Protocatechuic acid protects mice from influenza A virus infection

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Abstract
Influenza A virus (IAV) H1N1 infection remains a great challenge to public health and causes great burden over the world. Although there are anti-viral agents available, searching for effective agents to treat H1N1 infection is still in urgent because of the emergence of resistant strain. Protocatechuic acid (PCA) is a biological agent with multiple functions. In present study, we explored the effects of PCA on H1N1 infection. Mice infected with mouse adapted influenza strain A/Font Monmouth were administrated with PCA. The body weight change, mortality, lung index, viral titer, immune cell infiltration, and cytokine production in the lung were monitored. The activation of toll-like receptor 4 (TLR4) and nuclear factor kappa light chain enhancer of activated B cells (NF-κB) pathway was investigated. PCA treatment prevented H1N1 infection-induced mice body weight loss and death. PCA reduced the lung index, viral titer, infiltration of immune cells, and cytokine level in the lung, as well as suppressed H1N1-induced TLR4/NF-κB activation. PCA protects mice against H1N1 infection and could be a potential therapeutic agent to treat influenza.

Keywords Protocatechuic acid · Influenza · Protection · Mice

Introduction
Due to its severe morbidity and mortality, influenza infection causes great public health and economics burdens [1]. Patients with influenza infection could develop respiratory complications which are caused by cytokine storm, inflammation, and tissue damage [2]. The cytokine level is correlated to the severity of pneumonia during influenza infection. Therefore, anti-inflammation agents, together with antiviral agents such as neuraminidase inhibitors (NAIs), have been utilized to treat severe influenza [3].

Influenza A virus H1N1 (A/H1N1) is a subtype strain of influenza A virus (IAV) which widely spreads in humans. In history, there are 3 well known outbreaks of H1N1 strain in humans including 1918 flu pandemic, 1977 Russian flu pandemic, and 2009 swine flu pandemic. In many cases of 2009 H1N1 influenza pandemic, NAI treatment is not sufficiently effective [4, 5]. Therefore, searching for new therapeutic target of influenza pathogenesis is still in urgent.

Protocatechuic acid (PCA) belongs to phenolic acid and is widely distributed naturally. PCA is found in many fruits such as grapes, grains, and other human diet [6]. PCA has also been isolated from some traditional Chinese herbal medicines. PCA has multiple biological functions including anti-inflammation [7], anti-oxidation [8], anti-bacteria [9], anti-virus [10], and hepato-protection [11]. PCA has also been described to prevent H9N2 influenza infection [12]. These previous reports drive us to explore the effects of PCA on H1N1 infection.

Materials and methods

Mice infection and treatment
Six-week-old specific-pathogen-free BALB/c mice (body weight from 18 to 22 g, GemPharmatech, Nanjing, China) were used in this study. The influenza strain A/Font Monmouth/47 (H1N1, FM1), a mouse-adapted strain, was plaque purified and amplified in chicken embryos. The 50% lethal dose (LD_{50}) titers were measured following previous protocols [13].

Mice were divided into 6 groups including negative control (NC) group (mice were without infection or treatment),
virus control group (mice were infected only), oseltami-
vir (Ose) (Sigma, St. Louis, MO, USA) group (mice were
infected and injected with 10 mg/kg oseltamivir intraperi-
toneally), protocatechuic acid (PCA) 10 mg/kg group (mice
were infected and injected with 10 mg/kg PCA intra-
toneally), PCA 20 mg/kg group (mice were infected and
injected with 20 mg/kg PCA intraperitoneally), and PCA
40 mg/kg group (mice were infected and injected with 40
mg/kg PCA intraperitoneally). The doses of PCA chosen
for this study were based on the publications [12, 14]. Mice
were anaesthetized and intranasally injected with 15 × 50%
LD_{50} of influenza virus in 50 μL phosphate-buffered saline
(PBS). Mice of NC group were injected with 50 μL PBS.
Two hours post infection, mice were treated with oseltamivir
or protocatechuic acid daily for 5 consecutive days. The mice
mortality was recorded every day for 15 days. Mice were
sacrificed on day 6 post infection (pi) and samples were har-
vested for analysis. This study was approved by the ethical
committee of Cangzhou Central Hospital.

