Full Length Research Paper

Tetramethylpyrazine attenuates oleic acid-induced acute lung injury/acute respiratory distress syndrome through the downregulation of nuclear factor-kappa B (NF-κB) activation in rats

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The aim of this study was to investigate the anti-inflammatory activities of tetramethylpyrazine (TMP) in the mice model of acute lung injury/acute respiratory distress syndrome (ALI/ARDS) induced by oleic acid (0.15 ml/kg, intravenous (i.v.)). We report here that TMP (given at 160 mg/kg intraperitoneal (i.p.)) exerts potent anti-inflammatory effects in this model. After 4, 8 and 12 h, animal model reaction was observed and bronchoalveolar lavage fluid was obtained to measure proinflammatory cytokines (tumor necrosis factor α, interleukin 1β) and proteins by enzyme-linked immunosorbent assay and bicinchoninic acid protein assay kit, respectively. The protein expression of NF-κB in the lung was measured by immunohistochemistry and Western blotting. The results showed an increase in tumor necrosis factor α and interleukin 1β in the ALI/ARDS rat models. The activation of NF-κB was suppressed by TMP in the ALI/ARDS rats. The suppression of those molecules is controlled by NF-κB. These findings support the hypothesis that TMP inhibits the inflammatory process in the lung tissue through the suppression of NF-κB activation. Nevertheless, further investigations are required to determine the potential clinical usefulness of TMP in the adjunctive therapy for ARDS.

Key words: Tetramethylpyrazine, acute lung injury/acute respiratory distress syndrome, inflammatory factors, NF-κB.

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is a common clinical manifestation of acute lung injury (ALI)

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Abbreviations: ALI, Acute lung injury; **ARDS,** acute respiratory distress syndrome; **BALF,** bronchoalveolar lavage fluid; **BCA,** bicinchoninic acid; **C group,** control group; **ELISA,** enzyme-linked immunosorbent assay; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **IL-1β,** interleukin 1β; **NF-κB,** nuclear factor-kappa B; **OA,** oleic acid; **OD,** optical density; **TMP,** tetramethylpyrazine; **TNF-α,** tumor necrosis factor α.

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86.2 of 100,000 person-years, a mortality of 38.5% and a with a high mortality rate, an age adjusted incidence of significant morbidity among survivors (Hug and Lodish, 2005). It remains as one of the major challenges in adult intensive care units (Estenssoro et al., 2002; Rocco et al., 2001). ALI/ARDS can lead to sepsis, trauma and severe pneumonia, among which sepsis and in particular, pneumonia are the leading causes of ALI (Dreyfuss and Ricard, 2005). The molecular mechanisms underlying the inflammation include pattern recognition receptors, the coordinated expression of proinflammatory cytokines [tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) and adhesion molecules ICAM-1 and VCAM-1(Martin et al., 1992, 2000; Puneet et al., 2005)]. The presence of infiltrating leukocytes is the hallmark of pulmonary inflammation associated with ALI. The activation of
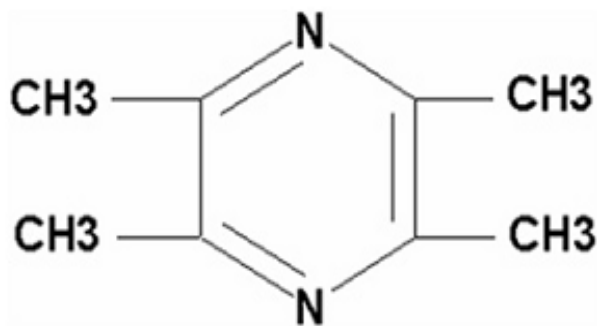
Figure 1. The structure of 2,3,5,6-TMP.

endothelial and infiltrating inflammatory cells results in the production of injurious cytokines and chemokines and the disruption of the alveolar epithelial and capillary barriers contributes to the accumulation of protein-rich fluid in the alveoli, decreasing lung compliance (Cristina et al., 2004).

Increased nuclear translocation of the transcription factor NF-κB contributes to the pathogenesis of ALI. NF-κB is a transcriptional regulatory protein that modulates the expressions of immunoregulatory genes related to critical organ injury. NF-κB activation in the lung tissue is associated with cytokine and chemokine production, lung neutrophilia, epithelial permeability and lipid peroxidation (Blackwell et al., 1996; Liu et al., 1999), with increased expression of NF-κB–regulated proinflammatory genes, including TNF-α and IL-1β. Intraalveolar and intravascular fibrin deposition is frequently found in the setting of ALI or ARDS (McDonald et al., 1990; Idell et al., 1994). Procoagulant activity is enhanced in the lungs of patients with ARDS (Fuchs Buder et al., 1996). Fibrin deposits enhance inflammatory response by increasing vascular permeability, activating endothelial cells to produce proinflammatory cytokines and other mediators, inducing the accumulation of activated neutrophils and modulating immunoregulatory responses in the lung (Edward Abraham, 2000).

