JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Tangeretin from *Citrus reticulate* Inhibits Respiratory Syncytial Virus Replication and Associated Inflammation *in Vivo*

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ABSTRACT: Human respiratory syncytial virus (RSV) is a common pathogen that causes pneumonia and bronchiolitis in infants and young children. Our previous study showed that tangeretin from *Citrus reticulate* possessed potent *in vitro* anti-RSV effects comparable to that of ribavirin. Therefore, in this study, we investigated the *in vivo* anti-RSV activity of tangeretin in 3-week-old male BALB/c mice. A plaque reduction assay and fluorescence quantitative polymerase chain reaction (FQ-PCR) showed that tangeretin inhibited RSV replication in the lung of mice. Moreover, a luminex assay indicated tangeretin relieved RSV-induced lung inflammation by attenuating interleukin (IL)-1β secretion. Possible anti-inflammatory mechanisms of tangeretin were preliminarily explored using a RSV-infected macrophage model. A FQ-PCR, enzyme-linked immunosorbent assay (ELISA), and luciferase assay revealed that tangeretin inhibited RSV-induced inflammation by suppressing nuclear factor-κB (NF-κB) activation. This study demonstrates that tangeretin inhibited RSV replication and RSV-induced lung inflammation *in vivo* and may be useful in preventing and treating RSV infections and inflammation.

KEYWORDS: *tangeretin, in vivo antiviral activity, respiratory syncytial virus replication, RSV-induced inflammation, NF-κB*

INTRODUCTION

Human respiratory syncytial virus (RSV) infection is a leading cause of lower respiratory tract diseases in infants and young children, with pneumonia and bronchiolitis as the main clinical symptoms.[1] Palivizumab and ribavirin are approved for the prevention and treatment of RSV infection. Prophylactic treatment with palivizumab, a neutralizing monoclonal antibody, is used to protect high-risk infants.[2] However, this antibody does not improve lung histopathology and inflammation.[3] Ribavirin is a broad antiviral agent that is used primarily in infants and immunocompromised patients with serious RSV infections; however, its use is limited as a result of its variable efficacy and toxicity,[4] and a suitable treatment for RSV infections remains a major concern.

The mechanisms of RSV-induced diseases remain elusive. Experimental evidence suggests that an excessive inflammatory response triggered by the host plays a major role in the development of the clinical manifestations of RSV infection.[5] Following primary RSV infection of the respiratory tract, alveolar macrophages and epithelial cells are likely to be infected, resulting in the enhancement of pro-inflammatory cytokines, including interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and chemokines. Additionally, macrophages are crucial in initiating antiviral adaptive immunity by inducing T helper lymphocyte differentiation, including Th1, Th2, Th17, and regulatory T lymphocytes (Treg) subsets. These T lymphocyte subpopulations are responsible for lung immune injury following RSV infection.[6−9] The nuclear factor-κB (NF-κB) transcription factor plays an essential role in immune and inflammatory responses triggered by stimuli through regulating the expression of genes encoding numerous pro-inflammatory cytokines and chemokines. RSV infection persistently activates NF-κB and leads to excessive NF-κB-mediated inflammatory gene expression.[10,11] Therefore, NF-κB is considered a preferred target in the development of therapeutic interventions aimed at limiting the inflammatory responses induced by RSV infection.

Our previous study found that the supercritical fluid extract from the pericarps of *Citrus reticulate* possessed *in vitro* anti-RSV activity and tangeretin, a major polymethoxylated flavone in this active extract, possessed a potent *in vitro* anti-RSV effect comparable to the positive control ribavirin.[12] We also demonstrated that tangeretin downregulated the expression of RSV phosphoprotein (P protein).[12] On the other hand, tangeretin has been reported to possess significant anti-inflammatory activity in lipopolysaccharide (LPS)-induced RAW264.7 cells by suppressing NF-κB activity.[13,14] Therefore, the present study was conducted to investigate the *in vivo* antiviral activities of tangeretin against RSV replication and RSV-induced inflammation using male 3-week-old BALB/c mice as the animal model. Moreover, we preliminarily explored the possible anti-inflammatory mechanism of tangeretin in a RSV-infected macrophage model. The results indicated that tangeretin inhibited RSV replication and relieved RSV-induced lung inflammation by suppressing NF-κB activation.

Received: July 16, 2015
Revised: October 14, 2015
Accepted: October 15, 2015
Published: October 15, 2015

