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Inhibition of curcumin on influenza A virus infection and influenzal pneumonia *via* oxidative stress, TLR2/4, p38/JNK MAPK and NF-κB pathways

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ABSTRACT

Oxidative stress, Nrf2-HO-1 and TLR-MAPK/NF-κB signaling pathways have been proved to be involved in influenza A virus (IAV) replication and influenzal pneumonia. In the previous studies, we have performed several high-throughput drug screenings based on the TLR pathways. In the present study, through plaque inhibition test, luciferase reporter assay, $TCID_{50}$, qRT-PCR, western blotting, ELISA and siRNA assays, we investigated the effect and mechanism of action of curcumin against IAV infection *in vitro* and *in vivo*. The results showed that curcumin could directly inactivate IAV, blocked IAV adsorption and inhibited IAV proliferation. As for the underlying mechanisms, we found that curcumin could significantly inhibit IAV-induced oxidative stress, increased Nrf2, HO-1, NQO1, GSTA3 and IFN-β production, and suppressed IAV-induced activation of TLR2/4/7, Akt, p38/JNK MAPK and NF-κB pathways. Suppression of Nrf2 *via* siRNA significantly abolished the stimulatory effect of curcumin on HO-1, NQO1, GSTA3 and IFN-β production and meanwhile blocked the inhibitory effect of curcumin on IAV M2 production. Oxidant H_2O_2 and TLR2/4, p38/JNK and NF-κB agonists could significantly antagonize the anti-IAV activity of curcumin *in vitro*. Additionally, curcumin significantly increased the survival rate of mice, reduced lung index, inflammatory cytokines and lung IAV titer, and finally improved pulmonary histopathological changes after IAV infection. In conclusion, curcumin can directly inactivate IAV, inhibits IAV adsorption and replication; and its inhibition on IAV replication may be *via* activating Nrf2 signal and inhibiting IAV-induced activation of TLR2/4, p38/JNK MAPK and NF-κB pathways.

1. Introduction

Influenza A virus (IAV) is a highly contagious respiratory virus. Severe IAV infection usually causes acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) with substantial morbidity and mortality. Currently, IAV vaccine production has not yet been satisfactory owing to the continuous evolution of IAV major antigens. Classical anti-IAV drugs, such as amantadine, rimantadine, oseltamivir and zanamivir, have also been limited in use due to their side effects and the continual emergence of resistant viral strains. And so far, the development of safe, effective and inexpensive anti-IAV drugs is still urgent [1].

IAV infection can cause severe oxidative stress. Inactivated H5N1 avian IAV can rapidly induce the formation of oxidized phospholipid, which can further induce ALI through the TLR4-TRIF-TRAF6-NF-κB pathway [2]. Oxygen free radical (ROS) has already been proved to play an important role in H5N1-induced ALI in mice [3]. Nuclear factor erythroid 2-related factor 2 (Nrf2) - heme oxygenase-1 (HO-1) is a classic anti-oxidative pathway and also possesses antiviral activity. Up-regulation of HO-1 expression can suppress the infection of many species of viruses, including human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), enterovirus 71 (EV71) and ebola virus (EBOV) [4,5]. Moreover, Kesic M.J. et al. have reported that IAV infection can significantly reduce the expressions of Nrf2 and HO-1, and Nrf2 activators can significantly increase the production of RIG-I, IFN-β and MxA and finally decrease IAV titer [6].

IAV infection also leads to activation of toll-like receptor (TLR) signaling pathways. Excessive activation of TLR signal is detrimental to IAV-induced ALI. Mauad et al. have reported that TLR3 is lastingly activated in 21 patients who have died in the 2009 pandemic [7].

