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Novel Role of Calcium-Sensitive Receptors in Chronic Hypoxia-Induced Proliferation of Pulmonary Vein Smooth Muscle Cells

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Abstract: *Objective:* Vascular remodeling due to chronic hypoxia (CH) occurs not only in the pulmonary arteries but also in the pulmonary veins. Pulmonary vascular remodeling arises from the proliferation of pulmonary vascular myocytes. However, the mechanism by which CH induces the proliferation of pulmonary vein smooth muscle cells (PVSMCs) is unknown. This study aimed to investigate the mechanism by which CH affects the proliferation of PVSMCs. *Methods:* PVSMCs were isolated from rat distal pulmonary veins and exposed to CH (4% O_2, 60h), and the expression of the calcium-sensitive receptor (CaSR) was detected by Western blotting and immunofluorescence. MTT assay was used to detect the proliferation viability of the cells, and the changes in the intracellular calcium concentration were detected by laser confocal scanning technique. *Results:* CaSR expression was present in rat distal PVSMCs, and CaSR protein expression was upregulated under hypoxia. The positive regulator spermine not only enhanced CH-induced CaSR upregulation but also enhanced CH-induced increase in cell viability and calcium ion concentration. The negative CaSR regulator NPS2143 not only attenuated CH-induced CaSR upregulation but also inhibited CH-induced cell viability and calcium ion concentration. *Conclusion:* CaSR-mediated hyperproliferation is a novel pathogenic mechanism for the development of proliferation in distal PVSMCs under CH conditions.

Keywords: Hypoxia; Calcium-sensitive receptor (CaSR); Pulmonary hypertension; Cell proliferation; Calcium ions

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1. Introduction

Pulmonary vascular remodeling due to alveolar hypoxia is an important cause of chronic hypoxic pulmonary hypertension (CHPH) formation. The pulmonary vasculature consists of a network of arteries, veins, and capillaries. Pulmonary veins, as one of the important components of the pulmonary vasculature, participate in the pulmonary circulation. Compared with the pulmonary artery, which is in a state of long-term hypoxia, the partial pressure of oxygen in the pulmonary vein is more variable and has a higher response threshold

to hypoxia and it should be the most reasonable site for sensing the oxygen level as well as triggering the response to hypoxia. Experiments have shown that the contraction of the pulmonary vein is greater than that of the pulmonary artery in hypoxia and that 50% of the total resistance generated by the pulmonary vasculature in hypoxia originates from the pulmonary vein, which increases with the exacerbation of hypoxia [1,2]. The pulmonary vasculature has been shown to be a major contributor to the development of CHPH in a wide variety of species, including humans [3,4] and rats [5,6] have seen pulmonary vein vascular remodeling. Therefore, studies of the pulmonary circulation response to hypoxia also need to focus on the pulmonary veins. However, the mechanism of pulmonary vein remodeling due to chronic hypoxia is currently unclear.

Abnormal proliferation of pulmonary vein smooth muscle cells (PVSMC) is the key pathological basis of pulmonary vein remodeling. Therefore, it is critical to recognize the mechanism of pulmonary vein cell proliferation under hypoxia.

Recent studies have found that the CaSR protein expression level of PASMC (IPAH-PASMC) in idiopathic pulmonary arterial hypertension (IPAH) patients was elevated compared with that of the normal group and the proliferation of IPAH-PASMC was inhibited by treatment of IPAH-PASMC with siCaSR [7]. In animal model experiments, the CaSR expression level of PASMC in wild larkspur alkaloid-induced pulmonary arterial hypertension (PAH) rats and chronic hypoxia-induced CHPH mice were significantly up-regulated, and CaSR function was enhanced, whereas intraperitoneal injection of the CaSR blocker NPS2143 significantly inhibited pulmonary arterial remodeling and prevented the PAH development and right ventricular hypertrophy [8]. The above studies suggest that cell proliferation caused by the upregulation and enhancement of CaSR expression may be a new mechanism for the proliferation of IPAH-PASMC and the pathogenesis of IPAH. In addition, hypoxia-induced up-regulation of CaSR expression in PASMC of CHPH mice and significant inhibition of pulmonary artery remodeling and right ventricular hypertrophy by CaSR blockers also suggested that CaSR was involved in the pathogenesis of CHPH [9]. Chattopadhyay *et al.* [10] also found that hypoxia up-regulated CaSR expression and promoted cell proliferation in rat PASMC and that the effects of CaSR agonist and blocker had enhanced and attenuated effects on hypoxia-induced cell proliferation, suggesting that hypoxia-activated CaSR is involved in regulating the proliferation of hypoxic PASMCs. However, the CaSR expression in pulmonary veins is not known, and its involvement in cell proliferation under hypoxia has not been reported in China. The study hypothesizes that CaSR exists in distal PVSMCs and that CaSR is involved in chronic hypoxic cell proliferation. The study reports on these issues and elaborates on the mechanism of hypoxic lung cell proliferation from a new perspective.

