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A study on circadian rhythm disorder of rat lung tissue caused by mechanical ventilation induced lung injury

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ABSTRACT

Ventilator-induced lung injury (VILI), the most serious complication of mechanical ventilation therapy, is an excessive inflammatory response in lung tissue characterized by infiltration of inflammatory cells and overproduction of inflammatory mediators. The pathogenesis of VILI is very complex. It is becoming increasingly evident that disruption of circadian rhythm affects the immune response. Whether the pathogenesis of VILI is associated with circadian rhythm disruption has not been reported. In this study, we establish VILI model in SD rat by performing an endotracheal intubation and placing the rat on a mechanical ventilator (tidal volume of 40 ml/kg or 10 ml/kg without positive end-expiratory pressure). To examine the effect of VILI on clock gene expression, real-time quantitative PCR was performed to measure bmal1, clock, per2 and Rev-erbα mRNA expression. We found that Rev-erbα mRNA was significantly decreased in high tide volume mechanical ventilation group compared with spontaneous group, the same as REV-ERBα protein product which was tested by Western blot approach. Stimulation of REV-ERBα activity by SR9009 greatly diminished VILI-induced lung edema, inflammatory cell infiltration and the production of the pro-inflammatory cytokine TNF-α. Collectively, our findings are the first to show that REV-ERBα plays an important role in VILI and inflammation, and circadian rhythm disorder in inflammation response may be a novel pathogenesis of VILI.

1. Introduction

Circadian clocks provide organisms with an internal mechanism to maintain temporal order in a rhythmic environment. In mammals, circadian rhythms are driven by a complex of feedback loops centered on the transcriptional activators CLOCK and BMAL1, and transcriptional repressors PERIOD (PER), and REV-ERBα. These feedback loops generate a repetitive transcriptional and translational oscillator with a period of 24 h. Two of the loops are the most important ones: 1) transcription factors CLOCK and BMAL1 form heterodimer through bHLH-PAS domain, bind to the E-box of the circadian gene Perl-3 [1] and Cryl-2 upstream promoters [2], and thus activate the genetic transcription. The expression products PER and CRY proteins move from cytoplasm to nucleus, PER and CRY proteins can be used as negative elements, directly interact with CLOCK or BMAL1 to inhibit the transcription of Perl1-3 and Cryl-2 by suppressing the activity of CLOCK–BMAL1 [3]; and 2) CLOCK/BMAL1 heterodimer activates the transcription of the orphan nuclear receptor Rev-erbα while activating the transcriptions of Per and Cry series genes [4]. In turn, REV-ERBα protein binding to Bmal1 promoter represses transcription of Bmal1.

Daily rhythms have been shown to influence peripheric organ biological processes. Pulmonary ventilation function, functional residual capacity, and pulmonary airway resistance have been discovered to exhibit regular oscillations [5]. Regulation of circadian clock gene expression in lungs is necessary for maintaining normal lung function [6]. The nature of the processes carried out by lung and the expression of a large number of genes that regulate these processes may be under direct or indirect control of circadian oscillators for the efficient functioning of the organ. Furthermore, disruption of this regulation could be a cause or an effect of lung pathologies [7]. Research has shown that mice with chronic lag due to the abnormity of clock gene expression in the lung tissue could result in pulmonary function changes [8].

Mechanical ventilation is essential for the support of critically ill patients, but may aggravate lung damage, leading to ventilator-induced lung injury (VILI). Injurious mechanical ventilation with high tidal volume or high pressure could cause an increase in microvascular permeability, lung edema and hyaline membrane formation [9]. Current evidence suggested that mechanical ventilation can provoke an excessive inflammatory response within the lung, which is characterized by the infiltration of inflammatory cells and overproduction of pro-inflammatory mediators, so-called biotrauma [10]. The mechanism of biotrauma is very complex, and has become the focus in the VILI. A large number of evidences demonstrated that light and daily rhythms have a strong influence on immune function. Rat spleen macrophages,

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dendritic cells, B cells [11] and NK cell [12] contain a variety of Clock genes (Per1, Per2, Bmal1, and Clock) and Clock regulate gene (Rev-erbα and Dbp). These genes are of great significance in maintaining normal immune system function. Many diseases exhibit circadian rhythmicity in their pathology, and lifestyles that disrupt inherent timing systems, such as chronic shift work, are associated with an increased risk of metabolic disease [13], cardiovascular disease [14] and cancer [15,16]. Further evidence showed that the circadian and immune systems are intertwined [17]. In the form of jet lag or night shift work can cause lymphocytes and NK cells secrete in abnormal rhythm [18,19], and induce the expression of inflammatory factors like IL-6, and TNF-α [20]. In the immune system, many functions and parameters vary based on the time of day, including variation in susceptibility to infection and course of immunologically mediated diseases is highlighting the importance of the circadian clock. VILI is an acute inflammation of lung tissue, therefore, we hypothesize that the pathogenesis of VILI is associated with immune function to the circadian clockwork. The aim of this study is to determine whether VILI is under clock gene control.

2. Materials and methods

2.1. Animals

The study protocol was approved by the Institutional Animal Care Committee at the southern medical University. Pathogen-free Sprague–Dawley rats weighing 200 to 250 g were used in this study. All rats were maintained under standard conditions with free access to water and rodent laboratory food and maintained on a 12-h light/dark (LD) cycle. Artificial light was present from 7 A.M. to 7 P.M. Each day, room temperature was maintained between 22 °C and 24 °C.