Although improvements in supportive care and ventilatory strategies have contributed significantly to the decline in the mortality rate, a specific treatment is still lacking (Ware et al., 2000). An improved understanding of the pathogenesis of ALI/ARDS and a search for effective and specific therapies are still urgently needed, ideally with conventional pharmaceutical drugs. Tetramethylpyrazine (TMP) is an alkaloid contained in ligustrazine, which has been used in traditional Chinese medicine for centuries. Its molecular formula is $C_8H_{12}N_2 \cdot HCl \cdot 2H_2O$ (Figure 1). It is widely used to treat cardiovascular and cerebrovascular diseases and occasionally used to treat various causes of acute and chronic respiratory failure (Hang and Zhou, 2000). TMP has also been reported to possess a diverse array of pharmacologic functions in the

modulation of arterial resistance, cerebral blood flow, platelet function, microcirculation and capillary permeability (Feng et al., 1988). Moreover, ligustrazine was reported to possess a very broad spectrum of pharmacologic actions, such as antioxidant, anti-inflammatory (Zheng et al., 2006), antifibrosis (Yan et al., 1996), antitumor and immunomodulatory effects (Wei et al., 2004).

Therefore, the present investigation was designed to evaluate its effect on ALI/ARDS. Different preclinical models of ALI/ARDS have been developed by utilizing different reagents including, but not limited to bleomycin, bacterial lipopolysaccharide (LPS) and oleic acid (OA) (Julien et al., 1986; Beilman, 1995; Hayashi et al., 2002; Kin and Ulrich, 2005; Zhou et al., 2002; Li et al., 2006). OA has been reported to induce ALI in a manner characteristic of acute respiratory distress syndrome (ARDS). In particular, structural and functional alterations consistent with ARDS, including severe alveolar attentuation and oedema, can be reproducibly attained in rats exposed to OA (Zhou et al., 2002). So we have chosen the oleic acid (OA)-induced ALI in rats as a model, with a hypothesis that the use of TMP may be one of the useful novel options for ALI/ARDS management.

MATERIALS AND METHODS

OA was from the Wuxi Chemical Co., Ltd., whereas TMP was from Suzhou Second Leaf Pharmaceutical Factory (batch H20040483). Rabbit monoclonal antibody anti–NF-κBp65, anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH), recombinant human TNF-α and IL-1β (enzyme-linked immunosorbent assay, ELISA) were purchased from Sigma Chemical. Horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology. Bicinchoninic acid (BCA) protein assay kit was purchased from Beijing BioTeke Biotechnologies, Inc. (Beijing, China). Electrophoresis apparatus and electric switch slot were from Bio-Rad. Image intensity and optical density (OD) analyses were done using Quantity One and ImagePro Plus image analysis software, respectively.

Animals

Male Sprague-Dawley rats (180 to 220 g) were provided by the Center of Laboratory Animal Science of Chongqing Medical University. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Chongqing regulations on the protection of animals used for experimental and other scientific purposes.

Experimental groups and animal model of ALI/ARDS

A total of 60 rats were randomly divided into control group (C group) and OA-treated, OA + saline–treated and OA + TMP–treated groups. The animals were anesthetized with 3.5% chloralhydrate and kept in the supine position with spontaneous breathing. ALI was induced by the intravenous (i.v.) injection of OA (dose, 0.15 ml/kg). The TMP treatment was administered 30 min after the OA injection at a dose of 160 mg/kg in 10% glucose intraperitoneal (i.p.). The dose of TMP was chosen according to our pilot study, in which we

found that a higher dose (160 mg/kg) of TMP led to pathology, only to improve significantly after the OA challenge. The rats in OA + saline treatment received an equal volume of 10% glucose. At each designated time point (4, 8 and 12 h after the OA challenge); five rats from each group were killed.

Animal model reaction

Faster breathing frequency, distressed respiration, reduced activity, erect hair and cyanotic lips and face were observed in the rats, which mean that the animal model was successful.

Lung wet/dry weight ratio

As an index of lung edema, the amount of extravascular lung water was calculated. The middle lobe of the right lung was excised and the wet weight was recorded. The lung was then placed in an incubator at 80°C for 24 h to obtain the dry weight. The wet/dry weight ratio was calculated by dividing the wet weight by the dry weight.

