Original Article

Ferulic acid regulates miR-17/PTEN axis to inhibit LPS-induced pulmonary microvascular endothelial cells apoptosis through activation of PI3K/Akt pathway

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ABSTRACT — Acute lung injury (ALI) is mainly mediated by the damage of pulmonary microvascular endothelial cells (PMVECs). LPS is one of the pathogenic factors leading to microcirculatory abnormalities of ALI. Ferulic acid (FA) exhibits therapeutic effects against various diseases. During lipopolysaccharide-induced acute respiratory distress syndrome, FA, when given beforehand, could depress inflammation and oxidative stress. However, the concrete role and underlying mechanism of FA in ALI have not been well characterized. Ten µg/mL Lipopolysaccharide (LPS) was used to treat rat PMVECs for 24 hr. qRT-PCR was used to detect the level of miR-17 and phosphatase and tensin homolog deleted on chromosome ten (PTEN). Western blot was used to analyze the associated proteins in the PI3K/Akt pathway, and the apoptosis-related proteins. Flow cytometric analysis was performed to detect the apoptosis of PMVECs. MTT assay was constructed to detect the cell viability. Luciferase assay was conducted to detect the target gene of miR-17 and PTEN. A cell model for *in vitro* studying the role of FA in ALI was established using PMVECs. Our data demonstrate that FA up-regulates miR-17 and declines apoptosis induced by LPS. FA inhibits apoptosis mediated by up-regulating miR-17. Furthermore, we found miR-17 targeted PTEN negatively. FA inhibits cleaved caspase-3 and Bax expression through the PI3K/Akt pathway mediated by up-regulating miR-17. Over-expression of PTEN could contribute to the similar expression trend of the PI3K/Akt signal pathway protein compared to miR-17 inhibitor transfected cells. FA inhibits PMVECs apoptosis induced by LPS via miR-17/PTEN to further regulate the activation of the PI3K/Akt pathway in ALI. We anticipate that our data will provoke additional studies for ALI clinical therapy.

Key words: Ferulic acid, Apoptosis, miR-17, PTEN, PI3K/Akt, PMVECs

INTRODUCTION

Acute respiratory distress syndrome (ARDS) is an important cause of mortality and morbidity in critically ill patients worldwide (Ashbaugh *et al.*, 1967). ARDS leads to approximate 45–45% mortality (Phua *et al.*, 2009) and costs about \$53,300 equally per year for intensive care unit (ICU) admission (Marti *et al.*, 2016). There are many pathogenic factors for ARDS, and all these eventually

lead to acute lung injury (ALI), which is mainly mediated by the damage of pulmonary microvascular endothelial cells (PMVECs) (Yao *et al.*, 2017). According to previous studies, LPS is one of the pathogenic factors leading to microcirculatory abnormalities of ALI (Yang *et al.*, 2018; Menden *et al.*, 2015). Preventing endothelial injury has been developed as a potential therapeutical strategy for treating ALI (Bai *et al.*, 2015). Despite the use of medical advances and cares (Spragg *et al.*, 2010), including

oxygen inhalation and endotracheal intubation for ventilator breathing, the mortality rate is still high. The pathological core of ARDS is the insight into the damage or apoptosis of pulmonary capillary endothelial cells (Wang *et al.*, 2016; Menden *et al.*, 2015; Yang *et al.*, 2018; Bai *et al.*, 2015), which can be used as a target for the treatment of ARDS in clinic and would be positive significance.