2.2. Animal preparation

All animals were anesthetized with 10% chloral hydrate 350 mg/100 g intraperitoneally, a catheter was inserted into the trachea under direct vision via oral cavity. Catheters were also inserted into the femoral artery and vein. The femoral artery catheter was inserted for collection biological signals to continuous monitoring mean arterial pressure (MAP) and heart rate (HR). Lactated Ringer's solution was infused at a rate of 10 ml/kg/h through the venous catheter. During the experiments, the rats were placed under a warming light and rectal temperature was maintained close to 37 °C.

2.3. Animal models

First, to evaluate expression of clock genes after spontaneous and mechanical ventilation, we first separated eighteen rats into three groups according to tidal volume. The control group ($n = 8$) underwent spontaneous breath. The second group (LV group) ($n = 8$) was mechanically ventilated with 10 ml/kg tidal volume MV for 2 h, whereas rats in the third group (HV group) ($n = 8$) were mechanically ventilated with 40 ml/kg tidal volume for 2 h. Mechanically ventilated rats were paralyzed by a continuous infusion of 0.1 ml/kg/h vecuronium bromide. The respiratory rate was set to 40 times per minute for the LV group, and 80 for the HV group. Rats were released to the cage and kept separately from each other for 24 h after the treatment was finished.

Second, to evaluate the effect of the REV-ERBα specific agonist SR9009 (EMD Millipore Corporation, Billerica, Massachusetts) on VILI, we assigned 16 rats into another two groups of 8 animals having treatment with SR9009 or not. The treatment group (name as SR9009) was injected 50 mg/kg SR9009 before anesthesia, while the other group (non-SR9009) was injected the same amount of normal saline (NS) replace. Both groups suffered 40 ml/kg tidal volume mechanically ventilation for 2 h, and the respiratory rate was set to 40 times per minute. Mechanically ventilated rats were paralyzed by a continuous infusion of 0.1 ml/kg/h vecuronium bromide. Two hours later, all rats were free separately for 24 h.

2.4. Preparation of lung tissues

24 h after the successful establishment of animal model, all rats were painlessly killed by chloral hydrate anesthesia and whole lung tissues were isolated from thoracic cavity. All of the left low lobes were collected to measure the wet-to-dry ratios, and the left upper lobe of lungs for histopathologic examination. The others were stored at −70 °C until the protein and RNA assays were performed.

2.5. Histopathologic examination and the wet-to-dry ratios

We measured pulmonary wet/dry weight ratio to evaluate pulmonary congestion and edema. The left low lobes were weighed and then dried to a constant weight at 80 °C for 24 h. The left upper lobe of lungs was fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 6 um thickness, and stained with hematoxylin and eosin. We graded the degree of the lung injury using a five-point scale according to a combined assessment of alveolar and interstitial edema, hemorrhage, accumulation and aggregation of immune cells based on a previously reported system: 0 = minimum damage, pulmonary vascular, alveoli and pulmonary interstitial are normal; 1 = mild damage, pulmonary interstitial is infiltrated with small amounts of neutrophils, and the range of alveolar hemorrhage and edema is less than 25%; 2 = moderate damage, pulmonary interstitial and a part of alveolar infiltrated with more neutrophils, interstitial broadening, alveolar cavity hemorrhage and edema are in the range between 25% and 50%; 3 = severe damage, most of the alveoli and interstitial infiltrated with mass of neutrophils, interstitial broadening obviously, alveolar cavity hemorrhage and edema are in the range between 50% and 75%; and 4 = maximum damage, almost all the entire field of vision is infiltrated with neutrophils and the range of alveolar cavity hemorrhage and edema is more than 75%.

2.6. Quantitative real-time PCR

To extract RNA from the lungs, 100 mg of frozen lung tissue was homogenized with 1 ml of Trizol (Takala Biotechnology, Dalian, China) in a ribonuclease (RNase)-free tube at 4 °C. The RNA isolation was performed according to the manufacturer's procedure. After extraction, total RNA was submitted to a reverse transcription by using a First Strand cDNA Synthesis Kit from Takala Biotechnology, according to the manufacturer's instruction. The final product complementary DNA (cDNA) was stored at −20 °C. cDNA was amplified on a Bio-Rad CFX-96 real-time system using SYBR Green qPCR Master mix from Takala. Specific primers against Per2:5′ GGGAAACACCACGAGAATGAGA 3′ (sense) 5′ CATAGCCTGAGTGTACCCTCTCTGC 3′ (antisense), Bmal1: 5′ GGGGAAATACGGGTGAAGTCTA 3′ (sense) 5′ TTCCGCAAGGTGTCCTAT ATCATC 3′ (antisense), Clock: 5′ CAGTTCTTACAGACATCTCGGTTGC 3′ (sense) 5′ GTGGTGCGAAGGAGGGAAA 3′ (antisense), Rev-erbα: 5′ CCTTTTCACGGCAGTGGTACTTG 3′ (sense) 5′ CAGTTTGGTGAAGCGGGA AGT 3′(antisense), β-actin:5′TGGGTATGGAATCCTGTGGCA 3′ (sense) 5′ TGTTGGCATAGAGGTCTTTACGG 3 (antisense) were purchased from Generay Biotechnology(Songjiang District, Shanghai). Relative expression of clock genes was normalized to the β-actin levels.

2.7. West blotting and ELISA analysis

For protein analysis, 100 mg of frozen lung tissues was homogenized in Protein Extraction Reagent (Thermo Scientific, Tianhe District, Guangzhou). Protein samples were subjected to standard SDS-PAGE electrophoresis, transferred to a PVDF membrane, and probed with Rev-erbα and beta Actin polyclonal antibody (Santa Cruz Biotechnology, Dallas, Texas). After chemiluminescence detection using ECL Western blot detection kit (PerkinElmer, Waltham, Massachusetts),