DOI: 10.1021/acs.jafc.5b03482
J. Agric. Food Chem. 2015, 63, 9520−9527

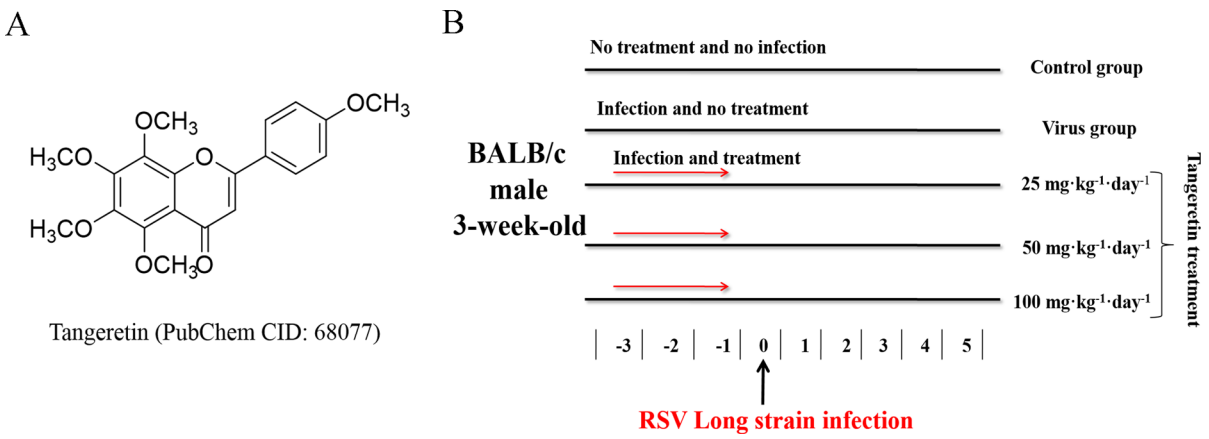

Figure 1. Structure of tangeretin and feeding protocols in mouse experiments: (A) structure of tangeretin and (B) animal treatments. Male 3-week-old BALB/c mice in the tangeretin-treated group were intragastrically administered tangeretin for 3 days consecutively at doses of 25, 50, or 100 mg/kg/day, while control and virus-treated groups were administered saline. Then, mice in the virus- and tangeretin-treated groups were slightly anesthetized with diethyl ether and intranasally infected with 6.7×10^6 PFU of RSV Long strain for 4 days, and the control mice were sham-infected with an equal volume of medium. On day 5 post-infection, mice were euthanized for anti-RSV tests.

MATERIALS AND METHODS

Reagents. Tangeretin (purity > 97%; Figure 1A) was isolated from *C. reticulate* pericarps, and the chemical structure was identified using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analysis in our laboratory as previously described.[15] Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Invitrogen, Carlsbad, CA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and ribavirin were obtained from Sigma-Aldrich (St. Louis, MO). The pGL-NF-κB-luciferase plasmid was purchased from Han Heng Biology Company (Shanghai, China). RAW264.7 [American Type Culture Collection (ATCC) TIB-71], human larynx epidermoid carcinoma (HEp-2, ATCC CCL-23), and human embryonic kidney 293 (HEK293, ATCC CRL-1573) cells and human RSV Long (ATCC VR-26) strain were purchased from the Medicinal Virology Institute of Wuhan University.

Animals and Treatment Protocol. Male 3-week-old BALB/c mice [specific pathogen-free (SPF) grade, certified number SCXK (Guangdong) 2003-0001] were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). The mice were maintained in an air-conditioned, pathogen-free room (temperature of 24 ± 2 °C, with a 12 h light/dark cycle from 6:00 am to 6:00 pm) with free access to food and water. Mice were randomly divided into five groups (n = 10) as follows: normal (control), RSV-challenged, and three treatment groups administered 25, 50, or 100 mg/kg/day tangeretin dissolved in saline. The control and RSV-challenged groups received equal volumes of saline. During the experiment, mice in the treatment groups were intragastrically administrated tangeretin for 3 days consecutively before RSV stimulation. Mice were lightly anesthetized with diethyl ether and intranasally challenged with RSV Long strain [6.7×10^6 plaque-forming units (PFU)] on day 4 after tangeretin treatment, while the control group was sham-infected with an equal volume of HEp-2 cell lysate, which was centrifuged under the same conditions as the viral suspensions. The mice were weighed during the experiment and sacrificed on day 5 post-infection after anesthetizing them with chloral hydrate (Figure 1B). The lung tissues were removed and weighed, and the lung index was calculated using the following formula: lung index = lung weight/body weight. All mice were handled in strict adherence to the Guidelines for Laboratory Animal Use and Care of the Chinese Center for Disease Control and Prevention (CDC) and the Rules for Medical Laboratory Animal of the Ministry of Health, China. The animal protocols were approved by the National Institute for Communicable Disease Control and Prevention and the Ethics Committee of Jinan University.

Plaque Reduction Assay. Viral titers of the lung tissue were determined using a plaque reduction assay.[16] Briefly, lung tissue was homogenized and centrifuged for 5 min at 600g, and supernatants were added to HEp-2 cell monolayers in a 24-well plate. After incubation for 2 h, the medium was removed and the cell monolayers were washed with phosphate-buffered saline (PBS) twice, followed by overlaying with agarose overlay medium. After agarose had solidified, the maintenance medium was added to each well and the plate was further incubated for 4−5 days to allow for plaque formation. The cells were fixed and stained, and the number of plaques was counted.

Luminex Assay. Cytokine levels in the lung were quantified using a multiplex immunoassay (Linco Research, Inc., St. Charles, MO) according to the instructions of the manufacturer. Lung tissue samples were lysed with lysis buffer, and the samples were assayed with appropriate standards and controls for each cytokine. Beads were added to the samples, and the plate was incubated overnight at 4 °C. Detection antibodies were added, and the plate was incubated on a shaker, followed by the addition of streptavidin−phycoerythrin to each well. After washing, sheath fluid was added and the plate was run on a Bio-plex200 (MILLIPLEX map kit, Millipore, Watford, U.K.). The median fluorescence intensity was analyzed using Milliplex analysts, and the concentration of each cytokine was calculated.

MTT Assay. The effect of tangeretin on RAW264.7 cells was determined using a MTT assay as previously reported.[13] Briefly, RAW264.7 cells (1×10^4 cells/well) were seeded in a 96-well plate for 24 h and treated with different concentrations of tangeretin (6.3−50.0 μM) and dimethyl sulfoxide (DMSO) (vehicle control, 0.01 and 0.1%) for 10 or 48 h. The absorbance was measured at 570 nm using an enzyme immunoassay (EIA) reader (Thermo Scientific, Waltham, MA), and cell viability (%) was calculated as follows: [(absorbance of the test group − absorbance of the blank control)/(absorbance of the control group − absorbance of the blank control)] × 100.