MicroRNA (miRNA) is an endogenous non-coding small RNA and has about 22 nucleotides. miRNAs function in the post transcriptional level regulation of gene expression by pairing to the 3' untranslated region (3' -UTR) of target messenger RNA (mRNA) to form an RNA-induced silencing complex (Lee *et al.*, 1993). miR-17, belongs to miR-17-92 cluster, which is known as typical oncogene-type miRNAs (Zhao *et al.*, 2015). miR-17 was found to act on CD4 + T-cells and strengthen Th1 responses during inflammation (Liu *et al.*, 2014). In Staphylococcal enterotoxin B-induced inflammatory lung injury, miR-17-92 cluster was found to play important roles for the first time (Rao *et al.*, 2015). In vascular endothelial growth factor (VEGF) stimulation-induced pro-angiogenic environment, VEGF triggers the phosphorylation of Elk-1 via mitogen-activated protein kinase (MAPK), increases expression of miR-17-92 cluster members and represses phosphatase and tensing homolog (PTEN) ultimately, thereby supporting endothelial cell (EC) proliferation (Fiedler and Thum, 2016). In lung cancer and pancreatic cancer, miR-17 was identified as an oncogene-associated with a poor prognosis of patients, and participates in cancer cell proliferation and invasion (Yu *et al.*, 2010; Chen *et al.*, 2013). Particularly in lung cancer, miR-17 might also serve as a therapeutic target for the induced apoptosis in lung cancer cells when using antisense oligonucleotides to depress miR-17 (Ebi *et al.*, 2009). Interestingly, miR-17 can also act as a tumor suppressor in lung cancer (Lin *et al.*, 2012; Chatterjee *et al.*, 2014). In acid-induced ALI, epithelial MV shuttle miR-17/221, which in turn further modulates macrophage β 1 integrin recycling, promoting macrophage recruitment and eventually contributing to the inflammation (Lee *et al.*, 2017). miR-17 was also found to down-regulate in lipopolysaccharide-induced acute lung injury and alveolar type II epithelial cells injury, and target FoxA1 expression (Xu *et al.*, 2014). However, the mechanism of miR-17 in the injury of PMVECs has not been studied.

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA), a natural compound, is widely found in plants (Pan *et al.*, 2016). FA was reported to have low toxicity and exhibit therapeutic effects against a variety of diseases like tumors, diabetes, and cardiovascular and neurodegenerative diseases (Pellerito *et al.*, 2020). The multiple ther-

apeutic effects of FA were owed to its biological activities, such as scavenging free radicals, anti-oxidation, anti-platelet aggregation, neuroprotection, and strengthening immune function (Barone *et al.*, 2009; Mancuso and Santangelo, 2014). Furthermore, FA also was found to against myocardial ischemia/reperfusion (I/R) injury via inhibiting mitophagy which is dependent on PINK1/Parkin (Luo *et al.*, 2020a). In melanoma cells, FA was observed to be combined with 2-methoxyestradiol to induce cell death by inhibiting Hsp60 and Hsp90 (Kamm *et al.*, 2019). FA induced the apoptosis of cervical carcinoma cells through the Phosphatidylinositol 3-Kinase (PI3K)/Akt Signaling Pathway (Luo *et al.*, 2020b). However, during lipopolysaccharide-induced acute respiratory distress syndrome, FA, when given beforehand, could depress inflammation and oxidative stress (Zhang *et al.*, 2018). Here, we focus on the mechanism of FA regulating PMVECs apoptosis and whether mediated by miR-17.

In our study, we found FA inhibited the apoptosis of PMVECs induced by LPS through the PI3K/Akt pathway and the increased miR-17 by targeting PTEN in FA administration followed by LPS stimulation. For the first time, we observed the necessity of miR-17 during FA relief of cell apoptosis induced by LPS, and its potential regulatory mechanism was also explored.

MATERIALS AND METHODS

Cell culture and cell transfection

Rat PMVECs were obtained from the BeNa Culture Collection (Beijing, China). PMVECs were cultured with M-1168 medium (Cell Biologics, Chicago, IL, USA) in a humidified atmosphere of 5% CO₂. Cells were treated with serial dilution (0, 0.1 μ g/mL, 1 μ g/mL, 10 μ g/mL, 100 μ g/mL) of FA (Sigma-Aldrich, St. Louis, USA) for 24 hr. In FA and LPS (Sigma-Aldrich) double stimulation model, we stimulated PMVECs with FA before, and 2 hr later we gave LPS (10 μ g/mL) for 24 hr. According to the manufacturer's instructions, cells were transfected with miR-17 inhibitor, or miR-17 mimic and PTEN over-expressed plasmid (oe-PTEN) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). miR-17 inhibitor, miR-17 mimic and oe-PTEN were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Then the cells (transfection efficiency > 90%) were further stimulated and used for further study.

Total RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from treated cells was extracted with the RNeasy Micro Kit (Qiagen, Frankfurt, Germany)

according to the manufacturer's instructions. qRT-PCR was processed on iCycler iQ (Bio-Rad, Hercules, CA, USA) using the Quantitect SYBR green PCR kit (Qiagen). Primers for qPCR were designed using Primer Express 5.0 software (Applied Biosystems, Foster City, CA, USA). Relative gene level was analyzed by using the $2^{-\Delta\Delta C_t}$ method. The following primers were used for the qRT-PCR: miR-17 forward primer, 5'-GCG-CAAAGTGCTTACAGTGC-3' and reverse primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCG-CACTGGATACGACCTACCT-3'; U6 forward primer, 5'-CTCGCTTCGGCAGCACA-3' and reverse primer, 5'-AACGCTTCACGAATTTGCGT-3'. Melting curves were used to validate and confirm the products and all assays were repeated in duplicate. miRNA expression was normalized to U6.

