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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 and myocardial metabolism drug that modifies metabolic function [8]. It has also been found TMZ is cardiovascular effectiveness to heart disease mainly through the inhibition of fatty acid beta oxidation [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-

dence has implied that mTOR and SGK$_1$ 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/ADRS 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/SGK$_1$ signaling pathway in ALI/ADRS 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 ug/ml) streptomycin at 37°C in a humidified atmosphere with 5% CO_2. 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 manufactory manual at 4°C overnight. The membrane was then incubated with the secondary antibody at dilution of manufactory 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 dewaxinged with xylene and hydrated with ethanol, and then it was treated with 3% H_2O_2 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).

Immunostainning

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

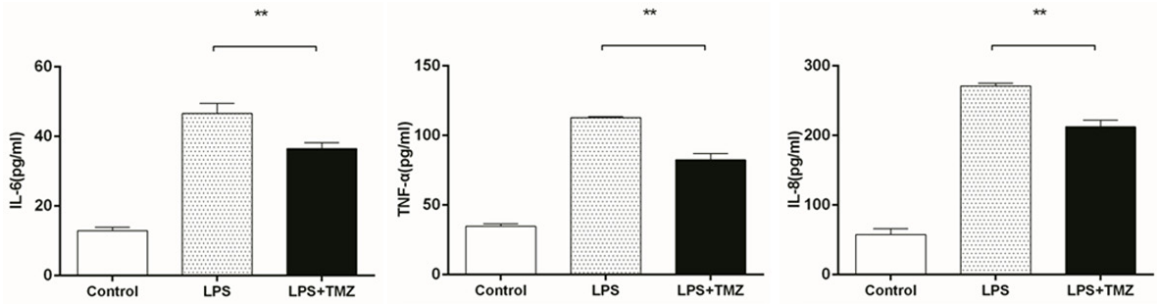

Figure 1. TMZ down-regulated LPS induced IL-6, IL-8 and TNF-α secretion in A549 cells. A549 cells were treated with 10 ug/ml LPS for 2 h then treated with 10 uM TMZ for another 2 h. Cytokines from supernatant were also measured by ELISA assay. Data are presented as mean ± SD. N = 3. **P<0.01.