Nitric Oxide (NO) Production Assay. The NO production assay was carried out according to a previously described method.[17] RAW264.7 cells (4×10^4 cells/well) were cultured in a 48-well plate for 24 h. The cells were treated with varying concentrations (6.3−50.0 μM) of tangeretin, DMSO (vehicle control, 0.01 and 0.1%), and RSV [multiplicity of infection (MOI) = 1] for 48 h. The supernatant (50 μL) was mixed with Griess reagent (50 μL) for 10 min, and the absorbance was measured at 540 nm with an EIA reader. NO levels were calculated using a calibration curve constructed with $NaNO_2$ concentrations of 0.78−100 μM.

Fluorescence Quantitative Polymerase Chain Reaction (FQ-PCR) Assay. FQ-PCR was performed to determine the mRNA expression levels of IL-1β, IL-6, and TNF-α in RAW264.7 cells following exposure to RSV (MOI = 1) for 10 h and RSV P protein, IL-1β, TNF-α, IL-6, IL-4, IL-17a, and IL-10 in lung tissues. Total RNA from both RAW264.7 cells and lung tissues was extracted using Trizol isolation reagent (Invitrogen, Carlsbad, CA), quantified, and reverse-

DOI: 10.1021/acs.jafc.5b03482
J. Agric. Food Chem. 2015, 63, 9520−9527

Table 1. Primers Used in FQ-PCR

genes	primers	accession number	length
IFN-γ	5′-ATGAACGCTACACACTGCATC-3′ (forward)	NM_008337.3	182
	5′-CCATCCTTTTGCCAGTTCCTC-3′ (reverse)		
IL-1β	5′-GAAATGCCACCTTTTGACAGTG-3′ (forward)	NM_008361.3	116
	5′-TGGATGCTCTCATCAGGACAG-3′ (reverse)		
IL-4	5′-GGTCTCAACCCCCAGCTAGT-3′ (forward)	NM_021283.2	102
	5′-GCCGATGATCTCTCTCAAGTGAT-3′ (reverse)		
IL-6	5′-TAGTCCTTCCTACCCCAATTTCC-3′ (forward)	NM_031168.1	76
	5′-TTGGTCCTTAGCCACTCCTTC-3′ (reverse)		
IL-10	5′-GCTCTTACTGACTGGCATGAG-3′ (forward)	NM_010548.2	105
	5′-CGCAGCTCTAGGAGCATGTG-3′ (reverse)		
IL-17a	5′-TTTAACTCCCTTGGCGCAAAA-3′ (forward)	NM_010552.3	165
	5′-CTTTCCCTCCGCATTGACAC-3′ (reverse)		
TNF-α	5′-AGCCCACGTCGTAGCAAACCACCAA-3′ (forward)	NM_013693.3	447
	5′-AACACCCATTCCCTTCACAGAGCAAT-3′ (reverse)		
RSV P	5′-TCAAAGAAGACCCTACGCCAAGTGA-3′ (forward)	NC_001803.1 (2331−3220)	131
	5′-ACCATCCCGAGCAGATGTAGGTCC-3′ (reverse)		
GAPDH	5′-AGGTCGGTGTGAACGGATTTG-3′ (forward)	NM_001289726.1	123
	5′-TGTAGACCATGTAGTTGAGGTCA-3′ (reverse)		

