Original Article

Trimetazidine protects against LPS-induced acute lung injury through mTOR/SGK\(_1\) pathway

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Abstract: The production of inflammatory cytokines and alveolar fluid clearance rate are critical for the initiation and extension of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) and the outcome. Trimetazidine (TMZ), an anti-ischemic and myocardial metabolism drug that has been used to treat heart disease for years. It is found that TMZ can also reduce the inflammation by regulation of cytokines secretion. However, the effect of TMZ in ALI/ARDS remains unknown. In this study, we found TMZ could not only down-regulated inflammatory cytokines IL-6, IL-8 and TNF-α secretion but also reduced pulmonary edema via increasing of ENaC expression in LPS induced A549 cells and animal model. Interestingly, the effect of TMZ on ALI/ARDS might be through regulation of mTOR/SGK\(_1\) signaling pathway. Taken together, our study provided evidence to show TMZ is a promising drug for ALI/ARDS therapy.

Keywords: Acute lung injury, trimetazidine, acute respiratory distress syndrome, ALI/ARDS

Introduction

Acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) are the high incidence and associated with higher mortality \([1-3]\). ALI/ARDS leads to severe capillary damage resulting to non-cardiogenic pulmonary edema. Evidence has shown that excessive production of inflammatory cytokines is critical for the initiation and extension of ALI/ARDS and the outcome \([4, 5]\). Importantly, pro-inflammatory mediators, such as, interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α), have been reported a direct response of cellular and tissue injury which contribute to ALI/ARDS malignancy and poor outcome \([6, 7]\). Alleviation of inflammation becomes an important avenue to improve the treatment of ALI/ARDS.

Trimetazidine [1-(2,3,4-trimethoxybenzyl) piperazine; TMZ] is an anti-ischemic and myocardial metabolism drug that modifies metabolic function \([9]\), promote the oxidation of glucose \([10]\), thereby reducing generation of ATP required oxygen consumption. Interestingly, Akan et al. reported that in acute pancreatitis, the IL-1β, IL-6 and TNF-α levels were significantly lowered by TMZ treatment, TMZ was considered to protect pancreatic tissue \([26]\). Recently, TMZ was found to protect the energy status after ischemia and reduces reperfusion injury in a rat single lung transplant model \([11]\). However, the effect of TMZ on acute pulmonary edema, has not been studied yet.

Mammalian target of rapamycin (mTOR), a multifunctional kinase plays an important role in cell proliferation, and metabolic reprogramming by regulating nutrient availability, cellular energy levels, oxygen levels, and mitogenic signal \([12]\). Serum and glucocorticoid-regulated kinase 1 (SGK\(_1\)) is a downstream of mTOR pathway. It plays a fundamental role in ion and solute transport processes in epithelia \([13]\). SGK\(_1\) is essential for normal sodium (Na\(^+\)) and potassium homeostasis in mice \([14]\) and for Na\(^+\) transport in cultured cells \([15]\). Accumulating evi-
evidence has implied that mTOR and SGK₁ play important roles in ALI pathogenesis in inflammation and autophagy [16, 17]. In addition to reduction of inflammation, the rate of alveolar fluid clearance (AFC) is also a crucial prognostic factor for ALI/ARDS patients since a lower AFC rate is relative with higher mortality in ARDS patients [18]. AFC is mediated by ion transporters, such as ENaC [19]. ENaC is a heteromultimeric protein composed of α, β and γ subunits. It has been found that ENaC plays an important role in the transepithelial absorption of sodium and fluid from alveolar spaces [17, 18].

The aim of the present study is to explore the effects of TMZ on LPS-induced ALI/ARDS model in vitro and in vivo. We investigated the role of TMZ on ALI-associated pulmonary edema, ENaC expression, and the regulation of the mTOR/SGK₁ signaling pathway in ALI/ARDS model.

Materials and methods

Cell culture and treatment

Non-small-cell lung cancer A549 cells were cultured in RPMI-1640 (Hyclone) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Cells were treated with 10 μg/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) for 2 h then treated with TMZ (Sigma-Aldrich, St. Louis, MO, USA) for another 2 h.