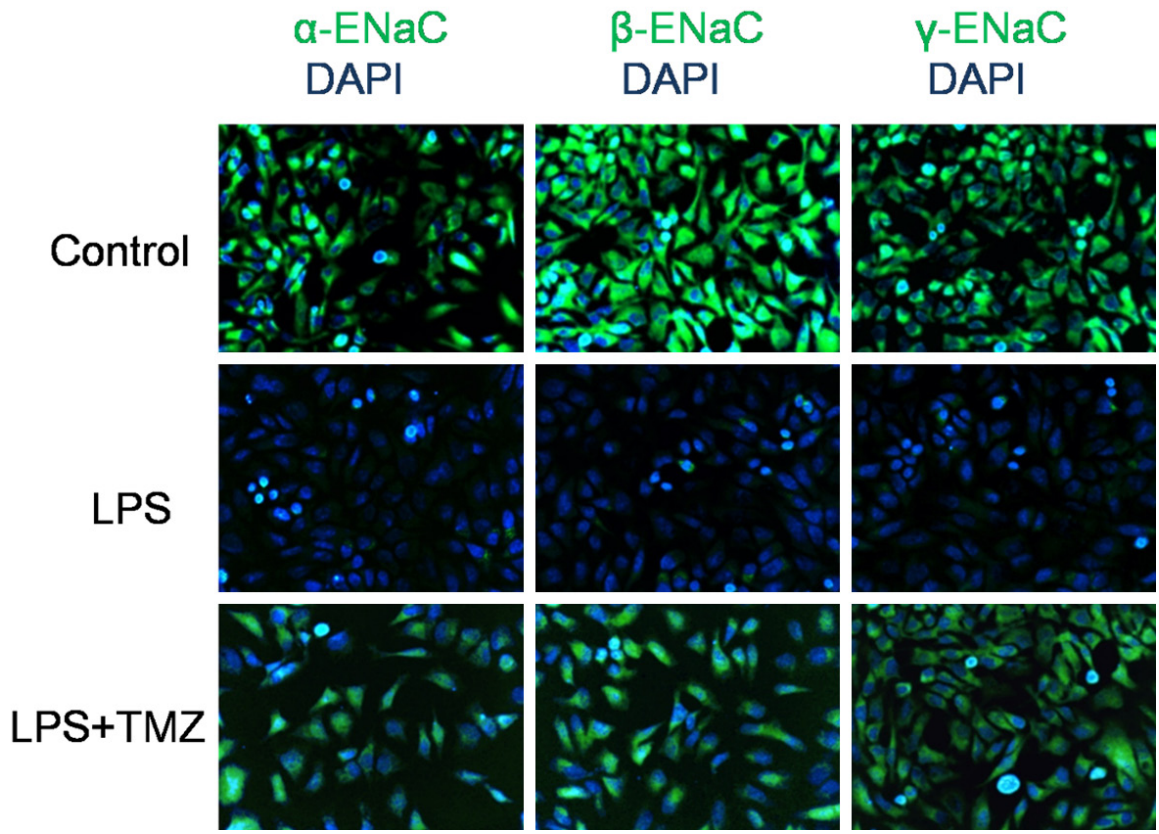

Figure 2. TMZ up-regulated A-ENaC, β and γ in LPS induced A549 cells. The expression and distributions of α-ENaC, β-ENaC, and γ-ENaC in A549 cells incubated with TMZ or PBS for 2 h were examined by fluorescence Microscopy.

and subsequently placed into oven at 80°C. 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

