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Salubrinal attenuates right ventricular hypertrophy and dysfunction in hypoxic pulmonary hypertension of rats

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ABSTRACT

The phosphorylation of eukaryotic translation initiation factor 2 alpha (p-eIF2α) is essential for cell survival during hypoxia. The aim of this study was to investigate whether salubrinal, an inhibitor of p-eIF2α dephosphorylation could attenuate pulmonary arterial hypertension (PAH) and right ventricular (RV) hypertrophy in rats exposed to hypobaric hypoxia. PAH of rats was induced by hypobaric hypoxia. Salubrinal supplemented was randomized in either a prevention or a reversal protocol. At the end of the follow-up point, we measured echocardiography, hemodynamics, hematoxylin-eosin and Masson's trichrome stainings. RNA-seq analysis is explored to identify changes in gene expression associated with hypobaric hypoxia with or without salubrinal. Compared with vehicle-treatment rats exposed to hypobaric hypoxia, salubrinal prevented and partly reversed the increase of the mean pulmonary artery pressure and RV hypertrophy. What's more, salubrinal reduced the percentage wall thickness (WT%) of pulmonary artery and RV collagen volume fraction (CVF) in both prevention and reversal protocols. We also found that salubrinal was capable of reducing endoplasmic reticulum stress and oxidative stress. The result of RNA-seq analysis revealed that chronic hypoxia stimulated the differential expression of a series of genes involved in cell cycle regulation and ventricular hypertrophy and so on. Some of these genes could be ameliorated by salubrinal. These results indicate that salubrinal could prevent and reverse well-established RV remodeling, and restore the genes and pathways altered in the right ventricles of rats exposed to hypobaric hypoxia.

1. Introduction

High altitude is an environmental condition with lower atmospheric pressure and air density. Exposure to chronic hypobaric hypoxia causes pulmonary arterial hypertension (PAH) characterized by progressive rise in pulmonary arterial pressure, pulmonary artery structural remodeling and further leading to right ventricular (RV) hypertrophy. What is more, prolonged hypoxia itself can also have a direct effect on cardiac myocytes, leading to myocardial dysfunction [1,2]. However, the underlying molecular mechanisms are not fully elucidated. The outcome of PAH is determined by RV adaption to the increased pressure [3]. Therefore, we should focus the treatment on the RV as a target of PAH induced by chronic hypobaric hypoxia.

Hypoxia as a stress can cause the accumulation of misfolded or unfolded proteins in endoplasmic reticulum (ER) and disrupt the cellular homeostasis. A series of adaptive responses including protein kinase RNA-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1) and activating transcription factor 6 (ATF-6) are touched off to restore normal ER function, collectively known as unfolded protein response (UPR) [4]. The PERK-eIF2α (eukaryotic initiation factor 2 submit α) arm of UPR serves as a central regulator of protein synthesis that globally monitors the balance between nascent cargo load and folding stress in the cell, and appears an important role in promoting cell survival during hypoxia [5–8]. Activation of PERK and PERK-mediated phosphorylation of eIF2α on ser51 can regulate mRNA translation through preventing the formation of eIF2-GTP-Met-tRNA ternary complexes and thereby inhibiting protein synthesis. It is reported that $PERK^{-/-}$ mouse embryo fibroblasts (MEFs) and MEFs with a transdominant, nonphosphorylatable mutant allele of eIF2α exhibited lower survival after prolonged exposure to hypoxia than did wild-type fibroblasts, suggesting the importance of PERK-eIF2α in hypoxic stress [9].

Salubrinal, a small molecule, prevents the dephosphorylation of p-eIF2α and prolongs the attenuation of translation [10]. It has been shown that salubrinal could protect against various cell injury [8,11,12]. Recently, our in vitro study has shown that both tunicamycin and hypoxia-induced rat cardiomyocyte injury could be ameliorated by

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salubrinal [13]. In the present study, we focused on the research of right ventricle exposure to chronic hypoxia and investigated the prevention and reversal effect of salubrinal on high altitude-associated PAH and RV remodeling. Genome-wide transcriptional profiling has been performed by the high-throughput RNA-sequencing (RNA-seq) approach in order to investigate genes of right ventricle in response to hypobaric hypoxia without or with salubrinal treatment.

2. Materials and methods

2.1. Animals and hypoxic exposure

All procedures used were approved to comply with recommendations of the Institutional Animal Care and Use of Laboratory Animals of Chinese PLA General Hospital. Adult male Sprague–Dawley rats weighing 200–250 g were obtained from Vital River Laboratory Animal Technology Company. They were kept at an ambient temperature of 20–24 °C with a 12–12 h light-dark cycle and allowed access to food and water ad libitum.

Rats in the hypobaric hypoxia groups were randomly divided into vehicle and salubrinal (Sal, 1 mg/kg/d) groups. They were kept in a hypobaric chamber with the pressure of 380 mm Hg to simulate 5500 m in altitude. Rats were further randomized to receive Sal in either a prevention (rats were kept in a hypobaric chamber for 2 weeks and Sal treatment was initiated when the animals were put into the hypobaric chamber) or a reversal (rats were kept in a hypobaric chamber for 4 weeks and Sal treatment was initiated on the third week of hypobaric hypoxia) protocol. Sal was dissolved in the vehicle (10% DMSO, 0.5% carboxymethyl cellulose sodium) and administered by intraperitoneal injection once a day for 14 days. There were 8–10 rats in each group. The chamber was opened for 1 h to deliver drugs, and to add food and water every day. Furthermore, another 12 rats that were kept at normoxic environment were also randomly divided into vehicle and Sal groups.

