科技部補助專題研究計畫成果報告
期末報告

研究芸香素對內毒素引發急性肺損傷的保護作用與分子調節機制

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中 華 民 國 103 年 11 月 01 日

中文摘要： 在國人的十大死因當中，與肺部相關的疾病就佔了15.5%；而其中，急性肺損傷為相當常見的急重症，當它惡化後會形成急性呼吸窘迫症候群，並伴隨著極高的死亡率（約40~60%)。造成大部分急性肺損傷的病人死亡的原因並非呼吸衰竭，而是由全身性發炎所引致的多發性器官衰竭。芸香素(Rutin) 是一種生物類黃酮，普遍存在於日常食用的蔬果之中，尤以蕎麥草中含量最高，近年來也被當作健康食品使用。芸香素具有相當多的生物活性與藥理作用，如抗發炎、抗高血壓、抗癌、血管保護與心血管保護作用。於本計畫研究證實芸香素有效降低由 lipopolysaccharide (LPS) 經氣管注射至小鼠所引發的急性肺損傷 (acute lung injury；ALI)的病理狀態，如嗜中性球浸潤、肺水腫、肺泡壁增厚、血氧下降、血中二氧碳濃度增加、血中酸鹼值降低等現象。進一步發現，芸香素降低LPS所引發的促發炎介質表現，如細胞激素 (TNF-脉, IL-1 刍, IL-6)、黏附因子 (VCAM-1)、cyclooxygenase-2 (COX-2)、inducible nitric oxide synthase (iNOS)、matrix metallopeptidase 9 (MMP-9)。同時，也發現芸香素可以抑制由LPS所引發的促發炎轉釋因子--nuclear factor (NF)-kB的活化與磷酸化作用。而NF-岂B上游調節因子I岂B經LPS刺激後所產生的降解作用也會被芸香素所抑制。再者可調節LPS所活化的NF-kB需經由磷酸化mitogen-activated protein kinases (MAPK) 路徑進行調節，而MAPK路徑包括 p38 MAPK, ERK1/2, JNK三種蛋白質。芸香素也可以降低由LPS所引發的 MAPK 路徑磷酸化。因此可以推斷芸香素所產生的抗發炎作用，主要是經由抑制 MAPK-NF-岂B 路徑活化所致。另一方面，我們也發現了芸香素可有效反轉由LPS所導致抗氧化酵素系統 (catalase, superoxide dismutase, glutathione peroxidase) 活性降低的現象，主要是經由促進抗氧化蛋白HO-1的表所致。綜合上述的成果，芸香素具有預防急性肺損傷的能力，同時具有潛力成為臨床上適用於急性肺損傷的預防或治療的化合物。

中文關鍵詞： 急性肺損傷、芸香素、嗜中性球、促發炎介質、抗氧化酵素、MAPK、NF-kB、HO-1

英文摘要： Acute lung injury (ALI) is a serious disease with unacceptably high mortality and morbidity rates. Up to now, no effective therapeutic strategy for ALI has been established. Rutin, quercetin-3-rhamnosyl glucoside, expresses a wide range of biological activities and pharmacological effects, such as anti-

inflammatory, anti-hypertensive, anti-carcinogenic, vasoprotective, and cardioprotective activities. Pretreatment with rutin not only inhibited histolopatholgical changes in lung tissues but also infiltration of polymorphonuclear granulocytes (PMNs) into bronchoalveolar lavage fuild (BALF) in LPS-induced ALI. In addition, LPS-induced inflammatory responses, including increased secretion of proinflammatory cytokines and lipid peroxidation, vascular cell adhesion molecule (VCAM)-1, and inducible nitric oxide synthase (iNOS)were inhibited by rutin in a concentration-dependent manner. Furthermore, rutin suppressed phosphorylation of NF-κB and MAPK, and degradation of IκB, a NF-κB inhibitor. Decreased activities of antioxidative enzymes and hemeoxygenase (HO)-1 caused by LPS were reversed by rutin. At the same time, we found the amelioration of rutin is better than deferoxamine. These results indicated that the protective mechanism of rutin is by inhibition of MAPK-NFκB activation and up-regulation of antioxidative enzymes. Pre-administration with rutin inhibited LPS-induced arterial blood gas exchange and neutrophils infiltration in the lungs. The mechanism of rutin is down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation. Experimental findings support the potential use of rutin as a therapeutic agent for prevention of ALI associated with direct infection by gram-negative bacteria.

英文關鍵詞： Acute lung injury, Rutin, Neutrophils, Proinflammatory mediators, Antioxidant enzyme system, MAPK, NF-kB, HO-1

科技部補助專題研究計畫成果報告

（□期中進度報告/■ 期末報告）

研究芸香素對內毒素引發急性肺損傷的保護作用與分子調節機制

計畫類別：■個別型計畫　□整合型計畫
計畫編號：MOST　102－2320－B－040－009－
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計畫主持人：關宇翔
共同主持人：陳春榮, 林則彬
計畫參與人員：林附偉、曾璟琪、李妙卿、梁清惠、彭亞婷、周文安、駱啟文、曾培修

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□出席國際學術會議心得報告

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中　華　民　國　103　年 10 月 31 日

目錄

中文摘要

在國人的十大死因當中，與肺部相關的疾病就佔了 15.5%；而其中，急性肺損傷為相當常見的急重症，當它惡化後會形成急性呼吸窘迫症候群，並伴隨著極高的死亡率（約 40~60%）。造成大部分急性肺損傷的病人死亡的原因並非呼吸衰竭，而是由全身性發炎所引致的多發性器官衰竭。芸香素 (Rutin) 是一種生物類黃酮，普遍存在於日常食用的蔬果之中，尤以蕎麥草中含量最高，近年來也被當作健康食品使用。芸香素具有相當多的生物活性與藥理作用，如抗發炎、抗高血壓、抗癌、血管保護與心血管保護作用。於本計畫研究證實芸香素有效降低由 lipopolysaccharide (LPS) 經氣管注射至小鼠所引發的急性肺損傷 (acute lung injury; ALI)的病理狀態，如嗜中性球浸潤、肺水腫、肺泡壁增厚、血氧下降、血中二氧碳濃度增加、血中酸鹼值降低等現象。進一步發現，芸香素降低 LPS 所引發的促發炎介質表現，如細胞激素 (TNF-α, IL-1β, IL-6)、黏附因子 (VCAM-1)、inducible nitric oxide synthase (iNOS)、matrix metallopeptidase 9 (MMP-9)。同時，也發現芸香素可以抑制由 LPS 所引發的促發炎轉釋因子--nuclear factor (NF)-κB 的活化與磷酸化作用。而 NF-κB 上游調節因子 IκB 經 LPS 刺激後所產生的降解作用也會被芸香素所抑制。再者可調節 LPS 所活化的 NF-κB 需經由磷酸化 mitogen-activated protein kinases (MAPK) 路徑進行調節，而 MAPK 路徑包括 p38 MAPK, ERK1/2, JNK 三種蛋白質。芸香素也可以降低由 LPS 所引發的 MAPK 路徑磷酸化。因此可以推斷芸香素所產生的抗發炎作用，主要是經由抑制 MAPK-NF-κB 路徑活化所致。另一方面，我們也發現了芸香素可有效反轉由 LPS 所導致抗氧化酵素系統 (catalase, superoxide dismutase, glutathione peroxidase) 活性降低的現象，主要是經由促進抗氧化蛋白 HO-1 的表所致。綜合上述的成果，芸香素具有預防急性肺損傷的能力，同時具有潛力成為臨床上適用於急性肺損傷的預防或治療的化合物。

關鍵詞: 急性肺損傷、芸香素、嗜中性球、促發炎介質、抗氧化酵素、MAPK、NF-κB、HO-1

Abstract

Acute lung injury (ALI) is a serious disease with unacceptably high mortality and morbidity rates. Up to now, no effective therapeutic strategy for ALI has been established. Rutin, quercetin-3-rhamnosyl glucoside, expresses a wide range of biological activities and pharmacological effects, such as anti-inflammatory, anti-hypertensive, anti-carcinogenic, vasoprotective, and cardioprotective activities. Pretreatment with rutin not only inhibited histolopatholgical changes in lung tissues but also infiltration of polymorphonuclear granulocytes (PMNs) into bronchoalveolar lavage fuild (BALF) in LPS-induced ALI. In addition, LPS-induced inflammatory responses, including increased secretion of proinflammatory cytokines and lipid peroxidation, vascular cell adhesion molecule (VCAM)-1, and inducible nitric oxide synthase (iNOS)were inhibited by rutin in a concentration-dependent manner. Furthermore, rutin suppressed phosphorylation of NF-κB and MAPK, and degradation of IκB, a NF-κB inhibitor. Decreased activities of antioxidative enzymes and hemeoxygenase (HO)-1 caused by LPS were reversed by rutin. At the same time, we found the amelioration of rutin is better than deferoxamine. These results indicated that the protective mechanism of rutin is by inhibition of MAPK-NFκB activation and up-regulation of antioxidative enzymes. Pre-administration with rutin inhibited LPS-induced arterial blood gas exchange and neutrophils infiltration in the lungs. The mechanism of rutin is down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation. Experimental findings support the potential use of rutin as a therapeutic agent for prevention of ALI associated with direct infection by gram-negative bacteria.

Keywords: Acute lung injury, Rutin, Neutrophils, Proinflammatory mediators, Antioxidant enzyme system, MAPK, NF-κB, HO-1

Chapter 1

Rutin decrease lipopolysaccharide-induced acute lung injury via inhibition of oxidative stress and MAPK-NF-κB pathway

Introduction

Acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome, are critical illness whose clinical symptoms include rapid-onset of respiratory failure with bilateral pulmonary infiltrations that is associated with either intra- or extrapulmonary risk factors. The distinguishing features of ALI are noncardiogenic edema, severe systemic hypoxemia, alveolar hemorrhage, hyaline membrane formation, increasing tichkness of the alveolar wall, and pulmonary inflammation characterized by the up-regulation of alveolar capillary permeability, polymorphonuclear neutrophils (PMN) infiltration, and secretion of proinflammatory cytokines and transcription factors [1]. ALI often results from sepsis, shock, aspiration, and blood transfusion and presents a high mortality rate of 18 to 54.7% [2]. In fact, sepsis is the main cause of ALI in human. Administration of lipopolysaccharide (LPS), also called endotoxin, has been used for simulating sepsis-induced ALI in several animal models, such as mouse, rat, and sheep [3].

LPS, exists in the cell wall of the Gram-negative bacteria, not only is the most serious global problem is sepsis but also the most potent bioactivator for immunological system, especially in innate immunity [4]. Administration of LPS into mice through respitory tract stimulates the release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interlukin (IL)-1β, and IL-6 by the airway epithelial cells and alveolar macrophages[1]. Expression of proinflammatory cytokines is regulated by the activation of transcription factor, nuclear factor (NF)-κB, and the three mitogen-activated protein kinases (MAPK) pathways, which are extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 [5]. LPS and proinflammatory cytokines induce the generation of proinflammatory mediators and apoptosis of epithelial and endothelial cells which result in the damage of alveolar capillary barrier hence the increase permeability. These incidences lead to the activation of peripheral polymorphonuclear leukocytes (PMN) follow by their transmigration into the lung are crucial events in the early development of ALI [6]. Activated PMN contribute to the killing of the infectious pathogen through respiratory burst and degranulation. However, over-activation of PMN can mediate inflammatory responses and causes tissue damage through oxidative stress [6] whose primary source are respiratory burst and degranulation. The oxidants include superoxide anion ($O_2^{\bullet -}$) and its toxic metabolites, such as hydrogen peroxide (H_2O_2), hydroxyl radical, and hypochlorous acid (HOCl). *In vivo*, the lung tissue is protected against oxidative damage by antioxidative enzymes (AOE), including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and hemeoxygenase (HO)-1 [7].

Rutin, quercetin-3-rhamnosyl glucoside, is a natural polyphenolic flavanoid found in buckwheat seeds, citrus fruits, vegetables, and plant-derived beverages such as wine and tea. Rutin has a wide range of biological activities and pharmacological effects, such as anti-inflammatory, anti-hypertensive, anti-carcinogenic, vasoprotective, and cardioprotective activities [8, 9, 10]. These beneficial effects of rutin is due to its high radical scavenging activity and antioxidative capacity, thus also known as Vitamin P [11]. However, there is no evidence showing the preventive effect of rutin in LPS-induced ALI. Up to present, the therapeutic options for ALI are still restricted and the majority of treatment strategies are just supportive interventions [12]. The aim of this study is to investigate the anti-inflammation effect of rutin in LPS-induced ALI in *in vivo* animal model and the mechanism involved in consideration of rutin as a potential therapeutic

medication for ALI.

Materials and methods

Materials Lipopolysaccharide (LPS; *Escherichia coli*, serotype 0111:B4), dimethyl sulfoxide (DMSO), and other reagents, unless specifically stated elsewhere, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of DMSO in the reaction mixture was <0.5%. Antibodies against IκB, phospo-p65, phospho-ERK, phospho-JNK, p65, HO-1, p38 MAPK, JNK, ERK, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-p38 MAPK was purchased from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were obtained from Jackson Immuno Research Laboratories. (Baltimore, MD, USA). Myeloperoxidase (MPO) content assay kit, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity assay kits were products of Cayman (Ann Arbor, MI, USA); malondialdehyde (MDA) assay kit was manufactured by ZeptoMetrix (Buffalo, NY, USA). All these assay kits were obtained from the same distributor Excel Biomedical (Taipei, Taiwan).

Animals Adult male ICR mice (25-30 g) were purchased from BioLASCO (Taipei, Taiwan). All mice were fed a standard laboratory diet and water *ad libitum* and housed under standard laboratory conditions of controlled lighting (12 h light, 12 h dark) and temperature (21 ± 1°C). All animal cared and studied were approved by the Institutional Animal Ethics Committee of Chung Shan Medical University in accordance with the principles and guideline of the US National Institute of Health Guide for the Care and Use of Laboratory Animals.