Lung index and viral load

On day 6 post infection, mice were weighted. After sacrifice,
the lungs were isolated and weighted. The formula of lung
index was lung weight/body weight × 100. Equal amount of
lung tissues (50 mg) from each mouse were homogenized
to prepare the supernatant. Then the supernatant was diluted
serially from 1 to 10^{-7}. One hundred microliters of diluted
supernatant was injected into the allantoic cavity of embryon-
ated chicken eggs. Two days after injection, the hemag-
glutination titer of allantoic fluid was measured and the 50%
egg infective dose (EID_{50}) was calculated.

Quantitative polymerase chain reaction (qPCR)

To measure the viral load in the lungs using qPCR, the total
RNA from the lungs was extracted by NucleoSpin® RNA
Plus kit (Takara, Beijng, China). Then reverse transcrip-
tion was performed to get cDNA by using PrimeScript™
RT-PCR Kit (Takara, China). The primer sequences of IAV
M gene were sense 5’-AATGGTGCAAGCGATGAG-3’
and anti-sense 5’-TACTTGCAG CAACACGGAG-3’.
Primer sequences of GAPDH, the internal control, were sense 5’-CCTCTGCCCTCGAGACAAATG-3’
and anti-sense 5’-TGAAGTCAATGAGGCTG-3’. The quanti-
tative PCR was set up using TB Green® Advantage® qPCR
Premix (Takara, China) and samples were subjected to 7500
Fast Real-Time PCR System (Thermo Fisher, USA).

Myeloperoxidase (MPO) activity

The MPO activity was measured using Myeloperoxidi-
sable (MPO) Activity Assay Kit (Abcam, Beijng, China)
following the instructions. Briefly, 6 days pi mice were
sacrificed and lung tissues were homogenized in MPO
assay buffer provided in the kit. After centrifuge, the
supernatants were harvested.

Immune cell analysis in broncho-alveolar lavage
fluid (BALF)

Six days post infection, the BALF was collected as
described previously [15]. The cell numbers of lymphocytes,
macrophages, and neutrophils in BALF were counted using
an automatic blood cell analyzer.

ELISA

The lung tissues were harvested and homogenized. The levels
of interleukin (IL)-1β, tumor necrosis factor α
(TNF-α), interferon gamma (IFN-γ), IL-6, monocyte
chemoattractant protein-1 (MCP-1), and IL-10 in lung
homogenates were measured by corresponding ELISA
kits (Abcam, China).

Western blot

Lung tissues were homogenized in radioimmunopre-
cipitation lysis buffer (Abcam, China) to extract protein.
Extracted proteins were subjected to sodium dodecyl sul-
fate–polyacrylamide gel electrophoresis and transfer. After
blocking, primary antibodies were incubated at 4 °C over-
night. All primary antibodies were purchased from Abcam
(Beijing, China): anti-TLR4, anti-phosphor-NF-kB p65,
anti-p65, anti-phosphor-IκBα, anti-IκBα, and anti-β actin.
Next day, after washing, corresponding secondary anti-
bodies were added for incubation. The immuno-reactive
bands were visualized by adding the ECL Western Blotting
Substrate (Abcam, China). The western blot experiments
were repeated three times from pooled tissues. ImageJ was
used to quantitate the band intensity.

Statistical analysis

The statistical analyses were performed using GraphPad
Prism 8.0 software. One- or two-way ANOVA analysis fol-
lowed by a Dunn’s multiple comparisons test or Bonferroni
post hoc test was used for analysis. When p < 0.05, the
statistical difference was termed as significant.
Results

Protocatechuic acid protected mice from H1N1 challenge

First, we explored whether PCA protected mice after H1N1 infection. After H1N1 infection, we treated mice with different amounts of PCA and the mice mortality was compared among different groups. As shown in Fig. 1a, H1N1 infection caused obvious mouse death and all mice died at day 11 post infection. In contrast, mice administered with oseltamivir (Ose), an effective drug to treat influenza, had significantly enhanced survival rate. Mice treated with different amounts of PCA had significantly increased survival rate, indicating PCA protected mice from H1N1 challenge. Mice treated with the highest dose of PCA (40 mg/kg) had the highest survival rate, indicating the protection of PCA was in a dose-dependent manner. Similarly, H1N1 challenge resulted in decreased body weight in mice while PCA rescued H1N1-induced body weight loss (Fig. 1b). Taken together, these data demonstrated that PCA protected mice from H1N1 challenge.

