of residues, environmental contamination caused by the frequent use of these drugs have led to the need of other alternative control methods.

There are sufficient data to support the idea that the combination of antiviral compounds and anti-inflammatory modulators to control the propagation of viruses would be an efficient therapeutic strategy for eradicating infectious diseases (Pinto et al., 2011). Cytokines are

Abbreviations: IAV, Influenza A virus; MPMEC, Mice Pulmonary Microvascular Endothelial Cells; LPS, Lipopolysaccharide; TNF-α, Tumor necrosis factor-alpha; RT-PCR, Quantitative real–time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, Polyvinylidene difluoride; PBS, phosphate buffer saline; MOI, multiple of infection; ECL, emitter coupled logic; FBS, Fetal bovine serum; NF-κB, Nuclear transcription factor-kappa B; MAPKs, Mitogen-activated protein kinases; ERK, Extracellular regulated protein kinases; JNK, c-Jun N-terminal kinase; m.p., melting points; MS, mass spectrometry; $CCID_{50}$, cell culture infective dose 50%; CPE, cytopathic effect; NMR, Nuclear Magnetic Resonance Spectroscopy; IL-6, Interleukin-6; IL-1β, Interleukin-1β

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important for an effective antiviral response and excessive production of pro-inflammatory cytokines plays a critical role in the pathogenesis of IAV infection, especially in the case of HPAI virus infection. The excessive levels of pro-inflammatory cytokine production also named "cytokine storm" accompanying IAV infection has been suggested to be associated to the lethal dissemination of influenza virus (Beigel et al., 2005; Chan et al., 2005). The imbalance between pro-inflammatory and anti-inflammatory cytokine expression could lead to host immunologic injury (de Jong et al., 2006). Nuclear factor-B (NF-kB), a primary regulator of inflammatory responses, plays a critical role in a variety of physiological and pathologic processes (Asehnoune et al., 2005). Growing evidence supports the critical role of NF-kB signaling in efficient influenza virus replication and aberrant pro-inflammatory responses associated with fatal outcome during infection (de Jong et al., 2006; Nimmerjahn et al., 2004; Schmolke et al., 2009). It was also reported that apoptosis of infected cells could be mediated by NF-kB pathway, as well as viral ribonucleoprotein nuclear export could be enhanced (Wurzer et al., 2004). Additionally, nuclear transcription factor-kappa B (NF-κB) p65, the subunit of NF-kB was reported to be responsible for the synthesis of influenza viral RNA (Kumar et al., 2008).

Mitogen-activated protein kinases (MAPKs) are a family of proteins that serve as components of signaling pathways within cells in order to process and respond to extracellular stimuli (Raman et al., 2007). Studies have shown that influenza A virus infection is able to activate the MAPK signal pathways (Gaur et al., 2011; Pleschka, 2008). It was also reported that H1N1pdm infection could activate MAPK pathways that enhance virus replication (Wang et al., 2014). It has been reported that suppression of MAPK or NF-kB signaling pathway could block HSV replication (Faith et al., 2006). Pharmacological targeting of NF-kB and MAPK may not only limit viral transmission but also serve to reduce the viral-induced production of pro-inflammatory cytokines. Therefore, possible inhibitors of NF-kB and MAPK signaling pathways would be a good approach for the development of anti-inflammatory and even antiviral drugs.

Recently, the products derived from plants with different biological effects have attracted extensive attention for the treatment of numerous diseases. The roots of *Cynanchum paniculatum* (Bunge) Kitag. ex H.Hara (Apocynaceae), commonly called "XuChangQing" in Chinese, have been used in traditional medicine for the treatment of hectic fevers, acute urinary infection, and abscesses (Opal et al., 2003; Gu et al., 2010). Previous phytochemical investigations on *C. paniculatum* have revealed the presence of C_{21} steroidal glycosides (Dou et al., 2007), phenolic derivatives (Kim et al., 2013), alkaloids and polysaccharides (Niu et al., 2015). It has been reported to be rich in C_{21} steroids and their glycosides (Ju et al., 2013; Dou et al., 2007), whose chemical structures are classified into normal four-ring C_{21} steroid type and aberrant 13, 14:14, 15-diseco-pregnane-type. Pharmacological studies indicated that the chemical constituents or extracts of *C. paniculatum* had analgesia, sedative, antibiosis, anti-inflammatory, anti-allergic and anti-tumor, activities, among others. (Sugama et al., 1986; Chu et al., 2015). In our continuous efforts to find active compounds from *C. paniculatum* that contribute to its anti-inflammatory activity, we established a lipopolysaccharide (LPS)-induced anti-inflammatory model to screen active compounds from *C. paniculatum* by bioactivity-guided isolation. In addition, we further investigated those active compounds for their antiviral activity and studied their potential molecular mechanisms.