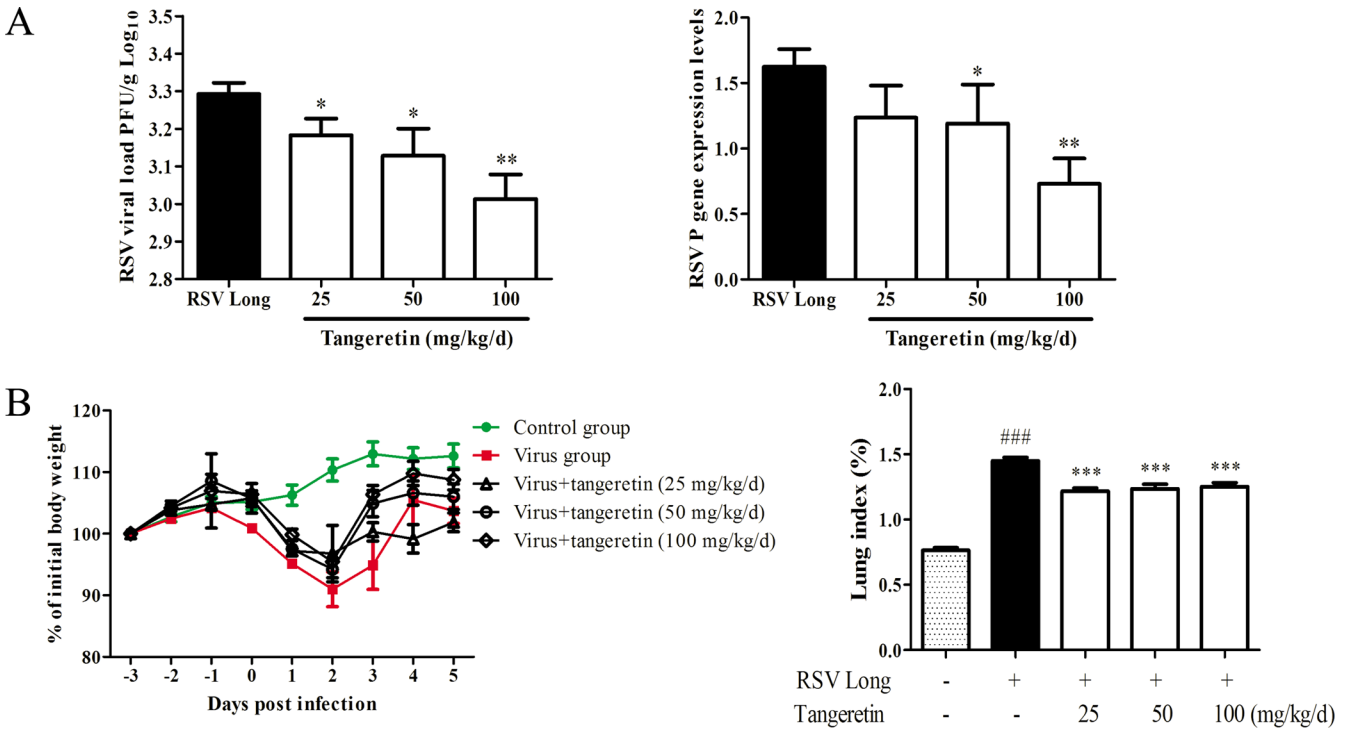

Figure 2. Effects of tangeretin on RSV replication and RSV-induced inflammation *in vivo*: (A) RSV viral load in lung tissue measured using two assays, namely, plaque reduction (left) and FQ-PCR (right), and (B) body weight gain (left) and lung index (right). Results were expressed as means ± SE (n = 10). Significant differences between tangeretin and virus control cells were observed at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, and significant differences between control and virus groups were observed at (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.001$.

transcribed to cDNA. Relative quantitation of gene expression was performed using the LightCycler 480 (Roche, Pleasanton, CA) system. The primer pairs for the PCR are shown in Table 1. The cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. A dissociation curve was generated using a cycle of 95 °C for 5 s, 67 °C for 1 min, and 97 °C for 15 s. Results were normalized to GAPDH as the reference gene. The data were analyzed by normalizing the resulting threshold cycle (CT) values of the target mRNAs to the CT values of the internal control (GAPDH) in the same samples ($\Delta CT = CT_{target} - CT_{GAPDH}$). Then, it was further normalized to the control ($\Delta\Delta CT = \Delta CT - CT_{control}$). The fold change in expression was then obtained as ($2^{-\Delta\Delta CT}$).

Enzyme-Linked Immunosorbent Assay (ELISA). The effect of tangeretin on cytokine (IL-1β, TNF-α, and IL-6) release in RAW264.7 cells was evaluated using an ELISA. Briefly, RAW264.7 cells were treated with varying concentrations of tangeretin and infected with RSV Long strain (MOI = 1) for 48 h. The concentration of IL-1β, TNF-α, and IL-6 in the supernatants was estimated using commercially available ELISA kits (Dakewei, China) according to the protocol of the manufacturer.

Luciferase Activity Assay. HEK293 cells (8×10^4 cells/well) were seeded in a 48-well plate and incubated for 24 h. Cells were transiently co-transfected with the pGL-NF-κB-luciferase reporter plasmid and the Renilla luciferase reporter plasmid pRL-TK using a

DOI: 10.1021/acs.jafc.5b03482
J. Agric. Food Chem. 2015, 63, 9520−9527

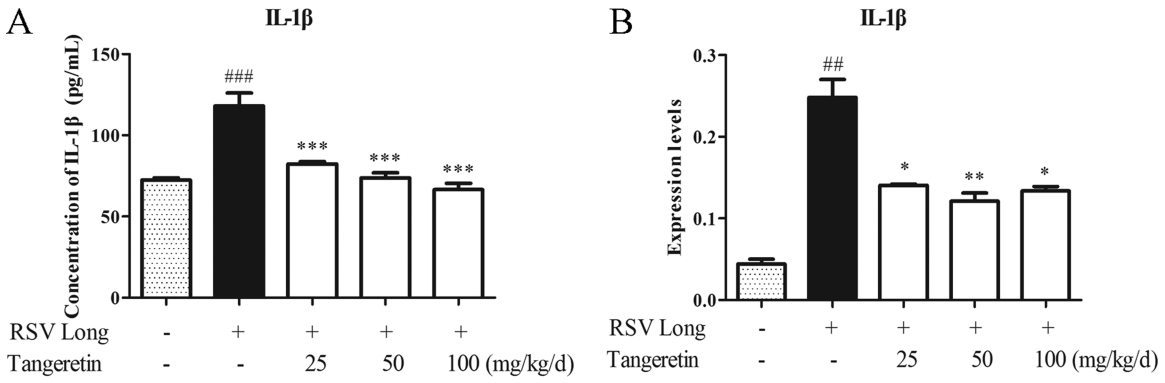

Figure 3. Effects of tangeretin on secretion and mRNA expression of IL-1β in lung tissue: (A) IL-1β secretion level and (B) IL-1β mRNA expression. Results were expressed as means ± SE (n = 10). Significant differences between tangeretin and the virus-treated control group were observed at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, and significant differences between control and virus-treated groups were observed at (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.001$.