2. Materials and methods

2.1. Experimental materials

CaSR was purchased from Santa Company, type II collagenase, spermine, and NPS3243 were purchased from Sigma Company, and the MTT kit was purchased from Roche Applied Science Company.

2.2. Methods

2.2.1. Smooth muscle cell culture

Rat PVSMC were extracted and cultured according to the literature [11]. The rat pulmonary artery was isolated using an in vivo microscope, and primary smooth muscle cells were extracted, separated, and cultured using collagenase digestion. The 3rd–6th generation cells with good growth status were selected for the experiments.

2.2.2. Establishment of cell hypoxia model and experimental groups

(1) The experiment was divided into 4 groups: normoxia, hypoxia, hypoxia + arginin, and hypoxia + NPS2143 group.
(2) Establishment of hypoxia model: Cells were de-serum and cultured for 24 h and then placed in a hypoxic incubator (4% O_2, 60 h). Spermine was used as a CaSR agonist at a concentration of 2 μmol/L, and NPS2143 was used as an inhibitor at a concentration of 10 μmol/L.

2.2.3.Detection of CaSR protein expression

Cells of each group were lysed in protein lysis solution for 30 min, centrifuged at 4°C for 30 min, and the supernatant was taken for protein quantification. 25 μg of protein samples were collected and SDS-PAGE electrophoresis was conducted with membrane transfer, closed at room temperature for 1.5 h, and primary antibody overnight at 4°C. The membrane was washed and incubated with alkaline phosphatase-labeled anti-IgG antibody for 2 h. The color was developed in the chromogenic solution and the bands were analyzed semi-quantitatively by absorbance scanning.

2.2.4. Detection of intracellular calcium ([Ca^{2+}]i) changes by laser confocal scanner

The cells were washed with Fluo-3/AM (5 μM) and incubated at 37°C for 40 min, avoiding light. The loading solution was discarded, washed, and stored in Krebs' solution. The changes of [Ca^{2+}]i were monitored in real-time by laser scanning confocal microscope (excitation wavelength: 488 nm).

2.2.5. Detection of cell viability by MTT assay

PVSMC were inoculated in 96-well plates with spent serum culture. The method was the same as before and replaced with 10% complete medium, placed in normoxic and hypoxic chamber (4% O_2), respectively, and took out the cell culture plate 3–4 hours before the termination point; 10 μL MTT reagent solution was added to each well, respectively, and continued to cultivate to the termination point of the time mentioned above, and the absorbance of each well was measured at the wavelength of 450 nm on the fully automated enzyme labeling instrument.

2.3. Statistical analysis

The data were statistically analyzed using SPSS 17.0 software. The experimental data were expressed as mean ± standard deviation (SD). Comparison of the means of two samples was performed by *t*-test, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. CaSR expression exists in primary cultured PVSMC, hypoxia promotes the up-regulation of CaSR expression

3.1.1. Protein level expression

As shown in **Figure 1A** and **Figure 1B**, Western-blot analysis showed that there was protein expression of CaSR in cultured PVSMC and the expression of CaSR in rat kidney tissues, which served as the positive control group, was significantly higher than that in smooth muscle cells.

3.1.2. CaSR immunofluorescence staining

Positive CaSR green immunofluorescence was seen in the cytomembrane and cytoplasm of PVSMC and

DAPI nuclear staining was seen in blue color (**Figure 1C**); whereas only nuclear blue staining was seen in the negative control group without CaSR primary antibody (**Figure 1D**).

3.1.3. Chronic hypoxia promotes upregulation of CaSR expression

Under chronic hypoxia, the study shows a significant increase in CaSR. Western blotting found that the relative amount of CaSR protein was 0.166 ± 0.02 in the normoxia group, and 0.412 ± 0.06 in the hypoxia group, which was significantly higher in the hypoxia group, $P < 0.05$. (**Figure 1E** and **Figure 1F**).