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Lasting activation of TLR3 is injurious to IAV-induced acute pneumonia [8]. Activation of TLR4 can modulate IAV entry and tropism through MyD88 expression and p38 MAPK activation [9]. Activation of TLR7 is also necessary for efficient replication of IAV [10]. PI3K/Akt and downstream cascades of TLRs, MAPK and NF-κB, are also important for IAV infection and replication [9,11–13]. Additionally, activation of Nrf-2-HO-1 signal can inhibit LPS-induced activation of TLR4-NF-κB pathway and improves LPS-induced ALI [14–17].

In our previous studies, we have performed several high-throughput drug screenings based on the TLR signaling pathways and found turmeric (*Curcuma longa* L.) possessing excellent activity. Curcumin is a major active compound of turmeric and is commonly used as a coloring agent and spice in foods in China and India [18]. Curcumin is a powerful antioxidant, possessing effective electron transfer capability and easily donating H-atom from two phenolic sites to scavenge intracellular smaller oxidative molecules such as H_2O_2, HON and ROON [19]. Curcumin is reported to possess anti-inflammatory, antibacterial, anti-carcinogenic and hepatoprotective activities [20], and can inhibit the infection of HIV, EV71, HBV, HCV and dengue virus (DENV) [21,22]. Curcumin also can inhibit IAV infection [23], but the mechanism of action is still poorly understood. In the present study, we have detected the antiviral effect of curcumin on 8 IAV strains, including avian and pandemic IAV strains, and explored the mechanism of action of curcumin, mainly focusing on the oxidative stress, Nrf2-HO-1 and TLR-MAPK/NF-κB signaling pathways.

2. Materials and methods

2.1. Reagents

Curcumin ($C_{21}H_{20}O_6$, 368.37, purity > 98%, #110823) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China, http://www.gjbzwz.com/zjsdzp/2333.html). MTT (#M2003), TPCK-trypsin (#4370285), ribavirin (#R9644) and sulforhodamine B (SRB, #230162) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Minimum essential medium (MEM), Lipofectamine 2000 reagent, Pfu DNA polymerase, DNase and TRIzol reagent were purchased from Invitrogen Life Technologies, Inc. (Carlsbad, CA, USA). Luciferase Reporter Assay Kit was bought from BD Biosciences Clontech (Franklin Lakes, NJ, USA). Agonists LAM-MS (#tlrl-lams), Poly(I:C) (#tlrl-pic), LPS-B5 (#tlrl-pb5lps), FLA-BS (#tlrl-pbsfla), R-848 (#tlrl-r848), CpG-ODN (#tlrl-2336) were purchased from InvivoGen (San Diego, California, USA). Agonists IGF-1 (#8917), EGF (#8916), anisomycin (#2222) and human antibodies, including MyD88 (#4283), TRAF6 (sc-8409), p-Akt (#10001), Akt (#11848), ERK1/2 (#8867), p-ERK1/2 (#13148), p-JNK (#3708), JNK (#4671), p-p38 (#4092), p38 (#14451), p65 (#4764), Hrf2 (#12721), HO-1 (#70081) and β-actin (#12262) were bought from Cell Signaling Technology® Inc. Company (Danvers, MA, USA). Human TLR2 (sc-21760), TLR3 (sc-517367), TLR4 (sc-293072), TLR7 (H-114, sc-30004), TLR8 (sc-373760), TLR9 (sc-47723), Lamin B1(sc-56144), NQO1 (sc-32793), GSTA3(sc-100547), secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human antibody TRIF (ab13810) and anti-influenza A virus M2 protein antibody (ab5416) were purchased from Abcam Inc. (Cambridge, England). Pierce™ ECL Plus Western Blotting Substrate (#32132) was purchased from Thermo Fisher Scientific™ (Cleveland, OH, USA). pARE-luc reporter plasmid (D2112) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals and solvents were commercially available and of analytical grade.

