

## Effect of Resveratrol on the Expression of p38 Mitogen Activated Protein Kinase and Intercellular Adhesion Molecule-1 in Rats with Lipopolysaccharide-Induced Acute Lung Injury

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**Abstract:** To study the expression of p38 Mitogen Activated Protein Kinase (MAPK) and Intercellular Adhesion Molecule-1 (ICAM-1) in LPS-induced Acute Lung Injury (ALI) and the intervention effect of resveratrol. Thirty two male Sprague-Dawley rats were randomly assigned to four groups (n = 8): saline group, resveratrol alone, Lipopolysaccharide (LPS) alone and resveratrol pretreatment (LPS + resveratrol). The rats were treated with resveratrol (5 mg kg<sup>-1</sup>, intraperitoneal injection (i.p) or saline, 30 min prior to LPS administration (6 mg kg<sup>-1</sup>, intravenous injection). About 6 h after LPS injection, samples of pulmonary tissue were collected. Blood gas analysis was carried out Wet/Dry ratios (W/D), Myeloperoxidase (MPO) activity, albumin in BALF and Tumor Necrosis Factor-alpha (TNF-α) and Interleukin-6 (IL-6) levels in serum were measured. Optical microscopy was performed to examine pathological changes in lungs and lung injury score was assessed. The pulmonary expression of p38 MAPK was analyzed by western blot. RT-PCR analysis was performed in lung tissue to assess gene expression of ICAM-1. PaO<sub>2</sub> in the lipopolysaccharide group significantly decreased compared with that in the saline group (p<0.01). The W/D ratio increased significantly in the LPS group (5.60±0.21) as compared with that in the saline group (4.21±0.12) (p<0.01) which was significantly reduced in the LPS + resveratrol group (4.58±0.39) (p<0.01). MPO levels increased significantly in the lipopolysaccharide group compared with that in the saline group which was reduced in the resveratrol + LPS group. The TNF-α and IL-6 level of pulmonary tissue increased significantly in the LPS group compared with that in the saline group protein (p<0.01). However, the increase of TNF-α level of serum was significantly reduced in the resveratrol + lipopolysaccharide group protein (p<0.01). The lung tissues from the lipopolysaccharide group were significantly damaged which were less pronounced in the resveratrol + lipopolysaccharide group. The expression of p38 MAPK, phospho-p38 and ICAM-1 was up-regulated significantly in the lung after rats were injected with LPS (p<0.01). Compared with LPS group resveratrol significantly inhibited the expression of p38 MAPK and ICAM-1 in the resveratrol + lipopolysaccharide group. The expression of p38 MAPK, phospho-p38 and ICAM-1 is up-regulated in rat lung with LPS-induced ALI. Resveratrol injection has a protective effect on ALI and its therapeutic mechanism may be related to inhibition of the phosphorylation of p38 MAPK and ICAM-1.

**Key words:** Acute lung injury, lipopolysaccharide, p38 mitogen activated protein kinase, intercellular adhesion molecule-1, resveratrol, China

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### INTRODUCTION

Acute Lung Injury (ALI) is a syndrome characterized by increased alveolar-capillary permeability and hypoxemia. Both ALI and its most severe form the Acute Respiratory Distress Syndrome (ARDS) are associated with an extensive and heterogeneous list of pulmonary and extrapulmonary insults. What these inciting

factors have in common is the ability to elicit an acute inflammatory reaction in the lung which culminates in parenchymal injury. Despite advances in supportive care the mortality rate of patients with ARDS remains high frequently in excess of 50%. Mitogen-Activated Protein Kinase (MAPK) intracellular signal transduction pathways are key processes in regulating the downstream proteins and the expressions of inflammatory mediators.

Endotoxin has been shown to activate members of the MAPK family including Extracellular signal-Regulated Kinases (ERK)-1/2, P38 and c-Jun N-terminal Kinases (JNKs) MAPK and to induce over-activating inflammatory cells and expanding inflammatory reaction (Kim *et al.*, 2006). Resveratrol (3, 4', 5-trihydroxy-trans-stilbene) is a phytoalexin found in root extract of the weed *Polygonum cuspidatum* and in grape skins and red wine. Indirect evidence suggests that presence of resveratrol in red wine may explain the reduced risk of coronary heart disease associated with moderate wine consumption (Kopp P. Resveratrol, a phytoestrogen found in red wine. A possible explanation for the conundrum of the French paradox?