Collection and analysis of the bronchoalveolar lavage fluid (BALF)

At the designated time points, BALF of the left lung was collected after tying off the right lung at the main stem bronchus. The rats were anesthetized by the i.p. injection of 3.5% chloralhydrate (350 mg/kg) and then, the lavage of the left lungs were done three times with 4 ml normal saline at room temperature using a polyethylene tube inserted into the left main stem bronchus. 60 to 80% of the 4 ml input volume was retrieved. The lavage fluid collected was centrifuged at 1,500 rpm for 10 min at 4°C and the supernatant was stored at -80°C until it was evaluated. The levels of TNF-α and IL-1β in the BALF were detected by ELISA kits following the manufacturer's instructions. Protein determination was detected by the BCA protein assay kit.

Histopathologic examination

After the lavage of the left lung, the middle lobe of the right lung tissue excised from each rat was fixed in 4% formalin and embedded in paraffin. 5 µm sections from blocks were stained with hematoxylin-eosin.

Immunohistochemistry

After the lavage of the left lung, the lower lobe of the right lung tissue excised from each rat was frozen in -80°C for 5 min. The fresh-frozen lung tissue sections (10 µm) were fixed in acetone for 20 min, soaked in phosphate buffer solution (PBS) for 3 min (three times), soaked in 0.3% Triton X-100 for 15 min, soaked in PBS for 3 min (three times), placed in goat serum for 20 min, incubated in NF-κBp65 (1:50) at 4°C overnight, incubated in fluorescein isothiocyanate goat anti-rabbit fluorescent secondary antibodies (1:50) at 37°C for 2 h, viewed under an inverted fluorescence microscope and subjected to perturbation.

Measurement of NF-κBp65 by Western blotting

After the lavage of the left lung, the right lung tissue samples of the rats were immediately frozen in liquid nitrogen except the middle lobe. Frozen lung tissue was homogenized in lysis buffer (50 mmol/l

Tris·HCl, pH 7.5; 100 mmol/l NaCl; 1% Triton X-100; 1 mmol/l EDTA; 1 mmol/l DTT; 0.1% SDS; 1× protease inhibitor cocktail) and protein concentration was determined using the BCA protein assay kit. Protein (50 µg) was solubilized in SDS sample buffer (50 mmol/l Tris·HCl, pH 6.8; containing 5% 2-mercaptoethanol, 2% SDS, 10% glycerol and 0.01% bromophenol blue), separated on 8% SDS-PAGE and transferred onto a nitrocellulose membrane at 100 V for 1 h. The membrane was then blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 for 1 h at room temperature and subsequently exposed to 1 µg/ml of monoclonal antibodies anti-NF-κBp 65 (1:1,000) and anti-GAPDH (1:5,000) overnight at 4°C. The next day, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,500) for 1 h at 37°C. Tagged proteins were detected using enhanced chemiluminescence assay and recorded on x-ray films. The OD value of the protein bands on the x-ray films were quantitated using Quantity One 4.31 analysis software. The relative OD value of NF-κBp65 proteins was defined as the ratio of OD NF-κBp65 to OD GAPDH.

Statistical analysis

All data were expressed as means ± SD. Statistical analyses were done using one-way ANOVA with the SPSS 10.0 software. The significant difference between the groups was assessed by Student's two-tailed t test. $p < 0.05$ was considered significant.

RESULTS

Symptoms

Performance of rats after OA injection

The symptoms of control rats were good and the respiratory rate (RR) was 40 to 50 times per minute. After OA injection, the RR went up to 135 to 160 times per minute. There was listlessness and decreased activity. They were significantly reduced after the administration of TMP compared with the ALI/ARDS rats ($p < 0.01$; Table 1).

