The Calcium-Sensing Receptor Mediates Hypoxia-Induced Proliferation of Rat Pulmonary Artery Smooth Muscle Cells Through MEK1/ERK1,2 and PI3K Pathways

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(Received 18 May 2010; Accepted 7 September 2010)

Abstract: Activation of the calcium-sensing receptor (CaSR) leads to an increase of intracellular calcium concentration and alteration of cellular activities. High level of intracellular calcium is involved in hypoxia-induced proliferation of pulmonary arterial smooth muscle cells (PASMCs). However, whether the CaSR is expressed in PAMSCs and is related to the hypoxia-induced proliferation of PASMCs is unclear. In this study, the expression and distribution of CaSRs were detected by RT-PCR, western blotting and immunofluorescence; the intracellular concentration of free calcium ([Ca2+]i) was determined by confocal laser scanning microscopy; cell proliferation was tested using an flow cytometric assay; cell cycle analysis was carried out using a flow cytometric assay; the expression of cell proliferation index induced by hypoxia and GdCl3 in PASMCs. Our results suggest that CaSR is expressed in rat PASMCs, and that CaSR activation through MEK1/ERK1,2 and PI3 kinase pathways is involved in hypoxia-induced proliferation of PASMCs.

Pulmonary arterial hypertension (PAH) is a complex disease that leads to right ventricular hypertrophy, right ventricular failure and death [1]. Pulmonary artery vasoconstriction and vascular remodelling contribute to a sustained elevation of pulmonary arterial pressure. Hypoxia-induced PAH is also closely associated with vasoconstriction and vascular remodelling, and especially with pulmonary arterial medial hypertrophy and muscularization because of hyperplasia of pulmonary artery smooth muscle cells (PASMCs) [2]. Both proliferation and contraction of PASMCs are associated with alterations in intracellular Ca2+ homoeostasis[3]. Hypoxic pulmonary artery proliferation and contraction are caused by the increase of cytoplasmic Ca2+ in PASMCs via multiple mechanisms. Previous evidence has demonstrated that voltage-dependent Ca2+ channels and store-operated Ca2+ channels are involved in regulating intracellular calcium concentration ([Ca2+]i) of PASMCs in hypoxic PAH [4–6], but the molecular mechanisms responsible for the proliferation of PASMCs in hypoxia remain poorly understood.

The calcium-sensing receptor (CaSR) is a member of the G-protein-coupled receptor superfamily [7]. CaSR was first cloned and characterized in 1993 from bovine parathyroid gland by Brown et al. [8]. The CaSR is important in maintaining and regulating calcium homeostasis. However, the CaSR is also widely expressed in tissues that are not involved in calcium homeostasis, and it modulates various cellular functions, including the secretion of peptides, ion-channel/transporter activity, gene expression, proliferation, differentiation, apoptosis and chemotaxis [9].

Increasing evidence suggests that the functional CaSR is expressed in some vascular smooth muscle cells (VSMCs). Wonneberger et al.[10] and Ohanian et al.[11] demonstrated that the expression of CaSR is involved in the regulation of myogenic tone in the gerbil spiral modiolar artery and in rat subcutaneous arteries. Smajilovic et al. reported that CaSR mRNA and protein were expressed in rat aortic VSMCs, and extracellular calcium concentration ([Ca2+]o) stimulated proliferation of the cells through the mitogen-activated protein/ERK kinase 1 (MEK1)/extracellular signal-regulated protein kinase1,2 (ERK1,2) pathway [12]. Molostvov et al.

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found that stimulation of CaSR could lead to activation of the MEK1/ERK1,2 and PLC pathways and up-regulation of VSMC proliferation [13].

