Bergamottin alleviates LPS-induced acute lung injury by inducing SIRT1 and suppressing NF-κB

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Abstract
Acute lung injury (ALI) is associated with a high mortality due to inflammatory cell infiltration and lung edema. The development of ALI commonly involves the activation of NF-κB. Since bergamottin is a natural furanocoumarin showing the ability to inhibit the activation of NF-κB, in this study we aimed to determine the effect of bergamottin on ALI. RAW264.7 mouse macrophages were pre-treated with bergamottin and then stimulated with LPS. Macrophage inflammatory responses were examined. Bergamottin (50 mg/kg body mass) was intraperitoneally administrated to mice 12 h before injection of LPS, and the effect of bergamottin on LPS-induced ALI was evaluated. Our results showed that LPS exposure led to increased production of TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1), which was impaired by bergamottin pre-treatment. In vivo studies confirmed that bergamottin pre-treatment suppressed LPS-induced lung inflammation and edema and reduced the levels of pro-inflammatory cytokines in lung tissues and bronchoalveolar lavage fluids. Mechanistically, bergamottin blocked LPS-induced activation of NF-κB signaling in lung tissues. Additionally, bergamottin treatment reduced NF-κB p65 protein acetylation, which was coupled with induction of SIRT1 expression. In conclusion, our results reveal the anti-inflammatory property of bergamottin in preventing ALI. Induction of SIRT1 and inhibition of NF-κB underlies the anti-inflammatory activity of bergamottin.

Keywords
Acetylation, bergamottin, lung inflammation, NF-κB, SIRT1

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Introduction
Acute lung injury (ALI) is a serious health problem with a high incidence of mortality.¹ Although there are currently no specific pharmacological therapies available for ALI, numerous natural compounds have shown protective effects in managing this disease.²⁻³ ALI is manifested as massive inflammatory cell infiltration, increased pulmonary vascular permeability, and pulmonary edema. Infiltrated immune cells include macrophages and neutrophils. They can release a lot of inflammatory cytokines such as TNF-α, IL-6, and monocyte chemoattractant protein-1 (MCP-1), which consequently cause pulmonary structural damage.⁴

LPS, located in the outer membrane of most Gram-negative bacteria, is commonly used to generate animal models of ALI.⁵⁻⁶ Upon LPS stimulation, lung tissues exhibit a robust inflammatory response, with increased production of TNF-α, IL-6, and other pro-inflammatory cytokines. NF-κB plays an essential role in mediating LPS-induced inflammatory response.⁷⁻⁸ NF-κB is a potent transcription factor involved in many biological processes such as inflammation, immunity, and cell proliferation.⁸ It can bind to NF-κB responsive elements, driving the transcription of multiple inflammatory genes. Activation of NF-κB relies on phosphorylation of NF-κB p65, typically on Ser276. Besides phosphorylation, other posttranslational modifications such as acetylation also modulate NF-κB p65 activation.⁹ Inhibition of NF-κB has been reported to attenuate LPS-induced lung injury.¹⁰¹¹ Therefore, NF-κB represents a promising therapeutic target against ALI.

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Bergamottin (Figure 1A) is a biologically active furanocoumarin that can be abundantly produced by grapefruits.12 Bergamottin has attracted increasing interest because of its anti-cancer properties.13,14 It has been reported that bergamottin causes apoptotic death and suppresses proliferation and invasion in melanoma cells.14 Bergamottin also inhibits metastasis of lung cancer cells.15 Most importantly, bergamottin shows the ability to block NF-κB signaling activation.16 These findings suggested a hypothesis that bergamottin might have anti-inflammatory effects in ALI.

To address this hypothesis, we evaluated the effect of bergamottin on LPS-induced inflammatory response in Raw264.7 mouse macrophages. We also checked the capacity of bergamottin in modulating LPS-induced lung inflammation in a mouse model. In addition, the signaling pathway involved in the activity of bergamottin was explored.