Murine model and grouping of LPS-induced ALI The procedures for induction of ALI by LPS in mice were performed as described in previous studies [7, 13, 14]. Forty-eight mice were randomly divided into 7 groups, which are a control and five treatment groups. The mice of the control group were first received vehicle intraperitoneally (IP) for 30 min followed by intratracheal (IT) instillation of 50 μl saline; while the mice of the five treatment groups were injected with 0, 1, 10, or 100 μmol/kg of rutin, 1 mg/kg dexamethasone (DEX), or 20 mg/kg desferrioxamine (DFX) IP for 30 min respectively followed by IT instillation of 100 μg/50 μl LPS. After 6 hours, to collect the tissue samples, the mice were sacrificed by sodium pentobarbital (30 mg/kg) IP. In each group, the right lung was collected from 4 animals for Western blot assay and the left lung for lipid peroxidation, myeloperoxidase (MPO) content, and catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activity assay. For the other 4 animals, the right lung was collected for histopathological assay and bronchoalveolar lavage fluid (BALF) was collected from left lung for cytokines and leukocytes content assays.

Histopathological analysis After midsternal thoracotomy, the lung tissues of mice were excised and fixed via tracheal cannula with 4% isotonic paraformaldehyde in situ. The samples were dehydrated with graded ethanol series and xylene and then embedded in paraffin at 60°C. Serial paraffin sections (3 μm) were procured using a rotatory microtome and stained with hematoxylin-eosin (HE) using standard histological techniques. Evaluation of lung injury, such as alveolar congestion, haemorrhage, infiltration of leukocytes, change in tichkness of the alveolar wall, and formation of hyaline membrane were then performed using these histological preparations [13].

Bronchoalveolar lavage and Cell Counting Bronchoalveolar lavage was performed as previously described [7, 13, 14]. After euthanasia, the lungs were lavaged three times with 1 ml sterile saline each through tracheal cannula. BALF was collected on ice and centrifuged at 800 *g* for 10 min at 4 ℃. The cell-free supernatant was stored at -20 ℃ for cytokines concentration assay. Total leukocytes content was determined by counting

the cells in the pellet with Geimsa stain.

Measurement of MPO contents MPO contents assay was performed as previousely described [7, 14]. MPO was extracted from the lungs with phosphate buffer contained cetyltrimethylammonium bromide in the presence of guaiacol. Reaction was started by the addition of H_2O_2. A standard curve was generated in the same fashion using commercially available pure MPO. The absorbance of the sample was measured at 470 nm using a microplate reader. The specific activities of the MPO in the lung were expressed as U/mg of the tissue.

Measurement of lipid peroxidation A thiobarbituric acid reactive substances assay kit was used to measure the lipid peroxidation products, the malondialdehyde (MDA) equivalents, according to the manufacturer's instructions and perious study [14]. Briefly, the lungs were homogenized with phosphate buffered saline (PBS) containing reaction buffer (provided by the kit) and heated at 95 ℃ for 60 min. The homogenates were then cooled in ice bath and centrifuged at 2,500 × *g* for 15 min. The absorbance of the supernatant was measured at 532 nm using a microplate reader. The lipid peroxidation products are expressed in terms of MDA equivalents.

Measurement of antioxidative enzymes activities CAT, SOD, and GPx activities were determined using commercially available assay kits [14]. After tissue homogenization, activities of these enzymes were determined following the procedures provided by the respective manufacturer. The measurement of CAT activity is based on the peroxidation of CAT with methanol in the presence of an optimal concentration of H_2O_2. The generated formaldehyde is assayed spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as chromogen. The measurement of SOD activity utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. The GPx activity was measured indirectly by its coupled reaction with reductase. Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced state by reductase and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease at A_{340} is directly proportional to the GPx activity. The specific activities of the various enzymes in the lung are expressed in mol/mg of respective protein.

Measurement of cytokines concentrations The levels of TNF-α, IL-1β, and IL-6 in BALF were measured using a commercially available ELISA kit (R & D Systems, Minneapolis, MN). These concentrations were interpolated from the standard curves for recombinant TNF-α, IL-1β, and IL-6.

Western blot analysis of lung tissue The lungs were harvested, with extrapulmonary structures removed then frozen in liquid nitrogen immediately until homogenization. Tissue extracts were homogenized in tissue protein extraction solution (T-PER; Pierce, Rockford, IL) containing 1% proteinase inhibitor cocktail. After centrifugation, the protein concentration in the supernatant was determined by Bradford assay. Each well was loaded with 100 μg of protein, separated by SDS–PAGE, and electrophoretically transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% (w/v) nonfat dried milk for 1 h at room temperature to reduce nonspecific binding, washed with PBS containing 0.1% Tween-20 (PBST), then probed with antibodies including β-actin, IκB, HO-1, and phosphorylated and non-phosphorylated forms of p65, p38 MAPK, ERK, and JNK. After the membranes were washed again with PBST, a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 h, and the blots were developed using ECL western blotting reagents [14].

Statistical analysis Statistical analyses were performed using ANOVA followed by the Bonferroni *t* test for multigroup comparisons; $p<0.05$ was considered significant for all tests. Data are expressed as mean ±

standard deviation.

Results

Effects of rutin on lung histopathology in LPS-induced ALI

To evaluate the effect of rutin on ALI, we observed the histopathological changes occurred in the lung of LPS administered mice pretreated with or without rutin. In control group, the lungs presented normal structure, no histopathological change was seen under the light microscope (Fig 1A). In contrast, lung tissues from the LPS administered groups without rutin pretreatment expressed extensive morphological damages, such as haemorrhage, interstitial edema, thickening of the alveolar wall, and infiltration of polymorphonuclear granulocytes (PMN) into the parenchyma and alveolar spaces of lung (Fig 1B). While with 30 min of rutin pretreatment, LPS-induced histopathological damages were attenuated in a concentration-dependent manner (Fig 1 C, D, and E). In positive control group, pretreatment with dexamethasone also reduced LPS-induced histopathological damages (Fig 1F). The results indicated that rutin has the ability to improve the histopathological conditions of lung caused by LPS-induced ALI.

Effects of rutin on the infiltration and activation of PMNs in LPS-induced ALI

Infiltration of PMN into the lung is an important feature of LPS-induced ALI [6]. To evaluate the effects of rutin on ALI, we evaluated the alteration of leukocytes infiltration occurred in lung of LPS administered mice with or without rutin pretreatment. The number of infiltrating leukocytes was counted by Giemsa stain performing on BALF. As shown in figure 2A, administration of LPS for 6 h without rutin pretreatment caused extensive leukocytes infiltration. While with 30 min rutin pretreatment, LPS-induced leukocytes infiltration was inhibited in a concentration-dependent manner, significant inhibitory effect began at 10 μmol/kg (p<0.05). Furthermore, we measured the ability of rutin to influence MPO activity, which is a marker for PMN infiltration and activation, and found MPO activity was siganificantly up-regulated in lung tissue by the LPS challenge. On the other hand, the up-regulation of MPO activity was prevented in animals pretreated with rutin (Fig 2B). These results demonstrated that rutin expressed protective effect in LPS-induced ALI mice by inhibition of PMN infiltration and activation.

Effects of rutin on lipid peroxidation in LPS-induced ALI

MDA, a product of lipid peroxidation, is the biomarker for estimating the status of oxidative stress. The generation of MDA was induced by LPS in ALI. The lipid peroxidation in lungs was significantly higher in LPS-treated group as compared with the control (p<0.05). Pretreatment with rutin reduced LPS-induced accumulation of MDA in a concentration-dependent manner. At both 10 and 100 μmol/kg, rutin significantly attenuated lipid peroxidation in lung tissue (p<0.05) (Fig. 3B). These results indicated rutin inhibited LPS-induced lipid peroxidation.

Effects of rutin on TNF-α,IL-1β, and IL-6 production in LPS-induced ALI

Proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, are major mediators involved in recruitment of PMN into the lungs in LPS-induced pulmonary injury [1]. After LPS treatment, the concentration of TNF-α, IL-1β, and IL-6 were increased in BALF. The responses were inhibited by rutin in a concentration-dependent manner (Fig 4). These results indicated that rutin reduced the expression of proinflammatory cytokines which in turn improved the lung damages caused by LPS-induced ALI.

Effects of rutin on NFκB phosphorylation and IκB degradation in LPS-induced ALI

Phosphorylation of NFκB prompts the transcription of most proinflammatory cytokines including TNF-α, IL-1β, and IL-6, thus plays a pivotal role in the pathogenesis of ALI [15]. The effects of rutin on phosphorylation of NFκB p65 on serine-536 residue were measured by western blotting. The serine phosphorylation of NFκB p65 in lung increased significantly after LPS administration as compared with the control group. Pretreatment with rutin reduced LPS-induced serine phosphorylation of NFκB p65 in a concentration-dependent manner, significant inhibitory effects started at 10 μmol/kg (p<0.05). In parallel with serine phosphorylation of NFκB p65, the effect of rutin on IκB degradation was also investigated. Similar to the serine phosphorylation on NFκB p65, LPS administration induced an elevation in degradation of IκB. The increase degradation was significantly attenuated by rutin in a concentration-dependent manner, significant inhibitory effects also started at 10 μmol/kg (p<0.05) (Fig 5).

Effects of rutin on MAPK activation in LPS-induced ALI

The three MAPK pathways, ERK, p38 MAPK, and JNK, have been demonstrated to participate in NFκB activation in LPS-induced ALI [5]. The effect of rutin on phosphorylation of ERK (on residue tyrosine-204), JNK (on residues threonine-183 and tyrosine-185), and p38 MAPK (on residues threonine-180 and tyrosine-182) in LPS-induced ALI was analyzed by western blotting. The results had shown LPS stimulation significantly increased MAPK phosphorylation and rutin inhibited LPS-induced phosphorylation of p38 MAPK and JNK in a concentration-dependent manner, the significant inhibitory effect started at 1 μmol/kg (p<0.05) (Fig 6A and C). In addition, rutin also inhibited LPS-induced phosphorylation of ERK in a concentration-dependent manner, but with significant inhibitory effect started at 10 μmol/kg (p<0.05) (Fig 6B). These data indicated that rutin relieved LPS-induced ALI by inhibiting the activity of MAPK pathways.

Effects of rutin on antioxidative enzymes on LPS-induced ALI

Oxidative stress exerted by activated PMN is critically important in the pathophysiology of ALI. Antioxidative enzymes, such as SOD, CAT, and GPx, are consumed during amelioration of ALI [16]. SOD, CAT, and GPx activities were significantly decreased in LPS-treated mice as compared to untreated group (p<0.05). Pretreatment with rutin recovered LPS-induced reduction in the activation of these antioxidative enzymes in a concentration-dependent manner, significant inhibitory effect on SOD and CAT started at 10 μmol/kg (p<0.05) (Fig 7A and B), while on GPx, significant inhibitory effect started at 1 μmol/kg (p<0.05) (Fig 7C).

Effects of rutin on HO-1 expression on LPS-induced ALI

HO-1, an antioxidative protein, is expressed during amelioration of ALI [17]. The effect of rutin on HO-1 expression in LPS-induced ALI was analyzed by western blotting. While LPS stimulation significantly increased HO-1 expression, rutin further enhanced LPS-induced HO-1 expression in a concentration-dependent manner, significant effect started at 1 μmol/kg (p<0.05) (Fig 8). This result suggested the ability of rutin to reduce LPS-induced oxidative stress.

Comparison of the Effects of rutin and desferrioxamine on LPS-induced ALI

DFX is an iron chelator which reduces the oxidative stress and provides beneficial effect on the

LPS-induced ALI [18, 19]. Although LPS-induced PMN infiltration was reduced by both rutin and DFX, rutin expressed a higher efficiency than DFX (Fig 9A). In addition, rutin not only could reduce the secretion of TNFα, IL-1β, and IL-6 but also the activity of CAT, while DFX had no effect (Fig 9B and C). Nevertheless, SOD and GPx activities were recovered by both rutin and DFX. These results suggested the amelioration of rutin is better than DFX on LPS-induced ALI.

Discussion

The major course of ALI correlates with infection of LPS-containing gram-negative bacteria [1]. In mouse model of LPS-induced ALI, the syndromes presented are similar to the pathological characteristics of ALI in human [3]. Therefore, the mouse model for the development of ALI induced by way of intratracheal LPS administration is well suited for the study of potential preliminary preventive or therapeutic compounds against ALI in human [7, 13, 20]. Lipid A is a structural section of LPS which can trigger the activation of endothelial cells, epithelial cells, and cellular innate immune system, including macrophages and PMN [21]. The exudative or early phase of LPS-induced ALI is characterized by respiratory failure, hypoxemia, PMN activation and infiltration, proinflammatory cytokines generation, changes in alveolar-capillary, and pulmonary edema [22]. So far, an effective therapy for ALI is still in search. At present, we carefully explored the potential effect of rutin in treating ALI using the murine model of LPS-induced ALI. The results of histopathological examination had shown pretreatment with rutin improved the LPS-induced symptoms, such as PMN infiltration, hyaline membrane formation, increased alveolar septum thickness, alveolar congestion, and hemorrhage significantly in this murine model of LPS-induced ALI. This indicated the potential therapeutic effect of rutin against ALI.

ALI is one of the inflammatory disorders in lung caused by pneumonia, sepsis , trauma, or aspiration [1]. In which, PMN is the major component to participate in the inflammation and pathogenesis processes [6]. Therefore, PMN is the predominant cell type in BALF and histological specimens from patients with ALI [1]. In murine model, PMN are rapidly activated and migrated into alveolar space and interalveolar septum in response to the intratracheal administration of LPS. MPO, stored in azurophilic granules of naive PMN, can serve as a marker for content and activation of PMN in tissue [7, 23]. Chemotaxis of PMN induced by formyl-L-methionyl-L-leucyl-L- phenylalanine or phorbol 12-myristate 13-acetate in *in vitro* assay is reduced by rutin [24]. In murine model of surgically induced oesophagitis, rutin inhibits MPO activation in oesophagus, which means the infiltration of PMN is inhibited by rutin [25]. Furthermore, pre-treatment with rutin significantly inhibits the PMN infiltration into mucosal tissues in indomethacin-induced gastric ulcers [26]. At present, we also found pretreatment with rutin prevented PMN infiltration into pulmonary tissue in LPS-induced ALI.