We suggested that CaSR is expressed in PASMCS and that CaSRs in hypoxia conditions may lead to proliferation of PASMCS. Proliferating cell nuclear antigen (PCNA) is an essential substance in the DNA synthesis phase of eukaryotic cells and is also an important index for evaluating the status of cell proliferation [14]. Further, it is well known as a cycle marker protein. To demonstrate our hypothesis, we detected the CaSR expression in cultured rat PASMCS in normal and in hypoxic conditions, and observed the effect of the CaSR agonist and antagonist on \( [\text{Ca}^{2+}] \), and the cell proliferation in hypoxia. We further observed the involvement of both MEK1/ERK1,2 and PI3K pathways during these processes.

Materials and Methods

Cell preparation and culture. Male Wistar rats were purchased from the Animal Center of Harbin Medical University and treated in accordance with the Guide for Care and Use of Laboratory Animals published by the China National Institutes of Health. Primary cultures of PASMCS were prepared as previously described [15,16]. Briefly, PASMCS were obtained from rat pulmonary artery. The isolated distal arterial rings were incubated in Hank’s balanced salt solution containing 1.5 mg/ml of collagenase II (Sigma, St. Louis, MO, USA) for 20 min. After incubation, the connective tissue and a thin layer of the adventitia were carefully stripped off with fine forceps, and the endothelium was removed by gently scratching the intimal surface with a surgical blade. The remaining smooth muscles were then digested with 1.0 mg/ml of collagenase II for 120 min. at 37°C. The cells were cultured in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 20% FBS, penicillin (100 units/ml), streptomycin (100 units/ml), and incubated in a humidified incubator with 5% CO2 for 3-5 days at 37°C. The passage 2-4 cells with typical hill-and-valley morphology were used in all experiments. The purity and identity of PASMCS were verified by immunocytochemistry staining using specific mouse monoclonal antibodies against smooth muscle cell alpha-actin and showed a reaction in 80% of the cells.

Experimental protocol. The PASMCS were starved in serum-free DMEM for 24 hr and were then exposed to one of the following six different experimental conditions for 24 hr: (i) control: 21% O2/5% CO2/balance N2; (ii) hypoxic: 2.5% O2/5% CO2/balance N2; (iii) hypoxic with 300 μM GdCl3 (an agonist of CaSR); (iv) hypoxic with 10 μM NPS2390 (antagonist of CaSR); (v) hypoxic with 20 μM M NPS2390 (antagonist of CaSR); (v) hypoxic with 20 μM M L Y294002 (a PI3K inhibitors) plus 300 μM GdCl3.

RT-PCR. Total RNA from PASMCS was extracted according to the Trizol reagent (Invitrogen, USA) protocol and redissolved in 20 μl of 0.1% diethylpyrocarbonate water before being stored at -70°C. RNA was spectrophotometrically quantified by measuring the optical density of samples at a wavelength of 260-280 nm. The nucleotide sequences of the primers used (TakaRa Co.Ltd.) were as follows: (i) CaSR: sense 5'-ttccgcatcacagctg-3', antisense 5'-tgagaatgattgcttctc-3'; (ii) glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5'-cttcaactatctgtca-3', antisense 5'-tgagtgctatcag-3', yielding predicted products of 234 and 420 bp, respectively. RT-PCR was performed using RT-PCR kit from Promega, Madison, WI, USA. Cycling conditions were as follows: 35 cycles of denaturation at 94°C for 20 sec., annealing at 55°C for 40 sec. and polymerization at 72°C for 40 sec. Aliquots (5 μL) of PCR products were electrophoresed through ethidium bromide-stained 1.2% agarose gels and visualized with ethidium bromide. Identity was confirmed by sequencing (Shanghai Sangon Biological Engineering Technology & Services Co. Ltd., Shanghai, China) [17,18].

