MitoKATP regulating HIF/miR210/ISCU signaling axis and formation of a positive feedback loop in chronic hypoxia-induced PAH rat model

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Abstract. In the present study, we studied the mechanism of mitochondrial ATP-sensitive potassium (mitoKATP) channels regulating hypoxia-inducible factor (HIF)-1α/microRNA (miR)-210/mitochondrial iron-sulfur protein integrin (ISCU) signaling axis and forming a positive feedback loop in chronic hypoxia-induced pulmonary arterial hypertension (PAH) by using *in vivo* animal model. Two hundred healthy adult SPF Sprague-Dawley rats were randomly divided into five groups: Control, a mimic miR-210 agent (mimic-210) intervention, a miR-210 inhibitor (anti-210) intervention, a chronic PAH and an anti-210 intervention PAH groups, with 40 rats in each group. After the chronic PAH rat model was successfully established, the rats were intervened with mimic-210 and anti-210. The pulmonary artery smooth muscle cells (PASMCs) of rats in each group were acutely isolated and the activity of mitoKATP and mitochondria-derived oxygen free radicals reactive oxygen species (ROS) was detected. RT-qPCR was used to detect the gene of HIF-1α/miR-210/ISCU and western blot analysis was used to detect the protein of HIF-1α and ISCU. The gene and protein expression were detected again after mitoKATP-specific opener diazoxide and blocker 5-HD was given via tail vein and took effect on each group of rats, respectively. Additionally, the indicators were detected again after ISCU recombinant protein was given via tail vein and ISCU small interfering RNA (siRNA) via nasal feeding and took effect on each group of rats, respectively. It was found that the activity of mitoKATP and ROS and the gene and protein levels of HIF-1α/miR-210/ISCU of the mimic-210 group were significantly higher than those of the control group while that of the anti-210 group was significantly reduced ($P<0.05$). The indicators in the chronic PAH group were significantly higher than those of the control group while those of the anti-210 intervention PAH group were significantly reduced ($P<0.05$). The indicators of all the groups were increased after being given mitoKATP specific opener diazoxide. The indicators of all the groups were significantly reduced after receiving blocker 5-HD ($P<0.05$). The indicators of all the groups were significantly reduced after given ISCU recombinant protein. The indicators of all the groups increased following ISCU siRNA, and there was a statistically significant difference ($P<0.05$). In conclusion, the mechanism of mitoKATP regulating the HIF-1α/miR-210/ISCU signaling axis and formation of a positive feedback loop exists in the PAH rat model.

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Introduction

Chronic hypoxia-induced pulmonary arterial hypertension (PAH) is an important pathogenesis mechanism in the progress of chronic obstructive pulmonary disease to chronic pulmonary heart diseases (1). PAH can be caused by chronic hypoxia-induced pulmonary vasoconstriction and reconstruction but the exact mechanism is unclear (1). There are not enough effective clinical interventions. Under hypoxia, mitochondrial electron transport chain (ETC) regulates the production of mitochondrial-derived oxygen-free radicals reactive oxygen species (ROS) (2), especially H_2O_2, which is permeable and can activate the transcription factor, low oxygen-induced factor hypoxia-inducible factor (HIF)-1α (3), causing the mitochondrial ATP-sensitive potassium (mitoKATP) channel of pulmonary artery smooth muscle cells (PASMCs) to be open and activated (4). It gives a positive feedback on ROS level and the expression of HIF-1α (5), promoting the proliferation of PASMCs and inhibiting their apoptosis (6) and taking part in the remodeling process of chronic hypoxic pulmonary blood

vessels. In addition, ~6% of human microRNAs (miRNAs) including the miR-210 have HIF binding sites in the promoter region, with which HIF can combine to regulate the expression of miRNA (7). It is confirmed that the expression of HIF-1α and miR-210 increases and forms a positive feedback loop (8) when human mesenchymal stem cells are under hypoxia. The highly expressed miR-210 under hypoxia stress is able to make target regulation to the mitochondrial iron-sulfur protein integrin (ISCU) of tumor cells, vascular endothelial cells and many other cells, affecting the circulation of Krebs, electron transport, ion metabolism and the production of ROS, thus affecting the function of mitochondria (9). Previous studies confirmed that the ETC complex II inhibitor can activate mitoKATP and mitoKATP-specific openers can also inhibit the activity of complex II (10). Those two are closely related, suggesting that ETC complex II may be a component of mitoKATP, composition or an regulatory factor (10).

Based on the above, we found in *in vitro* cell experiments that the mechanism of the mitoKATP regulating HIF-1α/miR-210/ISCU signaling axis forming a positive feedback loop in PAH is involved in the remodeling process of chronic hypoxic pulmonary blood vessels (11). However, to the best of our knowledge, there are few studies *in vivo* on animals. The current study examined the relationship between the mitoKATP and the signaling axis from the PAH rat model to provide strong evidence for clinical treatment.

