Dihydrokaempferol attenuates LPS-induced inflammation and apoptosis in WI-38 cells

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Received 7 August 2023; Accepted 23 August 2023
Available online 1 November 2023

Abstract

Background: Globally, pneumonia has been associated as a primary cause of mortality in children aged less than 5 years. Dihydrokaempferol (DHK) has been proposed for being correlated with the process of various diseases. Nevertheless, whether DHK has a role in the progression of infantile pneumonia remains unclear. This study aimed at exploring whether DHK was involved in the progression of infantile pneumonia.

Methods: Human fibroblast cells WI-38 were treated with lipopolysaccharide (LPS). The viability of WI-38 cells was measured via Cell counting kit-8. Reverse transcription-quantitative polymerase chain reaction was used to evaluate the levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α). Western blot analysis revealed the protein levels of IL-1β, IL-6, TNF-α, Bax, and cleaved-caspase 3. Flow cytometry was applied for exploring the apoptosis of WI-38 cells. The concentrations of IL-1β, IL-6, and TNF-α were assessed via enzyme-linked-immunosorbent serologic assay.

Results: DHK modulated the viability of WI-38 cells in infantile pneumonia. Furthermore, we identified that DHK treatment inversely changed LPS induction-mediated elevation on the levels of inflammation biomarkers. Besides, DHK counteracted LPS-induced production of reactive oxygen species (ROS) in WI-38 cells. DHK also decreased LPS-induced elevation of WI-38 cells apoptosis and mediated the levels of apoptosis-associated indexes. Moreover, modulating sirtuin-1 (SIRT1) protein level was lowered by the induction of LPS, and was reversed by DHK treatment. In addition, DHK counteracted LPS induction-mediated elevation of p-p65 and phosphorylated inhibitor of nuclear factor kappa-B kinase subunit alpha (p-IκBα) protein levels.

Conclusion: DHK alleviated LPS-induced WI-38 cells inflammation injury in infantile pneumonia through SIRT1/NF-κB pathway. The results shed light on the implications of DHK on the prevention and treatment of infantile pneumonia.

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KEYWORDS
dihydrokaempferol; infantile pneumonia; SIRT1; inflammation injury; lipopolysaccharide

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https://doi.org/10.15586/aei.v51i6.971
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Introduction

Globally, pneumonia has been associated as a primary cause of mortality among children aged <5 years, with a mortality rate of 14%. Although the pneumonia-related mortality in China has decreased, it remains a significant contributor to morbidity among children. According to the World Health Organization (WHO) data, pneumonia is responsible for more than 900,000 child deaths globally. Infantile pneumonia is still a significant clinical and public health problem. It is still imperative to identify reliable biomarkers associated with infantile pneumonia to decrease the incidence and mortality of this disease.

As a natural compound from Chinese herbal plant Bauhinia championii (Benth.), dihydrokaempferol (DHK) (CAS Number: 480-20-6) is a type of flavonoid with a variety of effects, such as anti-inflammatory and antioxidant stress. DHK is found to have significant potential as a therapeutic and pharmaceutical agent, making it an important candidate for the future medicine.

Previously, DHK was proposed for being correlated with the process of various diseases. For instance, DHK mediates Kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2 (Keap1/Nrf2) pathway to ameliorate the progression of severe acute pancreatitis. CC14-induced hepatic fibrosis is weakened by DHK by suppressing poly(ADP-ribose) polymerase 1 (PARP-1) and its downstream cytokines and pathways. DHK exerts a protective function on liver injury induced by acetaminophen-via modulating sirtuin-1 (SIRT1). Nevertheless, whether DHK has a role in the progression of infantile pneumonia remains unclear.

The aim of this work was to appraise the regulatory impacts of DHK on lipopolysaccharide (LPS)-triggered WI-38 cells inflammation injury in infantile pneumonia. This study, DHK’s participation in the development of infantile cells inflammation injury in infantile pneumonia through modulating SIRT1/NF-κB pathway. The findings underlined the potential importance of DHK in infantile pneumonia.