Materials and methods

RAW264.7 cell culture and treatment

RAW264.7 cells were cultured in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (100 IU/ml), and streptomycin (100 µg/ml). To induce inflammatory responses, RAW264.7 cells were exposed to Escherichia coli LPS (100 ng/ml; Sigma-Aldrich, St Louis, MO, USA). Twenty-four h later, the cells and culture media were harvested and tested for gene expression. To evaluate the activity of bergamottin, RAW264.7 cells were pre-treated with bergamottin (20 and 50 µM; Sigma-Aldrich) 12 h before LPS challenge.

Quantitative real-time PCR analysis

Total RNA was extracted from RAW264.7 cells and lung tissue samples using TRIzol reagent (Thermo Fisher Scientific). The first strand cDNA was synthesized by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Quantitative PCR was performed using the SYBR Green PCR Master Mix Kit (Thermo Fisher Scientific). The PCR primer sequences are summarized as follows: TNF-α forward: 5′-CCACCCAGCTCTTCTGTCTAC-3′, reverse: 5′-AGGGTCTGGGCCATAGAACT-3′; IL-6 forward: 5′-AGTTGCTCTTCTGGGACTGA-3′, reverse: 5′-GTTGCCTTCTTGGGACTGA-3′; MCP-1 forward: 5′-CTTCTGCGG CTGCTGTCA-3′, reverse: 5′-CCAGCCTACTCATTTGGG ATCA-3′; SIRT1 forward: 5′-AGTTCCAGCCGTCTCTGTG TGT-3′, reverse: 5′-CTCCACGGAAGCCTGCTACAA-3′; GAPDH forward: 5′-TCACCCACATGGAAGGC-3′, reverse: 5′-CCTAAGCACTGTGGTGGTGCA-3′. The relative gene expression was calculated by normalizing against GAPDH.17

Animal experiments

The animal experiments were approved by the Animal Care and Use Committee of Huazhong University of Science and Technology (Wuhan, China). C57/BL/6 mice (6–8 wk old) were housed in a specific-pathogen-free environment and had free access to food and water. Mice were randomly divided into four groups (n = 8 per group): control group injected with physical saline, LPS group injected with LPS alone, Bergamottin group injected with bergamottin alone, and LPS + Bergamottin group injected with LPS and bergamottin. LPS (5 mg/kg body mass) was administrated intratracheally using a 20-gauge catheter. Bergamottin (50 mg/kg body mass)18 was administrated intraperitoneally 12 h before injection of LPS. The mice were euthanized 6 h after LPS stimulation. Bronchoalveolar lavage fluids (BALFs) were collected and subjected to inflammatory cell counting and cytokine analysis. Lung tissues were resected and examined for pathological changes and gene expression.

Collection and analysis of BALFs

The trachea was exposed and repeatedly lavaged with 3 ml of cold PBS. The lavage fluids were centrifuged at 300 g for 10 min at 4°C. The pellets were re-suspended and tested for total and differential cell counts. Total cell count was measured using a hemocytometer. Neutrophils and macrophages were differentiated by the Wright-Giemsa staining method. The centrifuged supernatants were tested for inflammatory cytokine levels.

Histopathological analysis

The lung tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. Tissue sections were stained with haematoxylin and eosin (H&E). Pathological changes were observed under a light microscope.

Measurement of lung wet to dry (W/D) ratio

Fresh lungs were collected and weighted for wet mass. The lungs were dehydrated at 60°C for 72 h to get dry mass. The W/D ratio was then calculated.

Myeloperoxidase (MPO) activity assay

MPO activity was measured as previously described.19 In brief, homogenization of lung tissues was performed in 0.5% hexadecyltrimethylammonium bromide in 10 mM 3-N-morpholinopropanesulfonic acid. The tissue homogenates were centrifuged, and the supernatant was reacted with 1.6 mM tetra-methyl-benzidine and 1 mM hydrogen peroxide at 37°C. Absorbance was recorded at 650 nm. Protein concentrations of lung homogenates were...
determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). MPO activity was expressed as U/mg protein.