In the inflammatory site, proinflammatory cytokines such as TNFα, IL-1β, and IL-6 are secreted from pulmonary cells and alveaolar macrophages. These cytokines play an important role in the LPS-induced ALI [7, 13, 14]. TNFα and IL-1β are early response cytokines generated by activated alveaolar macrophages appear in BALF and plasma in the ALI. The secretion of TNFα and IL-1β in turn stimulates the neighboring cells to generate more effective proinflammatory cytokines and chemokines, such as IL-6, monocyte chemotatic protein, macrophage inflammatory protein, keratinocyte-derived chemokine, cytokine-induced neutrophil chemoattractant, and macrophage inflammatory protein-2, which subsequently mediate the recruitment of PMNs, macrophages, and lymphocytes [1]. Rutin reduces the generation of TNFα in human umbilical vein endothelial cells (HUVEC) induced by LPS, in acute liver damage in CCl_4 intoxicated mice,

and in cisplatin induced renal inflammation. [27, 28, 29]. Rutin inhibits the production of TNFα and IL-1β induced by β-amyloid42 in microglia, by brain ischemia/reperfusion injury in rat serum, and by high glucose in human monocytic THP-1 cells [30, 31, 32]. In murine model of LPS-induced ALI, rutin reduced the production of TNFα, IL-1β, and IL-6 in BALF. The results indicated rutin reduced leukocytes infiltration into lung via decreasing the expression of proinflammatory cytokines.

The transcription factor NF-κB is the crucial signal factor modulating proinflammatory cytokines in LPS-induced ALI [15]. There are five subunits, p65 (RelA), RelB, c-Rel, p50, and p52 to construct the homo- and hetero-dimers of NF-κB. The most abundant NF-κB in mammalian cells is the p50/p65 heterodimer. In unstimulated cells, the inactive NF-κB dimers are sequestered in the cytosol via non-covalent interactions with IκB, an inhibitor protein. After stimulation, the IκB is phophorylated by activated IκB kinases then the phosphorylated IκB is degradated by ubiquitin-dependent proteasome. During the process NF-κB is released, allowing rapid phosphorylation of p65 subunit and subsequently translocats into the nucleus [33]. Rutin suppresses both expression and activation of NF-κB and phosphorylation and degradation of IκB in LPS-stimulated HUVEC, cisplatin-induced nephrotoxicity, CCl_4-indcuded liver damage, and intracerebroventricular-streptozotocin–infused rat brain [25, 26, 27, 34]. At present, we found pretreatment with rutin prevented the phosphorylation of NF-κB p65 and degradation of IκB in the lung of LPS-induced ALI. Furthermore, dual phosphorylation of MAPK, one of the upstream kinases in NF-κB p65 phosphorylation pathway [5], at threonine and tyrosine residues within the kinase's activation loop is required for MAPK activation [35]. Phosphorylation of p38 MAPK and JNK induced by LPS are inhibited by Boehmeria nivea exerts, 40% of which is made up by rutin [36]. High glucose induced phosphorylation of ERK and p38 MAPK is inhibited by rutin in human monocytic THP-1 cells [32]. The present study demonstrated that intratracheal LPS instillation in mice resulted in phosphorylation of ERK, p38 MAPK, and JNK in lung tissue, while rutin pretreatment prevented these manifestations. Parallel trends were observed between suppression of both MAPK and NF-κB p65 phosphorylation. Therefore, the reduction of NF-κB p65 phosphorylation in lung resulted from rutin pretreatment was associate with MAPK activation.

MAPK also plays an important role in PMN activation, such as transmigration, degranulation, and respiratory burst [37]. During respiratory burst, the amount of oxygen consumed is converted into superoxide anions through nicotinamide adenine dinucleotide phosphate oxidase. SOD, which exists in the cytoplasm, can catalyze the reduction of superoxide anions into oxygen and hydrogen peroxide. MPO released via PMN degranulation can catatlyze hydrogen peroxide and chloride anions to form hypochlorous acid. These reactive oxygen species (ROS), including superoxide anions, hydrogen peroxide, and hypochlorous acid, cause oxidative stress which leads to tissue injury via lipid peroxidation, protein oxidation, and DNA damage [38]. MDA is the end-product of lipid peroxidation, indicating the destruction and damage of cell membrane caused by ROS [40]. In animal studies, rutin functions against MDA accumulation induced by ischemia/reperfusion in rat brain or kidney, and by cyclophosphamide in rat testes [11, 39]. In addition, rutin decreases the formation of MDA in SH-SY5Y neuroblastoma cells treated with β-amyloid42 [30]. In present study, we observed that with rutin pretreatment, MDA content in lung tissue was decreased when compared with the control group in LPS-induced ALI.

Furthermore, the formation of MDA acts as a marker for oxidative stress [38]. Under normal physiological conditions, improvement of oxidative damage is provided by antioxidative enxymes, such as SOD, CAT, and GPx. Superoxide anions are converted to hydrogen peroxide by SOD, which is then metabolized to water by CAT or GPx [39]. Rutin increases SOD and CAT levels in cerebral

ischemia-reperfusion injury in rats [40]. The activation of SOD, CAT, and GPx are elevated by rutin in 6-hydroxydopamine-induced neurotoxicity, ethanol- and CCl_4-induced liver damage, cyclophosphamide-induced reproductive toxicity, and cadium-induced testicular injury [41, 42, 43, 44]. For the present, we demonstrated that pretreatment with rutin raised the activation level of SOD, CAT, and GPx in LPS-induced ALI.

Heat shock protein 32, also called HO-1, is an inducible defense enzyme opposed to oxidative stress. In the process of oxidative degradation of heme to carbon monoxide, bilirubin, and ferrous ion, HO-1 is the rate limiting enzyme [45]. Carbon monoxide and bilirubin express cytoprotective effect. However, ferrous ion induces oxidative stress in mammlian cells [46]. In the rat model of LPS-induced ALI, the inflammatory respones evoked are decreased by the combination of N-acetylcysteine, an ROS scavenger and glutathione precursor, plus DFX, an iron chelator. Nevertheless, the use of isolated N-acetylcysteine or DFX has no effecet on LPS-induced ALI [18]. Although rutin is an impermeant extracellular iron chelators which exerts most of its antioxidative effects via inhibition of Fenton reactions catalysed by labile iron [47], our data had clearly suggested that exctracellular labile iron has a role in ALI perhaps following release from necrotic inflammatory cells. At present, we also found the amelioration of rutin is better than DFX. A previous study had suggested biliverdin, the marker of HO-1 activation, could improve injury occured in LPS-induced ALI, such as lung permeability, lung alveolitis, and generation of IL-6 [48]. Nuclear factor-erythroid-2-related-factor-2 (Nrf2), a transcription factor mediates HO-1 induction, has been demostrated to diminish damages caused by inflammation and oxidative stress [49]. Rutin reduces Nrf2 and HO-1 expressions in CCl_4 injured liver in BALB/c N mice [27]. Our data demonstrated that rutin prompted expression of HO-1 in LPS-induced ALI. These evidences should be noted in that rutin was capable of reducing serious lung damages through AOE.

In conclusion, we demonstrated that rutin effectively attenuated LPS-induced ALI by inhibiting histopathological changes and infiltration of leukocytes in lung. The mechanisms underlying this protective effect include (1) reduction of MPO activity; (2) decreasing of proinflammatory cytokines, such as TNFα, IL-1β, and IL-6 secretion; (3) inhibition of NF-κB phosphorylation and IκB degradation; (4) inhibition of MAPK phosphorylation; (5) diminution of lipid peroxidation and MDA formation; (6) elevation of antioxidative enzymes activity, such as SOD, CAT, and GPx; and (7) increased expression of HO-1 (Fig 9). Experimental findings support the potential use of rutin as a therapeutic agent for prevention of ALI associated with direct infection by gram-negative bacteria.

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Figure legends

Fig. 1. Effects of rutin pretreatment on histopathological changes of lung tissues in LPS-induced ALI (100X). (A) Control (B) LPS (C) 1 μmol/kg rutin + LPS (D) 10 μmol/kg rutin + LPS (E) 100 μmol/kg rutin + LPS (F) 1mg/kg dexamethasone + LPS.

Fig. 2. Effects of rutin on LPS-induced leukocytes infiltration into BALF. PBS or 1-100 μmol/kg of rutin were intraperitoneally injected for 30 min prior to intratracheal instillation of LPS (100 μg/50 μl saline) or saline into the mice. Six hours later, the mice were anesthetized and BALF was collected for PMNs count (A) and MPO activation in lung tissue (B). Values are expressed as mean ± S.D. (n=4 in each group). *Represents significant difference between the indicated and normal control group; $^{\#}$ between the indicated and LPS group, $p < 0.05$.

Fig. 3. Effects of rutin on LPS-induced MDA formation in lung tissue. Values are expressed as mean ± SD of 4 mice per group. *Represents significant difference between the indicated and normal control group; $^{\#}$ between the indicated and LPS group, $p < 0.05$.

Fig. 4. Effects of rutin on LPS-induced proinflammatory cytokines secretion in BALF. Values are expressed as mean ± S.D. (n=3-4 in each group). *Represents significant difference between the indicated and normal

control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 5. Effects of rutin on LPS-induced NF-κB p65 phosphorylation and IκB degradation in lung. Lungs were harvested from post-treated mice and homogenates of whole lung tissues were analyzed by Western blotting. The folds of NFκB p65 phosphorylation and IκB degradation between the treated and control groups were calculated. Values are expressed as mean ± S.D. (n=3-5 in each group). *Represents significant difference between the indicated and normal control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 6. Effects of rutin on LPS-induced MAPK phosphorylation. Lungs were harvested from post-treated mice and homogenates of whole lung tissues were analyzed by Western blotting. The folds of MAPK phosphorylation between the treated and control groups were calculated. Values are expressed as mean ± S.D. (n=3-5 in each group). *Represents significant difference between the indicated and normal control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 7. Effects of rutin on LPS-induced antioxidative enzymes activation in lung tissue. The antioxidative enzymes represented are (A) SOD (B) CAT (C) GPx. Values are expressed as mean ± SD of 4 mice per group. *Represents significant difference between the indicated and normal control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 8. Effects of rutin on LPS-induced HO-1 expression in lung. Lungs were harvested from post-treated mice and homogenates of whole lung tissues were analyzed by Western blotting. The folds of HO-1 expression between the treated and control groups were calculated. Values are expressed as mean ± S.D. (n=3 in each group). *Represents significant difference between the indicated and normal control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 9. Comparison of the Effects of rutin and DFX on LPS-induced ALI. (A) Effects of rutin (100 μmol/kg) and DFX (20 mg/kg) on LPS-induced leukocytes infiltration into BALF. (B) Effects of rutin and DFX on LPS-induced proinflammatory cytokines secretion in BALF. (C) Effects of rutin and DFX on LPS-induced antioxidative enzymes activation in lung tissue. Values are expressed as mean ± SD of 4 mice per group. *Represents significant difference between the indicated and normal control group; # between the indicated and LPS group, $p < 0.05$.

Fig. 10 Scheme for the mechanisms in the protective effect of rutin on LPS-induced ALI. The shaded parts indicate the molecules effected by rutin.

Fig 1

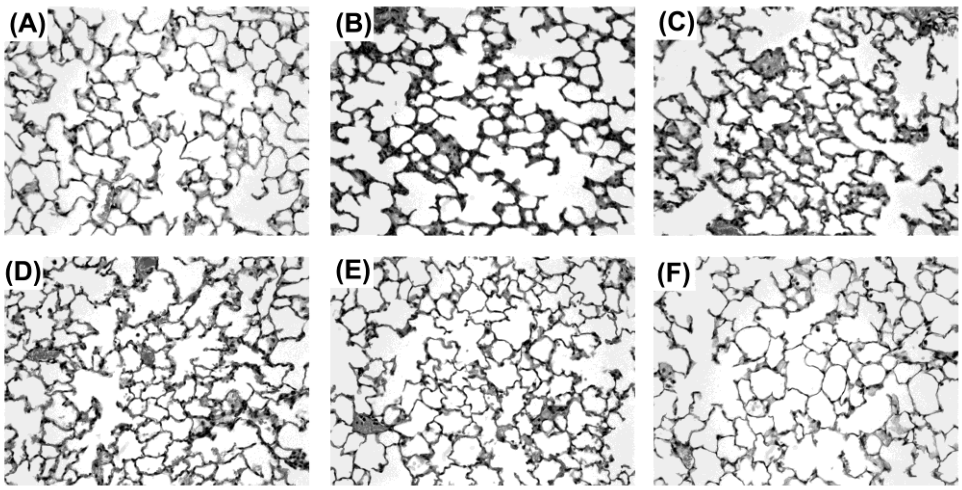
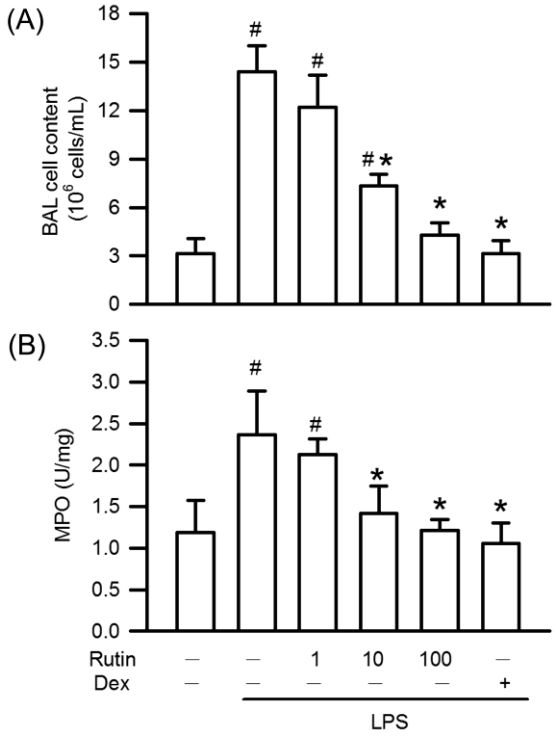