Materials and methods

Plant material

C. paniculatum was collected from Jilin Province, China, in May 2015, and then authenticated by Prof. X.L. He in Northwest A&F University, China. A voucher specimen (No. 6446) has been deposited in the Herbarium of College of Life Science of the University

Culture of Mice Pulmonary Microvascular Endothelial Cells

Isolation and culturing of Mice Pulmonary Microvascular Endothelial Cells (MPMEC) were conducted following our previous study (Hu et al., 2012). MPMEC were maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in 5% CO_2. MPMECs were cultured for 24 h before any treatment in all of the experiments.

Construction of NF-kB and MAPK signaling pathways-based screening model

MPMECs were seeded in 6-well plates and incubated in the presence of either LPS 1 μg/ml alone or LPS + fractions (2.0, 4.0 and 6.0 μg/ml) or active compounds (0.5, 1.0 and 2.0 μg/ml) isolated from *C. paniculatum* for 24 h. Total proteins from MPMEC were extracted using Mammalian Protein Extraction Reagent (M-PEK). The proteins were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto the polyvinylidene difluoride (PVDF) membrane. The proteins expression levels of NF-KBp65 and IκBα in NF-KB signal pathway, and levels of p38, ERK, JNK in MAPKs signal pathway were determined by Western blotting.

Isolation and identification of anti-inflammatory components from C. paniculatum

The anti-inflammatory component was isolated *via* an *in vitro* bioassay-guided fractionation based on NF-kB and MARK signaling pathways-based screening model. Only the fractions with strong inhabitation for LPS-induced inflammatory cytokines were further purified until the target component was obtained. Fractions and compounds with none inhabitation were abandoned.

Air-dried and powdered rhizomes of *C. paniculatum* were extracted with petroleum ether, ethyl acetate, chloroform, n-butanol, ethanol and water with increasing polarity. All the extracts were submitted to activity assays. Ethyl acetate extracts that showed the highest anti-inflammatory efficacy were subjected to open column chromatography on normal phase silica gel and sequentially eluted with chloroform and methanol with increasing polarity. Repetition of bioassay-guided chromatographic separations and recrystallization led to the isolation of two active compounds. The structures of active compounds were elucidated as cynanversicoside C (glaucogenin D 3-O-β-D-thevotopyranoside) and cynanversicoside A (glaucogenin C 3-O-β-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl–(1→4)-β-D-thevotopyranoside) (Supplementary Fig. 3) by analyzing their melting points (m.p.), mass spectrometry (MS) and nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR, Supplementary Figs. 1–2) (Fig. 1A).

Viral infection

Influenza virus A/FM/1/47 (adapted to mouse) was obtained from Chinese Center for Disease Control and Prevention (CDC, Beijing, China). When grown to 90% confluency, MPMECs were washed twice with PBS to remove residual fetal bovine serum (FBS) and were infected with virus at a MOI of 0.01. Viral stock was used in serum-free medium for 60 min at 37 °C to inoculate the MPMECs. The uninfected cells were used as control. MPMECs were subjected to active compounds as the experimental design.

Effect of active compounds on NF-kB and MAPK signaling pathways after virus infection

After virus infection and active compounds administration, the cells were washed 3 times in PBS and lysed on ice in the lysis buffer (RIPA) for 30 min. Subsequently, the lysates were centrifuged at 12,500 rpm at