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Attenuation of acute lung injury in mice by oxymatrine is associated with inhibition of phosphorylated p38 mitogen-activated protein kinase

G.L. Xu a, L. Yao a, S.Y. Rao b, Z.N. Gong a, S.Q. Zhang a, S.Q. Yu a,∗

a Center for New Drug Research and Development, College of Life Science, Nanjing Normal University, Nanjing 210097, China
b Pharmacology Department, China Pharmaceutical University, Nanjing 210009, China
c Molecular and Medical Biology Laboratory, College of Life Science, Nanjing Normal University, Nanjing 210097, China

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Abstract

Oxymatrine is one of the alkaloids extracted from Chinese herb Sophora japonica (Sophora flavescens Ait.) with activities of anti-inflammatory, inhibiting immune reaction, antivirus, protecting hepatocytes and antihepatic fibrosis. However, the effect of oxymatrine on acute lung injury (ALI) has not been known yet. In this study, the effect of oxymatrine on ALI was investigated using an oleic acid-induced ALI mouse model. Morphological findings showed that the oleic acid group demonstrated a marked lung injury represented by prominent atelectasis, intraalveolar and interstitial patchy hemorrhage, edema, thickened alveolar septum, formation of hyaline membranes and the existence of inflammatory cells in alveolar spaces. While in the oxymatrine/dexamethasone group, these changes were less severe and in the vicinity of the control group. Furthermore, pretreatment with oxymatrine significantly alleviated oleic acid-induced lung injury accompanied by reduction of lung index and wet-to-dry weight ratio, decreases in serum TNF-α levels and inhibition of phosphorylated p38 MAPK. These findings suggest that oxymatrine has a beneficial effect on acute lung injury induced by oleic acid in mice and may inhibit the production of proinflammatory cytokine, TNF-α, by means of the inhibition of p38 MAPK.

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Keywords: Oxymatrine; Acute lung injury; Oleic acid; p38 MAPK

1. Introduction

Acute lung injury (ALI) is characterized by an intense pulmonary inflammatory response, with neutrophil accumulation, interstitial edema, disruption of epithelial integrity and leakage of protein into the alveolar space (John et al., 2003). Acute respiratory distress syndrome (ARDS) is characterized by increased capillary endothelial permeability that leads to segmental accumulation of high protein content in interstitial edema. Fibrin and platelet plugs occlude the microvasculature of the lung and neutrophil sequestration and activation in the interstitium leads to further segmental alveolar damage and flooding. These processes result in decreased pulmonary compliance, compromised gas exchange and extensive intra-alveolar shunting with concomitant ventilation-perfusion mismatching. At present, ARDS is often classified as a continuum of injury ranging from the milder form ‘acute lung injury (ALI)’ to the more severe form ‘ARDS’ (Christopher et al., 1999).

ALI/ARDS is associated with the development of multiple organ dysfunction syndrome (MODS), which plays an important role in the death of patients with sepsis, pneumonia, aspiration of gastric contents, trauma, multiple transfusions and ischemia-reperfusion. Severe acute respiratory syndrome (SARS) is a novel global infectious disease induced by a virus from the family Coronaviridae. Clinical investigation shows that pathological changes in SARS patients are similar to those of acute lung injury, as revealed by alveolar cell collapse, severe exudation, acute inflammatory reaction and hyaline membrane formation (Zhong, 2003; Du et al., 2004). Currently, no corrective therapy is available for the...
management of ALI/ARDS/SARS and only supportive therapy exists.

It is generally accepted that the development of MODS follows the gradual route of ALI–ARDS–MODS. During this pathway, the lung behaves as a central organ and is the first target subject to injury. Because pulmonary dysfunction may lead to severe hypoxemia and further other multiple organ dysfunction or failure, the treatment of the above mentioned diseases should concentrate on the development of ALI and take measures as soon as possible (Li et al., 2001). The research and development of anti-SARS drugs should also comply with this law. Potential new therapeutic strategies for SARS have been shown to include a wide search for drugs, which is conducive to reduction of lung injury and management of symptoms (Zhong, 2003; Du et al., 2004).