Materials and methods

Animals and model building. Two hundred healthy adult SPF SD rats, each weighing 150-200 g were fed normally. The rats were housed in a temperature controlled room (21 ± 2˚C) on a 12:12-h light:dark cycle (lights on at 06:00) with free access to water and food. The rats were randomly divided into five groups: A normal control, a mimic miR-210 agent (mimic-210) intervention, a miR-210 inhibitor (anti-210) intervention, a chronic PAH group and an anti-210 intervention PAH groups, with 40 rats in each group.

Establishment of the chronic PAH rat model. The rats were placed in an open normobaric low-oxygen cabin in which the oxygen concentration was kept at 10.0±0.3% and the carbon dioxide concentration was kept <3%. The low-oxygen condition was kept for 8 h daily, 4 weeks continuously. The intervention in rats with mimic-210 and anti-210 (both from R&D Systems, Minneapolis, MN, USA) was made by the nasal topical drug delivery method (compared with systemic drug delivery, nasal topically drug delivery is lower in dosage, works better in targeting and miRNA positioning, and can reduce side effects on other organs). Several studies have shown that small interfering RNA (siRNA) delivered topically though nasal cavity or trachea can have a significant target gene effect in the lungs (12).

Approval for the animal studies was received from Wuhan Central Hospital Affiliated to Huazhong University (Hubei, China).

Research methods. The rat PASMCs in each group were acutely isolated (each PASMC was obtained using the enzyme digestion method and its shrinkage was observed by phenylephrine to identify its physiological activity). The flow cytometry method was used to detect immunofluorescent activity of mitoKATP and ROS, the RT-qPCR assay was used to detect the gene of HIF-1α/miR-210/ISCU and western blot analysis was used to detect the protein of HIF-1α and ISCU. The gene and protein expressions were detected again after mitoKATP-specific opener diazoxide and blocker 5-HD (both from R&D Systems) was given via tail vein and took effect on each group of rats, respectively. The indicators above were detected again after ISCU recombinant protein was given via tail vein and ISCU siRNA (both from R&D Systems) via nasal feeding and took effect on each group of rats, respectively.

Detection methods

Flow cytometry. FITC-labeled anti-human R-123, ROS monoclonal antibody, immunoglobulin G (IgG) isotype control with corresponding fluorescence tags and a BD FACSAria sorting flow cytometer (both from BD Biosciences, Frankin Lakes, NJ, USA). Separately 10 μl of different fluorescently labeled R-123, ROS antibodies and isotype control was added to the tubes of the PASMC suspension. Three sets of peripheral blood were added with anticoagulant (50 μl each set) and was incubated at 25˚C, in the dark for 15 min. Hemolysin (1 ml) was added and reacted at room temperature in the dark for 15 min and washed with phosphate-buffered saline (PBS), followed by centrifugation at 1,200 x g for 5 min, and 500 μl of PBS was added for cell suspension. Flow cytometry and the CellQuest software (Version 3.1, BD Biosciences, San Jose, CA, USA) were used to detect and corresponding fluorescence-tagged IgG staining cells isotype control was used as a negative control. ROS is the oxygen sensing function indicator of the mitochondrial respiratory chain. R-123 fluorescence intensity is proportional to the mitochondrial membrane potential, indirectly detecting the activity of mitoKATP.

RT-qPCR assay. Primer sequences were synthesized by Shanghai Shenggong Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The fluorescence quantitative PCR instrument 7900HT was purchased from Applied Biosystems (Foster City, CA, USA). Conventional TRIzol reagent was used to extract total RNA, and the ultraviolet spectrophotometric method was used to detect the concentration and purity. The reverse transcription kit indicated the synthesis of cDNA, designed primer sequences and amplified PCR (Table I). The reaction system consisted of 2X Taq Master Mix (25 μl), forward and reverse primers (10 μM) of 2 μl, template DNA (4 μl) and ddH_2O (17 μl) to make a total reaction volume of 50 μl.

The amplification conditions were pre-denaturation at 94˚C for 4 min, denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, extension at 72˚C for 1 min for total of 35 cycles, with another extension at 72˚C for 10 min. The agarose gel electrophoresis was performed by loading 5 μl of PCR product in each well, with an voltage of 110V for 40 min. The gel image was captured under ultraviolet analyzer and the results were expressed as a ratio of the target gene and reference gene, $2^{-\Delta\Delta Cq}$.

Western blot analysis. Total protein was extracted according to protein extraction kit (BCA protein). The BCA protein

Table I. Primer sequences used in this study.