Resveratrol alters the synthesis and secretion of lipids and lipoproteins by liver cells, blocks human platelet aggregation and inhibits the synthesis of pro-aggregatory and proinflammatory eicosanoids by platelets and neutrophils, respectively. Resveratrol dose-dependently suppressed LPS-induced NO production in macrophages. It also blocked the increases in levels of mRNA for IFN-1, Tumor Necrosis Factor (TNF)- $\alpha$  and inducible Nitric Oxide Synthase (iNOS) that were induced by LPS (Kim *et al.*, 2011a). In this study, experiments were designed to evaluate the effects of Resveratrol on acute lung injury induced by intravenously administered LPS in rats and to explore its underlying mechanism.

## MATERIALS AND METHODS

**Animal experimental protocol:** Thirty two Sprague-Dawley rats (male, 210-230 g, supplied by Center of Experimental Animals of Xi'an Jiaotong University, China) were randomly divided into 4 experimental groups: saline control, resveratrol alone group, Lipopolysaccharide (LPS) alone group and resveratrol + LPS group. The saline control, resveratrol alone and LPS alone groups were injected with 0.5 mL of saline, 0.5 mL of 50 mg kg<sup>-1</sup> resveratrol (Sigma, USA) (ip) and 5 mg kg<sup>-1</sup> LPS (Sigma, USA) in 0.5 mL of saline, respectively. The resveratrol + LPS group received a 5 mg kg<sup>-1</sup> of resveratrol 30 min before the injection of 5 mg kg<sup>-1</sup> of LPS. For injection, rats were treated with soluble Dentobarbitone anesthesia. Each treatment group consisted of 8 animals. Animals were sacrificed with sodium pentobarbitone (200 mg kg<sup>-1</sup>, ip) 6 h after saline treatment or LPS challenge.

**Changes of PO<sub>2</sub>:** The arterial blood in rats was taken and kept in heparinized anticoagulant empty needle which was maintained airtight and PaO<sub>2</sub> was observed.

**Lung Wet to weight (W/D) ratio:** Six after saline or LPS injection, animals were euthanized and the thoracic cavity

was opened. The left upper lobe was removed after the experiment then weighted (wet weight) and dried in a 80°C oven until weight is constant (dry weight). The rate of wet/dry weight of the lung was calculated.

**Bronchoalveolar Lavage (BAL):** The light helium was ligated, BAL was performed on the right lung. The lung was lavaged with ice-cold saline (5 mL kg<sup>-1</sup>) delivered through the tracheal cannula and removed after a 30 sec interval. This procedure was done three times and samples were then pooled for each animal. BAL fluid was centrifuged at 500 r min<sup>-1</sup> for 6 min at 4°C and the supernatants were stored at -80°C. The total cell count and the percentage of neutrophils were measured under a light microscope. The protein content of the supernatant was assayed according to the method of Lory.

**Microscopic assessment of the lung tissue:** Immediately after the rats were killed, a piece of lung tissue taken from the left lower lobe was sampled, fixed in 10% formaldehyde, embedded in paraffin and sectioned. Sections were stained with hematoxylin-eosin and examined under standard light microscope.

**Myeloperoxidase (MPO) assay:** MPO activities in cell-free BAL fluid and lung homogenates were determined according to the method described by Luo and GuoL.

**Detection of lung IACM-1 mRNA (RT-PCR):** Total cellular RNA from rat lung recovered 6 h after the challenge was isolated according to the instructions of the total RNA extracting kit (Sigma, USA). First strand cDNA was synthesized using reverse transcriptase with oligo (dT). Total RNA was Reversely Transcribed (RT) for each sample. Specific primers were designed for PCR. The sequence for the 5' primer was cagggtgctttctcaaaag and the 3' primer was gggcatgagactcattggt. The PCR conditions were as follows: 35 cycles of denaturation at 94°C for 50 sec, annealing at 64°C for 30 sec and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCRs. The PCR products were determined by electrophoresis on 1.8% (w/v) agarose gel containing 0.05  $\mu$ g mL<sup>-1</sup> ethidium bromide and the PCR products were quantified by Kodak Software.

**Levels of TNF- $\alpha$  and IL-6 in serum:** Blood was collected and centrifuged (3000 rpm min<sup>-1</sup>, for 5 min). The serum was captured and stored at -80°C refrigerator. The levels of TNF- $\alpha$  and IL-6 in serum were determined using commercial ELISA kits. The absorbance at 450 nm was determined using an automated plate reader.

**Activation of MAPK in lung tissue:** The activation of MAPK (phosphor-MAPK) in lung tissue was assessed by Western immunoblotting. Lung samples were homogenized and lysed. Proteins were separated by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Pierce Biotechnology, USA).