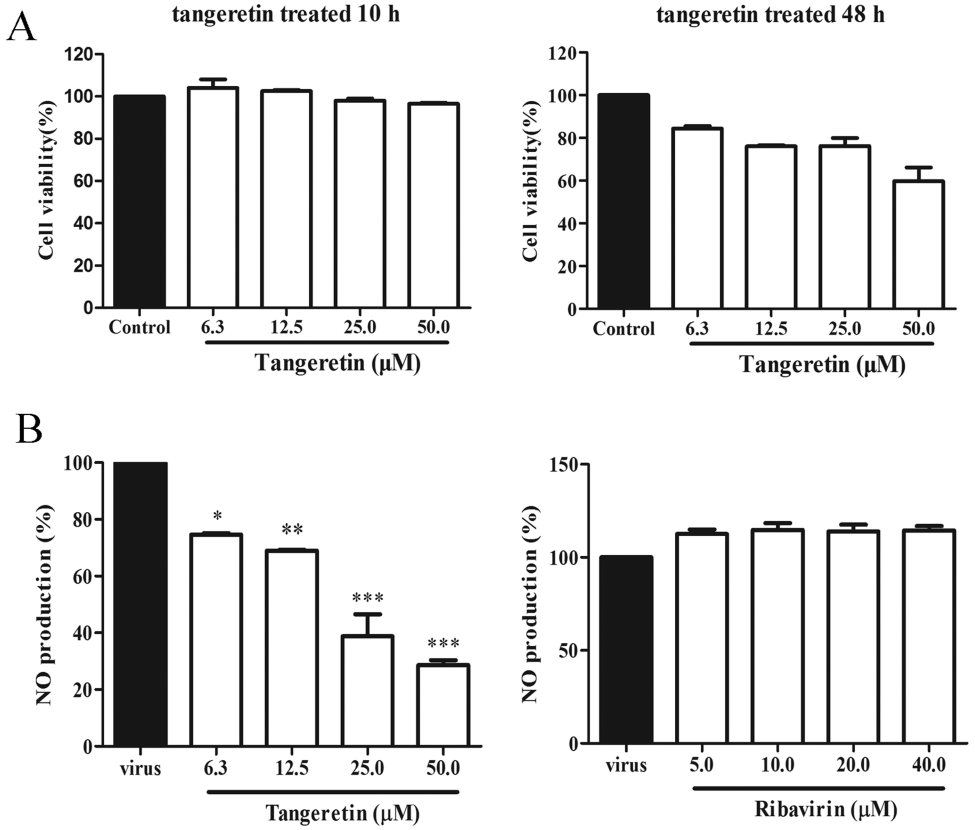

Figure 4. Effects of tangeretin on (A) cell viability and (B) NO production *in vitro*. Results were expressed as means ± SE (n = 6) of three independent experiments. Significant difference between tangeretin-treated and virus control cells was observed at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$.

Lipofectamine 3000 kit (Invitrogen, Carlsbad, CA). At 6 h following transfection, the cells were treated with varying concentrations of tangeretin and stimulated with RSV Long strain (MOI = 1) for 3, 6, 12, or 18 h. NF-κB luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) using a microplate reader (Thermo, Rockford, IL) according to the instructions of the manufacturer. Data were expressed by dividing the firefly luciferase activity by the Renilla luciferase activity.

Western Blot Analysis. RAW264.7 cells (1.6×10^6 cells/well) were seeded in a 100 mm dish for 24 h and then pretreated with varying concentrations of tangeretin (6.3–50.0 μM) or DMSO (vehicle control, 0.01 and 0.1%) for 3 h, followed by RSV infection for 30 min. Cells were collected, lysed, and then centrifuged at 15294g for 20 min at 4 °C. The supernatants were collected, and the protein concentrations of the lysates were measured using a bicinchoninic acid (BCA) protein assay. A total of 20 μg of total protein was separated using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electrotransferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA), blocked, and immunoblotted with specific rabbit polyclonal antibodies (1:1000) overnight at 4 °C. β-Actin served as the loading control. The blots were incubated with a goat anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h at 25 °C after being washed. The membrane was detected using an enhanced chemiluminescence western blot detection kit (Invitrogen, Carlsbad, CA) and Kodak X-ray film.