**ELISA**

Commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the concentrations of TNF-α, IL-6, and MCP-1 in conditioned media of RAW264.7 cells and in BALFs. Absorbance was read at 450 nm.

**Western blot analysis**

Protein samples were extracted from lung tissues and RAW264.7 cells using lysis buffer supplemented with protease inhibitors (Sigma-Aldrich). An equal amount of protein extracts was separated by sodium SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The following primary Abs were used in this study, which recognized NF-κB p65, phospho-p65, acetyl-p65, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubation with the primary Abs overnight at 4°C, the membranes were incubated with HRP-conjugated second Abs (Santa Cruz Biotechnology). The immune complexes were developed using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). The densitometry of protein bands was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

**Electrophoretic mobility shift assay (EMSA)**

NF-κB DNA-binding activity was determined by EMSA as described previously. In brief, lung nuclear homogenates were incubated with radiolabeled oligonucleotide probes containing a consensus NF-κB site (5′-AGTTGAGGGG ACTTTCGCCAGC-3′). The resultant DNA-protein complexes were separated from free oligonucleotides on a polyacrylamide gel.

**Statistical analysis**

Data are expressed as mean ± SD. Group differences were analyzed using one-way analysis of variance (ANOVA) and a Tukey post-hoc test $P < 0.05$ was considered statistically significant.

**Results**

**Bergamottin mitigates LPS-induced inflammatory response in RAW264.7 cells**

As shown in Figures 1B–1D, LPS treatment caused a significant increase in the abundance of TNF-α, IL-6, and MCP-1 transcripts in RAW264.7 cells. Consistently, the exposure of RAW264.7 cells to LPS led to increased release of TNF-α, IL-6, and MCP-1 proteins to the culture medium (Figures 1E–1G). When RAW264.7 cells were pre-treated with bergamottin, all the pro-inflammatory cytokines tested were reduced (Figure 1). Moreover, the effect of bergamottin had a concentration dependence.

**Bergamottin blocks LPS-induced lung injury in mice**

Given the anti-inflammatory effect of bergamottin in vitro, we performed animal experiments to test the activity of bergamottin in improving LPS-induced lung injury. Histopathological analysis revealed that LPS challenge resulted in severe inflammatory changes in the lung tissues (Figure 2A). Interestingly, bergamottin treatment attenuated LPS-induced inflammatory cell infiltration (Figure 2A). The lung W/D ratio was used to reflect the extent of lung edema. We found that bergamottin treatment prevented the increase of the lung W/D ratio induced by LPS stimulation (Figure 2B). In addition, we measured the lung MPO activity, which is used to evaluate the accumulation of neutrophils in the lung tissues. The lung MPO activity was significantly elevated upon LPS stimulation (Figure 2C). Notably, bergamottin treatment suppressed the induction of the MPO activity by LPS.

**Bergamottin suppresses inflammatory cell counts in BALF**

The numbers of total cells, neutrophils, and macrophages were significantly raised in the BALF from LPS-stimulated mice (Figure 3). However, bergamottin treatment remarkably counteracted the increase in total cells, neutrophils and macrophages in BALF after LPS stimulation. These data confirmed the anti-inflammatory property of bergamottin in a model of LPS-induced lung injury.