Genes	Forward primer	Reverse primer	Band length (bp)
HIF-1α	5'-CGTTCCTTCGATCAGTTGTC-3'	5'-TCAGTGGTGGCAGTGGTAGT-3'	143
miR-210	5'-CGCCTGTGCGTGTGACAGCG-3'	5'-GTGCAGGGTCCGAGGT-3'	71
ISCU	5'-GGCAAACCAGGCAGAGCCAGAG-3'	5'-GGATGGTACGGCCCGAGGTG-3'	95
β-actin	5'-CTGGAACGGTGAAGGTGACA-3'	5'-AAGGGACTTCCTGTAACAATGCA-3'	140

HIF, hypoxia-inducible factor; miR, microRNA.

assay kit was used for the determination of protein concentration to make up 5.0 mg/ml of concentration to store at -80˚C. SDS-PAGE electrophoresis method (Beijing Liuyi Instrument Factory, Beijing, China) was used to separate HIF-1α and the ISCU proteins. The gel was stained and placed in fast dye Coomassie brilliant blue to analyze with the gel imaging system (Syngene, Frederick, MD, USA) for the grayscale value, while using β-actin as the internal control.

Statistical analysis. SPSS 19.0 statistical software (Chicago, IL, USA) was used for inputting and analysis, all the quantitative data were expressed as mean ± standard deviation. The single factor ANOVA analysis was used for group comparison, and LSD or Bonferroni test was used for pairwise comparison; qualitative data were expressed as a number or percentage (%), χ^2 test was used for group comparison; P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the activity of mitoKATP and ROS. The activity of mitoKATP and ROS of the mimic-210 group was significantly higher than that of the control group while that of the anti-210 group was significantly reduced (P<0.05). The activity of mitoKATP and ROS of the chronic PAH group was significantly higher than that of the control group while that of the anti-210 intervention PAH group was significantly reduced (P<0.05) (Fig. 1).

The activity of ROS in all the groups increased after given mitoKATP specific opener diazoxide via the tail vein. ROS activity in all the groups reduced significantly after given blocker 5-HD (P<0.05). The activity of mitoKATP and ROS in all the groups reduced after given ISCU recombinant protein via the tail vein (P<0.05). In addition, the activity of mitoKATP and ROS in all the groups significantly increased after given ISCU siRNA via nasal feeding (P<0.05).

Gene level of HIF-1α/miR-210/ISCU. The levels of HIF-1α/miR-210/ISCU of the mimic-210 group were significantly higher than that of the control group while that of the anti-210 group was significantly reduced (P<0.05). The gene level of HIF-1α/miR-210/ISCU of the chronic PAH group was significantly higher than that of the control group while that of the anti-210 intervention PAH group was significantly reduced (P<0.05) (Fig. 2).

The gene level of HIF-1α/miR-210/ISCU in all the groups increased after diazoxide was given. The gene level of HIF-1α/miR-210/ISCU in all the groups decreased following 5-HD, with a statistically significant difference (P<0.05). The gene level of HIF-1α/miR-210/ISCU in all the groups decreased following ISCU recombinant protein, whereas the gene level of HIF-1α/miR-210/ ISCU in all the groups

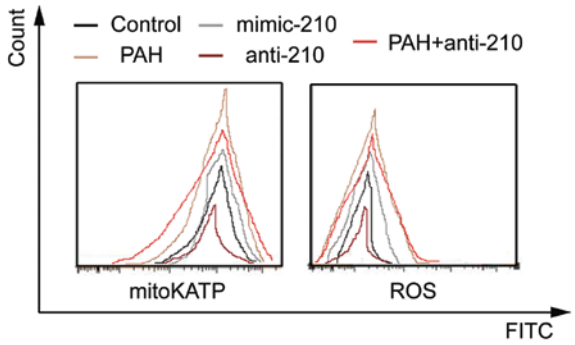

Figure 1. Activity of mitoKATP and ROS detected by flow cytometry method (control, mimic-210, anti-210, chronic PAH and anti-210 intervention groups). mitoKATP, mitochondrial ATP-sensitive potassium; ROS, reactive oxygen species.

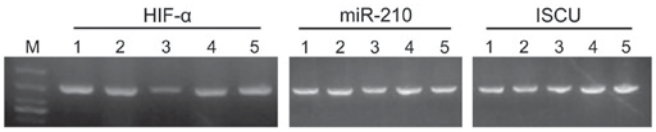

Figure 2. Levels of HIF-1α/miR-210/ISCU detected by RT-qPCR assay. HIF-1α, miR-210 and ISCU; lane 1, control (β-actin); lane 2, mimic; lane 3, anti-210; lane 4, chronic PAH; and lane 5, anti-210 intervention groups. HIF, hypoxia-inducible factor; PAH, pulmonary arterial hypertension.