Western blotting analysis. Total cellular protein was extracted using radio immunoprecipitation assay (RIPA) extraction buffer (0.2 ml) for the lysis of 105 cells [19,20]. Total protein was quantified with a Protein Assay Kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (Sigma) as a standard. Samples of 0.08 mg of protein from different experimental groups were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes by electroblotting (300 mA for 2 hr). The membranes were blocked in TBS-T [137 mM NaCl, 20 mM Tris (pH 7.6) and 0.1% (v/v) Tween 20] containing 5% (w/v) skim milk at 37°C for 1 hr. The membranes were then incubated overnight at 4°C with antibodies against CaSR, PCNA, ERK1,2, p-ERK1,2, AKT, p-AKT and anti-GAPDH (1:500), respectively. The membrane of the negative controls was incubated with the antigen-antibody complex. Primary antibodies and antigenic peptides were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The membranes were incubated with the secondary antibody AP-IgG (Promega), diluted 1:5000 in TBST, for 1 hr at room temperature. Antibody-antigen complexes were detected using western red (Promega). The volumes of the protein bands were quantified by a Bio-Rad Chemi DocTM EQ densitometer using Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). GAPDH was used as an internal control for a semi-quantitative assay.

Immunofluorescence assay. The isolated PASMCS were placed onto coverslips, which were covered in 24-well culture plates with polylysine. After 72 hr at 37°C, the PASMCS were washed with PBS, fixed with 4% formaldehyde in PBS for 10 min. and blocked in 1% BSA for 30 min. The cells were incubated with an antibody against CaSR. (Santa Cruz, CA, USA) (1:100) overnight at 4°C, and incubated with secondary IgG (Santa Cruz) (1:1000) conjugated with fluorescein isothiocyanate (FITC), for 1 hr at 37°C and finally washed in PBS and 0.1% Tween 20. DAPI (4,6-diamidino-2-phenylindole; final concentration of 6 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) was included to label nuclei. Fluorescence images were collected using a fluorescence microscope (Leica, Germany).

Measurements of [Ca2+]i. After the treatment described above, PASMCS were incubated with 5 μmol/L Fluo-3/AM for 30 min. at 37°C. PASMCS were rinsed twice with Ca2+-free PBS to remove the remaining dye and further incubated in DMEM. Changes in the [Ca2+]i were represented as fluorescence intensity (FI). To determine minimum and maximum levels of the intracellular calcium, the cells were dyed in DMEM, where extracellular calcium was removed and a calcium-ionophore (10 μm ionomycin) was added. During the experiment, the FI of Fluo-3 in PASMCS was recorded for 5 min. using a confocal laser scanning microscope (Olympus, Tokyo, Japan) with excitation at 488 nm. The FI was observed in eight randomly chosen cells to calculate the mean FI for all PASMCS.

Cell viability assay. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously[21]. Cells were cultured in 96-well plates. MTT (final concentration, 0.5 mg/ml) was added to each well under sterile conditions, and the plates were incubated for 4 hr at 37°C. The supernatant was removed, and dimethyl sulfoxide (150 μl/well) was added. The plates were then agitated on a plate shaker. The absorbance of each well was measured at 490 nm with a Bio-Rad automated ELA analyzer (Bio-Rad Laboratories).

DNA bromodeoxyuridine (BrdU) incorporation assay. Cell proliferation was quantified using a DNA BrdU incorporation assay (Roche Applied Science, Burgess Hill, UK). The amount of incorporated BrdU is a measure of the rate of DNA synthesis of the cells and thus indirectly of cell proliferation. Briefly, PASMCS were seeded into 96-well culture plates at a density of 3500 cells/well. After the cells

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were exposed to drugs and hypoxia, BrdU labelling solution was added to each well at a final concentration of 10 μM. At the end of the stimulation period, the cells were fixed (60 min.) and then incubated for 90 min. at room temperature, with 1/100 dilution of peroxidase-labelled anti-BrdU antibody. The wells were then washed three times and incubated for 5 min. at room temperature with substrate solution, and the luminescence was measured using a Fluorostar plate reader (Hercules, CA, USA) [22].