Rat acute lung injury models

The experimental Male Sprague Dawley rats (220 g to 240 g) were injected with 5 mg/kg LPS (055: B5, Sigma, USA) dissolved in 0.3 mL PBS for 4 h while the control mice received 0.3 ml PBS. At the time of LPS exposure, TMZ was administrated in different dose (20 mg/Kg, 10 mg/kg, 5 mg/kg) in different groups. The lungs were removed and immediately homogenized in ice-cold lysis RIPA buffer (50 mM Tris base PH7.5, 150 mM NaCl, 0.1% SDS and protease inhibitors) for 30 min in ice. The lysate was centrifuged (14,000 rpm, 10 min) at 4°C, supernatants were subjected to SDS PAGE.

Western blot analysis

Proteins were separated on 8% SDS PAGE and transferred onto polyvinylidene difluoride membranes. After incubation in a blocking solution (5% nonfat dried milk in TBST) for 1 h at room temperature, the membrane was incubated with the primary antibody at dilution of manufacturer manual at 4°C overnight. The membrane was then incubated with the secondary antibody at dilution of manufacturer manual at room temperature for 0.5 h. All of the antibodies were purchased from Abcam, USA.

Cytokine measurements

Cell medium containing cytokines was centrifuged 1000 rpm for 5 min at 4°C, supernatant was collected and stored at 80°C. Blood from experimental rats was centrifuged at 4°C, 3000 rpm for 5 min, serum was collected and stored at 80°C. Concentration of IL-6, IL-8 TNF-α were measured by an ELISA kit following the manufacturer’s protocol (R&D Systems, USA).

Immunocytochemistry

The sections were fixed by 10% formalin solution and embedded in paraffin. The paraffin was dewaxeding with xylene and hydrated with ethanol, and then it was treated with 3% H₂O₂ to quench the endogenous peroxidase activity for 15 minutes and rinsed with PBS (pH 7.6). The sections were blocked with BSA for 30 minutes and incubated with primary antibodies to ENaC family (anti A-ENaC1: 100; anti ENaC-β1: 100; anti ENaC-γ1: 100) at 4°C overnight then incubated with biotinylated anti-rabbit IgG (Sigma) for 1 h at room temperature following by incubation with avidin-biotin-peroxidase complex (Sigma) for 30 minutes and staining with DAB (Sigma).

Immunostaining

Cells were washed cells PBS twice and fixed with 4% paraformaldehyde for 10 min. After 3 washes with PBS, cells were permeabilized with 0.2% Triton-X-100 for 15 min. Cells were washed with PBS twice following blocked by 5% BSA in PBS for 1 h. Incubation with 100 ul primary antibody (anti A-ENaC1: 100; anti ENaC-β1: 100; anti ENaC-γ1: 100) was carried out for 1 h and 100 ul secondary antibody dilution (containing a fluorescent label). After incubation with DAPI for 8 minutes, slides were washed with PBS and mounted.

Lung wet/dry weight ratio (W/D ratio)

The W/D ratio was applied to measure pulmonary edema. The isolated lung was weighed
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Forty-eight hours later, the lung was weighed again to obtain its dry weight.

Statistical analysis

All data is presented as the mean ± SD. Comparisons among the groups were analyzed by unpaired T-test, a one factor analysis of variance (ANOVA) and post hoc comparison (Fisher’s protected least significant difference). The level of significance was defined to P<0.05.

Results

**TMZ down-regulated LPS induced IL-6, IL-8 and TNF-α secretion in A549 cells**

To investigate the role of TMZ in ALI/ARDS, an in vitro system was established by applying...
LPS, a major component of the outer membrane of gram-negative bacteria, to induce pulmonary inflammation [20-23], simulate ALI/ARDS in A549 cell. Cells were treated with LPS for 2 h following by TMZ treatment for 2 h. We used pro-inflammatory mediators IL-6, IL-8 and TNF-α as indicator of inflammation of ALI/ARDS malignancy. Cell supernatant was collected, IL-6, IL-8 and TNF-α were measured by ELISA assay. We found that the IL-6, IL8 and TNF-α were highly increased in LPS induction indicated that our model was well established (Figure 1). Importantly, the inflammation effect of ALI was alleviated by TMZ treatment as the levels of IL-6, IL8 and TNF-α were significantly reduced in TMZ group (Figure 1).

**TMZ up-regulated A-ENaC, β and γ in LPS induced A549 cells**

ENaC is considered a positive factor to ALI/ARDS which accelerates AFC process that increases Na⁺ transportation and keeps alveolar spaces free of edema fluid. We wonder whether TMZ also affected the expression of ENaC, a mechanism independent of the alleviation of inflammation. Immunostainning assay showed that all of α-, β-, and γ-ENaC protein in the plasma membrane were decreased in LPS induced group and were recovered by TMZ treatment (Figure 2), indicating that TMZ could not only alleviate the inflammation but also increase Na⁺ transportation by increasing ENaC family.