Protocatechuic acid ameliorated lung injury and decreased viral burden

Next, we evaluated the effects of PCA on lung index and lung viral burden. H1N1 infection resulted in significantly increased lung index (Fig. 2a). In contrast, Ose treatment significantly decreased the lung index after infection. PCA treatment also decreased the lung index and 20 mg/kg and 40 mg/kg treatments significantly decreased the lung index (Fig. 2a). We detected high viral load in the lung using viral burden (Fig. 2b) and viral gene expression (Fig. 2c) after infection. Ose treatment significantly decreased the viral load in the lung (Fig. 2b and c). Mice treated with all 3 doses PCA had significantly decreased viral load and the decreasing of viral load correlated to the PCA dose. Mice treated with 40 mg/kg PCA had the lowest viral load when compared to mice treated with 10 and 20 mg/kg PCA. H1N1 infection resulted in significantly elevated MPO activity in
the lung (Fig. 2d). Ose and PCA treatment significantly suppressed the elevation of MPO after H1N1 infection.

**Protocatechuic acid suppressed immune cell infiltration in the lung**

Furthermore, we evaluated immune cell infiltration in the lung after PCA treatment. H1N1 infection significantly increased the total cell number (Fig. 3a), macrophage number (Fig. 3b), neutrophil number (Fig. 3c), and lymphocyte number (Fig. 3d) in BALF, indicating infection induced immune cell infiltration in the lung. In contrast, mice treated with 40 mg/kg PCA had significantly decreased total cell number (Fig. 3a), macrophage number (Fig. 3b), neutrophil number (Fig. 3c), and lymphocyte number (Fig. 3d) in BALF.

**Protocatechuic acid inhibited inflammatory cytokine production in the lung**

We further evaluated the effects of PCA on the expression of inflammatory cytokines after infection. H1N1 infection induced the expression of IL-1β (Fig. 4a), TNF-α (Fig. 4b), IFN-γ (Fig. 4c), IL-6 (Fig. 4d), and MCP-1 (Fig. 4e) while did not change the expression of IL-10 (Fig. 4f). Infected mice which were treated with 40 mg/kg PCA had significantly decreased level of IL-1β (Fig. 4a), TNF-α (Fig. 4b), IFN-γ (Fig. 4c), IL-6 (Fig. 4d), and MCP-1 (Fig. 4e) while had significantly increased level of IL-10 in the lung when compared to infected mice.

**Protocatechuic acid suppressed the activation of TLR4/NF-κB signaling pathway**

TLR4 has been implicated in influenza pathogenesis [16]. Therefore, we detected whether PCA treatment affects the activation of TLR4/NF-κB signaling pathway after H1N1 infection. As shown in Fig. 5a, compared to control mice, mice infected with H1N1 had obviously increased protein level of TLR4, p-IκBα, IκBα, p-p65, and p65 in the lung. Infected mice treated with 40 mg/kg PCA had dramatically decreased TLR4, p-IκBα, IκBα, p-p65, and p65. After quantitation, H1N1 infection resulted in significantly increased expression of TLR4 (Fig. 5b), p-IκBα (Fig. 5c), IκBα (Fig. 5d), p-p65 (Fig. 5e), and p65 (Fig. 5f). The upregulation of TLR4 (Fig. 5b), p-IκBα (Fig. 5c), IκBα (Fig. 5d), p-p65 (Fig. 5e), and p65 (Fig. 5f) was prevented by PCA treatment. Collectively, these results indicated that PCA prevented H1N1-induced activation of TLR4/NF-κB signaling pathway.
Discussion

In present study, we established a H1N1 mice infection model and administrated different amount of PCA to the infected mice. We found that PCA improved mice survival rate, suppressed lung inflammation, and decreased viral burden. Our study demonstrated the anti-H1N1 activity of PCA, strongly suggesting that PCA could be an effective therapeutic agent to treat influenza.