Methods

Cell culture and treatment

Added to fetal bovine serum (FBS) (10%; Gibco, Grand Island, NE, USA), gentamycin (50 μg/mL; Invitrogen, Carlsbad, NM, USA), antibiotic/antimycotic solution (100 units; Invitrogen), and Na pyruvate (1 mM), Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, MO, USA) was employed for culture of normal human fibroblast cells WI-38 (ATCC, Manassas, VA, USA). WI-38 is a diploid human cell line composed of fibroblasts derived from female fetal lung tissues during the third trimester of pregnancy. WI-38 is a fibroblast that produces collagen. The cells were incubated in a humidified atmosphere containing 5% CO₂ at 37°C. Seeded in six-well plates and incubated overnight, LPS (10 μg/mL) was supplemented to mimic cell model for infantile pneumonia, while cells without LPS treatment served as a negative control. After 24 h, cell suspension was harvested.

DHK (purity > 98.5%) was bought from Meilune (Dalian, China). Different concentrations of DHK (5, 10, and 20 μM) were utilized for treating WI-38 cells.

Cell counting kit-8 (CCK-8) assay

WI-38 cells were seeded in 96-well plates and cultured for 24 h at 37°C in 5% CO₂. Subsequently, each well was treated with 10 μL of CCK-8 reagent (Beyotime, China) and incubated for 1 h. The absorbance at 450 nm was evaluated via a microplate reader.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TRizol reagent (Invitrogen) was used for extracting RNA from cells. TIANScript RT kit (Tiangen Biotech, Beijing, China) was used for reverse transcription of complementary DNA (cDNA), followed by RT-qPCR using the SYBR® Premix Dimmer Eraser kit (Takara, Dalian, China) on the CFX96 Touch™ RT-PCR detection system (Bio-Rad Laboratories, Hercules, USA). The 2-ΔΔCt method was applied for calculating the relative expression of interleukin (IL)-1β, IL-6, tumor necrosis factor-α (TNF-α), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as control, as follows:

\[ \text{IL-1β:} \quad F: 5′-AATCTCACAGAGCATCCTGCAAAGC-3′ \]
\[ \quad \text{R:} 5′-TCCACGGAAAGACATAGGTCG-3′ \]
\[ \text{IL-6:} \quad F: 5′-ATCTGCTTTCTGGGACTGATGG-3′ \]
\[ \quad \text{R:} 5′-GGATCCTGTGAAATGCTTCTC-3′ \]
\[ \text{TNF-α:} \quad F: 5′-CAGTCTTTCCGACTGAATCC-3′ \]
\[ \quad \text{R:} 5′-GCCGCTAGGCGTTGCTACTC-3′ \]
\[ \text{GAPDH:} \quad F: 5′-AATGGTGGGTGCAACGGGTTC-3′ \]
\[ \quad \text{R:} 5′-GAGGCGCTGTGAAACGGG-3′ \]

Enzyme-linked immunosorbent serological assay (ELISA)

Concentrations of IL-1β, IL-6, and TNF-α were assessed via the following ELISA kits (MultiSciences; Biotech Co., Hangzhou, China): IL-6 (Cat. No. EK206/3-96), IL-1β (Cat. No. EK201B/3-96), and TNF-α (Cat. No. EK282/3-96).

Flow cytometry

Fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) was applied for investigating the apoptosis of WI-38 cells. Digested by ethylenediaminetetraacetic acid (EDTA)-free trypsin, WI-38 cells were rinsed in PBS at 4°C. Dyed with FITC Annexin V (5 μL) and propidium iodide (5 μL PI) for 15 min at indoor temperature without light, WI-38 cells were observed
using the FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). The results were analyzed via FlowJoV 1.8.1 (Becton, Dickinson, Franklin Lakes, NJ, USA).

Assessment of oxidative stress

The 2′,7′-DCF diacetate (DCF-DA; Sigma-Aldrich) fluorescence was applied for assessing oxidative stress. Cultured with 2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) at 37°C for 15 min without light, WI-38 cells were rinsed in a fresh medium and re-suspended using PBS (20 mM, pH 7.0). Fluorescence microscopy (Leica Microsystems GmbH, Wetzlar, Germany) was utilized for visualization, with fluorescence levels detected through a fluorescence microplate reader at 488-nm excitation and 525-nm emission wavelengths.