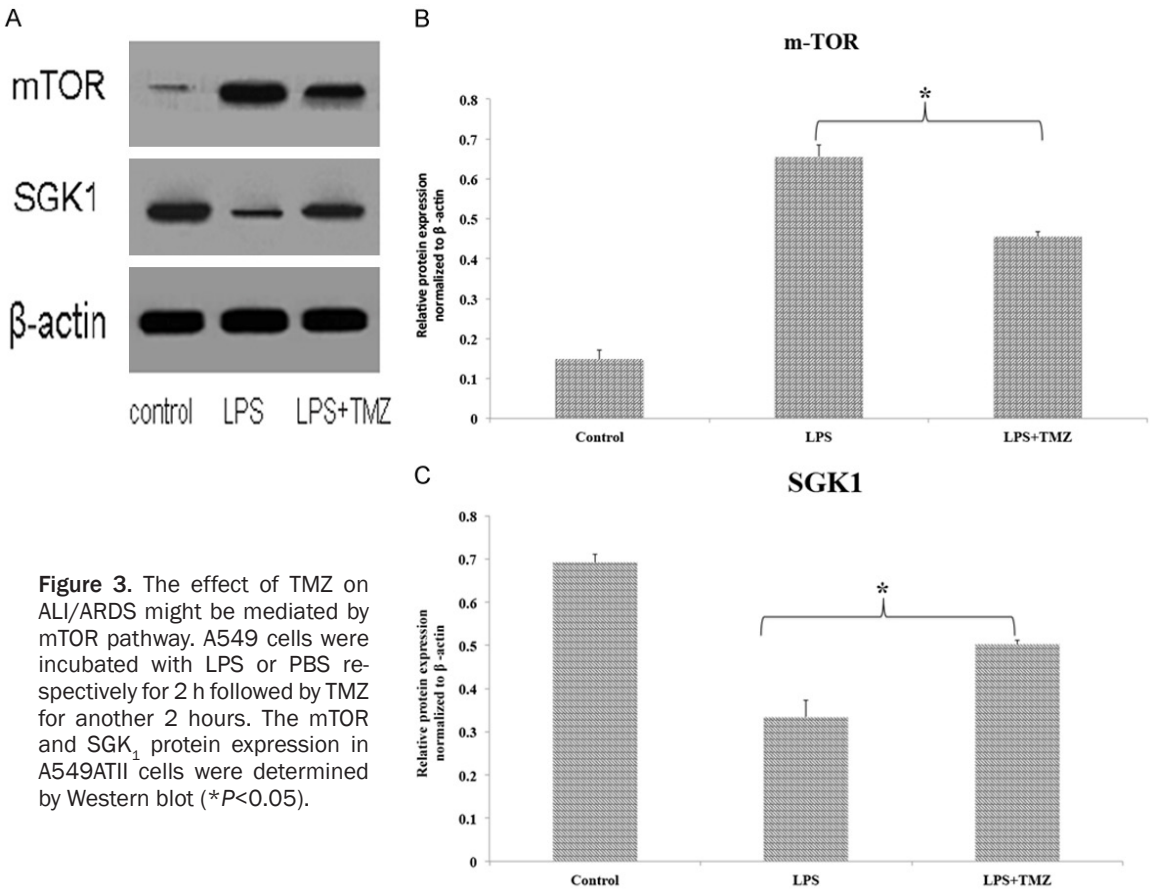

Figure 3. The effect of TMZ on ALI/ARDS might be mediated by mTOR pathway. A549 cells were incubated with LPS or PBS respectively for 2 h followed by TMZ for another 2 hours. The mTOR and SGK$_1$ protein expression in A549ATII cells were determined by Western blot (*P<0.05).