As a traditional Chinese medicine, Sophora japonica has been used for treatment of many diseases for thousands of years. Matrine-like alkaloids have been found to be the chief active components of Chinese herb Sophora japonica including matrine, oxymatrine, sophocarpine, sophoramine, sophoridine et al. The content of oxymatrine in the composite of Sophora japonica is up to 2.8% (Qi et al., 1996). Basic and clinical researches have shown that oxymatrine has the following pharmacological effects: anti-inflammation, inhibiting immune reaction, antivirus, protecting hepatocytes and antihypertrophic fibrosis (Liu et al., 1994, 2003; Liu and Chiu, 1996; Chen et al., 2001; Dong et al., 2002; Xiang et al., 2002; Yang et al., 2002). Chinese Bureau of Science and Technology had announced during the outbreak of SARS that the composite Sophora japonica injection has distinct effects in the treatment of SARS (Zhong, 2003). Considering the pharmacological effects of oxymatrine, we speculate that oxymatrine may play a central role in the composite injection although the effect of oxymatrine on acute lung injury was investigated using an ALI mouse model in this study. We also studied whether the p38 MAPK intracellular signal pathway was involved in the development of ALI and discussed whether oxymatrine could become a therapeutic candidate drug for ALI, ARDS and SARS.

2. Materials and methods

2.1. Reagents

Oxymatrine was obtained from Yixin Pharmaceutical Co. Ltd. (Zhejiang, China) with HPLC purity >98%. Oleic acid was purchased from Linfeng Chemical Co. Ltd. (Shanghai, China). Albumin was obtained from Sigma. Rabbit polyclonal antibodies for phosphorylated and nonphosphorylated p38 MAPK were provided by Cell Signaling Technology. Tween 20 and Glycine were purchased from Amresco Co. polyvinylidene difluoride (PVDF) transfer membranes for Western blotting were obtained from Roche Molecular Biochemicals (Quebec, Canada). Protein assay dye reagent was purchased from Fiancheng Bioengineering Co. (Nanjing, China). HRP Conjugate anti-rabbit IgG and PIPA lysis buffer were purchased from Shengeng Bocai Biotech Inc. (Shanghai, China). Mouse TNF-α ELISA assay kit was obtained from Jingmei Biotech Co. (Guangdong, China). All other reagents were of the highest grade available commercially.

2.2. Animals and experimental design

Male Kunming strain mice weighing 18–24 g were obtained from Laboratory Animal Center, School of Medicine, Southeast University (Nanjing 210009, China). The mice were housed in climate-controlled quarters (24–18 °C at 50% humidity) with a 12 h light/dark cycle and free access to food and water. All experiments were conducted according to the “Guide for the Care and Use of Laboratory Animals”, published by the National Institutes of Health (NIH publication 86-23, revised 1986). Mice were randomly assigned into six groups. The oleic acid group received 0.3 ml/kg, i.v. of oleic acid (mixed with 0.1% bovine serum albuman). The control group received 0.3 ml/kg, i.v. of saline. The three oleic acid + oxymatrine groups were treated with oxymatrine for three consecutive days before oleic acid injection (oxymatrine; 12.5, 25 and 50 mg/kg, i.p.) (Xiang et al., 2002). The dexamethasone group received intraperitoneal injection of dexamethasone at dose of 2.0 mg/kg 2 h before oleic acid injection. The oleic acid and control groups were treated with an equivalent volume of saline instead of oxymatrine. Mice were sacrificed 6 h after oleic acid injection. Immediately after each animal had been sacrificed, the thorax was opened and the lung was removed en bloc by observers unaware of the nature of the experiment.

2.3. Histopathological examination

The left lower lobe was excised and fixed in 10% buffered formalin. The lungs were embedded in paraffin, and the sections stained with hematoxylin and eosin were examined by light microscopy for evidence of lung injury, as described in the following: alveolar congestion, hemorrhage, edema, infiltration/aggregation of neutrophils in the airspace or vessel wall, thickness of the alveolar wall and hyaline membrane formation.

2.4. Electron microscopy

Electron microscopy of lungs was carried out on samples fixed in phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and post-fixed in osmium tetroxide. Transmission electron micrographs were produced with a Hitachi H-7000 electron microscope.
2.5. Lung index and wet-to-dry weight ratio measurements

The wet weight of the whole lung was weighed on an automatic electric balance and the lung index was calculated according to the following formula: lung index (%) = the wet weight of the whole lung/body weight × 100%. Subsequently, the right lung was excised and weighed to obtain the ‘wet’ weight. The lung was then dried in an oven at 80 °C for 7 days to obtain the ‘dry’ weight. To assess tissue edema, the ratio of ‘wet’ weight to ‘dry’ weight (w/d ratio) was calculated. The left lung was cut into pieces and subjected to histological examination and electron microscopy observation.