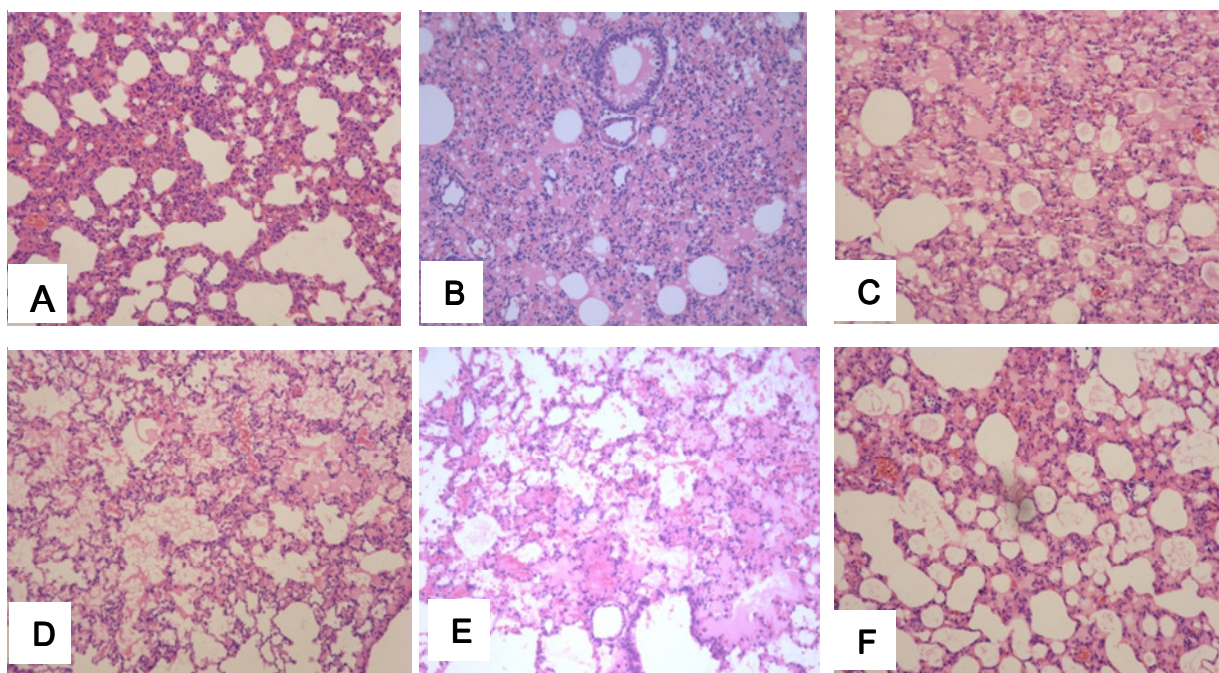### TMP reduced morphologic damage in lungs exposed to OA

As illustrated in Figure 2, the lungs of mice exposed to OA showed marked inflammatory alterations characterized by the thickening of the alveolocapillary membrane, the presence of alveolar hemorrhage, and the massive extravagation of monomorphonuclear and polymorphonuclear leukocytes into the alveolar spaces (Figure 2b) when compared with those of mice (Figure 2a). In contrast, histologic damage was less pronounced in the TMP-treated mice and was dose dependent (Figures 2c, d and e). The amount of erythrocytes and leukocytes in the alveolar spaces was clearly reduced in the TMP-treated mice. According to the earlier mentioned data, we decided to use 160 mg/kg of TMP on follow-up examination.

Representative histologic sections of the lungs harvested

Table 1. RR, wet/dry weight and protein content of the BALF of the rats ($n = 5$).

Group	Time (h)	RR	W/D	Protein (µg/µl)
C	4	43.2 ± 2.23	2.32 ± 0.19	0.14 ± 0.01
	8	44.60 ± 2.07	2.42 ± 0.83	0.15 ± 0.01
	12	46.20 ± 1.79	2.45 ± 0.19	0.14 ± 0.02
OA	4	142.8 ± 7.53*▲	6.80 ± 0.31*▲	0.54 ± 0.03*▲
	8	133.2 ± 3.11*	6.26 ± 0.21*	0.52 ± 0.04*
	12	131.2 ± 1.92*	6.04 ± 0.27*	0.50 ± 0.05*
OA + saline	4	138.8 ± 5.40*	6.70 ± 0.31*	0.55 ± 0.03*
	8	134.0 ± 3.67*	6.44 ± 0.31*	0.51 ± 0.02*
	12	130.8 ± 2.77*	6.08 ± 0.49*	0.51 ± 0.03*
OA + TMP	4	97.8 ± 1.78△	5.23 ± 0.14△	0.38 ± 0.43△
	8	93.8 ± 2.39	4.26 ± 0.21	0.30 ± 0.02
	12	88..2 ± 1.30*	4.16 ± 0.11*	0.29 ± 0.03*

Notes:*, $p < 0.001$ versus C group; ▲, $p < 0.05$ versus OA group at 8 and 12 h; △, $p < 0.05$ versus OA + saline at 4, 8 and 12 h; ★, $p < 0.05$ versus OA + TMP at 4 and 8 h. W/D, wet/dry weight; RR, respiratory rate.