2.2. Viruses and cells

The virus stocks of IAV subtypes A/ShanTou/169/06 (ST169, H1N1), A/PuertoRico/8/34 (PR8, H1N1), A/ShanTou/1233/06 (ST1233, H1N1), A/Quail/HongKong/G1/97 (HKG1, H9N2), A/Chicken/ Guangdong/A1/03 (GDA1, H9N2), A/Chicken/Guangdong/1/05 (GD105, H5N1), A/ShanTou/602/06 (ST602, H3N2) and A/ShanTou/364/05 (ST364, H3N2) were prepared in MDCK cells or 10-day-old embryonating eggs. Virus titer was determined by a plaque formation assay [24]. The cytotoxicity of curcumin on MDCK and A549 cells was determined using a MTT assay [25]. The concentration of curcumin required to lower cell viability by 50% (IC_{50}) was calculated using Origin 8.0 software. All experiments with IAV were performed in biosafety level 3 containment.

2.3. Plasmid construction

To construct the promoter luciferase reporter plasmids of human TLRs-MyD88/TRIF-TRAF6 pathway genes, human TLR2 (NG_016229.1), TLR3 (NT_016354.19), TLR4 (NT_008470.19), TLR7 (NG_012569.1), TLR8 (NG_012882.2), TLR9 (NG_033933.1), MyD88 (NT_022517.18), TRIF (NT_011255.14) and TRAF6 (NT_009237.18) promoters were amplified by Pfu DNA polymerase using A549 cell genome DNA as template, PCR productions were cloned into a pGL3-basic vector and named pTlr2-luc, pTlr3-luc, pTlr4-luc, pTlr7-luc, pTlr8-luc, pTlr9-luc, pMyd88-luc, pTrif-luc and pTraf6-luc, respectively. All constructs were verified by double enzyme digestion and DNA sequencing assays. The primers were presented in Supplementary Table 1. The results of double enzyme digestions were shown in Supplementary Fig. 1.

2.4. Transfection and luciferase assay

A549 cells were transfected with different plasmids using lipofectamine 2000 reagent in antibiotic free medium. After 8 h, virus (ST169, H1NH) was introduced (MOI = 2.0), and the cells were grown in virus growth medium (VGM) containing MEM, 0.5 μg mL^{-1} TPCK-trypsin and 0.125% (wt/vol) bovine serum albumin (BSA). At the same time, the cells were treated with the test drugs and incubated for 24 h. Transfection efficiency was normalized by co-transfection of Renilla luciferase reporter plasmid. Luciferase activity was determined using Luciferase Reporter Assay Kit (BD Biosciences Clontech) and presented in fold change after normalization to renilla luciferase activities. Every assay included five independent experiments.

2.5. TCID50 and antiviral assay by Sulforhodamine B (SRB) method

IAV stock solution was diluted with VGM in serial dilutions, after incubation with MDCK cells for 48 h, $TCID_{50}$ was calculated following the method of Reed and Muench [24]. Antiviral activities were also evaluated by a SRB method using CPE reduction [1]. Briefly, MDCK cells were seeded in 96-well plate. 0.09 mL of virus suspension (50 × $TCID_{50}$) and 0.01 mL medium containing various concentrations of test compounds were added. At 48 h, after washing, 100 μL − 20 °C 70% acetone was added. After removing acetone, plates were dried, and added 100 μL 0.4% (w/v) SRB, after washing, plates were dried and added 100 mL 10 mM Tris-base solution. OD was read at 562 nm. Three wells were used each for negative (virus-infected non-drug-treated) and mock controls (non-infected non-drug-treated). 0.5% (v/v) DMSO was used in each group. Percent protection of test compounds (cell viability) was calculated as following:

$$\text{Protection of test compound}(\%) = \frac{\overline{OD_{test}} - \overline{OD_{Negative}}}{\overline{OD_{Mock}} - \overline{OD_{Negative}}} \times 100\%$$

Concentration of 50% protection was defined as EC_{50}. Antiviral index (AI) was defined as IC_{50}/EC_{50}. The cell viability of negative control was expressed as the percent to the mock group. Every assay included at least five independent experiments.