**Bergamottin alleviates inflammatory cytokine levels in BALF and lung tissues**

Next, we determined the effect of bergamottin on the production of inflammatory cytokines. As shown in Figures 4A–4C, the lung tissues from LPS-treated mice had greater levels of TNF-α, IL-6, and MCP-1 transcripts than those from control mice. LPS-induced up-regulation of TNF-α, IL-6, and MCP-1 was significantly relieved by bergamottin treatment. The levels of TNF-α, IL-6, and MCP-1 in BALF were increased by 15.2-, 18.6-, and 20.4-fold, respectively, after LPS stimulation (Figures 4D–4F). Of note, bergamottin treatment markedly decreased the levels of TNF-α, IL-6, and MCP-1 in BALF.
Figure 1. Bergamottin mitigates LPS-induced inflammatory response in RAW264.7 cells. (A) Chemical structure of bergamottin. (B–D) Measurement of TNF-α, IL-6, and MCP-1 mRNA levels in RAW264.7 cells with indicated treatments. (E–G) Analysis of TNF-α, IL-6, and MCP-1 levels in the culture media of RAW264.7 cells with indicated treatments. BER (20) and BER (50) mean 20 and 50 μM bergamottin, respectively. *P < 0.05.
Figure 2. Bergamottin blocks LPS-induced lung injury in mice. Mice were treated with LPS and bergamottin (BER) alone or in combination, and lung injury was evaluated. (A) Histopathological analysis of lung tissues from each group. Scale bar = 100 μm. (B) Measurement of lung wet to dry (W/D) ratio for each group (n = 4). (C) Analysis of MPO activity for each group (n = 4). *P < 0.05.

Figure 3. Bergamottin suppresses inflammatory cell counts in BALF. Mice were treated with LPS and bergamottin (BER) alone or in combination, and bronchoalveolar lavage fluids (BALFs) were collected. (A) Representative images of Wright-Giemsa staining are shown. Arrows and arrowheads indicate macrophages and neutrophils, respectively. Scale bar = 10 μm. (B) Total cells, (C) neutrophils, and (D) macrophages in BALFs were counted (n = 4). *P < 0.05.
**Bergamottin inhibits LPS-induced NF-κB signaling activation in the lung tissues**

LPS has been shown to exert pro-inflammatory effects through NF-κB signaling.\(^7,8\) Therefore, we examined the effect of bergamottin on NF-κB signaling activation. We demonstrated that bergamottin inhibited phosphorylation and acetylation of NF-κB p65 protein in the lung tissues from LPS-treated mice (Figure 5A). To assess the DNA-binding activity of NF-κB, we prepared nuclear extracts of lung tissues and performed EMSA experiments. The results showed that LPS stimulation enhanced DNA-binding activity of NF-κB in the lung tissues (Figure 5B). Bergamottin treatment reversed LPS-mediated induction of NF-κB DNA-binding activity. These results suggested that bergamottin blunts LPS-induced activation of NF-κB signaling.

**Bergamottin suppresses NF-κB signaling through up-regulation of SIRT1**

It has been documented that SIRT1 is involved in deacetylation of NF-κB and inhibition of NF-κB activation.\(^{21,22}\) Next, we checked whether bergamottin could promote SIRT1 expression to inhibit NF-κB signaling. LPS stimulation decreased the mRNA level of SIRT1 in the lung tissues of mice (Figure 5C). Notably, bergamottin treatment

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**Figure 4.** Bergamottin alleviates inflammatory cytokine levels in BALF and lung tissues. (A–C) Quantification of TNF-α, IL-6, and MCP-1 transcripts in the lung tissues from each group (n = 4). (D–F) Measurement of TNF-α, IL-6, and MCP-1 levels in BALFs (n = 4). *P < 0.05.
restored the expression of SIRT1 in the lung tissues from LPS-treated mice. Similarly, bergamottin increased SIRT1 expression and reduced NF-κB phosphorylation and acetylation in LPS-treated RAW264.7 cells (Figures 6A and 6B). Taken together, bergamottin showed the ability to regulate the SIRT1/NF-κB axis.

**Discussion**

In this study, we showed that bergamottin treatment prevented LPS-induced production of inflammatory cytokines in mouse macrophages *in vitro*. Consistently, in a mouse model, bergamottin ameliorated LPS-induced lung inflammation and suppresses inflammatory cell infiltration into the lung and reducing lung edema. Moreover, neutrophils and macrophages were the main inflammatory cell types affected. When mice were pre-treated with bergamottin, LPS-elicited release of inflammatory cytokines in BALF was attenuated. These findings collectively indicated the anti-inflammatory property of bergamottin.