Western blot analysis

Total protein was extracted in RIPA buffer (Beyotime, Shanghai, China) with 1% phenylmethanesulfonyl fluoride (PMSF) (Solarbio, Beijing, China). The lysates were separated by SDS-PAGE. Separated protein was transferred to polyvinylidene membranes (Millipore, Billerica, MA, USA), and probed with antibodies after blocking for 1 hr. Primary antibodies against PTEN (1:1000), PI3K (1:2000), p-PI3K (1:1000), Akt (1:2000), p-Akt (1:1000), cleaved-caspase-3 (1:1000), Bax (1:1000), Bcl-2 (1:1000) and β -actin (1:5000) were obtained from Cell Signaling Technology (CST, Boston, MA, USA). After probing with the primary antibodies, the blots were incubated with HRP-conjugated secondary antibody and were visualized with enhanced chemiluminescence (ECL) kit.

Cell viability assay

Cell viability was detected with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay kit (Biosharp Life Sciences, Anhui, China). Cells were seeded in 96-well plates (1×10^4 cells/well, 100 μ L) for 24 hr and used for cell viability assay after FA administration. The spectrophotometric absorbance at 490 nm was analyzed with SPECTRAmax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

Flow cytometric analysis

Apoptosis was investigated with an Annexin V-fluorescein isothiocyanate (FITC) apoptosis kit (BD Biosciences, San Jose, CA, USA). We added 5 μ L FITC-labeled Annexin V and 5 μ L propidium iodide (PI) to the binding buffer with the cells. Finally, we used a flow cytometer (BD Biosciences) to detect the stained cells. The annex-

in V+/ PI- cells represent the early apoptotic cells. The annexin V-/ PI+ cells represent the necrosis. The data could be analyzed using FlowJo software v10.0.7 (Tree Star, Inc., Ashland, OR, USA).

Luciferase assay

We construct a luciferase reporter vector firstly. We inserted the PTEN 3'-UTR fragment containing putative binding sites for miR-17 into the downstream of the luciferase gene in the pmirGLO vectors (Promega, Shanghai, China). PTEN-WT represents the reporter constructs containing the entire 3'UTR sequences of PTEN. PTEN-MUT represents the reporter constructs containing mutated nucleotides. According to standard operation, cells were collected to measure the Luciferase and Renilla signals using a dual luciferase reporter assay kit (Promega) and measure. Firefly luciferase values were normalized to Renilla.

Statistical analysis

All experiments were performed in at least three biological replicates. Statistical evaluation was performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). All data were expressed as means $\pm$ standard deviation (SD) of at least three experiments. Student t-test was performed to analyze comparison between two groups. One-way analysis of variance (ANOVA) followed by Tukey's test was conducted to analyze comparison among three or more groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Toxicity detection of ferulic acid

We used ferulic acid (FA) at different dosages (0.1 μ g/mL, 1 μ g/mL, 10 μ g/mL, 100 μ g/mL) to stimulate PMVECs for 24 hr to detect its toxicity to cells. MTT assay results showed the cell viability decreased in 100 μ g/mL FA treatment cells, but not in other dilutions (Fig. 1). Later we use 10 μ g/mL FA to construct the experiment.

FA suppresses apoptosis induced by LPS and up-regulates the expression of miR-17

We stimulated PMVECs with LPS (10 μ g/mL) for 24 hr to induce cell apoptosis. Further we detected the apoptosis variance when FA was pretreated for 2 hr before LPS stimulation. Flow cytometric analysis showed the enhanced apoptosis after LPS treatment and the decreased apoptosis gradually after FA pre-treatment in a concentration-dependent manner (Fig. 2A). Western blot results also showed the decreased protein level of

Fig. 1. Toxicity detection of ferulic acid. Ferulic acid (FA) at different dosages (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL) was used to stimulate PMVECs for 24 hr to detect its toxicity by MTT assay. The data are the means $\pm$ SD of three independent sets of analyses. *** $P < 0.001$.

cleaved-caspase-3 and Bax, and the enhanced expression of Bcl-2 after FA pre-treatment (Fig. 2B). Together, FA declined cell apoptosis induced by LPS. Next, we detected the reduced miR-17 level after LPS administration in

PMVECs (Fig. 2C). Interestingly, with the increase of FA dose, FA restored the down-regulated expression of miR-17 (Fig. 2C). Combining the results, we hypothesized FA might ameliorate the apoptosis induced by LPS and also up-regulate the expression of miR-17.