2.2. Echocardiography and hemodynamics

Echocardiography was measured at the end of the follow-up point using a Vevo 2100 imaging system (Visual Sonics Inc., Canada). The parameters of right ventricular anterior wall (RVAW) and pulmonary artery acceleration time (PAAT) were recorded. Hemodynamic parameters were made in anesthetized rats at the end of the echocardiogram. A polyethylene tubing catheter, connected to a pressure transducer, was inserted via the right external jugular into the right ventricle and preceded to the pulmonary artery. The right ventricular systolic pressure (RVSP) and mean pulmonary artery pressure (mPAP) were monitored by an MP150 polygraph system (Biopac, USA).

2.3. MDA, SOD, ET-1 and BNP measurements

Blood samples were collected from the catheter before heart excision and clotted for 2 h at room temperature before centrifugation for 10 min at 3000*g*. The serum was removed and then stored at −70 °C. The Elisa kits of Endothelin-1 (ET-1) (R&D Systems, USA) and brain natriuretic peptide (BNP) (Abcam, USA) were used to determine the concentrations of ET-1 and BNP, respectively. Malondialdehyde (MDA) content and superoxide dismutase (SOD) activity were estimated using thiobarbituric acid and Xanthine oxidase methods, respectively, according to the manufacturer's instructions (Nanjing Jiancheng, China).

2.4. Morphometric analysis

The hearts and lungs were harvested. Right ventricle (RV), left ventricle and interventricular septum (LV + IS), and lungs were weighed. The values of RV / (LV + IS), RV to body weight (BW) and Lung/BW were calculated. Lung sections from inferior lobe of right lung were processed with hematoxylin-eosin (HE) staining using a standard protocol. Masson's trichrome staining was performed to assess RV fibrosis. Slices of tissue were observed under light microscopy. At 400× magnification small pulmonary vessels of at least 3 animals in each group ranging from 50 to 100 μm in internal diameter were recorded. The percentage wall thickness (WT%) calculated by the following formula: WT% = (medial thickness × 2) / (external diameter) × 100. The index of collagen volume fraction (CVF) as the percentage of fibrosis was also calculated.

2.5. Western blot analysis

Western-blot was performed as previously described [14]. Briefly, total proteins from rat right ventricles in Sal prevention protocol were extracted using lysis buffer [62.5 mmol/l Tris-HCl (pH 6.8 at 25 °C), 2% w/v SDS (sodium dodecyl sulfate), 10% glycerol and 50 mmol/l DTT (dithiothreitol)]. The concentrations of protein samples were measured by the bicinchoninic acid assay. Then, samples were separated on a gradient gel (Bio-Rad, USA). The primary antibodies used were p-eIF2α (Cell Signaling Technology, USA, 1:1000), eIF2α (Cell Signaling Technology, USA, 1:1000), CHOP (Cell Signaling Technology, USA, 1:1000), GRP78 (Abcam, USA, 1:100), caspase-12 (Santa, USA, 1:200) and calreticulin (Santa, USA, 1:200). β-actin was used as internal calibration to account for differences in protein. Membranes were then incubated with the appropriate horseradish peroxidase conjugated secondary antibodies (Zhongshan Golden Bridge Biotechnology Company, China) at a 1:5000 dilution. Protein bands were detected with Alpha Imager 5500 system (Alpha Innotech, USA).

2.6. RNA-seq alignment and differential expression

RNA-seq of RV tissues in Sal reversal protocol was performed as previously reported [15]. Briefly, total RNA was extracted and RNA concentration was measured spectrophotometrically. The mRNA was enriched by using the oligo(dT) magnetic beads, and reverse transcription was performed to synthesize cDNA. Agilent 2100 Bioanalyzer was used to qualify and quantify of the cDNA library. The library products were ready for sequencing via Illumina HiSeqTM 2000. Data filtering of raw reads was carried out to obtain high quality reads as clean reads. Clean reads were mapped to reference genome and reference gene set using SOAPaligner/SOAP2. We applied NOIseq method [16] to screen differentially expressed genes (DEGs) between two groups. The filtering condition was fold change ≥ 1.5 and probability ≥ 0.6. Comparisons were made between the hypoxia-treated group (vehicle) and the control group, and between the hypoxia group (vehicle) and the hypoxia plus Sal reversal group (Sal). GO analysis was performed to identify the biological processes altered during hypoxia without or with Sal treatment.

2.7. Quantitative PCR validation of RNA-seq results

For quantitative real-time PCR (qPCR), the reaction mixture (10 μl) consisted of 3 μl cDNA, 5 μl SYBR® Premix Ex Taq™ (TaKaRa, China), 1 μl forward primer and 1 μl reverse primer of the genes studied. The quantification of gene expression was performed using the 7300 system SDS software. Sequences for the PCR primers were listed as follows: Alox15 sense: GATGGGTGTCTACCGCATCC, antisense: CCTCTCCATGC TGTCCAACC; Loxl1 sense: TACTTGCCTGTGCGAAACTCT, antisense: GTGGATGCCTGCACGTAGTT; Loxl2 sense: GCATGGATTTGGCATGACTG, antisense: GCACACTCGTAACTCTTCTG; Ubd sense: ACCAGATCCTTCTGC TAGAC, antisense: AGGCACAGCAGTCACATTC; β-actin sense: TAAAGA CCTCTATGCCAACACAGT, antisense: CACGATGGAGGGGCCGGACTCATC.