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Phloretin attenuates LPS-induced acute lung injury in mice via modulation of the NF-κB and MAPK pathways

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ABSTRACT

Phloretin, which can be isolated from apple trees, has demonstrable *anti*-inflammatory and anti-oxidant effects in macrophages. We previously reported that phloretin could inhibit the inflammatory response and reduce intercellular adhesion molecule 1 (ICAM-1) expression in interleukin (IL)-1β-activated human lung epithelial cells. In the present study we now evaluate whether phloretin exposure could ameliorate lipopolysaccharide (LPS)-induced acute lung injury in mice. Intra-peritoneal injections of phloretin were administered to mice for 7 consecutive days, prior to the induction of lung injury by intra-tracheal administration of LPS. Our subsequent analyses demonstrated that phloretin could significantly suppress LPS-induced neutrophil infiltration of lung tissue, and reduce the levels of IL-6 and tumor necrosis factor (TNF)-α in serum and bronchoalveolar lavage fluid. We also found that phloretin modulated myeloperoxidase activity and superoxide dismutase activity, with decreased gene expression levels for chemokines, proinflammatory cytokines, and ICAM-1 in inflamed lung tissue. Phloretin also significantly reduced the phosphorylation of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK), thus limiting the inflammatory response, while promoting expression of heme oxygenase (HO)-1 and nuclear factor erythroid 2-related factor 2, both of which are cytoprotective. Our findings suggest that, mechanistically, phloretin attenuates the inflammatory and oxidative stress pathways that accompany lung injury in mice via blockade of the NF-κB and MAPK pathways.

1. Introduction

Acute lung injury (ALI) is a severe pulmonary inflammatory disease, characterized by neutrophil accumulation in the lung, which leads to immersion pulmonary edema, reduced lung capacity, and severe hypoxemia [1]. ALI also causes acute respiratory distress syndrome (ARDS), with fluid accumulating in the alveoli, which leads to difficulty in breathing, a shortness of breath, and rapid breathing; all of which increase the mortality rate [2,3]. Previous studies have found that many factors induce ALI, including the inhalation of toxic gas, bacterial and viral infections, and severe sepsis [1]. In the ALI patient, lung tissue can release proinflammatory cytokines and chemokines, which exacerbate harmful immune responses including potentially injurious oxidative stress [4,5].

Occasionally ALI also occurs as a result of bacterial infection or early sepsis in the respiratory tract [6]. Lipopolysaccharide (LPS) is an important component of the cell wall in gram-negative bacteria, and stimulates innate immunity as part of the early host response to contain pathogenic bacteria [7]. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Haemophilus influenzae* are the most common gram-negative pathogens underlying bacterial pneumonia and ALI in clinical patients [8–11]. The *P. aeruginosa* infected airway has also been employed as an induced lung injury model in mice [9]. However, the intra-tracheal administration of LPS to induce acute lung inflammation is a more commonly used pharmacological research animal model of ALI.

LPS binds to Toll-like receptor 4 and triggers a cascade of inflammatory signaling, resulting in the activation of nuclear factor kappa B (NF-κB) to induce the production of proinflammatory cytokines and inflammatory mediators [12]. LPS also results in the generation of reactive oxygen species (ROS) that further contribute to cellular injury [13]. Many studies have found that NF-κB is suppressed by IκB, with exposure to LPS inducing the activity of inflammatory signals that provoke IκB phosphorylation and degradation. These events release NF-κB, such that this

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transcription factor can translocate to the nucleus and promote the transcription of inflammatory mediators [14]. Furthermore, mitogen-activated protein kinase (MAPK) signaling pathways are also stimulated by inflammatory gene expression in the lung tissue of ALI mice [15]. Consequently, reducing both MAPK and NF-κB activity may be a plausible strategy with which to ameliorate inflammatory responses in ALI.

Phloretin is a natural dihydrochalcone found in apple and pear trees [16].

Recent studies have found that phloretin has anti-inflammatory and anti-obesity properties [16,17]. Previously studies have shown that phloretin could improve the function of glucose transporters (SGLTs and GLUTs) in hepatocytes and renal cells [18,19]. Phloretin also decreased fasting plasma glucose, triglycerides, total cholesterol in diabetic mice [20]. Other study found that phloretin reduced oxidative damage for neuroprotective effects in cerebral ischemia of rats [21]. Phloretin also been demonstrated that has antitumor effect by inducing apoptosis in non-small cell lung carcinoma, breast cells, human leukemia cells, and ovarian cancer cells [22–25]. We also found that phloretin could decrease expression of the intercellular adhesion molecule 1 (ICAM-1), and proinflammatory cytokine expression, by its blockade of the NF-κB and MAPK pathways in interleukin (IL)-1β-activated human lung epithelial cells [26]. However, whether phloretin could lessen the effects of acute lung injury were uncertain. In the present study, we investigated whether phloretin could prevent the acute lung injury provoked by intra-tracheal LPS administration in mice, and evaluated the molecular mechanisms of inflammation in this animal model.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6–8 weeks, weight 20–25 g) were purchased from the National Laboratory Animal Center in Taiwan. Mice were fed a standard chow diet with water, and were maintained in an animal house equipped with thermostat controlled central air-conditioning. Care and housing protocols for mice and all experimental procedures were approved by the Laboratory Animal Care Committee of Chang Gung University of Science and Technology.