Western blot analysis

Bicinchoninic acid (BCA) protein kit was used for determining protein concentrations. Proteins were loaded and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferring to polyvinylidene fluoride (PVDF) membranes. Post-sealed by bovine serum albumin (BSA, 5%) at 25°C for 1 h, primary antibodies against IL-1β (1:5000; ab254360), IL-6 (1:5000; ab303458), TNF-α (1:5000; ab183218), Bax (0.477 µg/mL; ab270742), p-p65 (1:1000; ab76302), p65 (1:1000; ab32536), phosphorylated inhibitor of nuclear factor kappa-B kinase subunit alpha (p-IκBα; 1:10,000; ab133462), IκBα (1:1000; ab32518), and β-actin (1:5000; ab8226) (Abcam, Shanghai, China) were supplemented to the membranes overnight at 4°C. Membranes were washed with phosphate-buffered saline solution (PBS), and horseradish peroxidase (HRP)-conjugated affinipure goat anti-rabbit Immunoglobulin G (IgG) secondary antibody was supplemented on the next day. Images of the band were observed using the enhanced chemiluminescence (ECL) detection kit and a chemidoc XRS Imaging system (Bio-Rad Laboratories).

Statistical Analysis

GraphPad Prism 8 was used for data analysis. The data were represented as mean ± standard deviation (SD), with the Student’s t-test used for comparing differences between groups. The data were analyzed attributing to the normal distribution and homogeneity of variance. Differences between multiple groups were analyzed by one-way Analysis of Variance (ANOVA) and Tukey’s multiple comparisons post-test; P < 0.05 was considered statistically significant.

Results

DHK modulated cell viability in infantile pneumonia

The chemical structure of DHK is displayed in Figure 1A. The viability of WI-38 cells was assessed via CCK-8 assay. The viability of WI-38 cells was evidently low because of LPS treatment (from 99% to 70%; P < 0.001; Figure 1B). Furthermore, the viability of WI-38 cells was evidently lowered due to LPS treatment (from 99% to 43%, P < 0.001), and 10- and 20-µM DHK treatment reversed the effect of LPS on the viability of WI-38 cells (from 43% to 74% and 85%; P < 0.001; Figure 1C). To sum up, DHK modulated the viability of WI-38 cells in infantile pneumonia.

DHK attenuated inflammation in WI-38 cells induced by LPS

The effect of DHK on inflammation in infantile pneumonia was evaluated. The messenger RNA (mRNA) levels of IL-1β, IL-6, and TNF-α were elevated because of LPS induction (P < 0.001), and the effects were inversely changed by 5-, 10-, and 20-µM DHK treatment (P < 0.05; Figure 2A). Similarly, the increased concentrations of IL-1β, IL-6, and TNF-α because of the induction of LPS was offset by 5-, 10-, and 20-µM DHK treatment (P < 0.01; Figure 2B). Altogether, DHK attenuated inflammation in WI-38 cells induced by LPS.

DHK inhibited reactive oxygen species (ROS) induced by LPS

The treatment of 5-, 10-, and 20-µM DHK counteracted LPS-induced production of ROS in WI-38 cells (from 1.05 to 5.83; P < 0.001), and there was a tendency to increase amelioration with the increased dose of DHK (from 5.83 to 4.16, 3.19, and 2.15; P < 0.001 relative to LPS). Hence, DHK inhibited ROS induced by LPS.

DHK restrained cell apoptosis induced by LPS

Next, the apoptosis of WI-38 cells was detected. The results of flow cytometry revealed that DHK (5 µM, 10 µM, and 20 µM) treatment reversed LPS induction that caused increase in WI-38 cells apoptosis (from 32.98% to 26.46%, 20.56%, and 14.86%; P < 0.01; Figure 4A). In addition, LPS treatment elevated the protein levels of Bax and
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was lowered with the induction of LPS (P < 0.001), and was reversed by 5-, 10-, and 20-μM DHK treatment (P < 0.001; Figure 5A). The treatment of 5-, 10-, and 20-μM DHK counteracted LPS induction-mediated elevation of p-p65 and p-IκBα protein levels (P < 0.01; Figure 5B). To conclude, DHK modulated infantile pneumonia via SIRT1/NF-κB pathway.