DOI: 10.1021/acs.jafc.5b03482
J. Agric. Food Chem. 2015, 63, 9520–9527

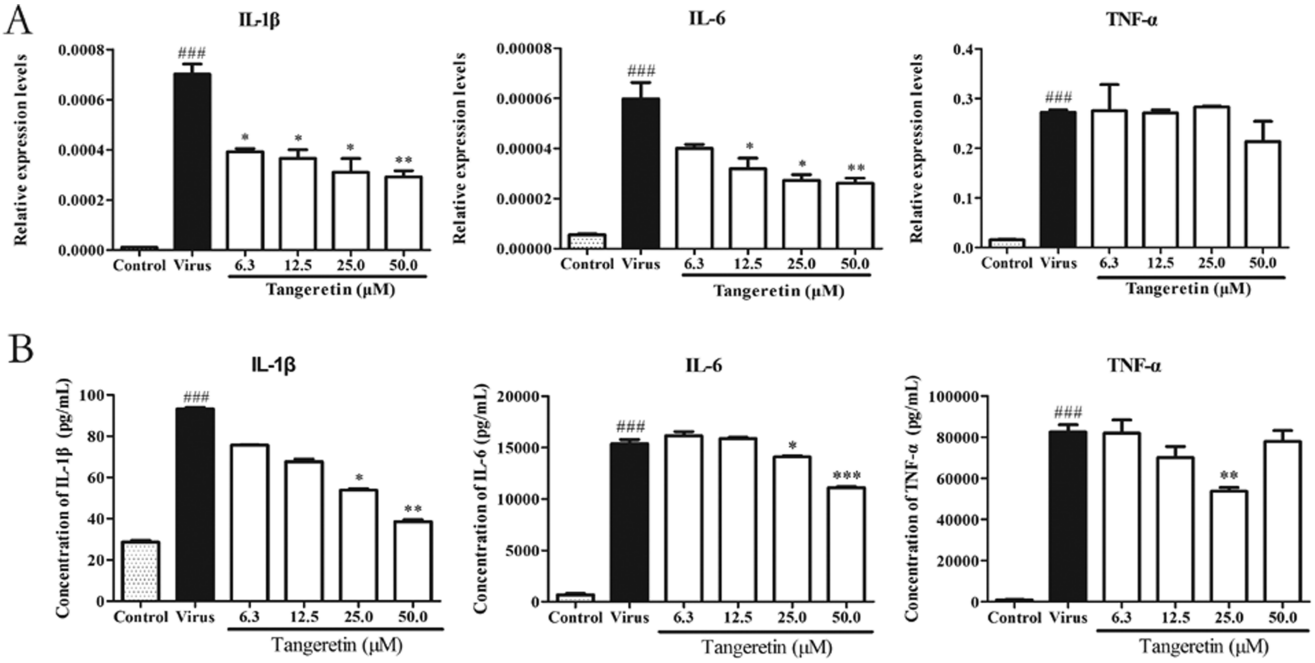

Figure 5. Effects of tangeretin on (A) mRNA expression and (B) secretion of IL-1β, IL-6, and TNF-α. Results were expressed as means ± SE ($n = 6$) of three independent experiments. Significant difference between tangeretin and virus-treated control cells was observed at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, and significant difference between control and virus-treated group cells was observed at (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.001$.

Statistical Analysis. Data are presented as the means ± standard error (SE), and the statistical analyses were performed using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test using the GraphPad Prism 5.0. A p value of <0.05 was considered significant.

RESULTS

Effect of Tangeretin on RSV Replication *in Vivo*. The *in vivo* anti-RSV activity of tangeretin was investigated in male 3-week-old BALB/c mice infected intranasally with RSV Long strain. Viral loads in the lung tissues of the mice were determined using a plaque reduction assay when viral replication attained the peak in the lungs, 5 days post-inoculation. As showed in Figure 2A, the viral loads following treatments with 25, 50, and 100 mg/kg/day tangeretin were 3.183 ± 0.044, 3.128 ± 0.072, and 3.013 ± 0.065 PFU/g $\log_{10}$, respectively, representing a significant decrease compared to that of the RSV-infected mice (3.300 ± 0.973 PFU/g $\log_{10}$). Our previous study indicated that tangeretin affected RSV replication in Hep-2 cells by downregulating the expression of RSV P protein.[12] Therefore, we determined the gene expression level of RSV P protein in the lung tissue of the mice using a FQ-PCR assay. The results revealed that the expression of RSV P protein was dose-dependently inhibited by tangeretin (from 1.240 ± 0.244 to 0.731 ± 0.237) compared to that of the RSV-treated mice (1.624 ± 0.072; Figure 2A).

Effect of Tangeretin on RSV-Induced Inflammation *in Vivo*. First, we assessed the histological changes in mouse lung tissue samples using lung index. As showed in Figure 2B, the lung index of RSV-infected mice (1.449 ± 0.026) was significantly higher than that of the control group (0.765 ± 0.020), indicating that pneumonia was successfully induced in this model. Tangeretin significantly decreased the lung indices at the tested doses (Figure 2B), suggesting that this compound might prevent RSV-induced pneumonia. To explore the inhibitory effect of tangeretin on lung inflammation in the mice, we evaluated the mRNA expression levels and secretions of some inflammatory cytokines, including IL-1β, IL-4, IL-6, IL-10, IL-17a, interferon (IFN)-γ, and TNF-α, in lung homogenates. There was no difference in IL-4, IL-6, IL-10, IL-17a, IFN-γ, or TNF-α expression and secretion between the RSV-challenged and tangeretin-treated mice (data not shown). Notably, both the mRNA expression level and secretion of IL-1β in lung homogenates were dramatically attenuated by tangeretin compared to those in the RSV-challenged group (Figure 3). These results suggest that tangeretin might suppress RSV infection-induced inflammation *in vivo* by attenuating IL-1β secretion.

Effects of Tangeretin on Cell Viability and NO Production. The above results proved that tangeretin effectively inhibited lung inflammatory response induced by RSV infection. Activated macrophages are the main source of pro-inflammatory mediators, and therefore, we determined the anti-inflammatory effect and preliminary mechanism of action of tangeretin using the RSV-infected macrophage model. To study the effects of tangeretin on NO production in RSV-stimulated RAW264.7 cells, cell viability was first evaluated using the MTT assay following treatment with varying tangeretin concentrations. No difference was observed between the control and tangeretin-treated groups after 10 h. However, cell viability was decreased following treatment with the same doses for 48 h (Figure 4A). As shown in Figure 4B, RSV Long strain infection significantly increased NO production compared to the unstimulated control. Furthermore, tangeretin dose-dependently inhibited NO production, with an IC_{50} of 21.7 μM and a selective index (SI) of 2.8.