FA inhibits apoptosis induced by LPS in PMVECs via miR-17

To further confirm the reduced apoptosis mediated by miR-17, when FA was administrated, we transfected miR-17 inhibitor or inhibitor NC into PMVECs followed by LPS stimulation. We observed the 57.33% declined miR-17 level in miR-17 inhibitor transfected cells (Fig. 3A). Later when the transfected cells were given FA (10 µg/mL) and LPS stimulation, the enhanced miR-17 was depressed in miR-17 inhibitor transfected cells (Fig. 3B). Flow cytometric analysis also showed the significant rebound of apoptosis rate in miR-17 inhibitor transfected cells, relative to the reduced apoptosis in inhibitor NC transfected cells (Fig. 3C). And western blot results also showed the rising protein level of cleaved-caspase-3 and Bax again in miR-17 inhibitor-transfected cells, when compared to inhibitor NC-transfected cells (Fig. 3D).

Fig. 2. FA declines apoptosis induced by LPS and up-regulates the expression of miR-17. PMVECs were stimulated with LPS (10 µg/mL) and different concentrations of FA (0, 0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL) for 24 hr. (A) Apoptosis was determined by flow cytometric analysis after different stimulation. The annexin V+/PI- cells represent the early apoptotic cells. The annexin V-/PI+ cells represent the necrosis. (B) Western blot analysis of Bax, cleaved-caspase-3, Bcl-2 protein expression in cells incubated with LPS and/or FA at different dosages. β -actin was used as a loading control. (C) qRT-PCR analysis of miR-17 level. The data are the means $\pm$ SD of three independent sets of analyses. ** $P < 0.01$, *** $P < 0.001$.

Ferulic acid reduces apoptosis by upregulating miR-17

Fig. 3. FA inhibits apoptosis induced by LPS in PMVECs via miR-17. (A) qRT-PCR analysis of miR-17 in inhibitor NC and miR-17 inhibitor transfected cells. PMVECs were transfected with miR-17 inhibitor and its negative control and then stimulated with LPS (10 µg/mL) and FA (10 µg/mL) for 24 hr. (B) qRT-PCR analysis of miR-17 in the transfected cells, which were given FA (10 µg/mL) and LPS stimulation. (C) Apoptosis was determined by flow cytometric analysis in the treated PMVECs. The annexin V+/PI- cells represent the early apoptotic cells. The annexin V-/PI+ cells represent the necrosis. (D) Western blot analysis of Bax, cleaved-caspase-3, Bcl-2 protein expression in inhibitor NC or miR-17 inhibitor transfected cells followed by LPS and/or FA. The data are the means $\pm$ SD of three independent sets of analyses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Similarly, the return of reduced Bcl-2 protein level was also be observed (Fig. 3D). These results suggested that FA inhibited the apoptosis of PMVECs mediated by the enhanced expression of miR-17.

MiR-17 negatively targets PTEN

To further explore the targets of miR-17, we constructed over-expressed cells by transfecting miR-17 mimic (Fig. 4A). Using the TargetScan database, we found PTEN might be a target gene of miR-17 (Fig. 4B). Further luciferase activity assay showed the reduced lucif-

5C). In PTEN overexpressed cells, cleaved-caspase-3 and Bax protein level were increased; Bcl-2 protein level was decreased at the same time (Fig. 5C). Apoptosis rate also showed the rising apoptotic cells in PTEN overexpressed cells (Fig. 5D). These results indicated that PTEN overexpression could relieve the anti-apoptosis effect of FA on LPS stimulation in PMVECs. Furthermore, FA activated the PI3K/Akt signal pathway to exhibit its anti-apoptosis via miR-17/PTEN in PMVECs.

DISCUSSION

Many clinical diseases, such as septicemia, pneumonia, trauma and so on, may develop into ALI (Yao *et al.*, 2017). ARDS, as an important global health problem and crucial cause of mortality and morbidity, leads to serious economic pressure especially in middle countries (Adhikari *et al.*, 2010). According to the data, the ICU patients account for 10.4% among the incidence of ARDS (Bellani *et al.*, 2016). However, the subsequent fatality rate is as high as 11%–87% (Máca *et al.*, 2017). So it is important to gain insight into the pathological changes and find possible new agents for the therapy of ARDS.