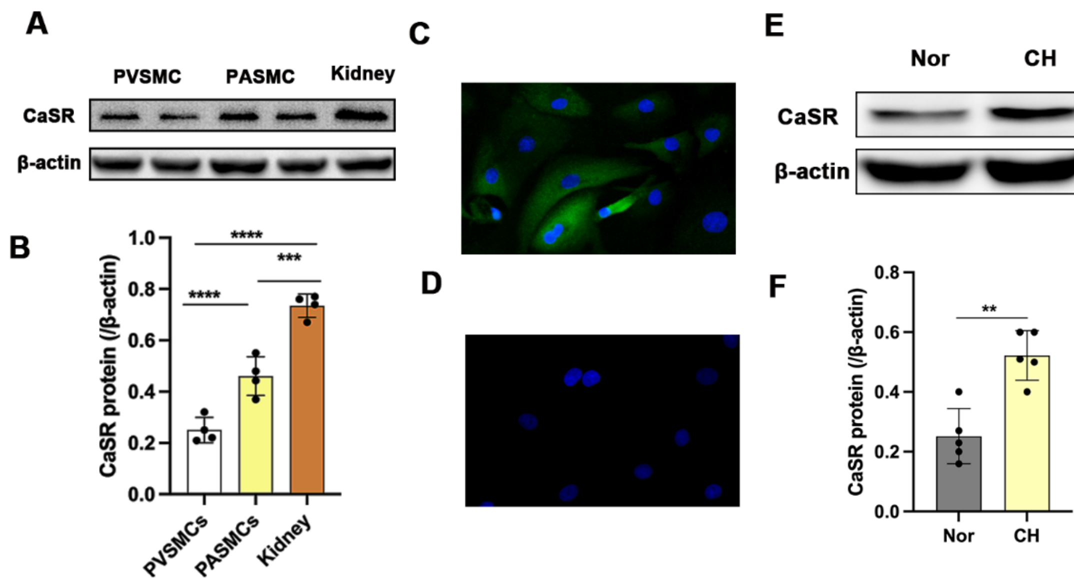

Figure 1. The presence of CaSR expression in primary cultured PVSMC, hypoxia promotes the upregulation of CaSR expression. A–B: Western blotting to detect CaSR protein expression in PVSMC, $n = 4$. (PASMC: rat pulmonary artery smooth muscle cells; Kidney: kidney tissues); C: PVSMC stained positively with CaSR immunofluorescence, CaSR immunofluorescence was green, and nuclei stained with DAPI were blue; D: PVSMC incubated without CaSR primary antibody were negative; E-F: CaSR protein expression under chronic hypoxia. $^{**}P < 0.05$ vs PVSMC, $^{****}P < 0.01$.

3.2. CaSR agonists upregulate PVSMC CaSR under chronic hypoxia and promote cell proliferation, while CaSR blockers downregulate CaSR expression and inhibit cell proliferation

3.2.1. Western-blot

The results showed that PVSMC CaSR protein expression was increased in the hypoxia group compared with the normoxia group (N: 0.15 ± 0.05, CH: 0.41 ± 0.07, $P < 0.05$); CaSR protein expression in the hypoxia + spermine (2 μM) group was further increased to 0.79 ± 0.03, and $P < 0.01$ when compared with that of the CH group; and the CaSR protein expression in the hypoxia + NPS2143 (10 μM) group both had a significant decrease in CaSR protein expression at 0.26 ± 0.06 (both $P < 0.05$ compared with the CH group) (**Table 1**). It indicated that CaSR agonists upregulated PVSMC CaSR under chronic hypoxia, while CaSR blockers downregulated CaSR expression.

3.2.2. MTT cell viability

Analysis showed (**Table 1**) that cell viability was significantly increased in the hypoxia group compared with the normoxia group [N: (100.00 ± 10)%, CH: (149.00 ± 12.23)%, $P < 0.05$]; cell viability was further enhanced in both hypoxia + spermine (2 μM) groups at (223.33 ± 28.10)%, and compared with the hypoxia group, $P < 0.01$;

while the cell viability of both hypoxia + NPS2143 (10 μM) group both had significantly reduced cell viability at (110.83 ± 25.50)% compared to hypoxia group, $P < 0.05$.