The blots were incubated with anti-phospho-P38 antibody (CST, USA). After having been incubated by horseradish peroxidase secondary antibody, bands were visualized by chemi-luminescent substrates and Kodak X-ray films and the results were quantified by densitometry using the image system. To ensure equal loading the blots were striped and reprobed with anti-P38 antibody for the levels of P38 and densitometrically quantified by using the Bio Image system (Syngene, UK).

**Statistical analysis:** Values were expressed as mean±SD of n independent observations. Statistical comparisons were made using one way ANOVA. All statistical analyses were performed by using SPSS for windows Version 11.0 (SPSS). A p<0.05 was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**Changes of PaO<sub>2</sub>, lung W/D-weight ratio, BAL protein content and MPO activities:** Changes of PaO<sub>2</sub>, the W/D-weight ratio, BAL protein content and MPO activities were significantly higher in the LPS alone (p<0.01) and LPS + resveratrol groups (p<0.05) than those in the saline control group. Pretreatment with resveratrol significantly inhibited or lowered LPS-induced the W/D-weight ratio, BAL protein content and MPO activities (Table 1).

**Lung ICAM-1 expression:** There is a constitutive expression of ICAM-1 mRNA in rat lungs which was not significantly changed by resveratrol alone. The ICAM-1 expression in lung tissue was significantly increases in LPS alone group as compared with saline control (p<0.01). Pretreatment with resveratrol was found to significantly inhibited the increase in ICAM-1 mRNA expression induced by LPS (Table 2).

**Pathological examination:** Light microscopic examination showed that the morphology of lung tissues was basically normal in the saline group. Similar to that in the resveratrol alone group and that edema, hemorrhage, increased thickness of alveolar wall and infiltration of inflammatory cells into lung tissues were observed in the LPS alone group (Fig. 1). These changes caused by LPS were less pronounced in the LPS + resveratrol group (Fig. 2).

Table 1: Changes in the W/D-weight ratio and BAL protein content (mean±SD)

Groups	PaO <sub>2</sub> (kPa)	W/D	BAL (g L <sup>-1</sup> )	MPO (U mL <sup>-1</sup> )
Saline control	14.31±1.22	4.21±0.12	0.18±0.03	1.43±0.02
Resveratrol alone	14.40±0.26	4.20±0.13	0.17±0.02	1.45±0.02
LPS alone	8.81±0.68**	5.60±0.21**	0.90±0.04**	12.05±0.03**
LPS + resveratrol	12.01±0.81**	4.58±0.39**	0.35±0.03**	6.03±0.03**

Table 2: Lung tissue ICAM-1 mRNA expression and comparison of p38 MAPK expression (mean±SD)

Groups	ICAM-1 mRNA expression	p38 MAPK expression
Saline control	0.58±0.24	3212±685
Resveratrol alone	0.56±0.23	3264±806
LPS alone	2.46±0.41**	15686±935**
LPS + resveratrol	1.17±0.54**	10236±869**

\*p<0.05 and \*\*p<0.01 versus the saline group; <sup>ΔΔ</sup>p<0.01 versus the LPS alone group

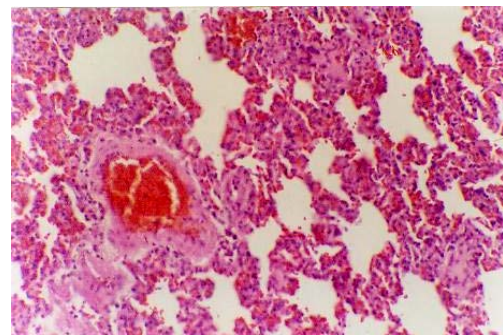


Fig. 1: LPS group edema, hemorrhage, increased thickness of alveolar wall and infiltration of inflammatory cells into lung tissues (10x20)



Fig. 2: LPS + resveratrol group: less edema, hemorrhage and infiltration of inflammatory cells into lung tissues (10x20)

**Levels of TNF-α and IL-6 in serum:** The serum levels of TNF-α in LPS alone group and LPS + resveratrol group were significantly higher than those in saline group (p<0.01). Compared with the LPS alone group, the serum levels of TNF-α were significantly decreased in the LPS + resveratrol group (p<0.05) (Table 3).