Fig 2


(A)
BAL cell content
(10^6 cells/mL)
18
15
12
9
6
3
0
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(B)
MPO (U/mg)
3.5
3.0
2.5
2.0
1.5
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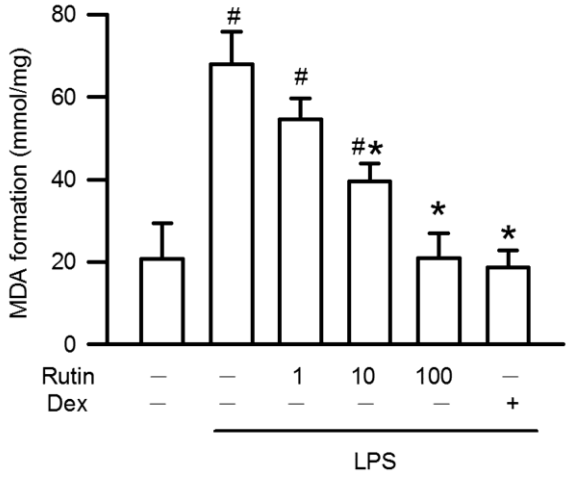
Rutin – – 1 10 100 –
Dex – – – – – +
LPS

Fig 03


MDA formation (mmol/mg)
80
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40
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0
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Rutin – – 1 10 100 –
Dex – – – – – +
LPS

Fig 4

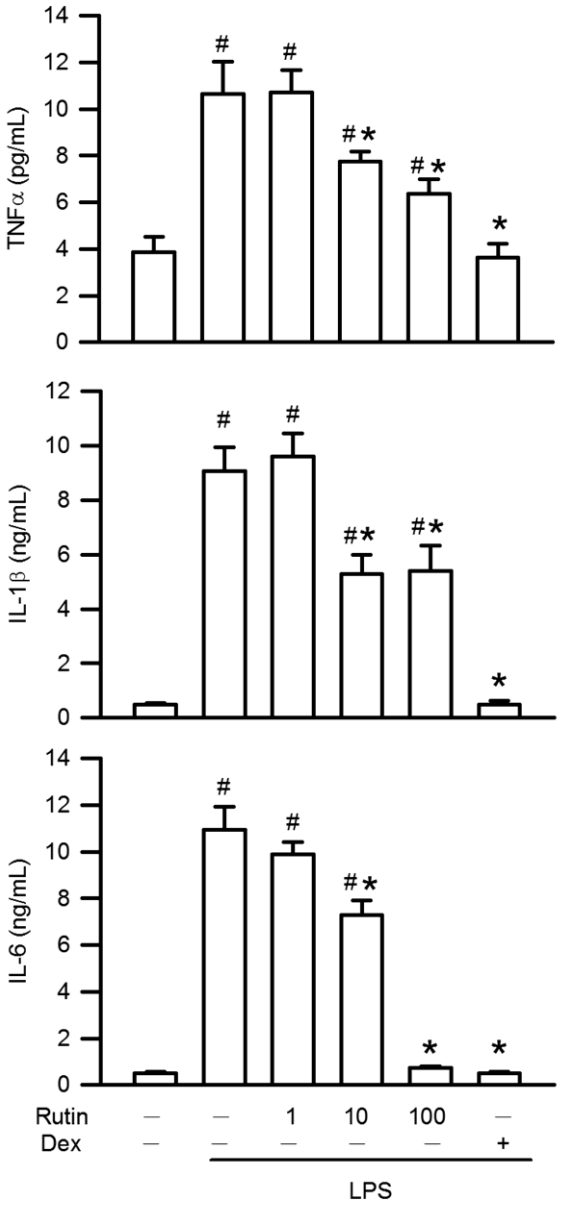
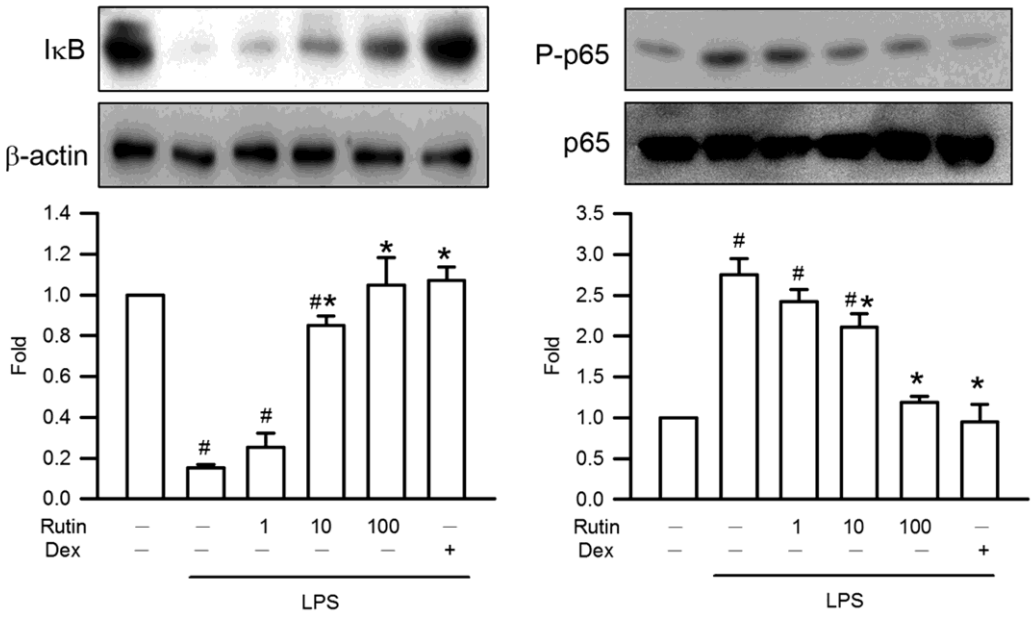

TNFα (pg/mL)
IL-1β (ng/mL)
IL-6 (ng/mL)
Rutin
Dex
LPS
1
10
100

Fig 5


IκB
β-actin
P-p65
p65
Fold
Rutin
Dex
LPS
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10
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Fig 6

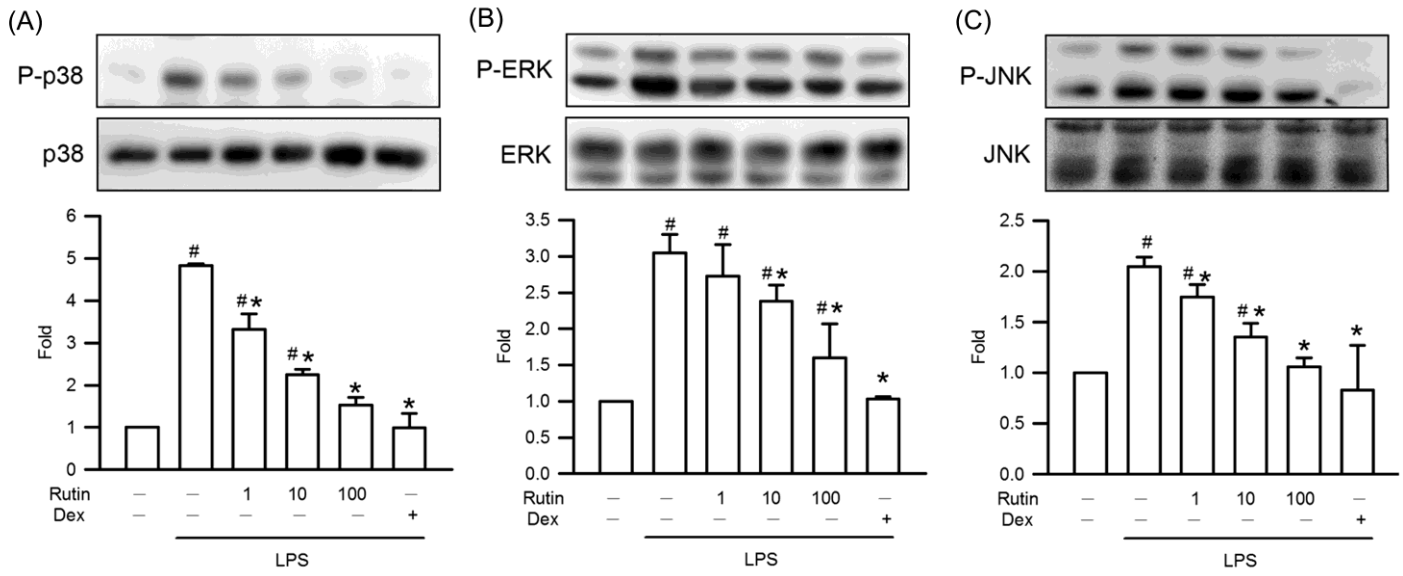

(A)
P-p38
p38
Fold
Rutin
Dex
LPS
(B)
P-ERK
ERK
Fold
Rutin
Dex
LPS
(C)
P-JNK
JNK
Fold
Rutin
Dex
LPS

Fig 7

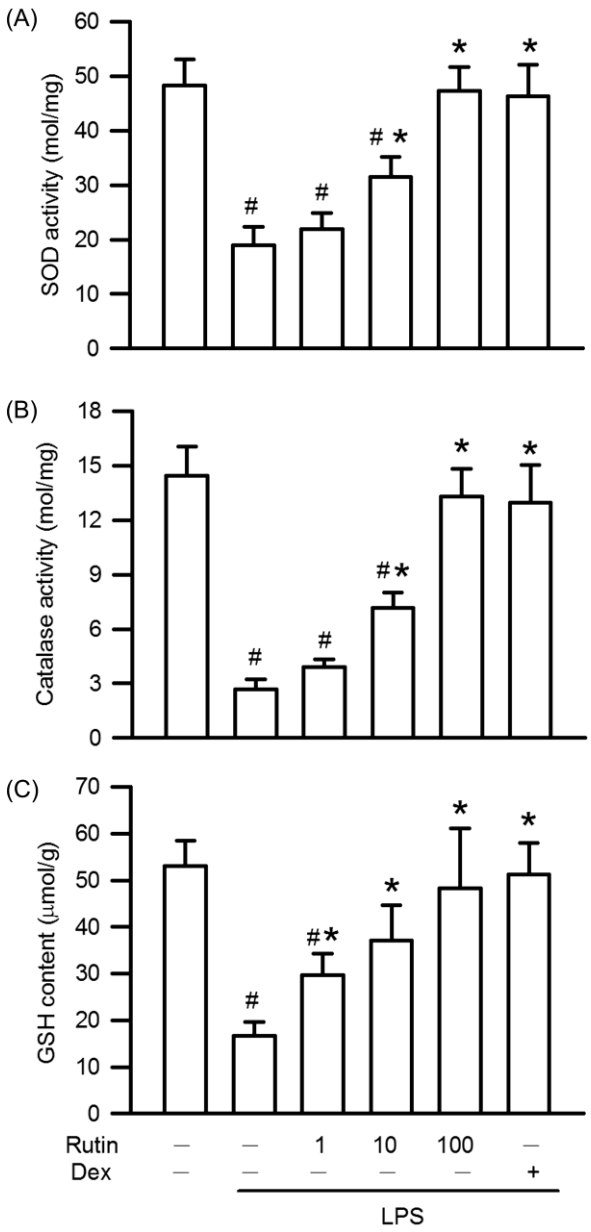
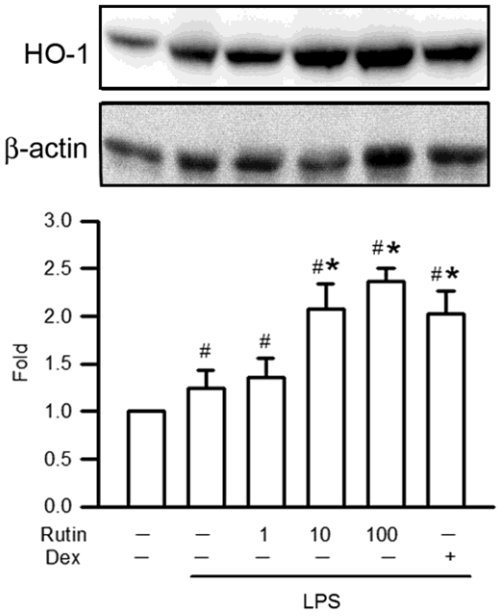

(A)
SOD activity (mol/mg)
(B)
Catalase activity (mol/mg)
(C)
GSH content (μmol/g)
Rutin
Dex
LPS

Fig 8


HO-1
β-actin
Fold
3.0
2.5
2.0
1.5
1.0
0.5
0.0
#
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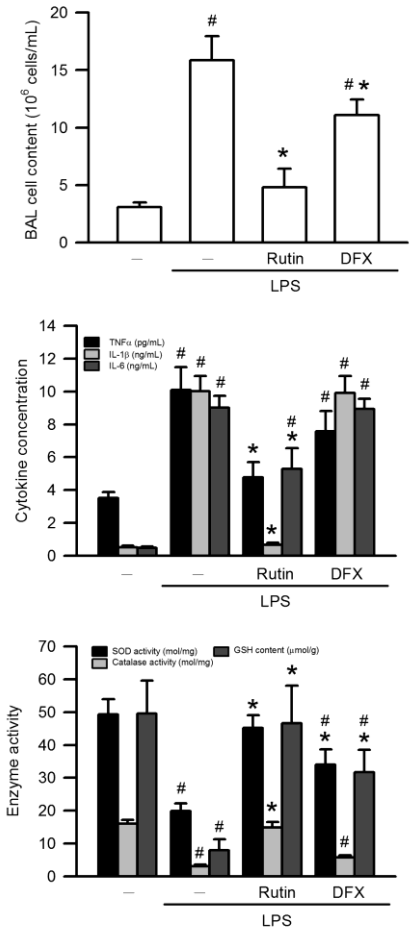
Rutin – – 1 10 100 –
Dex – – – – – +
LPS