Figure 2. Lung morphology after the mice was challenged with OA or TMP.

at 4 h (control; A); after the intratracheal instillation of OA (B), 40 mg/kg TMP (C), 80 mg/kg TMP (D) and 160 mg/kg TMP (E); and after the challenge with OA at 4 h (D) and 12 h (F). The morphologic alterations induced by (LPS) were reduced by TMP treatment. Pictures are representative of $n = 5$ mice per treatment group. Original magnification, × 200.

Wet/dry weight ratio and protein content in BALF

Compared with the normal groups, RR increased significantly in the OA groups ($p < 0.05$). The indicators all decreased in the OA + TMP–treated groups (Table 1). A hallmark of ARDS is the development of high permeability edema, characterized by high protein content
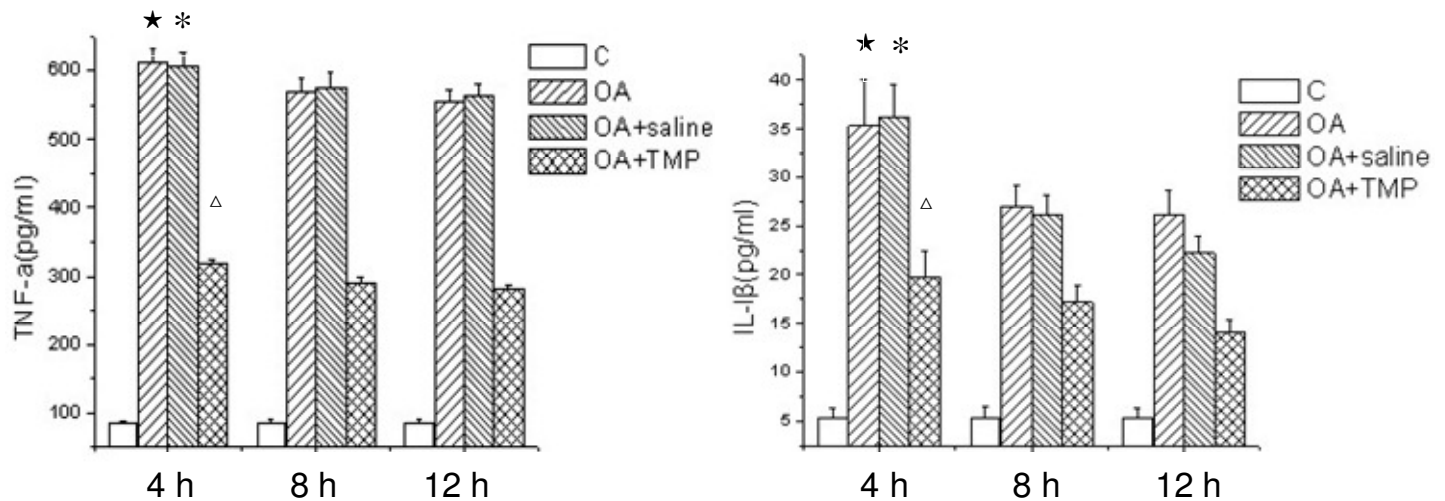
Figure 3. Concentrations of proinflammatory cytokines in the BALF. Cytokines were measured in the BALF obtained at 4, 8 and 12 h after the i.v. instillation of OA. Mice receiving LPS were treated with TMP. The concentrations of TNF-α (A) and IL-1β (B) were detected by ELISA. ★p < 0.001 versus C group; *p < 0.05 versus OA group at 8 and 12 h; △p < 0.05 versus OA + saline group; ▲p < 0.05 versus OA + TMP group at 4 and 8 h.

content in the edema fluid. Such abnormalities were noted in the lungs of the mice exposed to OA. The protein concentration in the BALF from the control mice had increased from 0.14 ± 0.01 μg/μl (4 h) to 0.54 ± 0.03 μg/μl (4 h), 0.50 ± 0.04 μg/μl (8 h) and 0.50 ± 0.05 μg/μl (12 h) when the mice were challenged with OA (Table 1).

Respectively, the concentration of protein in the BALF was significantly lower in the OA-treated mice that received TMP. The most obvious time point was at 12 h (p < 0.05; Table 1).

Effect of TMP on the expression of proinflammatory TNF-α and IL-1β in BALF

A substantial increase in TNF-α and IL-1β production was found in the BALF (Figure 3a and b, respectively) collected from rats at 4, 8, and 12 h and peaked at 4 h after OA administration. In contrast, the levels of these proinflammatory cytokines were significantly lower in the OA-treated mice treated with TMP, which peaked at 12 h after OA administration.