Resveratrol, a stilbene type compound identified in wine and fruit juice is an effective component of some Chinese traditional medicines and has been found to

Table 3: The levels of cytokine TNF- $\alpha$  and IL-6 in serum (mean $\pm$ SD)

Groups	TNF- $\alpha$	IL-6
Saline control	36.90 $\pm$ 0.52	182.68 $\pm$ 2.85
Resveratrol alone	35.57 $\pm$ 0.44	179.05 $\pm$ 3.01
LPS alone	257.40 $\pm$ 4.52**	510.73 $\pm$ 4.95**
LPS + resveratrol	189.52 $\pm$ 3.03***	369.91 $\pm$ 1.86***

\*\*\*p<0.01 versus the saline group; \*\*p<0.01 versus the LPS alone group

exhibit various pharmacological activities such as anti-oxidative, anti-cancerous, anti-inflammatory and anti-aging effects. Although, numerous papers have explored the pharmacology of resveratrol in one particular cellular action how this compound can have multiple effects simultaneously has not been fully addressed. Resveratrol dose-dependently suppressed LPS-induced NO production in macrophages. It also blocked the increases in levels of mRNA for IFN-1, Tumor Necrosis Factor (TNF)-alpha and inducible Nitric Oxide Synthase (iNOS) that were induced by LPS. Resveratrol has also various pharmacological effects including antiinflammatory properties, modulation of lipid metabolism and prevention of cancer. Its antiinflammatory effect is related to inhibiting oxidation, leukocyte priming and expression of inflammatory mediators. Recently it has been found to prevent and cure cardiovascular disease and improve significantly microcirculatory disorders by protecting the blood vessel endothelium and inhibiting platelet aggregation (Hung *et al.*, 2004).

However, the specific mechanisms underlying their protective effects remain unclear. Some researchers aimed to investigate the different effects of three representative flavonoids-hesperidin, naringin and resveratrol on Intracellular Adhesion Molecule-1 (ICAM-1) induction in Human Umbilical Vein Endothelial Cells (HUVECs) by using High-Glucose (HG) concentrations and the possible underlying molecular mechanisms. In HG-induced HUVEC cultures, the effects of three different flavonoids on ICAM-1 production and p38 phosphorylation were examined in the presence or absence of inhibitors targeting the MAPK signal transduction pathway. HG stimulation of HUVECs increased the levels of the Adhesion Molecules ICAM-1, Vascular Cell Adhesion Molecule-1 (VCAM-1) and Endothelial selection (E-selectin). Pretreatment with all the three flavonoids drastically inhibited ICAM-1 expression in a dose- and time-dependent manner but did not alter VCAM-1 and E-selectin expressions. Moreover, the author investigated the effects of flavonoids on the MAPK signal transduction pathway because MAPK families are associated with vascular inflammation under stress. These flavonoids did not block HG-induced phosphorylation of Extracellular signal-Regulated Kinase (ERK) and c-Jun N-Terminal Kinase (JNK) but completely inhibited the HG-induced phosphorylation of p38 MAPK. SB202190 an

inhibitor of p38 MAPK also, inhibited the HG-induced enrichment of ICAM-1. This study demonstrated that hesperidin, naringin and resveratrol reduced the HG-induced ICAM-1 expression via the p38 MAPK signaling pathway, contributing to the inhibition of monocyte adhesion to endothelial cells (Kim *et al.*, 2011a, b). Other study confirmed the anti-inflammatory properties of resveratrol and demonstrated the prevention of injury to the intestinal barrier in the rat SAP model (Jha *et al.*, 2011).

Recent experimental studies have showed some light on the intracellular signaling pathway in the inflammatory cascade. It was reported that LPS-mediated responses on cytokine transcription and biosynthesis bifurcate at the level of Ras/Raf G-coupled proteins into major signaling pathways: One that runs through the Nuclear Factor- $\kappa$ B (NF- $\kappa$ B)/Inhibitory- $\kappa$ B (I $\kappa$ B) which activates I $\kappa$ B Kinase (IKK) leading to I $\kappa$ B phosphorylation/degradation and subsequently allowing NF- $\kappa$ B complex to translocate to the nucleus and promoting gene expression and another which is mediated through the ERK and p38 MAP kinase which involves the phosphorylation of MAPK Kinase (MKK) which phosphorylates and activates p38 MAPK.