Influenza is an acute and recurring respiratory disease which causes severe illness [17]. After infection, IAV triggers innate immune response, activates multiple downstream signaling pathways, and finally results in expression of pro-inflammatory cytokines [18]. These cytokines contribute to the pathology in IAV infection. These pro-inflammatory cytokines can also recruit immune cells into the lung and amplify the immune response. In present study, we also found that after H1N1 infection, there were obvious immune cell infiltration and robust pro-inflammatory cytokines production. In contrast, PCA treatment remarkably prevented infiltration of immune cell and suppressed pro-inflammatory cytokine production. Our findings were consistent to previous report about the anti-inflammatory activities of PCA. Wang et al. showed that PCA reduced the monocytes infiltration into the abdominal cavity in apolipoprotein E-deficient mice [19]. PCA also suppressed the inflammation in diabetic rats and ameliorated their neurobehavioral deficits [20].

TLR4 is one of the pathogen-associated molecular patterns (PAMPs) which are involved in IAV-induced inflammation [18]. IAV infection induces oxidative and produces oxidized phospholipids, which could activate TLR4 [21]. Nhu et al. found that TLR4-deficient mice were resistant to IAV-induced lethality, suggesting that targeting TLR4 could protect against IAV infection [22]. Shirey et al. reported that the TLR4 antagonist eritoran prevented lethal influenza infection in mice. Eritoran also decreased lung pathology and cytokine production [16]. These reports strongly suggested that suppressing TLR4 is a promising strategy to prevent IAV infection. Our present study demonstrated that PCA treatment significantly decreased the expression of TLR4 and inhibited the activation of NF-κB, indicating PCA targeted TLR4 signaling pathway. These activities of PCA could contribute the anti-influenza effects. The inhibitory effects of PCA on activation of NF-κB have been described previously. Wang and colleagues reported that PCA prevented LPS-induced production of IL-6 and IL-8 by suppressing NF-κB activation in human fibroblasts [23]. Kaewmool and colleagues described that PCA inhibited inflammatory response in LPS-treated microglia by regulating NF-κB pathway [24].

Besides NF-κB signaling pathway, MAPK signaling pathway is another downstream pathway mediated by TLR4 [25]. IAV infection activated MAPK signaling pathway while MAPK inhibitor ameliorated IAV infection outcomes in mice, suggesting that MAPK is another therapeutic target for IAV treatment [26]. The inhibitory effects of PCA on MAPK have been widely described [27–29]. It should be interesting to explore whether PCA also inhibit MAPK activation in...
our model and it will not be surprising that PCA also target MAPK signaling pathway in our H1N1 infection in mice. In addition, we demonstrated that PCA treatment resulted in significantly decreased viral load in the lung after infection. Although our findings were consistent to previous report which described the anti-virus activates of PCA [30, 31], the underlying mechanisms are still need to be further determined. It is interesting to determine whether PCA can directly affect the translation/expression of viral proteins.

**Conclusion**

In present study, we demonstrated that PCA ameliorated H1N1 infection-induced outcomes and suppressed the lung inflammation by targeting TLR4/NFκB signaling pathway. Our results strongly suggest that PCA could be an effective therapeutic agent to treat H1N1 infection.
Fig. 5 Effects of protocatechuic acid on TLR4/NF-κB activation. a Lung tissues were homogenized. The protein expressions of TLR4, p-IκBα, IκBα, p-p65, and p65 in lung tissues were determined by western blot. The expressions were normalized to NC group (b–f). n = 3 for each group. **p < 0.01 and ***p < 0.001 compared to influenza A virus infection control group.

Author contribution Did the experiments and analyzed the data: Qian Wang, Xiaojuan Ren, Jinhua Wu, Hongrong Li, Liu Yang, Yan Zhang, Xin Wang, and Zhicun Li; designed the study and wrote the manuscript: Qian Wang. All the authors have accepted responsibility of the content of this submitted manuscript and approved submission.

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Availability of data and material The data could be obtained upon request to the corresponding author.

Declarations

Ethics approval This study was approved by the ethics committee of Cangzhou Central Hospital.

Consent to participate Not applicable.

Consent for publication Current study is available from the corresponding author on reasonable request.

Conflict of interest The authors declare no competing interests.
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