Immunohistochemistry for the activation of NF-κBp65

Immunohistochemical analysis was carried out for *in situ* visualization and it was very clear from the immuno-histochemical staining that NF-κBp65 expression. Nuclear localization of NF-κBp65 was observed in OA group, positive cells of the C group were less than those of the OA group. A complete absence of NF-κBp65 expression was observed in OA group. However, positive cells decrease after TMP administration (Figure 4). The quantification of staining intensity was showed in Figure 5.

Effects of TMP on the activation of NF-κB p65

The rats in OA and OA + slaine saline groups expressed higher NF-κB p65 than the normal control rats (p < 0.05; Figure 6) at 4, 8 and 12 h, which peaked at 4 h after OA administration. TMP significantly suppressed NF-κBp65 protein at 4, 8 and 12 h, which peaked at 12 h after TMP administration (p < 0.05; Figure 6). The relative OD value of NF-κBp65 Western blot in each group is shown in Figure 7.

DISCUSSION

ARDS is characterized by the development of inter-connected inflammatory cascades, with proinflammatory cytokines playing a central role in the initiation and propagation of the inflammatory response leading to lung injury. TNF-α and IL-1β are considered pivotal mediators of lung inflammation in ARDS (Agouridakis and Meduri, 2002). TNF-α and IL-1β released by alveolar macrophages stimulate target cells (notably epithelial and endothelial cells) to produce additional mediators, thereby amplifying the initial inflammatory response. High TNF-α and IL-1β concentrations are found in the BALF of ARDS patients, particularly those who succumb to the disorder.

This study showed that TMP exerted potent anti-inflammatory and immunomodulatory effects in the lungs exposed to OA. The symptoms of rats improved in the TMP-treated. The respiratory rate became smooth and the cyanosis of skin and mucous membrane improved. TMP reduced the infiltration of activated poly-morphonuclear neutrophils in the airways and decreased
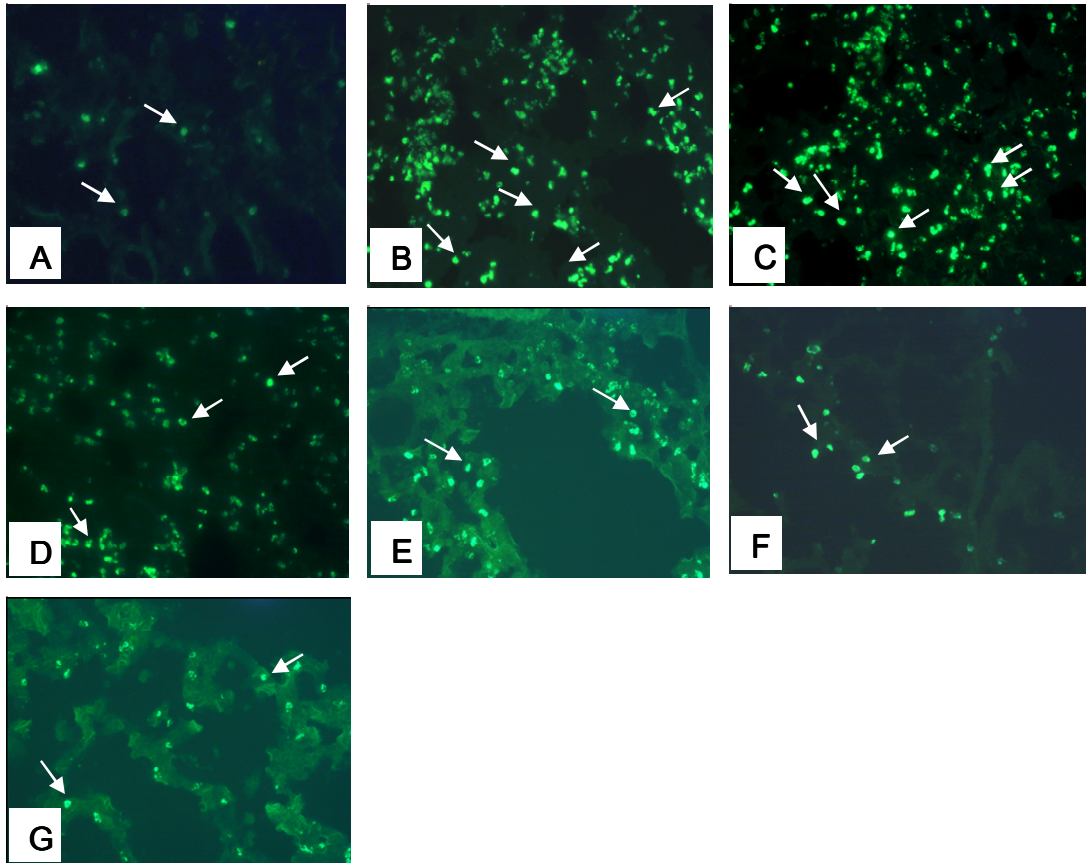
Figure 4. Immunohistochemistry for NF-κBp65 with positive staining (arrows) mainly localized along the alveolar nuclei of the rats. At 4 h (B) after OA administration, the intensity of the positive staining for NF-κBp65 was significantly more than that of the C (A), 8-h (C) and 12-h (D) groups. It was reduced in the lungs of TMP-treated mice after TMP administration at 4 h (E), 8 h (F) and 12 h (G). This is representative of all the animals in each group.
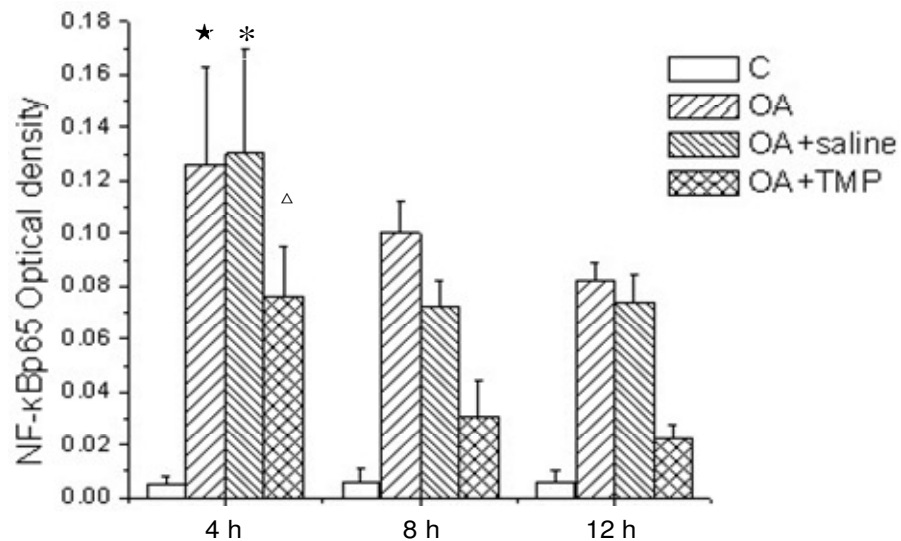