Fig 9


BAL cell content (10^6 cells/mL)
20
15
10
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*
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– – Rutin DFX
LPS
Cytokine concentration
TNFα (pg/mL)
IL-1β (ng/mL)
IL-6 (ng/mL)
14
12
10
8
6
4
2
0
– – Rutin DFX
LPS
Enzyme activity
SOD activity (mol/mg)
Catalase activity (mol/mg)
GSH content (μmol/g)
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60
50
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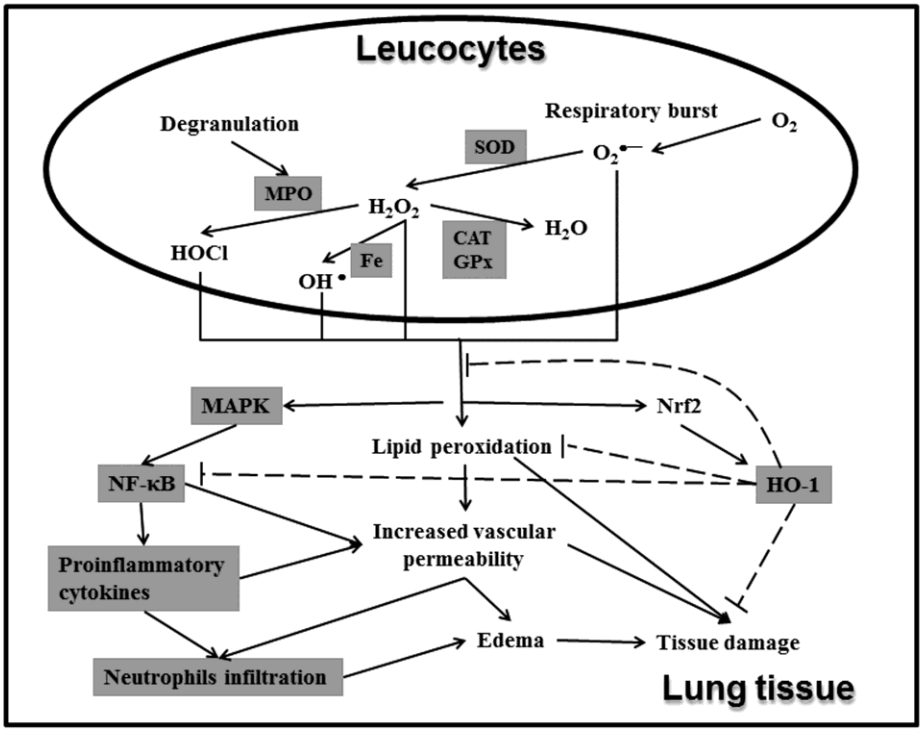
30
20
10
0
– – Rutin DFX
LPS

Fig 10


Leucocytes
Degranulation
Respiratory burst
O_2
SOD
$O_2^{\bullet-}$
MPO
H_2O_2
H_2O
CAT
GPx
HOCl
Fe
$OH^{\bullet}$
MAPK
Nrf2
Lipid peroxidation
NF-κB
HO-1
Increased vascular permeability
Proinflammatory cytokines
Edema
Tissue damage
Neutrophils infiltration
Lung tissue

Chapter 2

Protective effect of rutin on LPS-induced acute lung injury via down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation

1. Introduction

Acute lung injury (ALI) is a clinical disease characterized by the acute onset of severe hypoxemia accompanied with life-threatening inflammation. There are several pathogenic factors participate in the induction of ALI, such as sepsis, pneumonia, smoke, transfusions, and toxic gas inhalation. Among them, sepsis accounts for approximately 23 to 40 % of total cases [1]. The endotoxin, also called lipopolysaccharide (LPS), is the component of the outer membrane of the cell wall of Gram-negative bacteria which is an important pathological factor in sepsis-related ALI [2]. So far the therapeutic protocols for ALI still stop in supportive care with no effective medicine established as yet [3].

The inflammatory responses of ALI, including neutrophils infiltration, hypoxaemia, and expression of matrix metalloproteinase (MMP)-9 are induced by lipopolysaccharide (LPS) [4]. Nuclear factor (NF)κB plays the major role in the expression of proinflammatory mediators in LPS-induced ALI [5, 6]. In resting cells, the inactive NFκB is binding to unphophorylated inhibitor of NFκB, which is IκB, in cytoplasm. After stimulation, IκB is phosphorylated by protein kinase B (PKB/Akt) and then degraded by proteasome to free NFκB [7]. The free type of NFκB can be translocated to the nucleus and activates transcription of target genes [8].

The flavonoids are a family of polyphenolic compound and the major dietary constituents of plant-based food. Rutin is one of the flavonoids in many plants, such as buckwheat seeds, citrus fruits, vegetables, and plant-derived beverages such as wine and tea. Rutin belongs to vitamin P and has various beneficial bioeffects including anti-inflammation, anti-hypertension, anti-carcinogen, and cardiovasoprotection [9-12]. Recently, we have proposed that LPS-induced ALI is prevented by rutin through inhibition of oxidative stress and MAPK pathway in mice [13]. However, there has no study demonstrated that Akt participates in the suppression of proinflammatory mediator generation in the LPS-induced ALI by rutin. In present study, we aimed to investigate the protective effect of rutin in LPS-induced ALI and the mechanism involved Akt- NFκB pathway in mice.

2. Materials and methods

2.1 Materials

Antibodies against phospho-Akt and Akt were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were obtained from Jackson Immuno Research Laboratories (Baltimore, MD, USA). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cayman (Ann Arbor, MI, USA). LPS was from *Escherichia coli* Serotype 0111:B4. Dimethyl sulfoxide (DMSO) and other reagents, unless specifically stated elsewhere, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of DMSO in the reaction mixture was <0.5%.

2.2 Animals

Adult mice weighing 25-30 g were purchased from BioLASCO Taiwan (Taipei, Taiwan). All mice were housed under standard laboratory conditions and fed a standard laboratory diet and water *ad libitum*. All

animal procedures involving experimentation on ALI were reviewed by the Institutional Animal Ethics Committee of Chung Shan Medical University, which were approved on No. 1229.

2.3 Murine model of LPS-induced ALI

The scheme of LPS-induced ALI model was consisted of six experiment groups, in which vehicle or rutin was intraperitoneally given 0.5 hr before a single intratracheal injection of saline (50 μl) or LPS (100 μg 50 μl^{-1}). In the first group, the mice received vehicle followed by a saline administration. In the second group, the mice received vehicle followed by a LPS administration. In the third to fifth group, the mice received 1, 10, or 100 μmol kg^{-1} of rutin before LPS administration. In the sixth group, the mice received 1 mg kg^{-1} of dexamethasone (DEX) before LPS administration. After 6 hr, the mice were sacrificed, and samples were collected [14].

2.4 Bronchoalveolar lavage fluid (BALF) collection

The BALF was prepared as described previously [5]. Briefly, the lungs were flushing with sterile saline through a tracheal cannula for three times. After centrifugation, the supernatant was stored at -20 ℃ for macrophage inflammatory protein (MIP)-2. Resuspend the sediment cells in saline. Total cell count used the haemocytometer. The percent of neutrophils could be determined by Wright and Giemsa stain. The numbers of neutrophils were calculated by total cell count × the percent of neutrophils.

2.5 Blood gas analysis of arterial blood

Blood samples were collected from the abdominal aorta after euthanasia. The oxygen partial pressure (PaO_2), carbon dioxide partial pressure ($PaCO_2$), and acidity (pH) in arterial blood were measured immediately by Blood Gas Analyzer (Radiometer ABL 700 Blood Gas Analyzer, Copenhagen, Denmark.).

2.6 Measurement of MIP-2

Measurement of MIP-2 in BALF was performed by commercially available ELISA assay kits. The quantification of MIP-2 were calculated according to the manufacturer's instruction.

2.7 Zymography analysis of MMP9 activation

As previously described [14], the MMP-9 activation in BALF was measured using gelatin zymography protease assay. BALF was prepared with SDS sample buffer then subjected to 8% SDS-PAGE which contained 0.1% gelatin. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 1 hr, and incubated at 37°C for 16 hr in reaction buffer. The gels were then stained with Coomassie Brilliant R-250 and destained in a solution of 7.5% acetic acid and 5% methanol.

2.8 Western blot analysis of lung tissue

As previously described [15], lungs were harvested and immediately frozen in liquid nitrogen until homogenization. Tissue extracts were homogenized in tissue protein extraction solution (T-PER; Pierce, Rockford, IL, USA) containing 1% proteinase inhibitor cocktail and phosphatase inhibitor cocktail. Protein samples, 100 μg each, were separated by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with phosphate buffered saline containing 0.1% Tween-20 (PBST) and 5% non-fat milk (w/v) for 1 hr at room temperature. After washed with PBST, the membranes were probed with antibodies including non-phosphorylated forms of Akt. The membranes were washed again with PBST, then a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 hr, and the blots were developed using ECL western blotting reagents.

2.9 Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni's *t* test for multigroup comparisons; $p<0.05$ was considered significant for all tests. Data are expressed as mean ± standard deviation

(S.D.).

3. Results

3.1 Effects of rutin on arterial blood gas in LPS-induced ALI

To study the effects of rutin on LPS-induced ALI, the mice were pretreated with rutin for 30 min before LPS administration. As shown in Fig. 1, pH and PaO_2 were significantly decreased in the LPS-treated group. These effects were attenuated by rutin in a concentration-dependent manner, significant inhibitory effect began at 10 μmol kg^{-1} ($p<0.05$). On the other hand, $PaCO_2$ was significantly increased in the LPS-treated group. However, the effect was also attenuated by rutin in a concentration-dependent manner, but significant inhibitory effect began at 100 μmol kg^{-1} ($p<0.05$).

3.2 Effects of rutin on LPS-induced neutrophils infiltration

Neutrophils infiltration is an indicator of LPS-induced ALI, which was significantly increased in LPS-treated group as compared with control group ($p<0.05$). Pretreatment with rutin reduced LPS-induced neutrophils infiltration in a dose-dependent manner started at 1 μmol k g^{-1} ($p<0.05$, Fig 2).

3.3 Effects of rutin on LPS-induced MIP-2 generation

MIP-2 is an important cytokines in the recruitment of leukocytes into the lungs in LPS-induced ALI [16-18]. The effect of rutin on MIP-2 generation in BALF was analyzed by ELISA. The results had shown that the concentrations of MIP-2 increased significantly after the mice were treated with LPS for 6 hr as compared with control group ($p < 0.05$). Pretreatment of rutin reduced LPS-induced generation of MIP-2 in a dose-dependent manner started at 1 μmol kg^{-1} ($p<0.05$, Fig 3).

3.4 Effects of rutin on LPS-induced activation of MMP-9

MMP-9 released from activated neutrophil results in tissue damage in LPS-induced ALI [19]. After LPS administration, MMP-9 activation was significantly increased in BALF as compared with control group ($p<0.05$). Pretreatment with rutin reduced LPS-induced MMP-9 activation in a concentration- dependent manner started at 1 μmol kg^{-1} ($p<0.05$, Fig 4).

3.5 Effects of rutin on LPS-induced phosphorylation of Akt

Akt has been demonstrated as the upstream factor in NFκB translocation in LPS-induced ALI [20]. LPS stimulation significantly increased Akt phosphorylation when compared with control group ($p<0.05$). Rutin suppressed LPS-induced phosphorylation of Akt in a concentration-dependent manner started at 1 μmol kg^{-1} ($p<0.05$, Fig 5).

4. Discussion

LPS, also called endotoxin, is the major component of the cellular wall which is enveloped by the outer membrane in Gram negative bacteria. LPS acts as the causal factor in many serious infectious diseases, such as sepsis, atherosclerosis, Crohn's disease, arthritis, and cancer. In murine model, the pathogenic characteristics of ALI induced by intratracheal LPS administration are the most similar to human patients [21]. Clinically, patients of ALI present hypoxaemia, breathlessness, and diffuse pulmonary edema on the chest radiograph. Therefore, abnormalities of gas exchange are good candidates of ALI [22]. At present study, we found LPS not only significantly induced $PaCO_2$ up-regulation, but also PaO_2 and pH down-regulation in arterial blood. These results are similar as previous study [5]. But pretreatment with rutin prevented the concentration exchange of arterial blood gas. These evidences suggested rutin could ameliorate the gas exchange of ALI induced by endotoxin.

The breakdown of alveolocapillary barrier causes obstruction of gas exchange in ALI. The mechanism behind is the activation of neutrophils which increases the permeability of alveolocapillary barrier after LPS administration and results in the generation of reactive oxygen species (ROS). Although moderate amount of ROS is a signal wagging for defense system to kill pathogens, excessive amount can cause harmful effects including tissue damage, apoptosis, and necrosis to lung. The dysfunction of alveolocapillary barrier results in plasma-protein leakage into the alveolar space which then leads to lung edema [4]. In previous study, we have demonstrated that the protective effect of rutin in LPS-induced pathohistological changes, such as hyaline membrane formation, hemorrhage, alveolar septal thickening, and neutrophils infiltration [13]. At present study, we yet again confirmed that rutin not only reduced LPS-induced leukocytes infiltration but also demonstrated the most abundant of leukocytes were neutrophils. In addition, we also proposed that LPS-induced lung edema and protein leakage were suppressed by rutin. These results indicated rutin could prevent and ameliorate endotoxin induced ALI by reducing the neutrophils infiltration and the permeability of alveolocapillary barrier.