2.2. Phloretin treatment and acute lung injury

Phloretin (from apple wood, ≥99% by HPLC) was purchased from Sigma-Aldrich (Sigma, St. Louis, MO, USA), and then solubilized for use in DMSO.

Mice were assigned to four groups, each comprising 8 mice. These groups were termed the N group (normal control mice), the LPS group (mice challenged with LPS alone), the PT5 group (mice challenged with 5 mg/kg phloretin plus LPS), and the PT20 group (20 mg/kg phloretin plus LPS). On days 1–7, mice were intra-peritoneally injected with DMSO (N and LPS groups) or phloretin (PT5 and PT20 groups). On day 8 of the experiment, mice were anesthetized with isoflurane (Aesica, Kent, UK), with intra-tracheal administration of 50 μl LPS (1 μg/ml) or normal saline. Four hours later, mice were sacrificed and bronchoalveolar lavage fluid (BALF), lung tissue, and serum harvested.

2.3. Histological analyses of lung tissue

Lung tissue was fixed with formalin and paraffin embedded. Six-micron sections were stained with hematoxylin and eosin (H&E). The neutrophil infiltration assay was observed microscopically (Olympus, Tokyo, Japan), as described previously [27].

2.4. Serum collection

Blood was collected from the orbital vascular plexus of mice anesthetized with isoflurane. Samples were then centrifuged at 6000 rpm for 5 min as previously described [28], with serum subsequently stored at −80 °C.

2.5. BALF and cell count

All mice were sacrificed, and BALF collected as described previously [28]. Briefly, tracheae were intubated and the lungs flushed with normal saline. Cells were stained with Liu stain solution (Polysciences, Inc., Taipei, Taiwan), and neutrophils counted under the microscope (Olympus, Tokyo, Japan). Additionally, supernatants were assayed for levels of cytokines and chemokines.

2.6. Lung wet-to-dry (W/D) weight ratio

Mice were sacrificed, and the right lung excised and its wet weight (W) obtained. The lung was then dried in an oven at 80 °C for 48 h to obtain the dry weight (D). The ratio of W/D was calculated to evaluate lung edema.

2.7. Myeloperoxidase (MPO) activity

MPO activity was measured in the lung tissue using the myeloperoxidase fluorometric activity assay kit (Sigma). Lung tissue was homogenized in cool normal saline, and then processed according to the manufacturer's instructions. Briefly, the MPO could catalyze hypochlorous acid and taurine to form taurine chloroamine, which reacts with 2-nitro-S-thiobenzoic acid to form colorless 5,5′-dithiobis (2- nitrobenzoic acid) [29]. Next, MPO activity was measured using a multi-mode microplate reader (BioTek SynergyHT, Bedfordshire, United Kingdom).

2.8. Superoxide dismutase (SOD) activity

SOD activity was measured in the lung tissue using a SOD determination kit (Sigma).

Lung tissue was homogenized and SOD activity evaluated according to the manufacturer's instructions. SOD could catalyze the dismutation of the superoxide anion into oxygen and hydrogen peroxide. In the assay kit, utilizing water-soluble tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) produces a water-soluble formazan dye and reduced superoxide anion [30]. Optical density (OD) was measured at 450 nm using a microplate reader (Multiskan FC, Thermo).

Table 1
Primers used for real-time PCR analyses of mRNA expression.

Gene	Primer forward	Primer reverse
IL-1β	CACTACAGGCTCCGAGATGA	CGTTGCTTGGTTCTCCTTGT
IL-4	TCCGTGCTTGAAGAAGAACTC	GTGATGTGGACTTGGACTCATT
IL-6	AGGACCAAGACCATCCAATTCA	GCTTAGGCATAACGCACTAGG
IL-13	GCTCCAGCATTGAAGCAGTG	CGTGGCAGACAGGAGTGTT
TNF-α	GCACCACCATCAAGGACTC	AGGCAACCTGACCACTCTC
MCP-1	TTCCACAACCACCTCAAGCA	TTAAGGCATCACAGTCCGAGTC
CCL5	CGAAGGAACCGCCAAGTGT	AGGACTAGAGCAAGCAATGAC
CCL11	GGCTTCATGTAGTTCCAGAT	CCATTGTGTTCCTCAATAATCC
CCL24	AGGCAGTGAGAACCAAGT	GCGTCAATACCTATGTCCAA
iNOS	TTCCACAACCACCTCAAGCA	TTAAGGCATCACAGTCCGAGTC
COX-2	ACCAGCAGTTCCAGTATCAGA	CAGGAGGATGGAGTTGTTGTAG
ICAM-1	AACAGAATGGTAGACAGCAT	TCCACCGAGTCCTCTTAG
β-actin	AAGACCTCTATGCCAACACAGT	AGCCAGAGCAGTAATCTCCTTC