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/SGK$_1$ 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/SGK$_1$ pathway. We applied western blotting to measure the mTOR and SGK$_1$ protein levels to test this hypothesis. Indeed, TMZ could down-regulate mTOR in LPS induced cells, whereas SGK$_1$ was increased by TMZ, suggesting that TMZ could regulate the mTOR/SGK$_1$ pathway (**Figure 3**, *P<0.05).

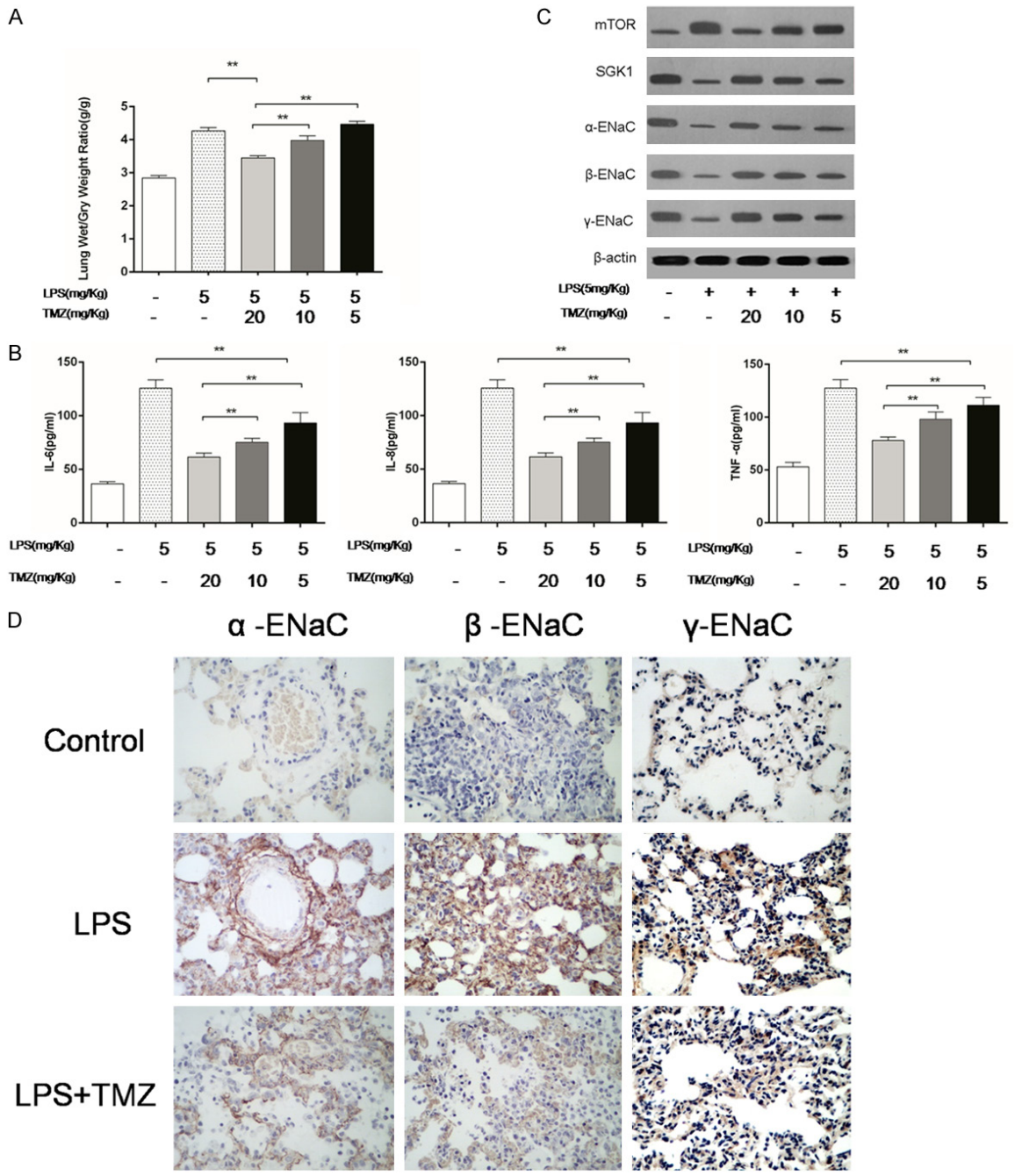

Figure 4. TMZ ameliorated pulmonary edema in vivo TMZ (20 mg/kg, 10 mg/kg, 5 mg/kg) was administered to Sprague-Dawley rats with LPS (5 mg/kg) stimulation. Lung tissue and blood serum were harvested. A. W/D ratio in LPS-induced ALI rats. B. TMZ decreased IL-6, IL-8 and TNF-α secretion in LPS-induced ALI rats. Cytokines from serums were also measured by ELISA assay. C. Lung cells isolated and were subject to SDS-PAGE. The mTOR, SGK$_1$ 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-

ing that TMZ could prevent LPS-induced edema (**Figure 4A**). Moreover, TMZ could decrease IL-6, IL-8 and TNF-α secretion in serum of LPS-stimulated rat (**Figure 4B**). In addition, TMZ activated mTOR and ENaC family expression and decreased SGK_1 expression in lung in dose dependent manner (**Figure 4C**). In immunohistochemical assay, we found 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 4D**). Taken together, our data showed that TMZ alleviate ALI/ARDS in vivo by protecting lung from pulmonary edema, reducing cytokines, increasing ENaC, regulating mTOR pathway which were consistent with our in vitro study. Our results showed that TMZ plays a protective role in ALI/ARDS.

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 SGK_1 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/SGK_1 pathway.

To sum up, our study provide evidence to show that TMZ may be a promising drug therary 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/SGK_1 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; SGK_1, Serum and glucocorticoid-regulated kinase 1; Na^+, normal sodium.

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References

[1] Avecillas JF, Freire AX, Arroliga AC. Clinical epidemiology of acute lung injury and acute respi-