This pathway is selectively blocked by the pyridinylimidazole SB203580. The activation of p38 MAPK pathway regulates the downstream activation of MAPKAP which phosphorylates the Hsp27 and activates the stability of transcripts of cytokines bearing the ARE. Increased understanding of the signal transduction pathways involved in the regulation of cytokine production and cytokine signaling in inflammatory cells has opened the door for the discovery of novel therapeutics useful for treating a variety of inflammatory diseases in which cytokine production or signaling is implicated (Hong-Shan *et al.*, 2011). MAPK pathways as the most important intracellular signaling pathways in mammals are involved in the regulation of inflammatory reaction. There are at least three groups of MAPK expression in humans including ERK1/2, P38 and NK. Various forms of stimuli can activate them and thereafter phosphorylations of MAPKs regulate all kinds of downstream proteins. Many studies have shown that both ERK and P38 kinases regulate the expression of cytokine genes. Carter *et al.* (1999) found that there was a positive interaction between ERK and P38 kinases in LPS-stimulated Alveolar Macrophages (AM) which both regulated the expression of cytokine genes such as TNF- $\alpha$  and IL-6 at the level of transcription. In this study, LPS induced acute lung injury as assessed by the W/D ratio albumin levels in the BALF and the pathological changes 6 h after LPS challenge pretreatment with resveratrol significantly not only attenuated the LPS-induced increases in the biological makers of the lung

injury but also lessened the extensive lung damage caused by LPS. It has previously been demonstrated that MPO activity is directly related to neutrophil number and can be used as a neutrophil marker.

Therefore, researchers used the MPO assay to assess the extent of neutrophil recruitment in lung tissue. LPS injection induced a marked increase in MPO content in lung homogenates which was significantly reduced by resveratrol pretreatment, consistent with the BAL neutrophil differential cell count. MPO activity in cell-free BAL is also quantified as an index of neutrophil degranulation and activation. LPS-induced MPO activity in cell-free BAL was significantly inhibited by resveratrol. The data suggest that the inhibitory effect of resveratrol on acute lung injury might be at least in part due to the inhibition of neutrophil recruitment and activation in the lung. Lung W/D as a classical method for evaluating pulmonary edema has been used for several years. In this study there was significant difference in lung W/D between the ALI group and resveratrol treatment group.

ICAM-1 is an adhesion molecule on the cellular surface and a member of immunoglobulin superfamily. In an inflammatory response, ICAM-1 plays a central role in the course of cells getting into contact cells, ICAM-1 has previously been shown to be a requirement for neutrophil recruitment after LPS challenge with peak levels of expression associated with maximum leukocyte adherence. Studies have shown protective effects of anti-ICAM-1 mRNA in models of acute lung injury which was attributed to inhibition of PMN sequestration in the lung. In agreement with published data, researchers found that lung ICAM-1 expression was up-regulated by LPS injection. Pretreatment with resveratrol significantly diminished ICAM-1 mRNA expression. Therefore, it is possible that reduced ICAM-1 expression by resveratrol may have contributed to impaired neutrophil recruitment. However, researchers cannot preclude any additional effects that resveratrol may have on PMN-endothelial cell interactions apart from the effect on ICAM-1 expression as demonstrated in the present studies. Indeed resveratrol may have additional effects on other endothelial cell adhesion molecules involved in lung leukocyte sequestration and these possibilities require further investigations. In this study, researchers investigated the effect of resveratrol on Acute Lung Injury (ALI) induced by LPS *in vivo* and we found that the pretreatment with resveratrol can effectively protect mice against LPS-induced ALI. Mice were pretreated with 5 mg kg<sup>-1</sup> resveratrol for 30 min before challenging with a dose of 5 mg kg<sup>-1</sup> LPS.

The histological result showed that resveratrol can suppress the edema, inflammatory cell infiltration and alveolar structure damage of lungs in ALI mice and a decrease in the lung W/D ratio was also observed in mice with resveratrol pretreatment. Additionally, resveratrol markedly decreased the production of inflammatory cytokines including IL-6 and TNF- $\alpha$  in serum. Furthermore, the pretreatment with resveratrol suppressed the expression of p38 MAPK and ICAM-1 in lung tissues which may be partly responsible for its effect on the ALI. In this study, LPS markedly increased the expressions of phosphor-P38 MAPK and ICAM-1 and resveratrol significantly inhibited the activation of P38 MAPK and ICAM-1 in lung tissues and decreased the levels of downstream cytokines TNF- $\alpha$  and IL-6. These findings suggest that resveratrol inhibit the activation of MAPK and down-regulate the inflammatory reaction thus improving the endotoxin-induced ALI.

## CONCLUSION

In this study, the results presented here may suggest resveratrol as a potential therapeutic agent for treating ALI in the future (Cao *et al.*, 2011). In the study, resveratrol inhibited the activation of P38 MAPK and ICAM-1 and down-regulated the levels of downstream cytokines TNF- $\alpha$  and IL-6 and that this effect of resveratrol is related to an inhibition of neutrophil influx and activation, relieved endothelial permeability and improved ALI in LPS-induced rats. Resveratrol has a very low toxicity in animal studies and also inhibit the inflammation and attenuated the LPS-induced ALI.

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