Effects of Tangeretin on mRNA Expressions and Secretions of IL-1β, IL-6, and TNF-α *in Vitro*. To further assess the effects of tangeretin on RSV-induced inflammation, we evaluated the mRNA expression of cytokines produced in infected RAW264.7 cells. As shown in Figure 5A, the mRNA

DOI: 10.1021/acs.jafc.5b03482
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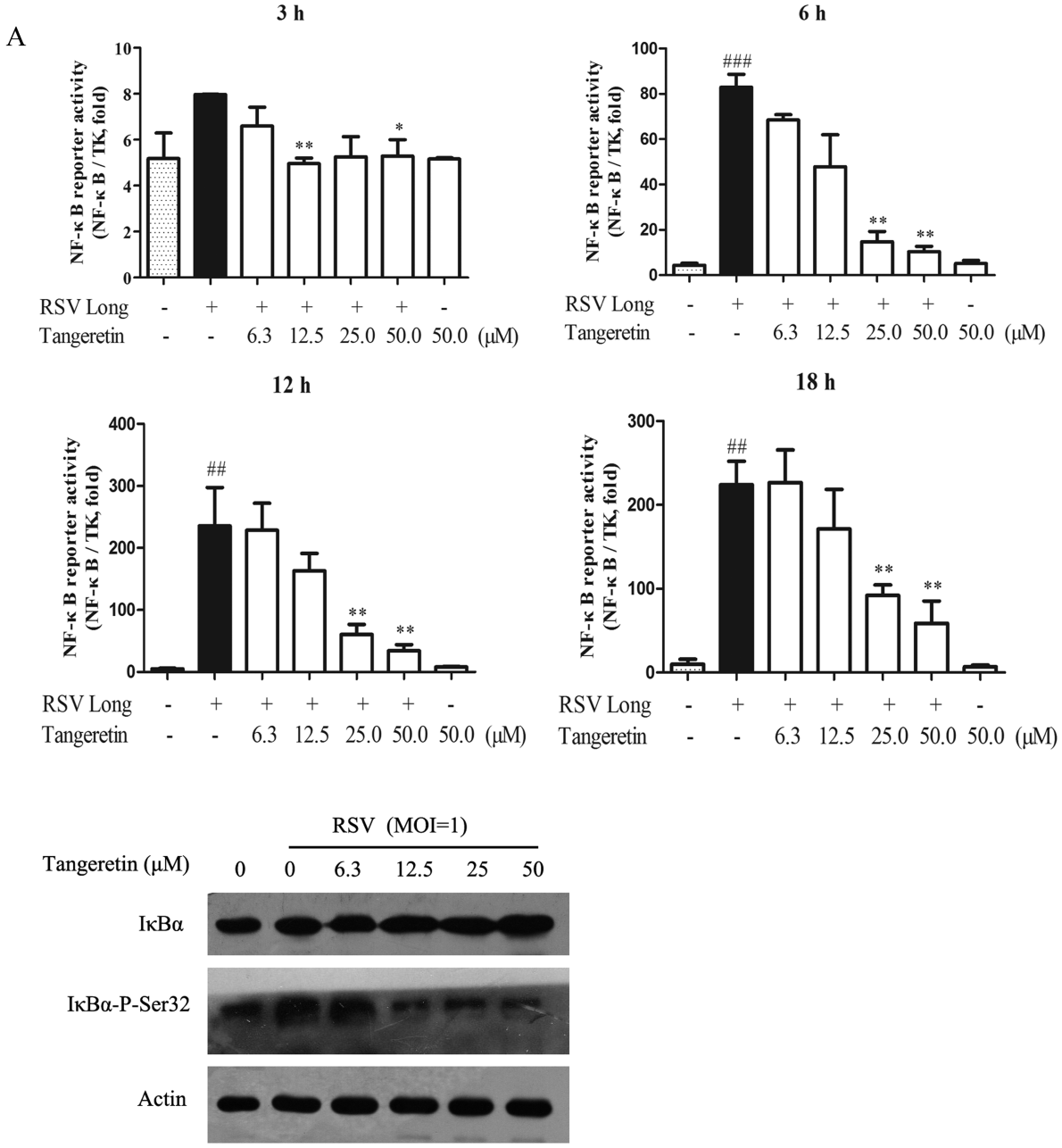

Figure 6. Inhibition of RSV Long strain-induced NF-κB activation by tangeretin. (A) NF-κB translocation in HEK293 cells induced by RSV was measured using a dual-luciferase reporter assay. Data represent three identical experiments. Significant differences between tangeretin and the virus-treated control were observed at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$, and significant differences between control and virus-treated groups were observed at (#) $p < 0.05$, (##) $p < 0.01$, and (###) $p < 0.001$. (B) Phosphorylation of IκBα was analyzed via western blot analysis.

expression levels of IL-1β, IL-6, and TNF-α were significantly increased after RSV infection. Following tangeretin treatment, RSV-induced IL-1β and IL-6 expressions were dose-dependently inhibited. In particular, the IL-1β mRNA expression decreased to 3.93×10^{-4} and 3.65×10^{-4} at tangeretin concentrations of 6.3 and 12.5 μM, respectively, compared to that of RSV-infected RAW264.7 cells (7.02×10^{-4}).

An ELISA was used to evaluate the effect of tangeretin on pro-inflammatory cytokine secretion in RSV-treated RAW264.7 cells. Tangeretin dose-dependently inhibited the secretion of IL-1β and IL-6 (Figure 5B), similar to the mRNA expression data. However, both the FQ-PCR and ELISA results showed no difference in TNF-α expression between the virus-infected and tangeretin-treated groups, even at 50.0 μM.

Effects of Tangeretin on RSV-Induced NF-κB Translocation in Cells. NF-κB is an essential transcription factor involved in immune and inflammatory responses, which regulates the production of several cytokines, including IL-1β.[18,19] To investigate whether tangeretin inhibits RSV Long strain-induced inflammation via the NF-κB pathway, we used a dual-luciferase reporter assay system to determine NF-κB activity. As shown in Figure 6A, no obvious changes in NF-κB activity were observed in the presence of tangeretin alone, whereas RSV infection sharply and time-dependently increased transcriptional activity. Moreover, RSV-induced activation of NF-κB was dose-dependently inhibited by tangeretin treatment. To determine the effect of tangeretin on the other components of the NF-κB pathway, the phosphorylation of IκBα at Ser32

DOI: 10.1021/acs.jafc.5b03482
J. Agric. Food Chem. 2015, 63, 9520−9527

was analyzed using western blotting. The result showed that IκBα phosphorylation was restored after treatment with tangeretin (Figure 6B).