The hallmark of ALI is excessive neutrophils infiltration into the lung [4]. Neutrophils are known to infiltrate into the lung through chemotaxis induced by proinflammatory cytokines. MIP-2 is a chemokine which acts as a chemoattractant to guide the migration of neutrophils produced by the endothelial cells in alveolocapillary barrier, alveolar macrophages, and activated neutrophils [23, 24]. After LPS administration into mice, the expression of MIP-2 is elevated in BALF [25]. Here, we had obtained the same results as previous findings. Rutin inhibited the LPS-induced generation of MIP-2 in BALF.

MMP-9 is a proteolytic enzyme which cleaves gelatin and type IV collagen present in basement membranes [26]. MMP-9 has been demonstrated to participate in the recruitment of neutrophils into the lung in a LPS-induced ALI model [14, 19, 27]. Rutin improves functional outcome of lung via reducing the level of MMP-9 in the photothrombotic focal ischemic rat model [28]. At present, we also found the up-regulation of MMP-9 activity in LPS treated mice. Rutin reduced the activation of MMP-9 in a concentration-dependent manner as measured by zymography assay. The above evidences indicated that the neutrophils infiltration reduced by rutin was due to inhibition of MIP-2 generation and MMP-9 activation.

NF-κB is a proinflammatory and inducible transcription factor involved in immune and inflammatory responses [8]. NF-κB controls the expression genes of several proinflammatory mediators, such as MIP-2, TNF-α, interleukin (IL)-1β, IL-6, adhesion molecules, MMP-9, iNOS, and COX-2 [8, 29]. Activation and translocation of NF-κB into nucleus are triggered by LPS via IκB phosphorylation [8]. After LPS administration, NF-κB activation and IκB phosphorylation are reduced by rutin in HUVEC and RAW264.7 cells [30, 31]. In previous study, we have demonstrated that rutin suppresses the LPS-induced ALI in mice via reduction of NF-κB phosphorylation and IκB degradation in a concentration-dependent manner [13]. Moreover, Akt participates in the activation of NFκB via IκB phosphorylation in LPS-induced ALI [7]. Akt, a downstream molecule of phosphoinositol 3-kinase, is involved in activation of alveolar macrophages, peripheral neutrophils, and alveolocapillary barrier cells induced by LPS [32]. Phosphorylation of Akt is induced by LPS administration which is in turn reduced by rutin. The inhibitory effect of rutin on Akt phosphorylation exhibited the parallel trend with inhibitions on NF-κB phosphorylation and IκB degradation. We also found the inhibitory concentration of rutin on Akt phosphorylation was similar to MIP-2 expression and MMP-9 activation. Based on the present results, we purposed that phosphorylation of Akt reduced by rutin was a primary signal pathway in LPS-induced ALI.

At present study, the similar protective effect on the LPS-induced ALI was observed between DEX at 1

mg kg^{-1} and rutin at 100 μmol kg^{-1}. ALI has been treated by DEX via inhibited activation phagocytes and related inflammatory process [33]. In clinical, long-term use of dexamethasone can cause serious advisor effect such as immune suppression, Cushing's disease, weight gain, impaired skin healing, etc. Up to now, there is no evidence show side effects on long-term use of rutin. It is for this reason for rutin could be able to replace DEX for protective effect of LPS-induced ALI.

In the present investigation, we had demonstrated that rutin significantly prevented the blood gas parameters exchanges and inflammatory responses, such as neutrophils infiltration on the mouse model of LPS-induced ALI. The mechanisms involved in the protective effect of rutin were: (1) decreased generation of proinflammatory cytokine, MIP-2; (2) decreased activation of MMP-9; (3) down-regulation of Akt phosphorylation. These results suggested the molecular mechanism of rutin to act as a preventive agent in LPS-induced ALI was via reversing the MIP-2 expression and MMP-9 activation via Akt activation.

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Figure Legends

Figure 1 Effect of rutin on changes of arterial blood gas in LPS-induced ALI. The parameters represented are PaO_2, pH, and $PaCO_2$. Values are expressed as mean ± S.D. of 5 mice per group. # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $p < 0.05$.

Figure 2 Effects of rutin on LPS-induced neutrophils infiltration and lung edema. Neutrophils infiltration determined by leukocyte counts in BALF. Values are expressed as mean ± S.D. (n=4 in each group). * Represents significant difference between the indicated and control group; # between the indicated and LPS groups, $p < 0.05$.

Figure 3 Effect of rutin on LPS-induced generation of MIP-2 in BALF. Values are expressed as mean ± S.D. of 4 mice per group. # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $p < 0.05$.

Figure 4 Effect of rutin on LPS-induced MMP-9 activation in BALF. The activation of MMP-9 was analyzed by gelatin zymography. The fold of MMP-9 activation between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n = 3 in each group). *Represents significant difference between the indicated and control group; #between the indicated and LPS groups, $p < 0.05$.

Figure 5 Effect of rutin on LPS-induced Akt phosphorylation in lung. Lung tissues harvested from post-treated mice were analyzed by Western blotting. The fold of Akt phosphorylation between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n=3-5 in each group). * Represents significant difference between the indicated and control group; # between the indicated and LPS groups, $p < 0.05$.

Figure 1

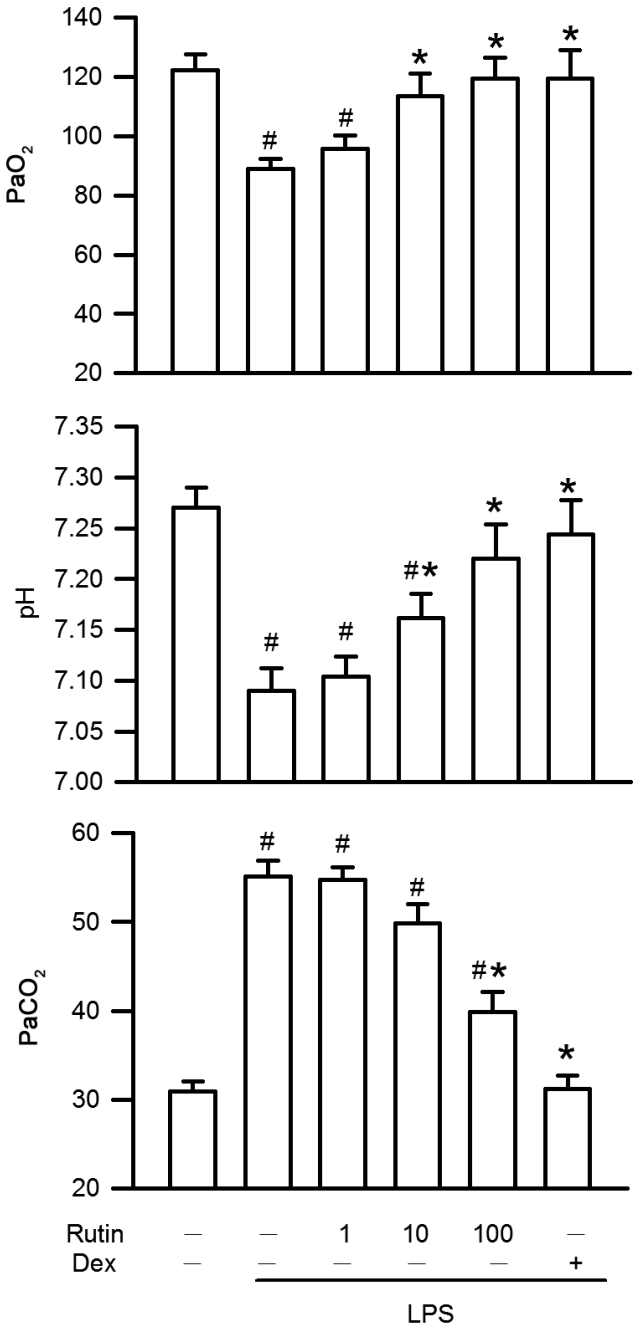
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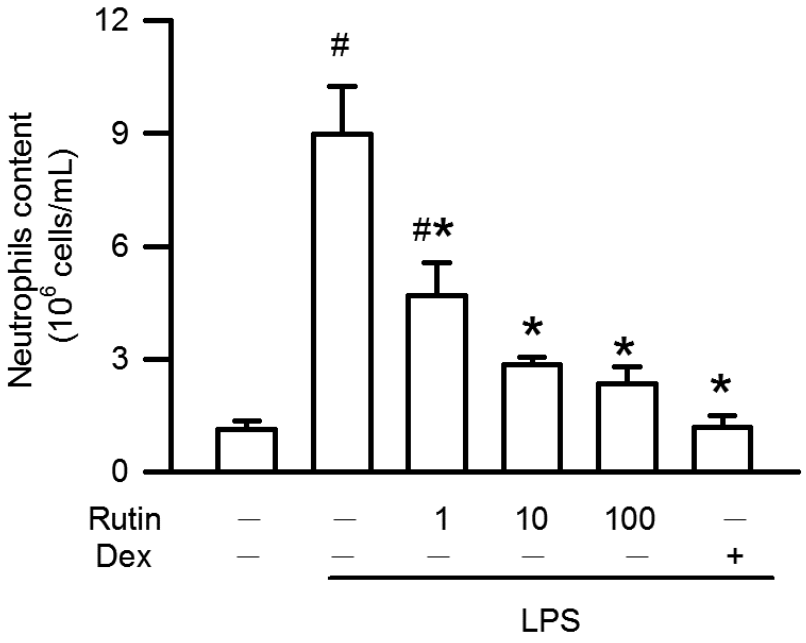
Figure 2



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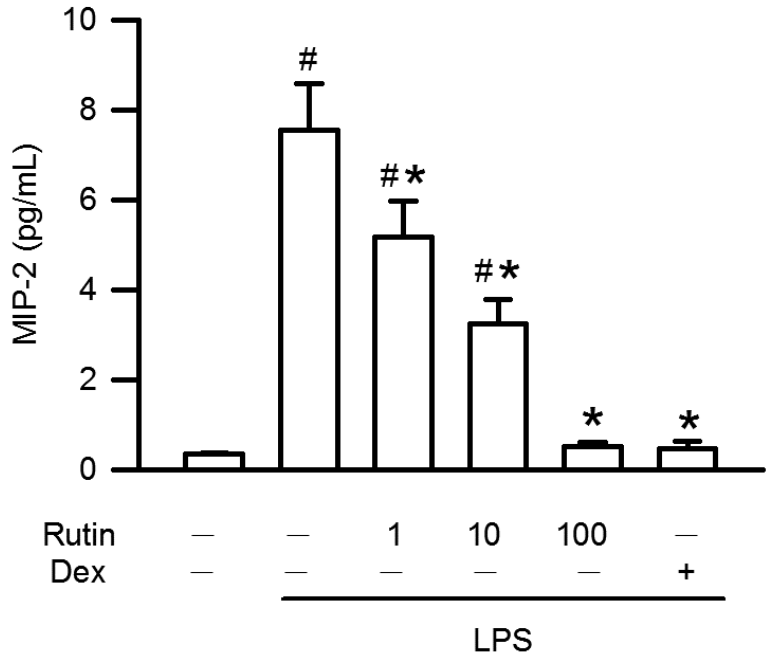

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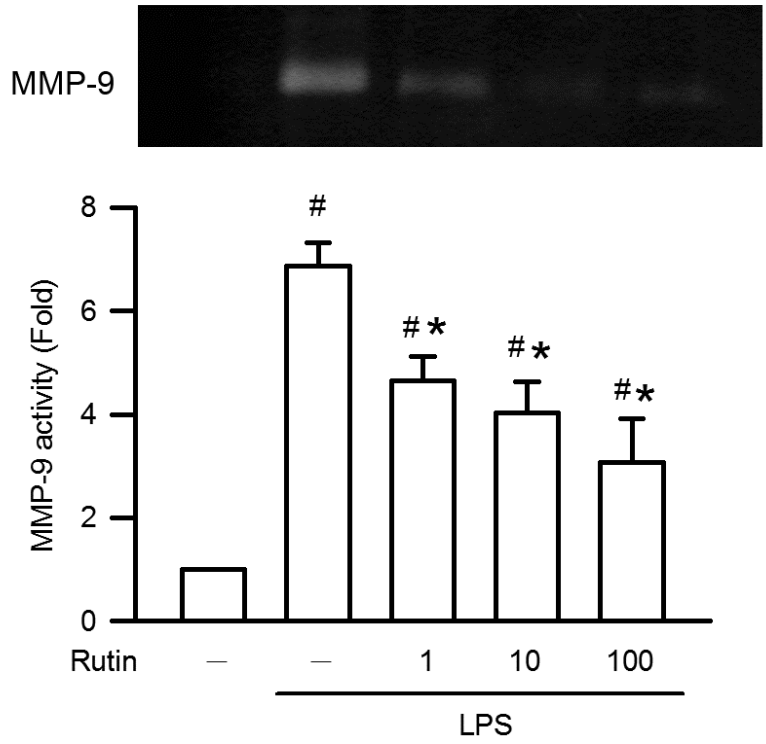
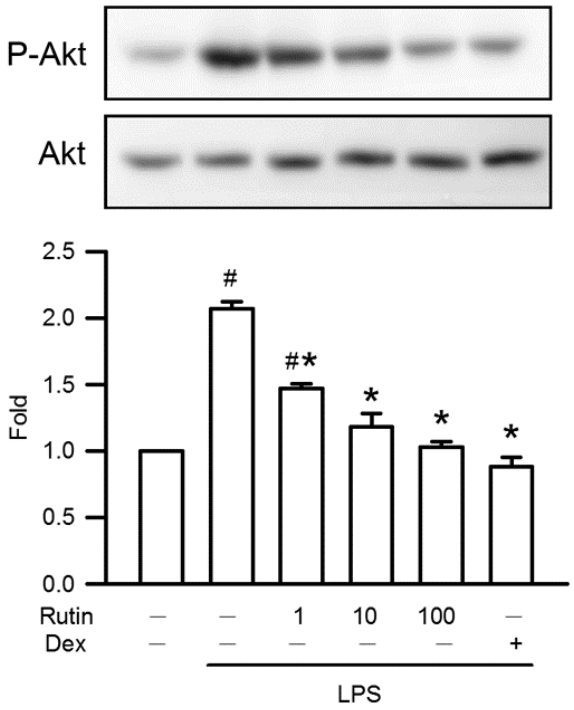


Figure 5



Chapter 3

Rutin improves endotoxin-induced acute lung injury via inhibition of iNOS and VCAM-1 expression

Introduction

Endotoxins are the inherent fraction of the outer membrane of the cell wall of Gram-negative bacteria and are composed of lipopolysaccharide (LPS) and its molecular weight is more than 100 kilo-Daltons (Rylander, 2002). LPS consists of three distinct regions: O-specific antigen forming the surface antigen, core polysaccharide, and lipid A. The lipid A portion, a hydrophobic region, is responsible for most of the immunological responses by the host immune system (Park and Lee, 2013). There are major sources of endotoxins including water pools, dust, humidifier systems, machining fluids due to the Gram-negative bacteria. Its fragments exist everywhere. Endotoxins are the causal factor in many serious diseases, such as fever, sepsis, multi-organ failure, meningococcemia, and severe morbidities like neurologic disability, and hearing loss (Park and Lee, 2013; Su et al., 2013). In addition, endotoxin is the major pathological factor in acute lung injury (ALI) (Rojas et al., 2005). The clinical features of ALI are acute onset, severe hypoxemia, bilateral infiltration of leukocytes, and pulmonary edema (Wheeler and Bernard, 2007). Therefore, ALI causes high morbidity and mortality in critically ill patients (Elizabeth and Michael, 2010). However, there are currently no appropriate therapeutic or effective medicines for ALI (Varisco, 2011).