The effect of TMZ on ALI/ARDS might be mediated by mTOR pathway

Since ENaC was the downstream gene of mTOR/SGK₁ pathway [24], it was reported that mTOR plays an important role in ALI/ARDS [16, 17]. This evidence promoted us to hypothesize that whether TMZ regulates ENaC through mTOR/SGK₁ pathway. We applied western blotting to measure the mTOR and SGK₁ protein levels to test this hypothesis. Indeed, TMZ could down-regulate mTOR in LPS induced cells, whereas SGK₁ was increased by TMZ, suggesting that TMZ could regulate the mTOR/SGK₁ pathway (Figure 3, *P<0.05).
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Figure 4. TMZ ameliorated pulmonary edema in vivo TMZ (20 mg/kg, 10 mg/kg, 5 mg/kg) was administered to Sprague-Dawley rats with LPS (5 mg/kg) stimulation. Lung tissue and blood serum were harvested. A. W/D ratio in LPS-induced ALI rats. B. TMZ decreased IL-6, IL-8 and TNF-α secretion in LPS-induced ALI rats. Cytokines from serum were also measured by ELISA assay. C. Lung cells isolated and were subject to SDS-PAGE. The mTOR, SGK₁, and ENaC α, β, γ protein expression were determined. D. Immunohistochemistry to measure ENaC protein in lung tissue. Brown cells represented positive cells. Data are presented as mean ± SD. N = 3. **P<0.01.

TMZ ameliorated pulmonary edema in vivo

To further verify the important effect of TMZ in ALI/ARDS, we employed LPS induced ALI animal model. To assess pulmonary edema in LPS treated rats, lung Wet/Dry ratios were calculated. As showed in Figure 4A, the lung W/D ratio of LPS treated rat was significant increased, however, it could be reduced by TMZ treatment in dose-dependent manner, indicat-
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Discussion

For many years, studies of ALI/ARDS were mainly focused on the aspects of inflammation. Although many basic and clinical researches have been conducted on glucocorticoid [10], the neutral granular cell protease inhibitors [25], antioxidants [9], vasodilators [26] and alveolar surface active agent [27], few of them could effectively reduce the mortality rate of ALI/ARDS.

In this study, we found that TMZ could alleviate pulmonary injury and edema in ALI/ARDS in vitro and in vivo model, providing a promising treatment for ALI/ARDS therapy. The proinflammatory cytokines IL-6, IL-8 and TNFα play crucial roles in the immuno-inflammatory response. Elevated serum levels IL-6, IL-8 and TNFα have been associated with poor outcome in a number of inflammatory conditions such as burn injury, trauma and sepsis [28-30]. We found that TMZ could alleviate ALI by decreasing the proinflammatory cytokines secretion in both LPS induced cells and rats model.

In addition to the inflammatory response in ARDS/ALI, proteinaceous pulmonary edema that floods the airspace and impedes gas exchange are considered to deteriorate the malignancy and outcome.

AFC is needed for better outcome of ALI/ARDS in which ENaC could remove the edema and maintain the efficiency of AFC [18, 19]. Therefore, exploring the mechanism of ENaC in AFC has more clinical value and practical significance. Importantly, TMZ also promoted AFC by increasing expression of ENaC, a mechanism independent of the alleviation of inflammation but dependent of ENaC. We hypothesize that TMZ could alleviate ALI in both inflammation and ENaC pathways. Interestingly, we found that TMZ decreased mTOR protein and increased SGK₁ protein expression in LPS induced model. MTOR plays important signaling pathways controlling center in cell growth, metabolism and survival while SGK-1 is a key regulator of ENaC. We suggested that TMZ increases ENaC protein level probably by promoting the mTOR/SGK₁ pathway.

To sum up, our study provide evidence to show that TMZ may be a promising drug therapy which could decrease the inflammation and effectively clear alveolar edema fluid leading to effective gas exchange which are extremely very important for the outcome of ALI/ARDS. In addition, our study also suggested that mTOR/SGK₁ might be a novel target of curing ALI/ARDS.

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Disclosure of conflict of interest

None.

Abbreviations

ALI, acute lung injury; ARDS, acute respiratory distress syndrome; TMZ, Trimetazidine; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-α; mTOR, Mammalian target of rapamycin; SGK₁, Serum and glucocorticoid-regulated kinase 1; Na⁺, normal sodium.

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