Discussion

Pneumonia is a globally prevalent infection causing mortality in children.14,15 Some other rehabilitation strategies, such as exercise regime, can diminish pneumonia;16 such a scheme has anti-inflammatory effects and could be a good strategy to treat pneumonia.17,18 Severe infantile pneumonia can lead to heart failure, encephalitis, and other complications, eventually causing death.19–21 Currently, it is important to find more reliable biomarkers linked to infantile pneumonia. DHK was identified as having an important function in severe acute pancreatitis,11 hepatic fibrosis,12 and liver injury,13 but the potential effect of DHK on infantile pneumonia was unknown. The present research examined the regulatory functions of DHK on cell viability, information, ROS, and cell apoptosis in LPS-triggered WI-38 cells. It further evaluated the role of DHK in infantile pneumonia. The data showed that 40-μM DHK treatment markedly reduced the viability of WI-38 cells, and DHK modulated the viability of WI-38 cells in infantile pneumonia. Furthermore, it was identified that DHK treatment inversely changed LPS-induced elevation in the levels of inflammation biomarkers, which suggested that DHK attenuated inflammation in WI-38 cells induced by LPS. DHK counteracted LPS-induced production of ROS in WI-38 cells. DHK also decreased LPS-induced elevation of WI-38 cells apoptosis and mediated the levels of apoptosis-associated indexes, indicating DHK restrained cell apoptosis induced by LPS. On the whole, DHK relieved LPS-induced WI-38 cells inflammation injury in infantile pneumonia.
Figure 4  DHK restrained cell apoptosis induced by LPS. (A) Apoptosis of WI-38 cells was detected by flow cytometry. ***p < 0.001 relative to control. **p < 0.01 and ***p < 0.001 relative to LPS. (B) Protein levels of Bax and cleaved-caspase 3 were evaluated via Western blot analysis. ***p < 0.001 relative to control. **p < 0.01 and ***p < 0.001 relative to LPS.

Figure 5  DHK modulated infantile pneumonia via SIRT1/NF-κB pathway. (A) Protein levels of SIRT1 were analyzed by Western blot analysis. ***p < 0.001 relative to control. **p < 0.01 and ***p < 0.001 relative to LPS. (B) Western blot analysis presented the protein levels of p-p65, p65, p-IκBα, and IκBα. ***p < 0.001 relative to control. **p < 0.01 and ***p < 0.001 relative to LPS.
SIRT1 is a widely expressed and extensively explored member of sirtuin family. SIRT1 is a NAD\textsuperscript{+}-dependent deacetylase that acts as an intracellular regulator of transcriptional activity and various protein functions.\textsuperscript{22} The protective effect of SIRT1 has been demonstrated in a variety of pathological conditions, including atherosclerosis,\textsuperscript{23} neurodegenerative diseases,\textsuperscript{24} and cerebral ischemia.\textsuperscript{25} Past studies showed that SIRT1 exacerbated pressure overload-induced hypertrophic heart failure by modulating the metabolism of energy.\textsuperscript{26} Treatment of pinocembrin (flavanone) regulates early brain injury after subarachnoid hemorrhage by modulation of SIRT1.\textsuperscript{27} Interestingly, SIRT1 is found to mediate several important key targets via deacetylation, including p53 and NF-κB.\textsuperscript{28} SIRT1 represses inflammatory response through deacetylation of p65 subunit and inhibiting NF-κB activity.\textsuperscript{29} SIRT1 participates in herpes simplex 1 (HSV-1)-mediated microglial inflammation via NF-κB signaling.\textsuperscript{30} Notably, SIRT1 is reported for being correlated with infections. For instance, SIRT1 inhibition impairs glycolysis in infectious challenge to potentiate endothelial dysfunction.\textsuperscript{31} SIRT1 has a role on regulatory mechanisms of bacterial, viral, and parasitic infections.\textsuperscript{32} However, whether SIRT1 is implicated in infantile pneumonia needs consideration. Herein, SIRT1 protein level was lowered with the induction of LPS, but was reversed by DHK treatment. What’s more, DHK counteracted LPS-induced elevation of p-p65 and p-IκBα protein levels. In summary, DHK modulated infantile pneumonia via SIRT1/NF-κB pathway.

**Conclusion**

This study initially validated that DHK lessened inflammation injury in LPS-induced WI-38 cells by modulating SIRT1/NF-κB pathway. The results could shed light on the implication of DHK for preventing and treating infantile pneumonia.

**Funding**

No funding was used in this study.

**Competing interests**

The authors stated that there was no conflict of interest to declare.

**Ethical approval**

This article did not require ethical approval, as it contained no research involving human participants or animals performed by any of the authors.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

**Author Contributions**

Qiao Wang designed and conducted the study. Qiao Wang, Liwen Zhang, and Ping Pang supervised data collection, analysis, and interpretation. All the authors prepared the manuscript for publication and reviewed its draft. Finally, all the authors read and approved the final manuscript.

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