2.6. TNF-α ELISA assay

Blood samples were harvested from the eyes before mice were killed. The blood was allowed to clot for approximately 1 h at room temperature and then centrifuged at 3500 × g for 5 min to obtain the serum. The serum was stored at −20 °C until assayed. The TNF-α was quantitated according to the instructions available in ELISA kits. The levels in mice samples were calculated from a standard curve generated from recombinant mice TNF-α. The detection range of this assay for TNF-α is 26–2000 ng/l. Samples with a concentration exceeding the limits of the standard curve were repeated after dilution.

2.7. Western blot analysis of p38 MAPK

Three mice in each group were adopted 30 min after oleic acid injection. The lungs were frozen in liquid nitrogen for measurements immediately after they were removed. Before the assay, the specimens were cleared of fat and debris. All specimens had a wet weight of 100 mg. Phenylmethylsulfonylfluoride (PMSF, 1 mmol) was added just before use. The samples were homogenized in 500 μl of PBS lysis buffer using a microhomogenizer on ice. All debris and nuclei were removed by centrifugation at 9000 × g for 10 min. The supernatant obtained was used as the whole cell lysate. Protein concentrations were determined with bovine serum albumin as the reference standard using a modified assay dye reagent. An 80 μg of proteins were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were first incubated with 5% nonfat milk in Tris-buffered saline (TBS). After washing three times in 0.1% Tween 20-TBS (TBST), the membranes were incubated with p38 MAPK and phosphorylated p38 MAPK antibodies (1:1000) for 1 h at room temperature and then washed three times in TBST. The membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG (1:10000) for 1 h at room temperature and detected with the TMB substrate for horseradish peroxidase.

2.8. Statistical analysis

Data from experiments are expressed as mean ± S.E.M. and statistically analyzed using the Student’s unpaired t-test. A value of P < 0.05 was considered significant.

3. Results

3.1. Observation under light microscopy

Light microscopic findings in the lung at 6 h after oleic acid injection demonstrated a marked lung injury resembling those seen in lung of patients with ALI/ARDS, represented by prominent atelectasis, intraalveolar and interstitial patchy hemorrhage, edema, thickened alveolar septum, formation of hyaline membranes and the existence of inflammatory cells in alveolar spaces (Fig. 1A), which were not observed in the control group (Fig. 1B). In oleic acid + oxymatrine groups, these changes were less severe than in the oleic acid group and were in the vicinity of the control group (Fig. 1C). In the oleic acid + dexamethasone groups, these changes were not pronounced and were close to the high dose oxymatrine-treated group (Fig. 1D).

3.2. Observation under electron microscopy

As shown in Fig. 2, electron microscopic findings in the lung after oleic acid injection included epithelial cell swelling (endoplasmic reticulum dilation and mitochondria swelling), the existence of blood cells and inflammatory cells in alveolar spaces (Fig. 2B–D), which were not observed in the control group (Fig. 2A). These changes were improved or not evident in oleic acid + oxymatrine/dexamethasone groups (Fig. 2E and F).

3.3. Lung index and wet-to-dry weight ratio

Values of the lung index and wet-to-dry weight ratio (w/d) in various groups of experimental animals were shown in Fig. 3. Oleic acid injection resulted in an increase in the lung index and w/d ratio of the lung, as compared to the control (P < 0.01). Both were attenuated by 25 mg/kg (P < 0.05, P < 0.01) and 50 mg/kg (P < 0.01, P < 0.01) oxymatrine treatment. In contrast, 12.5 mg/kg oxymatrine did not diminish the above two values significantly although they were lower than the oleic acid group. The lowest level was found in animals receiving dexamethasone treatment.

3.4. TNF-α measurements

As TNF-α plays a pivotal role in mediating oleic acid-induced ALI, we also assessed the regulation of TNF-α production by oxymatrine (Fig. 4). The serum TNF-α level in the oleic acid group was significantly higher than that in the control group (P < 0.01). Treatment with oxymatrine inhib-
Fig. 1. Effect of oxymatrine on lung tissue damage in mice at 6 h after oleic acid injection (100×).

Representative photomicrographs showing hematoxylin and eosin staining: alveolar damage with intralveolar and interstitial patchy hemorrhage; edema; thickened alveolar septum; formation of hyaline membranes; and inflammatory cell. Meanwhile, these damages were not identified or less severe in saline control group (B), oxymatrine (50 mg/kg) + oleic acid group (C) and dexamethasone (2 mg/kg) + oleic acid group (D).