DISCUSSION

RSV infection is an important cause of lower respiratory tract infection, leading to bronchiolitis and pneumonia.[20] Despite decades of efforts, there is no effective and safe drug for the treatment RSV infection. Ribavirin was reported to inhibit RSV replication in previous studies,[2,4] but the present data show that ribavirin treatment had no effect on the inflammatory response induced by RSV infection (Figure 4B). However, this study has revealed that RSV-induced local inflammation remains and is exacerbated, leading to the development of bronchiolitis and pneumonia even after the virus is cleared.[20] Therefore, except for inhibiting viral replication, anti-inflammatory therapy may represent an efficient treatment for RSV infection. Our previous study indicated that tangeretin possessed potent *in vitro* anti-RSV activity, mainly by affecting intracellular viral replication by downregulating the expression of RSV P protein.[12] The present study further investigated the antiviral activity of tangeretin in RSV-infected BALB/c mice. We selected male 3-week-old BALB/c mice as an *in vivo* model because the susceptible population to RSV infection is infants and children. The results revealed that tangeretin decreased the viral titer and reduced the mRNA expression of RSV P protein in the lung of RSV-infected mice (Figure 2A). The *in vivo* finding was consistent with the *in vitro* study, indicating that tangeretin inhibits viral replication.

Recent research studies have demonstrated that the immune response to RSV may result in airway inflammation and additional airway destruction.[21] In the present study, the preliminary histological results showed that tangeretin treatment significantly prevented RSV-induced pneumonia. The mRNA expression and protein secretion level of pro-inflammatory cytokines in lung homogenates were further studied to determine the effect of tangeretin on lung inflammation. In comparison to the RSV-challenged mice, tangeretin treatment significantly inhibited the mRNA expression and secretion of IL-1β in the lungs of RSV-infected BALB/c mice (Figure 3). Besides the involvement of innate immunity in the response against viral infections, the adaptive immune response is further induced after RSV infection, which is an obvious Th2 and Th17 response enhanced by repeated RSV challenges.[22,23] In our study, tangeretin did not inhibit the secretion and mRNA expression of IFN-γ, IL-4, and IL-17a cytokine production in the lung induced by the RSV challenge (data not shown). The results indicated that tangeretin alleviated inflammatory response induced by RSV by modulating innate but not adaptive immunity.

To explore the intrinsic mechanisms for the anti-inflammation activity of tangeretin, we used the RSV-infected macrophage model because a large number of macrophages are recruited to the lung and play an important role in mediating different immunopathological phenomena during inflammation.[24] After RSV infection, macrophages may overexpress toxic radicals, such as NO, and pro-inflammatory cytokines and inflammatory mediators, such as IL-1β, IL-6, TNF-α, and monocyte chemotactic protein-1 (MCP-1).[23,25,26] The results revealed that tangeretin suppressed the secretions of IL-1β and IL-6 in RSV-infected macrophages (Figure 5B), suggesting that tangeretin exerted its anti-inflammatory effect via inhibition of cytokine secretion in macrophages. The NF-κB pathway is an important discrete signal that induces the gene expression of pro-IL-1β following pattern recognition receptor (PRR) activation by pathogen-associated molecular patterns (PAMPs) in macrophages.[19,27] The dual luciferase reporter assay showed that tangeretin significantly inhibited RSV-induced NF-κB activity dose- and time-dependently (Figure 6), indicating that one possible mechanism mediating the inhibition of IL-1β secretion by tangeretin may be blockade of the NF-κB pathway. Besides the NF-κB pathway previously described, inflammasome complexes, such as the nod-like receptor family protein 3, can also be assembled to activate caspase-1, and the latter cleaves pro-IL-1β into IL-1β.[28,29] Nevertheless, the effect of tangeretin on the inflammasome complex assembly remains unclear, and further studies to elucidate this phenomenon are under way.

In conclusion, our study found that tangeretin inhibited RSV replication and suppressed RSV-induced inflammation *in vivo*, probably via the inhibition of NF-κB activation, which decreases IL-1β secretion. These results suggest that tangeretin may be potentially useful in the prevention and treatment of RSV infection and RSV-induced inflammation. Furthermore, the regular consumption of tangeretin at a dose of no more than 10 mg/day would be helpful in protecting children from RSV infections.

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Author Contributions

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Funding

This work was supported by grants from the Natural Science Foundations of China (81202427 and 81473116), the Natural Science Foundation of Guangdong Province (S2013020012864), the China Postdoctoral Science Foundation (2014M56225), and the International Science and Technology Cooperation Program of Guangdong Province, China (2013B051000061).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FQ-PCR, fluorescence quantitative polymerase chain reaction; HEK293, human embryonic kidney 293; HEp-2, human larynx epidermoid carcinoma; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NO, nitric oxide; NF-κB, nuclear factor-κB; PAMPs, pathogen-associated molecular patterns; P protein, RSV phosphoprotein; PRR, pattern recognition receptor; RSV, respiratory syncytial virus; TNF, tumor necrosis factor; Treg, regulatory T lymphocytes

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DOI: 10.1021/acs.jafc.5b03482
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DOI: 10.1021/acs.jafc.5b03482
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