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Fig. 5. FA inhibits apoptosis through the activation of PI3K/Akt pathway mediated by up-regulating of miR-17. (A) Western blot was used to detect the protein expression of PTEN, PI3K, p-PI3K, Akt and p-Akt in the indicated groups of cells. PMVECs were transfected with miR-17 inhibitor and its negative control and then stimulated with LPS (10 μg/mL) and FA (10 μg/mL) for 24 hr. (B) qRT-PCR analysis to confirm the mRNA level of PTEN in PTEN overexpressed cells. PMVECs transfected overexpression PTEN vector and its negative control. (C) Western blot was applied to check the expression of PTEN, PI3K, p-PI3K, Akt, p-Akt, Bax, cleaved-caspase-3 and Bcl-2 protein in PTEN overexpressed cells following by LPS and/or FA stimulation. PMVECs were transfected with overexpression PTEN vector and its negative control, and then stimulated with LPS (10 μg/mL) and FA (10 μg/mL) for 24 hr. (D) The indicated cells were determined by flow cytometric analysis. PMVECs were transfected with overexpression PTEN vector and its negative control, and then stimulated with LPS (10 μg/mL) and FA (10 μg/mL) for 24 hr. The annexin V+/PI- cells represent the early apoptotic cells. The annexin V-/PI+ cells represent the necrosis. The data are the means ± SD of three independent sets of analyses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

pathway (Kamm *et al.*, 2019; Luo *et al.*, 2020b). And in ARDS, FA was found to inhibit inflammation and oxidative stress (Zhang *et al.*, 2018). Here, we found FA inhibited the apoptosis of PMVECs induced by LPS. Interestingly, we observed the dynamic expression of miR-17 during the LPS and/or FA stimulation. miR-17, as the most prominent member in the miR-17-92 cluster, could function as an oncogene or tumor suppressor, although it was firstly known as typical oncogene-type miRNAs (Zhao *et al.*, 2015; Lin *et al.*, 2012; Chatterjee *et al.*, 2014). We used miR-17 inhibitor to acquire the evidence that FA relieved cell apoptosis after LPS administration mediated by miR-17. The results gave us the question to further explore the underlying mechanism of miR-17.

In lung cancer, miR-17 was found to target several pathways. PTEN is a direct target gene of miR-17 (Shan *et al.*, 2013; Lee *et al.*, 2017). miR-17 targets CDKN1A to regulate cell cycle (Zhao *et al.*, 2015). miR-17 also was found to target EZH1 and PTEN to mediate PI3K/Akt signal pathways (Osada and Takahashi, 2011; Zhang *et al.*, 2017). However, in ALI, the role of miR-17 and the targets of miR-17 are unknown. Whether miR-17 and its targets were involved in the anti-apoptosis function of FA remains unclear. In our study, we found miR-17 targeted PTEN. When we transfected over-expression PTEN plasmid, which could contribute to the similar expression trend of the PI3K/Akt signal pathway protein compared to miR-17 inhibitor transfected cells. These results combined to show that FA inhibits cell apoptosis induced by LPS via miR-17/PTEN to further regulate the activation of the PI3K/Akt pathway. Studies performed in other disease models suggested that NF- κ B were regulated by FA (Zhang *et al.*, 2018). Oppositely, it was previously reported that FA could regulate p38 MAPK signaling in different cells (Cheng *et al.*, 2016; Lin *et al.*, 2015; Koshiguchi *et al.*, 2017). FA also has an opposite effect in ERK signaling in lymphocytes, when given radiation or not (Ma *et al.*, 2011a, 2011b). However, the exact underlying mechanism in regulating the apoptosis of FA also remains for further investigation.

In our study, we chose LPS-induced cell injury model for the mechanism research of ALI. PMVECs apoptosis leads to destruction of the endothelial barrier, resulting in pulmonary edema (Li *et al.*, 2018). Therefore, we did research to explore FA function in LPS-induced PMVECs apoptosis, which also might be an effective strategy in the therapeutic management of ALI. In summary, we showed that FA decreased cell apoptosis induced by LPS and regulated the activation of the PI3K/Akt pathway via miR-17/PTEN. We hope that our data will be pursued with additional studies for ALI clinical therapy.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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