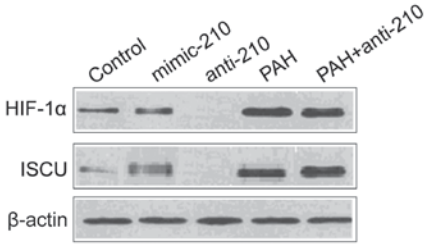

Figure 3. Protein level of HIF-1α and ISCU detected by western blot analysis. HIF-1α, and ISCU; lane 1, control (β-actin); lane 2, mimic-210; lane 3, anti-210; lane 4, chronic PAH; and lane 5, anti-210 intervention groups. HIF, hypoxia-inducible factor; PAH, pulmonary arterial hypertension.

increased following ISCU siRNA, with a statistically significant difference (P<0.05).

Protein level of HIF-1α and ISCU. The protein levels of HIF-1α and ISCU of the mimic-210 group were significantly higher than that of the control group while that of the ant-210 group was significantly reduced (P<0.05); the protein level of HIF-1α and ISCU of the chronic PAH group was significantly higher than that of the control group while that of the anti-210 intervention PAH group was significantly reduced (P<0.05) (Fig. 3).

The protein level of HIF-1α and ISCU in all the groups significantly increased after diazoxide was given, whereas the protein levels of HIF-1α and ISCU in all the groups were significantly reduced after 5-HD was given (P<0.05). The protein level of HIF-1α in all the groups significantly reduced after ISCU recombinant protein was given (P<0.05). In addition, the protein level of HIF-1α in all the groups significantly increased after ISCU siRNA was given (P<0.05).

Discussion

Our previous experiments in cell lines has confirmed that activated mitoKATP under hypoxia can increase the production of ROS, which can activate the HIF (13). The mitoKATP of PASMCs opens and regulates the signaling pathway of ROS/HIF/miR-210/ISCU under hypoxia, forming a positive feedback loop that continually induce a high expression of miR-210, thus repetitively stimulate the proliferation of PASMCs. The opening and activating of mitoKATP can cause an influx of potassium ions and mitochondrial swelling, being an important factor that affects the mitochondrial membrane potential. It can protect a variety of cells such as nerve cells and heart muscle cells and promote cell survival and inhibit apoptosis (14). Previous findings have shown that miR-210 has an anti-apoptotic function of hypoxic PASMCs (15). We assume that the opening and activating of mitoKATP changes oxygen-free radical levels by affecting the oxygen sensing function of mitochondrial respiratory chain complex, thus activating increased HIF and regulating the HIF/miR-210/ISCU signaling pathway while ISCU affects the activity of mitoKATP by affecting the activity of ETC compound II. In this way, a positive feedback loop is formed, repeatedly stimulating the proliferation of PASMCs to promote the remodeling of pulmonary blood vessels.

Through establishment of the PAH rat model and *in vivo* study, we found that the activity of mitoKATP and ROS and the gene, protein levels of HIF-1α/miR-210/ISCU of the mimic-210 group were significantly higher than that of the control group, while that of the anti-210 group was significantly reduced. These indicators of the chronic PAH group were all significantly higher than those of the control group while those of the anti-210 intervention PAH group was significantly reduced. The indicators of all the groups significantly increased after mitoKATP-specific opener diazoxide was given, and the indicators of all the groups significantly reduced after blocker 5-HD was given. The indicators of all the groups significantly reduced after ISCU recombinant protein was given, the indicators of all the groups significantly increased following ISCU siRNA. Thus, the mechanism of mitoKATP regulating HIF-1α/miR-210/ISCU signaling axis and formation of a positive feedback loop exists in the PAH rat model.

We further explored, giving mitoKATP-specific inhibitors *in vivo*, pulmonary arterial pressure and pathological changes in reversible pulmonary hypertension rats (16). In this way, we completely explained the mechanism of mitoKATP regulating HIF-1α/miR-210/ISCU signaling axis and the formation of a positive feedback loop in chronic PAH, providing theoretical basis for filtering new chronic hypoxic PAH therapeutic targets.

Currently there are no studies on chronic hypoxic PASMC mitoKATP regulating miR-210 and its target gene ISCU. This study explored that the chronic hypoxic PASMC mitoKATP regulating HIF/miR-210/ISCU signaling axis and forms a positive feedback loop, repeatedly stimulating the proliferation and migration of PASMCs. Since they horizontally regulate the expression of genes after transcription, miRNAs can regulate the function of mitochondria at the genetic level, providing experimental basis for chronic hypoxic pulmonary hypertension gene therapy. This *in vivo* study on the function of mitoKATP regulating HIF-1α/miR-210/ISCU signaling axis and formation of a positive feedback loop based on previous research has improved value and significance due to the complexity of *in vivo* experiments.

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