ratory distress syndrome: incidence, diagnosis, and outcomes. Clin Chest Med 2006; 27: 549-557; abstract vii.
[2] Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. N Engl J Med 2005; 353: 1685-1693.
[3] Phua J, Badia JR, Adhikari NK, Friedrich JO, Fowler RA, Singh JM, Scales DC, Stather DR, Li A, Jones A, Gattas DJ, Hallett D, Tomlinson G, Stewart TE, Ferguson ND. Has mortality from acute respiratory distress syndrome decreased over time?: A systematic review. Am J Respir Crit Care Med 2009; 179: 220-227.
[4] Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008; 295: L379-399.
[5] Mei SH, Haitsma JJ, Dos Santos CC, Deng Y, Lai PF, Slutsky AS, Liles WC, Stewart D. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 2010; 182: 1047-1057.
[6] Meduri GU, Kohler G, Headley S, Tolley E, Stentz F, Postlethwaite A. Inflammatory cytokines in the BAL of patients with ARDS. Persistent elevation over time predicts poor outcome. Chest 1995; 108: 1303-1314.
[7] Parsons PE, Eisner MD, Thompson BT, Matthay MA, Ancukiewicz M, Bernard GR, Wheeler AP; NHLBI Acute Respiratory Distress Syndrome Clinical Trials Network. Lower tidal volume ventilation and plasma cytokine markers of inflammation in patients with acute lung injury. Crit Care Med 2005; 33: 1-6; discussion 230-232.
[8] McClellan KJ, Plosker GL. Trimetazidine. A review of its use in stable angina pectoris and other coronary conditions. Drugs 1999; 58: 143-157.
[9] Lang JD, McArdle PJ, O'Reilly PJ, Matalon S. Oxidant-antioxidant balance in acute lung injury. Chest 2002; 122: 314S-320S.
[10] Meduri GU, Annane D, Chrousos GP, Marik PE, Sinclair S. Activation and regulation of systemic inflammation in ARDS: rationale for prolonged glucocorticoid therapy. Chest 2009; 136: 1631-1643.
[11] Inci I, Dutly A, Rousson V, Boehler A, Weder W. Trimetazidine protects the energy status after ischemia and reduces reperfusion injury in a rat single lung transplant model. J Thorac Cardiovasc Surg 2001; 122: 1155-1161.
[12] Liu P, Cheng H, Roberts TM, Zhao JJ. Targeting the phosphoinositide 3-kinase pathway in cancer. Nat Rev Drug Discov 2009; 8: 627-644.
[13] Lang F, Bohmer C, Palmada M, Seebohm G, Strutz-Seebohm N, Vallon V. (Patho)physiological significance of the serum and glucocorticoid-inducible kinase isoforms. Physiol Rev 2006; 86: 1151-1178.
[14] Wulff P, Vallon V, Huang DY, Völkl H, Yu F, Richter K, Jansen M, Schlünz M, Klingel K, Loffing J, Kauselmann G, Bösl MR, Lang F, Kuhl D. Impaired renal Na(+) retention in the sgk1-knockout mouse. J Clin Invest 2002; 110: 1263-1268.
[15] Chen SY, Bhargava A, Mastroberardino L, Meijer OC, Wang J, Buse P, Firestone GL, Verrey F, Pearce D. Epithelial sodium channel regulated by aldosterone-induced protein sgk. Proc Natl Acad Sci U S A 1999; 96: 2514-2519.
[16] Wang L, Gui YS, Tian XL, Cai BQ, Wang DT, Zhang D, Zhao H, Xu K. Inactivation of mammalian target of rapamycin (mTOR) by rapamycin in a murine model of lipopolysaccharide-induced acute lung injury. Chin Med J (Engl) 2011; 124: 3112-3117.
[17] Hu Y, Liu J, Wu YF, Lou J, Mao YY, Shen HH, Chen ZH. mTOR and autophagy in regulation of acute lung injury: a review and perspective. Microbes Infect 2014; 16: 727-734.
[18] Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2001; 163: 1376-1383.
[19] Hummler E, Planes C. Importance of ENaC-mediated sodium transport in alveolar fluid clearance using genetically-engineered mice. Cell Physiol Biochem 2010; 25: 63-70.
[20] Altemeier WA, Matute-Bello G, Gharib SA, Glenny RW, Martin TR, Liles WC. Modulation of lipopolysaccharide-induced gene transcription and promotion of lung injury by mechanical ventilation. J Immunol 2005; 175: 3369-3376.
[21] Gharib SA, Liles WC, Matute-Bello G, Glenny RW, Martin TR, Altemeier WA. Computational identification of key biological modules and transcription factors in acute lung injury. Am J Respir Crit Care Med 2006; 173: 653-658.
[22] Rojas M, Woods CR, Mora AL, Xu J, Brigham KL. Endotoxin-induced lung injury in mice: structural, functional, and biochemical responses. Am J Physiol Lung Cell Mol Physiol 2005; 288: L333-341.
[23] Kitamura Y, Hashimoto S, Mizuta N, Kobayashi A, Kooguchi K, Fujiwara I, Nakajima H. Fas/FasL-dependent apoptosis of alveolar cells after lipopolysaccharide-induced lung injury in mice. Am J Respir Crit Care Med 2001; 163: 762-769.
[24] Lu M, Wang J, Jones KT, Ives HE, Feldman ME, Yao LJ, Shokat KM, Ashrafi K, Pearce D. mTOR complex-2 activates ENaC by phosphorylating SGK1. J Am Soc Nephrol 2010; 21: 811-818.
[25] Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. Mol Mel 2011; 17: 293-307.
[26] Raghavendran K, Pryhuber GS, Chess PR, Davidson BA, Knight PR, Notter RH. Phar-

macotherapy of acute lung injury and acute respiratory distress syndrome. Curr Med Chem 2008; 15: 1911-1924.

[27] Donahoe M. Acute respiratory distress syndrome: A clinical review. Pulm Circ 2011; 1: 192-211.

[28] Hack CE, De Groot ER, Felt-Bersma RJ, Nuijens JH, Strack Van Schijndel RJ, Eerenberg-Belmer AJ, Thijs LG, Aarden LA. Increased plasma levels of interleukin-6 in sepsis. Blood 1989; 74: 1704-1710.

[29] Giannoudis PV, Hildebrand F, Pape HC. Inflammatory serum markers in patients with multiple trauma. Can they predict outcome? J Bone Joint Surg Br 2004; 86: 313-323.

[30] Faunce DE, Gregory MS, Kovacs EJ. Acute ethanol exposure prior to thermal injury results in decreased T-cell responses mediated in part by increased production of IL-6. Shock 1998; 10: 135-140.