3.5. p38 MAPK expression

As shown in Fig. 5, the level of p38 phosphorylation in the oleic acid group was significantly higher than that in the control group. Pretreatment with oxymatrine inhibited phosphorylation of p38 in a dose dependent manner. p38 Phosphorylation in the highest oxymatrine group was similar to that of the control group. In contrast, the total p38 protein remained unchanged.

4. Discussion

Xiang and colleagues used oxymatrine to evaluate effect of oxymatrine on fulminant hepatitis and hepatocyte apoptosis in mouse models and oxymatrine was administered at dose of 50 mg/kg intraperitoneally (i.d. × 3 days) (Xiang et al., 2002). Initially, we administered oxymatrine at the dose of 50 mg/kg intraperitoneally twice a day for three consecutive days, resulting in a protective effect that could be easily monitored by macroscopic and/or microscopic observation. Then we used, oxymatrine at doses of 25, 12.5 and 6 mg/kg to investigate the relationship between dose and effect. Results showed that no protective effects were observed at 12.5 mg/kg or below. So 50, 25 and 12.5 mg/kg were ultimately selected as the test dosage.

Our results showed that a series of pathological changes were observed under light and electron microscopy after an intravenous administration of oleic acid in mice, which mimicked the pathological changes of clinical ALI/ARDS satisfactorily. Furthermore, the lung index and wet/dry weight ratio were greater in ALI mice than in control group. These findings are in agreement with other reports (Kenji et al., 2001), demonstrating that the oleic acid-induced ALI mouse model is reproducible and our ALI model is successful.

The underlying mechanism of ALI induced by oleic acid is associated with cytokines releases such as TNF-α, which stimulates monocytes to produce IL-1. As the core of the cytokine-network, TNF-α and IL-1 play important roles not only in the production of other inflammatory cytokines, but also in the migration and adherence of neutrophils to endothelial cells (Yoshimi et al., 2000), which together with endothelial cell injury by cytokines result in the production of superoxide radicals and subsequently injure alveolar epithelial cells. All these cause ultimate pulmonary dysfunction.

In the present study, our data revealed that serum TNF-α level was higher in oleic acid group than that in control group. Oxymatrine evidently decreased serum TNF-α level, lung index as well as wet-to-dry ratio and reduced pulmonary injury induced by oleic acid. These findings not only corroborate the direct relationship between TNF-α and ALI but also suggest that oxymatrine have a protective effect on oleic acid-induced ALI.
Fig. 2. Ultrastructure of the lung tissue in mice without or with oleic acid injection (8000×). Representative photomicrographs showing: epithelial cell swelling (endoplasmic reticulum dilation and mitochondria swelling) (B), the existence of red blood cells (C) and inflammatory cells (D) in alveolar spaces were observed in oleic acid group. These changes were not observed in the control group (A) and improved or not evident in oleic acid + oxymatrine/dexamethasone groups (E, F): a, normal epithelial cell; b, swollen epithelial cell; c, red blood cell in alveolar spaces; d, inflammatory cell in alveolar spaces.

Fig. 3. Effect of oxymatrine on lung index and wet-to-dry weight ratio (w/d) in mice with lung injury induced by oleic acid. The mice were given oxymatrine (12.5, 25 and 50 mg/kg, i.p.) for three consecutive days and dexamethasone (2 mg/kg, i.p.) 2 h before injection of oleic acid. The control and oleic acid groups received normal saline. The mice were then sacrificed 6 h after oleic acid administration and lung index, wet-to-dry weight ratio (w/d) were calculated. Mean ± S.E.M., n = 6; **P < 0.01 when compared with control; #P < 0.05 and ##P < 0.01 when compared with oleic acid group.

Fig. 4. Effect of oxymatrine on serum TNF-α level in mice with lung injury induced by oleic acid. The mice were given oxymatrine (12.5, 25 and 50 mg/kg, i.p.) for three consecutive days and dexamethasone (2 mg/kg, i.p.) 2 h before injection of oleic acid. The control and oleic acid groups received normal saline. The mice were then sacrificed 6 h after oleic acid administration and serum TNF-α level were determined by ELISA assay. Mean ± S.E.M., n = 6; **P < 0.01 when compared with control; ##P < 0.01 when compared with oleic acid group.
markedly attenuated in the oxymatrine group compared with the oleic acid control group, whereas expression of phosphorylated p38 MAP kinase was augmented in the oleic acid group compared with the control group, whereas expression of phosphorylated p38 MAP kinase was markedly attenuated in the oxymatrine group compared with the oleic acid group.