Endotoxins cause neutrophil infiltration and pulmonary edema via up-regulation of pulmonary vascular permeability due to disruption of the alveolocapillary barrier in ALI (Grommes and Soehnlein, 2011). The alveolocapillary barrier is disrupted by overproduction of nitric oxide (NO) and prostaglandin (PG) E2 through induction of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2, respectively, which are induced by endotoxin administration (Grommes and Soehnlein, 2011). In addition, expression of adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, are induced by endotoxin and participate in neutrophil infiltration in lung (Reutershan et al., 2006, Li et al., 2009). Expression of iNOS, COX-2, and adhesion molecules is regulated by the pro-inflammatory transcription factor, Nuclear factor (NF)κB (Fan et al., 2001; Kuo et al., 2011; Li et al., 2012).

Rutin, also named quercetin-3-O-rutinoside or rutoside, is the polyphenol flavonoid commonly found in fruits, vegetables, and plant-derived drinks such as wine and tea. Many potential beneficial effects of rutin have been demonstrated including anti-inflammation, antioxidation, anti-hyperlipidemia, anti-platelet aggregation, anti-hypertension, anti-carcinogen, and cardiovasoprotection (Guo and Wei, 2008; Lee et al., 2000; Novakovic et al., 2006; Korkmaz, and Kolankaya, 2010). Oxidative stress generation and mitogen-activated protein kinase (MAPK) activation are suppressed by rutin in a LPS-induced ALI murine model (Yeh et al., 2014). However, no study has demonstrated that suppression of adhesion molecules, iNOS, and COX-2 participates in LPS-induced ALI by rutin. In the present study, we aimed to investigate the protective effect of rutin in LPS-induced ALI and its mechanism involving expression of the adhesion molecules, iNOS, and COX-2, in mice.

Materials and methods

Materials

Antibodies against COX-2, iNOS, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were obtained from Jackson Immuno Research Laboratories (Baltimore, MD, USA). NFκB p65 Transcription Factor Kit was obtained from Thermo Fisher Scientific (CA,

USA). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Cayman (Ann Arbor, MI, USA). LPS was from *Escherichia coli* Serotype 0111:B4. Dimethyl sulfoxide (DMSO) and other reagents, unless specifically stated elsewhere, were purchased from Sigma-Aldrich (St. Louis, MO, USA). The final volume of DMSO in the reaction mixture was <0.5%.

Animals

Adult male mice specific pathogen-free were purchased from BioLASCO Taiwan (Taipei, Taiwan). All mice weighing 25-30 g were maintained under standard laboratory conditions and provided with food and water at discretion. All experimental procedures on ALI were reviewed and approved by the Institutional Animal Ethics Committee of Chung Shan Medical University.

Murine model of LPS-induced ALI

Mice were randomly divided into six experimental groups, in which vehicle or rutin was intraperitoneally given 0.5 hr before a single intratracheal injection of saline (50 μl) or LPS (100 μg /50 μl). Mice in the control group received vehicle followed by a saline administration. The LPS group of mice received vehicle followed by LPS administration. The third to fifth groups of mice received 1, 10, or 100 μmol/kg of rutin before LPS administration. The sixth group of mice received 1 mg/kg of dexamethasone (DEX) before LPS administration. After 6 hr, the mice were killed and samples were collected (Huang et al., 2013).

Bronchoalveolar lavage fluid (BALF) collection

The BALF was collected as described previously (Kuo et al., 2011). Briefly, the lungs were lavaged three times with 1 ml ice-cold sterile saline through a tracheal cannula. After centrifugation, the supernatant was then stored at -20 ℃ for adhesion molecule and protein concentration analyses and the number of neutrophils was counted in the sediment of the cells.

Lung edema assessment

The lungs were dissected immediately after the mice were killed and excessive blood was removed before the wet weight was recorded. Then the lung tissues were placed in a drying oven at 80°C for 24 hr to obtain the dry weight. The ratios of wet weight to dry weight were calculated as an indicator of lung edema (Li et al., 2012).

Measurement of adhesion molecules

Measurement of ICAM-1 and VCAM-1 in BALF was performed by commercially available ELISA assay kits (Kuan et al., 2012). The quantification of cytokines and adhesion molecules was calculated according to the manufacturer's instructions.

Western blot analysis of lung tissue

As previously described (Yeh et al., 2013), lungs were homogenized. Tissue extracts were homogenized in tissue protein extraction solution (T-PER; Pierce, Rockford, IL, USA) including proteinase and phosphatase inhibitor cocktail. Proteins were separated by SDS-PAGE and electrophoretically transferred to membranes. The membranes were blocked with phosphate buffered saline containing 0.1% Tween-20 (PBST) and 5% non-fat milk (w/v) for 1 hr at room temperature. After washing with PBST, the membranes were probed with antibodies including β-actin, iNOS, and COX-2. The membranes were washed again with PBST, then a 1:10,000 (v/v) dilution of horseradish peroxidase-labeled IgG was added at room temperature for 1 hr and the blots were developed using ECL western blotting reagents.

NFκB Activation

As previously described (Yeh et al., 2013), NFκB activation in nuclear extracts was measured by NFκB

p65 Transcription Factor Kit (Thermo Science) according to the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed using ANOVA followed by the Bonferroni *t* test for multigroup comparisons; $p<0.05$ was considered significant for all tests. Data are expressed as mean ± standard deviation (S.D.).

Results

Effects of rutin on LPS-induced neutrophils infiltration

Neutrophil infiltration is the primary feature of LPS-induced ALI. The counts of neutrophils in the control and LPS group were $1.05 \pm 0.24 \times 10^6$ and $9.90 \pm 0.54 \times 10^6$ cells / ml. The infiltration of neutrophils was significantly increased in the LPS-treated group compared with the control group ($p<0.05$). The counts of neutrophils were $4.86 \pm 0.88 \times 10^6$, $2.73 \pm 0.39 \times 10^6$, $1.88 \pm 0.63 \times 10^6$ cells / ml in groups treated with rutin at 1, 10, or 100 μmol/kg before LPS administration, respectively. These results indicated that pretreatment with rutin reduced LPS-induced neutrophil infiltration in a dose-dependent manner starting at 1 μmol/kg ($p<0.05$).

Effects of rutin on LPS-induced lung edema

Lung edema, an indicator of LPS-induced ALI due to changes in barrier permeability, was significantly increased in LPS-treated mice as compared with the control group ($p<0.05$). Pretreatment with rutin reduced LPS-induced lung edema in a dose-dependent manner starting at 10 μmol/kg ($p<0.05$, Fig 1).

Effects of rutin on LPS-induced protein leakage in lung

Protein concentration, which serves as a pulmonary permeability barrier, was significantly increased in BALF increased in LPS-treated mice when compared with control mice ($p<0.05$) and was also reduced by pretreatment with rutin in a dose-dependent manner starting at 1 μmolg/kg ($p<0.05$, Fig 2).

Effects of rutin on LPS-induced VCAM-1 and ICAM-1 generation

VCAM-1 and ICAM-1 are important adhesion molecules in the recruitment of leukocytes into the lungs in LPS-induced ALI (Bhatia and Moochhala, 2004; Reutershan et al., 2006, Li et al., 2009). The effect of rutin on VCAM-1 and ICAM-1 generation in BALF was analyzed by ELISA. The results showed that the concentrations of VCAM-1 and ICAM-1 increased significantly after the mice were treated with LPS for 6 hr as compared with the control group ($p < 0.05$). Pretreatment with rutin reduced LPS-induced generation of VCAM-1 in a dose-dependent manner starting at 10 μmol/kg ($p<0.05$, Fig 3A), but had no effect on ICAM-1 even though the concentration of rutin was up to 100 μmol/kg (Fig 3B).

Effects of rutin on LPS-induced expression of COX-2 and iNOS

COX-2 and iNOS play an important role in LPS-induced ALI (Fukunaga et al., 2005). After LPS administration, COX-2 and iNOS were significantly expressed in the lung tissue when compared with the control group ($p < 0.05$). Pretreatment with rutin reduced LPS-induced iNOS expression in a concentration-dependent manner starting at 1 μmol/kg ($p<0.05$, Fig 4A). In addition, rutin at 100 μmol/kg significantly reduced LPS-induced COX-2 expression ($p<0.05$, Fig 4B).

Effects of rutin on LPS-induced NFκB activation and IκB phosphorylation

NFκB activation is critically required for expression of iNOS, COX-2, and adhesion molecules in LPS-induced ALI (Fan et al., 2001). After LPS administration, NFκB activation was significantly expressed in the lung when compared with the control group ($p < 0.05$). Pretreatment with rutin reduced the NFκB activation in a concentration-dependent manner starting at 1 μmol/kg ($p<0.05$, Fig 5).

Discussion

There are several pathological and physiological characteristics in ALI including neutrophil infiltration, leakage of plasma protein into the interstitium and alveolar space, pulmonary edema, and disruption of the alveolocapillary barrier (Zhou et al., 2005). Simulation of ALI using intratracheal administration of LPS triggers ALI in a mouse model, which is a well-established animal model for acute inflammatory responses and early pathological phenomena (Matute-Bello et al., 2008). Neutrophil infiltration into inflamed lung tissue is a crucial factor in the pathogenesis of ALI. In the present study, we also found that neutrophil infiltration into BALF is significantly induced by intratracheal administration of LPS in mice. A previous study reported a reduction in neutrophil infiltration by rutin in LPS-induced ALI through histopathological analysis (Yeh et al., 2014). In this study, rutin inhibition of LPS-induced neutrophil infiltration was also observed through Giemsa stain in an ALI mouse model.

The disruption of the alveolocapillary barrier, caused by permeability upregulation of microvascular endothelial cells, plays a major role in neutrophil infiltration in ALI. The disruption of the alveolocapillary barrier results in leakage of intravascular plasma fluid, formation of lung edema, and infiltration of neutrophils into the alveolar space (Wheeler and Bernard, 2007). After LPS administration, the lung edema and plasma protein leakage into the BALF were significantly increased compared with the control group. These results are in complete agreement with previous studies (Li et al., 2012). In LPS-activated human umbilical vein endothelial cells (HUVECs), rutin decreased LPS-induced barrier disruption in a concentration-dependent manner (Lee et al., 2012). Rutin reduced LPS and tumor necrosis factor (TNF)-α mediated hyperpermeability in the peritoneal cavity of mice. We found that LPS-induced lung edema and plasma leakage into the BALF were inhibited by rutin in a concentration-dependent manner. These results indicated that LPS-induced neutrophil infiltration is suppressed by rutin via down-regulation of the disruption of the alveolocapillary barrier.

Peripheral blood neutrophils are recruited into inflamed lung tissues through the alveolocapillary barrier via expression of adhesion molecules on endothelial cells, such as ICAM-1 and VCAM-1. ICAM-1 and VCAM-1, that participate in transmigration via binding to LFA-1 and VLA-4 (Dimasi et al., 2013). Rutin, one of the flavonoids isolated from *Lycium barbarum* Linnaeus, inhibited the expression of ICAM-1 and VCAM-1 induced by TNF-α in HUVECs (Wu et al., 2012). In the ALI murine model, we first proposed that LPS-induced expression of VCAM-1 was reduced by rutin, but expression of ICAM-1 was not influenced. Several studies have demonstrated the expression of VCAM-1 on endothelial cells and airway smooth muscle cells induced by LPS administration (van Oosten et al., 1995; Lazaar et al., 1994; Klein et al., 1995). VCAM-1 is a key mediator of leukocyte recruitment to inflammatory sites, especially in lung diseases (Sagara et al., 1997; Chin et al., 1997). These results indicated that VCAM-1 played an important role in the mechanism of the protective effect of rutin in LPS-induced ALI.

Proinflammatory mediators, such as PGE2 and NO, are involved in the lung tissue damages of LPS-induced ALI (Ejima et al., 2003; Farley et al., 2006). PGE2 and NO are derived from iNOS and COX-2 respectively. In the ALI model, LPS induced expression of COX-2 and iNOS in lung tissue (Li et al., 2012). In RAW264.7 macrophages and primary peritoneal macrophages, rutin inhibited LPS-induced generation of NO and protein expression of iNOS in a concentration-dependent manner. However, there was no effect on generation of PGE2 and protein expression of COX-2 (Shen et al., 2002). When LPS was administered intratracheally, rutin reduced protein expression of iNOS which has higher potency than COX-2 in mice. The

present results made it clear that rutin suppressed LPS-induced iNOS expression in the lung and prevented tissue injury.