3.2.3. Intracellular calcium ion concentration

The results showed that intracellular calcium ions were significantly increased in the hypoxia group compared to the normoxia group [N: (209 ± 17.90)%, CH: (412 ± 18.70)%, $P < 0.05$]; further increased in the hypoxia + spermine (2 μM) group at (537.50 ± 15.90)%; and significantly decreased in the hypoxia + NPS2143 (10 μM) group at (319.50 ± 33.31)%, $P < 0.05$ (**Table 1**).

Table 1. Effect of each clock treatment factor on CaSR, intracellular calcium ion concentration, and MTT cell viability

	CaSR/β-actin	Intracellular calcium ion concentration	MTT cell viability
Nor	110	103	+7
CH	223	214	+9
CH + R568	197	+77	
CH + NPS2143	134	121	+13

Abbreviation: Nor, normoxic group; CH, hypoxic group

4. Discussion

Chronic hypoxia leads to pulmonary vascular remodeling, which plays an important role in the mechanism of CHPH. Under chronic hypoxia, pulmonary venous vascular remodeling is of concern, and the excessive proliferation of pulmonary vascular smooth muscle cells is a key factor leading to pulmonary vascular remodeling.

CaSR belongs to the GPCR family, which consists of an amino extracellular domain, a transmembrane domain with seven transmembrane helices, and an intracellular carboxyl tail. It was cloned from bovine parathyroid gland in 1993 by Brown *et al.* [12]. Early studies focused on the involvement of CaSR in regulating and maintaining calcium homeostasis in the thyroid, parathyroid, kidney, bone, and other cells of the body. Further studies found that CaSR is also widely expressed in non-calcium-regulated tissues such as the central and peripheral nervous system, liver, pancreas, etc., and its function is not only involved in the maintenance of serum Ca^{2+} homeostasis but also plays an important role in physiological processes such as cell proliferation, differentiation, apoptosis, etc. [12]. Studies have recently confirmed that CaSR is also functionally expressed in rat cardiomyocytes, aortic endothelial cells, aortic smooth muscle cells, PASMC, and other circulatory system cells. In this experiment, by primary culture of rat distal PVSMC and using Western-blot and immunofluorescence staining methods, it is confirmed for the first time that CaSR was also expressed in rat distal PVSMC, and found that the expression level of CaSR in rat distal PVSMC was significantly lower than that in PASMC and renal tissues, which reveals that the expression of CaSR in cells of different tissues has tissue cell variability.

Pulmonary hypertension is a complex pathological process involving multiple genes and molecules, and the interaction of these factors activates the corresponding intracellular signaling pathways, ultimately leading to vascular remodeling. The main feature of vascular remodeling is hypertrophy of the middle layer of the vasculature due to increased proliferation or decreased apoptosis of pulmonary vascular smooth muscle cells. Several studies have shown that CaSR is involved in the pathogenesis of CHPH and plays an important role in

the proliferation of hypoxic PASMCs [13,14]. However, the question arises: "Does CaSR have a similar role in hypoxia-induced PVSMC proliferation?"

The study first looked at the difference in PVSMC CaSR expression under normoxia (60h, 21% O_2) and hypoxia (60h, 4% O_2). The results showed that chronic hypoxia increased CaSR expression, which was similar to previous findings in the literature on pulmonary artery smooth muscle [14]. The changes in CaSR expression under different treatment conditions were further observed after applying CaSR agonist and blocker interventions, and the results showed that spermine (CaSR agonist) further up-regulated the hypoxia-induced increase in CaSR, whereas NPS2143 (CaSR blocker) down-regulated this increase. Meanwhile, the study also detected changes in the viability of PVSMC and changes in intracellular calcium ion concentration under the above treatment conditions by MTT. The results confirmed that cell viability and intracellular calcium ion concentration were significantly increased under hypoxic conditions. This hypoxia-induced effect was further amplified by the use of spermine intervention, whereas the hypoxic effect was attenuated by NPS2143 intervention. It is suggested that CaSR is involved in mediating the onset of hypoxic PVSMC proliferation.

5. Conclusion

In conclusion, the study preliminarily identified CaSR expression in rat distal PVSMCs and demonstrated that hyperproliferation of PVSMCs mediated by up-regulated CaSR expression in PVSMCs is a novel pathogenic mechanism involved in the initiation and progression of proliferation of PVSMCs exposed to CH. The study may contribute to the development of new therapeutic approaches for the treatment of CH-associated PH, especially PH accompanied by pulmonary vein remodeling, by blocking CaSR by synthetic calcium-solubilizing agents or down-regulation of CaSR by siRNAs or specific microRNAs.

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Disclosure statement

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