Figure 5. Quantification of staining Intensity. For intensity measurements, each image was converted to grayscale before analysis using Olympus Micro Suite Five image processing software. ★, $p < 0.05$ versus C group; *, $p < 0.05$ versus OA group at 8 and 12 h; △, $p < 0.05$ versus OA + saline group; ▲, $p < 0.05$ versus OA + TMP group at 4 and 8 h.
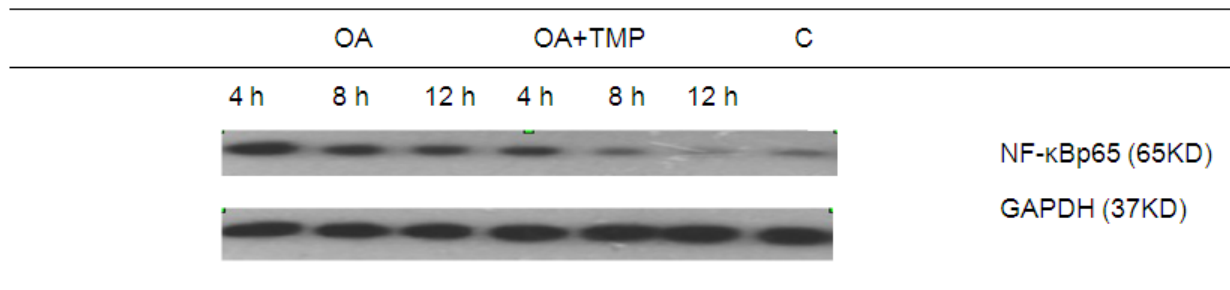
Figure 6. Expression of NF-κB p65 at 4, 8 and 12 h, after OA and TMP administration.
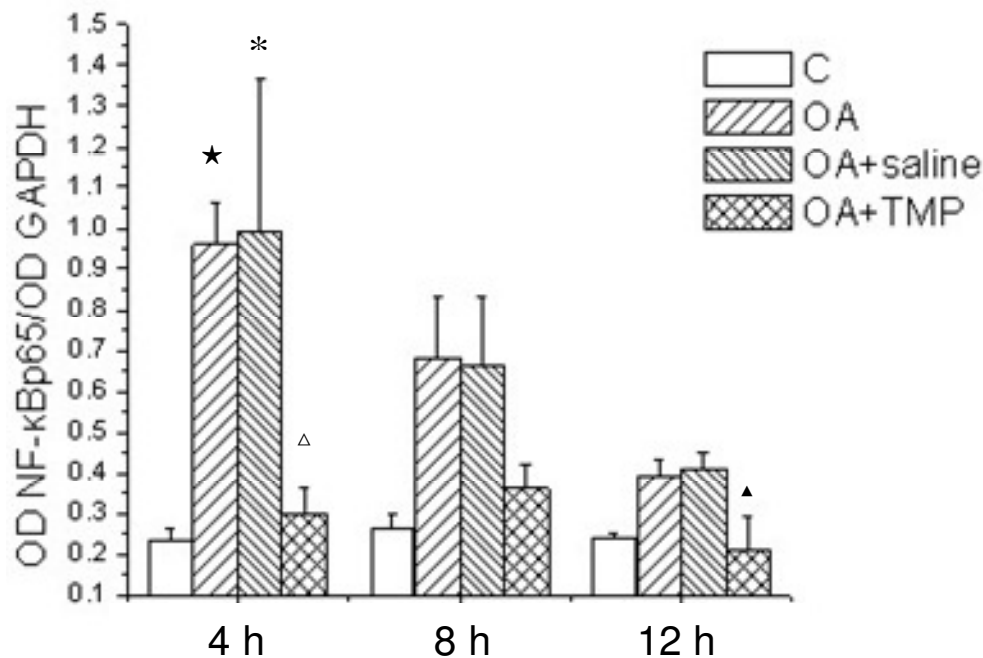