Protein expression of VCAM-1 and iNOS is regulated by NF-κB activation which is a proinflammatory transcription factor in the inflammatory responses (Said and Dickman, 2000). Several studies have demonstrated that NF-κB activation is reduced by rutin in LPS-treated HUVECs and RAW264.7 cells and in the LPS-induced ALI mouse model (Lee et al., 2012; Su et al., 2014; Yeh et al., 2014). In the present study, we also found that rutin reduced NF-κB activation induced by LPS in an ALI model. Parallel trends were observed in suppression of VCAM-1 expression, iNOS expression, and NF-κB activation. Therefore, the reduction in protein expression of VCAM-1 and iNOS in lung resulting from rutin pretreatment was associated with NF-κB activation.

In conclusion, rutin significantly reduced the neutrophil infiltration, lung edema, and plasma protein leakage into the BALF in the mouse model of LPS-induced ALI. The mechanism of rutin involves decreased expression of VCAM-1 in BALF, decreased expression of iNOS in lung tissue, and decreased activation of NF-κB in lung tissue. These results indicated that rutin had a protective role in LPS-induced ALI via reversing VCAM-1 expression, iNOS expression, and NF-κB activation.

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Figure legends

Figure 1 Effect of rutin on edema in LPS-induced ALI. Values are expressed as mean ± S.D. of 5 mice per group. # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $p < 0.05$.

Figure 2 Effects of rutin on LPS-induced protein leakage. The concentration of protein in BALF was determined by Bradford assay. Values are expressed as mean ± S.D. (n=4 in each group). * Represents significant difference between the indicated and control group; # between the indicated and LPS groups, $p < 0.05$.

Figure 3 Effect of rutin on LPS-induced generation of VCAM-1 and ICAM-1 in BALF. Values are expressed as mean ± S.D. of 4 mice per group. # Represents significant difference between the indicated and control group; * between the indicated and LPS groups, $p < 0.05$.

Figure 4 Effect of rutin on LPS-induced iNOS and COX-2 expression in lung. Lung tissues harvested from post-treated mice were analyzed by Western blotting. The fold-change of iNOS and COX-2 expression between the treated and control groups was calculated. Values are expressed as mean ± S.D. (n=3-5 in each group). * Represents significant difference between the indicated and control group; # between the indicated and LPS groups, $p < 0.05$.

Figure 5 Effect of rutin on LPS-induced NFκB p65 activation in lung. Lungs were harvested from

post-treated mice, and nuclear extracts from whole lung tissues were analyzed by p65 transcription factor assay to study the activation levels of NFκB. The fold of NFκB activation between the treatment and control groups was calculated. Values are expressed as mean ± S.D. (n=3-5 in each group). * Represents significant difference between the indicated and control group; # between the indicated and LPS groups, $p < 0.05$.

Fig 1

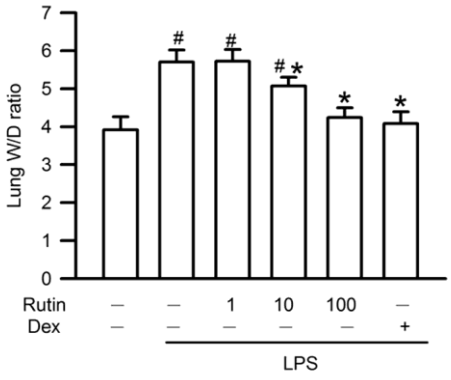


Fig 2

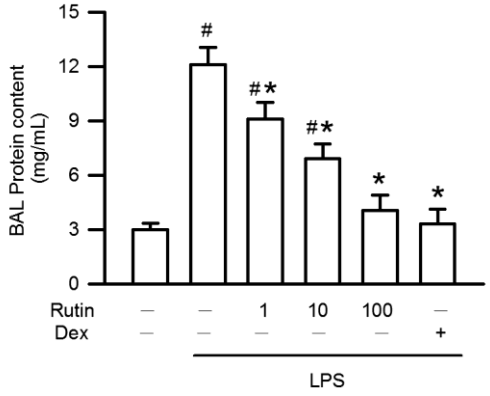


Fig 3

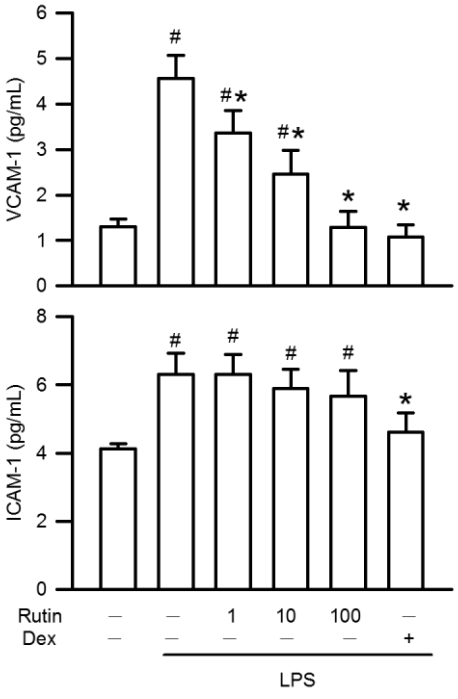
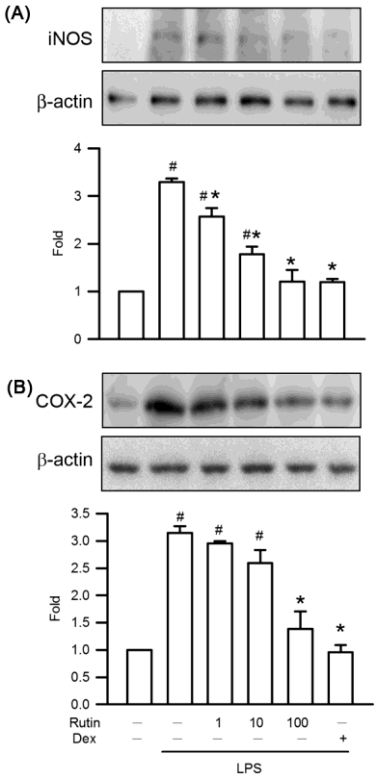


Fig 4


(A)
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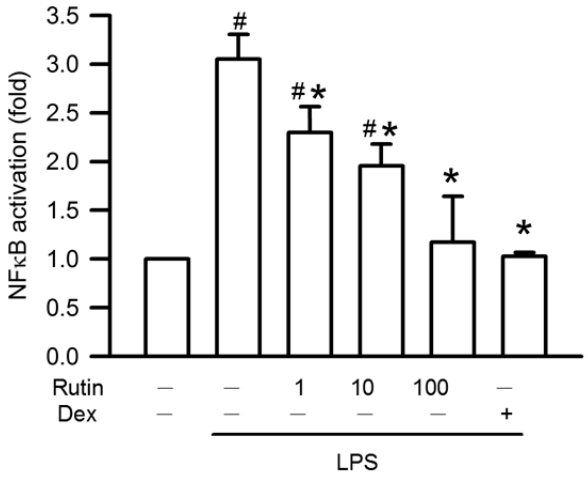
β-actin
Fold
(B)
COX-2
β-actin
Fold
Rutin
Dex
LPS

Fig 5


NFκB activation (fold)
Rutin
Dex
LPS

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否有嚴重損及公共利益之發現）或其他有關價值等，作一綜合評估。

1.請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■ 達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2.研究成果在學術期刊發表或申請專利等情形：

論文：■ 已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■ 無

技轉：□已技轉 □洽談中 ■ 無

其他：（以 100 字為限）

3.請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性），如已有嚴重損及公共利益之發現，請簡述可能損及之相關程度（以 500 字為限）

急性肺損傷 (acute lung injury; ALI) 為常見的急重症，惡化後成急性呼吸窘迫症候群，並伴隨著極高的死亡率。芸香素 (Rutin) 存在於常用的蔬果，以蕎麥草中含量最高，近年來當作健康食品使用。芸香素具有相當多的生物活性與藥理作用，如抗發炎、抗高血壓、抗癌、血管保護與心血管保護作用。本計畫研究證實芸香素有效降低由 LPS 經氣管注射至小鼠所引發的急性肺損傷的病理狀態，如嗜中性球浸潤、肺水腫、肺泡壁增厚、血氧下降、血中二氧碳增加等現象。發現芸香素降低 LPS 所引發的促發炎介質表現，如細胞激素 (TNF-α, IL-1β, IL-6)、黏附因子 (VCAM-1)、COX-2、iNOS、MMP-9。同時，也發現芸香素可以抑制由 LPS 所引發的促發炎轉釋因子 NF-κB 的活化與磷酸化作用。而 NF-κB 上游調節因子 IκB 經 LPS 刺激後所產生的降解作用也會被芸香素所抑制。再者可調節 LPS 所活化的 NF-κB 需經由磷酸化 MAPK 路徑進行調節。芸香素也可以降低由 LPS 所引發的 MAPK 路徑磷酸化。因此可以推斷芸香素所產生的抗發炎作用，主要是經由抑制 MAPK-NF-κB 路徑活化所致。另一方面，我們也發現了芸香素可有效反轉由 LPS 所導致抗氧化酵素系統活性降低的現象，主要是經由促進抗氧化蛋白 HO-1 的表所致。綜合上述的成果，芸香素具有預防急性肺損傷的能力，同時具有潛力成為臨床上適用於急性肺損傷的預防或治療的化合物。

附錄 1 本計畫已發表論文

1. Chen WY, Huang YC, Yang ML, Lee CY, Chen CJ, Yeh CH, Pan PH, Horng CT, Kuo WH, **Kuan YH***. Protective effect of rutin on LPS-induced acute lung injury via down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation. Int Immunopharmacol 2014 **(SCI, IF = 2.711) (PHARMACOLOGY & PHARMACY, Ranking: 95/254 = 37.40%).**
2. Chen WY, Huang YC, Yang ML, Lee CY, Chen CJ, Yeh CH, Pan PH, Horng CT, Kuo WH, **Kuan YH***. Protective effect of rutin on LPS-induced acute lung injury via down-regulation of MIP-2 expression and MMP-9 activation through inhibition of Akt phosphorylation. Int Immunopharmacol 2014 **(SCI, IF = 2.711) (PHARMACOLOGY & PHARMACY, Ranking: 95/254 = 37.40%).**
3. Huang YC, Horng CT, Chen ST, Lee SS, Yang ML, Lee CY, Kuo WH, Yeh CH, **Kuan YH***. Rutin improves endotoxin-induced acute lung injury via inhibition of iNOS and VCAM-1 expression. Environ Toxicol. 2014. **(SCI, IF = 2.562) (WATER RESOURCES, Ranking: 14/79 = 17.72%)**

科技部補助計畫衍生研發成果推廣資料表

日期:2014/11/01

科技部補助計畫	計畫名稱: 研究芸香素對內毒素引發急性肺損傷的保護作用與分子調節機制
	計畫主持人: 關宇翔
	計畫編號: 102-2320-B-040-009- 學門領域: 保健營養

無研發成果推廣資料

102 年度專題研究計畫研究成果彙整表

計畫主持人：關宇翔	計畫編號：102-2320-B-040-009-
計畫名稱：研究芸香素對內毒素引發急性肺損傷的保護作用與分子調節機制	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	300%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	2	2	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		
國外	論文著作	期刊論文	3	1	300%	篇	發表研究論文於具SCI國際知名期刊共計3篇。1篇發表於Free Radic Biol Med (SCI, IF=5.71) (Endocrinology, Diabetes and Metabolism, Ranking 15/123 =12.19%)。1篇發表於 Environ Toxicol. 2014. (SCI, IF = 2.562) (WATER RESOURCES, Ranking: 14/79 = 17.72%)。1篇發表於 Int Immunopharmacol 2014 (SCI, IF = 2.711) (PHARMACOLOGY & PHARMACY, Ranking: 95/254 = 37.40%)。
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		

		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)		訓練本校大學生共計 5 名學生。					

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

急性肺損傷 (acute lung injury； ALI) 為常見的急重症，惡化後成急性呼吸窘迫症候群，並伴隨著極高的死亡率。芸香素 (Rutin) 存在於常用的蔬果，以蕎麥草中含量最高，近年來當作健康食品使用。芸香素具有相當多的生物活性與藥理作用，如抗發炎、抗高血壓、抗癌、血管保護與心血管保護作用。本計畫研究證實芸香素有效降低由 LPS 經氣管注射至小鼠所引發的急性肺損傷的病理狀態，如嗜中性球浸潤、肺水腫、肺泡壁增厚、血氧下降、血中二氧碳增加等現象。發現芸香素降低 LPS 所引發的促發炎介質表現，如細胞激素 (TNF-a, IL-1b, IL-6)、黏附因子 (VCAM-1)、COX-2、iNOS、MMP-9。同時，也發現芸香素可以抑制由 LPS 所引發的促發炎轉釋因子 NF-kB 的活化與磷酸化作用。而 NF-kB 上游調節因子 IkB 經 LPS 刺激後所產生的降解作用也會被芸香素所抑制。再者可調節 LPS 所活化的 NF-kB 需經由磷酸化 MAPK 路徑進行調節。芸香素也可以降低由 LPS 所引發的 MAPK 路徑磷酸化。因此可以推斷芸香素所產生的抗發炎作用，主要是經由抑制 MAPK-NF-kB 路徑活化所致。另一方面，我們也發現了芸香素可有效反轉由 LPS 所導致抗氧化酵素系統活性降低的現象，主要是經由促進抗氧化蛋白 HO-1 的表所致。綜合上述的成果，芸香素具有預防急性肺損傷的能力，同時具有潛力成為臨

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