Previous studies have focused on the anti-cancer effect of bergamottin. It has been reported that bergamottin causes apoptotic death in tumor cells. Another study demonstrates that bergamottin evokes DNA damage in melanoma cells. These studies have confirmed the cytotoxic activity of bergamottin in malignant cells. However, bergamottin treatment had mild effect on the viability of non-malignant cells including macrophages and lung epithelial cells (Supplementary Figure S1). The mechanism by which bergamottin selectively kills malignant cells needs further investigation. Intriguingly, our results indicated that bergamottin reduced inflammatory response in macrophages upon LPS stimulation. *In vivo* studies validated the protective effect of bergamottin against LPS-induced lung injury, which was accompanied by suppression of inflammatory cytokine expression. Bergamottin has been found to inhibit NO generation in RAW264.7 cells induced by LPS and IFN-γ. NO can serve as an important mediator of inflammation. NO synthase deficiency protects mice from LPS-induced ALI. Based on these findings, we suggested that the anti-inflammatory activity of bergamottin might be ascribed to inhibition of NO formation.

NF-κB signaling activation is involved in many pathological processes including LPS-induced lung inflammation. In this study, we found that bergamottin was able to inhibit LPS-induced NF-κB p65 phosphorylation and

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Figure 5. Bergamottin inhibits LPS-induced NF-κB signaling activation in the lung tissues. (A) Western blot analysis of indicated proteins in the lung extracts from each group. Phospho-p65 and acetyl-p65 mean phosphorylated and acetylated NF-κB p65, respectively. (B) EMSA analysis of DNA binding activity of NF-κB p65 in nuclear extracts from the lung tissues of each group. (C) Analysis of SIRT1 mRNA levels in the lung tissues. *P < 0.05.
DNA binding activity. Therefore, inhibition of NF-κB signaling activation may represent an important mechanism for the anti-inflammatory activity of bergamottin. In agreement with our findings, bergamottin also exerts inhibitory effects on NF-κB signaling in tumor cells. In addition to inhibition of phosphorylation, bergamottin treatment led to reduction of p65 acetylation. SIRT1 is a protein deacetylase and has been shown to control p65 acetylation. Most importantly, SIRT1 shows the capacity to alleviate LPS-induced inflammation. Therefore, we speculated that bergamottin might regulate p65 activation through induction of SIRT1 and reduction of p65 acetylation. Indeed, we noted that bergamottin treatment caused an induction of SIRT1 in mouse macrophages and lung tissues even in the presence of LPS. Taken together, we provided evidence that bergamottin improved LPS-induced lung inflammation likely through modulation of the SIRT1/NF-κB signaling axis (Figure 6C).

Our data showed that bergamottin increased the mRNA level of SIRT1, suggesting an enhanced transcription. However, the mechanism by which bergamottin promotes the expression of SIRT1 remains to be further clarified. Although our data highlight the importance of NF-κB signaling, we cannot exclude other signaling pathways that might be involved in the activity of bergamottin. In future work, we could perform integrated transcriptome and proteome analysis to identify the direct mediator of bergamottin biological activity. Additionally, the therapeutic potential of bergamottin post-treatment warrants further investigation.

In conclusion, bergamottin pre-treatment mitigates LPS-induced macrophage inflammatory response and lung inflammation, which is likely associated with induction of SIRT1 and inhibition of NF-κB signaling. Thus, bergamottin may provide therapeutic benefits in ALI prevention.

Figure 6. Bergamottin suppresses NF-κB signaling through up-regulation of SIRT1. RAW264.7 cells were treated with LPS with or without bergamottin (BER) pre-treatment. (A) Analysis of SIRT1 mRNA levels. *P < 0.05. (B) Western blot analysis of indicated proteins. (C) Schematic model showing that bergamottin inhibits LPS-induced lung inflammation through induction of SIRT1 and inhibition of NF-κB activation.
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Supplemental material
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