Figure 7. Relative OD value of NF-κBp65 Western blot in each group. ★, $p < 0.05$ versus the C group; *, $p < 0.05$ versus OA group at 8 and 12 h; △, $p < 0.05$ versus OA + saline group; ▲, $p < 0.05$ versus OA + TMP group at 4 and 8 h.

pulmonary edema. The concentration of protein in the BALF was significantly lower in the OA-treated mice that received TMP. The most obvious time point was at 12 h (Table 1), with improved lung morphology (Figure 2) and downregulated expression of proinflammatory cytokines and chemokines TNF-α and IL-1β (Figures 3a and b).

What then is the mechanism by which TMP protects against inflammatory injury? A variety of findings support a role in the activation of the transcription factor NF-κB in the regulation of the inflammatory responses (Baldwin, 2004; Demoule et al., 2006). NF-κB is a general term used to describe a number of dimeric combinations of members of the Rel family of gene regulatory proteins that possess transcription-activating properties (Ghosh et al., 1998). In the NF-κB pathway, the presence of inhibitory factor κB (IκB) and the IκB/NF-κB interactions prevent the nuclear translocation of NF-κB by masking the nuclear localization sequences of the NF-κB dimers. The degradation of IκB leads to the activation of NF-κB, which is defined as the translocation of the NF-κB complex from the cytoplasm to the nucleus. Once in the nucleus, NF-κB binds specific promoter elements of the DNA and induces the transcription of relevant genes. The results of this study show that TMP inhibits, in particular, the DNA binding of the NF-κB dimer containing the p65 subunit, a complex that is transcriptionally active in comparison with the p50 homodimer (Cuzzocrea et al., 2004).

The present study has shown that there was a significant increase in TNF-α and IL-1β, which peaked at

4 h after OA administration (Figures 3a and b). Meanwhile, there was a significant increase in NF-κBp65, which peaked at 4 h after OA administration (Figure 4). TNF-α and IL-1β were significantly reduced by TMP (Figure 3). We showed that TMP attenuates TNF-α and IL-1β expression in the OA-treated lungs, probably because of its blockage of NF-κB. Therefore, the exact mechanisms by which TMP suppresses NF-κB activation in inflammation remain to be further elucidated. Its antioxidant property should be responsible for the ability to inhibit NF-κB. We further showed that the dose of NF-κB used (160 mg/kg i.p.) is sufficient to inhibit the activation of NF-κB in rats.

In conclusion, our results indicate that in the OA-induced ALI/ARDS model, TMP has strong anti-inflammatory properties, resulting in a reduced (a) respiratory rate; (b) pulmonary edema; (c) concentration of protein in the BALF; (d) lung tissue injury; (e) TNF-α and IL-1β production; (f) NF-κB activation.

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