Fig. 5. Expression of p38 MAP kinase and phosphorylated p38 MAP kinase.

Bands of p38 MAP kinase were identified in the five groups, and mean density levels in the five groups were almost the same. Phosphorylated p38 MAP kinase was augmented in the oleic acid group compared with the control group, whereas expression of phosphorylated p38 MAP kinase was markedly attenuated in the oxymatrine group compared with the oleic acid group.

It has been reported that oxymatrine concentration is higher in lung and heart than in other organs. This signifies the anti-ALI effect of oxymatrine has a pharmcokinetic basis (Wang and Wang, 2000). Additionally, dexamethasone as positive control exerted a significant preventive effect on oleic acid-induced ALI injury. This might be explained by its potent anti-inflammation effect described as follows: diminution of alveolocapillary permeability; reduction of alveolar epithelial response to pathogen; stability of cell and lysosome membrane; enhancement of surfactant release; prevention of microthrombogenesis and blockade of neutrophil activation (Wang et al., 2001; Su et al., 2004).

Under the stimuli of different ALI/ARDS pathogens, a wide and complicated signal transduction process occurs in many different cells. Although the detailed mechanism is still unknown, the p38 mitogen-activated protein kinase (MAPK) has been paid special attention. p38 MAPK is a cytokine-suppressive anti-inflammatory drug target first discovered by Lee et al. (1994). Most reports demonstrate that p38 MAPK is responsible for regulating inflammatory responses (Nick et al., 2000, 2002; Fang et al., 2002). On the other hand, Arcaroli et al. (2001) reported that p38 MAPK did not have a central role in the development of ALI after either hemorrhage or endotoxemia. Thus, the role of p38 MAPK in the development of ALI is still uncertain. As a result, p38 MAPK has not been proposed as a valid target for clinical management of ALI/ARDS. In order to examine the role of p38 MAPK in ALI, we then analyzed the expression of p38 MAPK and evaluated the effects of oxymatrine. The expression of p38 MAP kinase was shown using Western blotting analysis. Results showed that phosphorylated p38 MAP kinase was augmented in the oleic acid group compared with the control group, whereas expression of phosphorylated p38 MAP kinase was attenuated in the oxymatrine group compared with the oleic acid group in a concentration-dependent manner. This suggested that the expression of oxymatrine before ALI markedly attenuated the activation of p38 MAP kinase during lung injury. It has been reported that TNF-α is augmented via activation of the p38 MAP kinase-related intracellular signal pathway (Johnson et al., 1989; Lee et al., 1994). Oxymatrine markedly attenuated the phosphorylation of p38 MAP kinase and disturbed the mechanism for the production of TNF-α, thus resulting in the lower serum TNF-α level.

From these findings, we conclude that oxymatrine ameliorates ALI by attenuating the production of proinflammatory cytokines, and that this attenuation is associated with suppression of p38 MAP kinase activation. In addition, our results confirm that p38 MAP kinase does play an important role in the development of oleic acid-induced ALI. Based on our results and previous reports, we expect that p38 MAP kinase may become a promising target for clinical management of ALI, although it needs to be supported by further animal experiments and clinical data.

Reports have shown that there are at least three types of MAP kinase: extracellular signal-regulated protein kinase (ERK1/2; p42/p44); c-Jun N-terminal protein kinase (JNK); p38 MAP kinase (Davis, 1993; Kyriakis et al., 1994; Bogoyevitch et al., 1995; Han et al., 1995; Raingeaud et al., 1995; Xia et al., 1995). The NF-κB pathway is also reported to be involved in the process of ALI. Together with the three types of MAPK, NF-κB may create a network to regulate inflammatory responses in ALI. In this study, we only studied the effect of oxymatrine on p38 MAPK. Further study is needed to elucidate whether oxymatrine has modulatory effects on other pathways.

In China, pure oxymatrine injection has been available in hospital for treatment of hepatitis and tumor for many years. However, it has not been used for ALI/ARDS/SARS in clinic. Since our results indicate that oxymatrine prevents mice from oleic acid-induced ALI, we hope that oxymatrine can be used to treat ALI/ARDS/SARS although further research should be carried out on more animal experiments before clinical trial attempt.

In this paper, we found oxymatrine had a beneficial effect on ALI in mice for the first time. Although details of the mechanism of oxymatrine remain to be unraveled, the present results suggest that oxymatrine improves acute lung injury by attenuating the production of TNF-α, and that this attenuation is associated with suppression of p38 MAP kinase activation.

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