Cell cycle analysis. Propidium iodide staining was used to analyse DNA content and cell cycle distribution [23]. PASMCs were seeded into 6-well plates at the cell density of 1 × 10⁶ cells/well and incubated overnight. After the cells were exposed to drugs and hypoxia, they were collected and washed twice with PBS. Then, the cells were resuspended and fixed using 75% cold ethanol and were kept at 4°C overnight. Before detection, the fixed cells were centrifuged at 72·g for 5 min., and the supernatant fluid was discarded. Then, the cell precipitate was resuspended in 1 mL PBS, and 200 μL RNase A was added, and the suspension was then incubated at room temperature for 10 min. Subsequently, 200 μL of propidium iodide (125 μg/mL; Becton Dickinson, USA) was added and mixed, and the reaction was carried out at 4°C for 10 min. in a dark room. Finally, the cells were filtered once through 400-mesh sieves and detected by flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

Statistical analysis. The results were expressed as means ± S.E.M. and were analysed using SPSS v. 11.5 software (SPSS, Chicago, IL, USA). Comparisons among the groups were performed using a Kruskal–Wallis one-way ANOVA. Significance was set at p < 0.05.

Results

Expression of CaSR in PAMSCs and vascular tissues. A cDNA fragment of 234 bp corresponding to the selected CaSR mRNA sequence was detected in PAMSCs (fig. 1A). In the absence of reverse transcriptase, no PCR-amplified fragments could be detected, indicating that the tested RNA samples were free of genomic DNA contamination. Sequencing results were as follows: ttccaceccaaggtgtggcctcaacctgctctgtggtctcttcgccacctc-ctgtcacgtcctctctacacgccccctccctacgccgtgccaccctca-ctgtcaagaacaatcatctc. The sequence shared 100% identity with the rat CaSR sequence (GenBank/EMBL accession #U20289).

The protein form of CaSR with a relative molecular mass of 130kD was detected in rat-cultured PASMCs and whole PAs extracts, and there were no bands in the negative control groups (fig. 1B). Immunofluorescence staining showed that CaSR proteins were present in the cytoplasm and membrane of the PASMCs (fig 1C, D).

Effect of hypoxia on the expression of CaSR and PCNA. To further confirm the involvement of CaSR in hypoxia-induced cell proliferation, CaSR protein expression was examined by western blotting. Lowering of oxygen from 21% to 2.5% significantly increased CaSR expression (p < 0.05). When the cells were incubated with GdCl₃ during hypoxia, the CaSR expression level was further enhanced (p < 0.05 versus 2.5% O₂), while NPS-2390 inhibited CaSR expression. PD98059 and LY294002 had no influence on CaSR expression (fig. 2A).

The expression of PCNA was similar to the expression of CaSR in the 2.5% O₂ group, 2.5% O₂ + GdCl₃ group and 2.5% O₂ + NPS2390 group, but in the 2.5% O₂ + PD98059 + GdCl₃ group and 2.5% O₂ + LY294002 + GdCl₃ groups, the expression of PCNA was decreased (p < 0.05 versus 2.5% O₂); the expression of PCNA in the 2.5% O₂ + PD98059 + LY294002 group was markedly decreased compared to 2.5% O₂ group (p < 0.05), (fig. 2B).

Changes in [Ca²⁺]i in different groups. Lowering of oxygen from 21% to 2.5% significantly increased [Ca²⁺]i (p < 0.05 versus 21% O₂), and this increase in [Ca²⁺]i

![Fig. 1. The calcium-sensing receptor (CaSR) is expressed in pulmonary artery smooth muscle cells (PASMCs). (A) Detection of CaSR mRNA by RT-PCR in rat PASMCs in the absence or presence of reverse transcriptase and GAPDH. (B) Detection of CaSR protein by western blotting in cultured rat PASMCs and pulmonary arteries (PAs). (C, D) Immunofluorescence detection of CaSR in rat PASMCs in the presence of anti-CaSR Ig conjugated with FITC (C) and in the absence of anti-CaSR Ig (D), (magnification: 400×).](https://example.com/f1.png)
was up-regulated by GdCl₃ (\(p < 0.05\) versus 2.5\% O₂). \([\text{Ca}^{2+}]_{\text{i}}\) was decreased by adding NPS290 (\(p < 0.05\) versus 2.5\% O₂) (fig. 3).

The expression of phosphorylated ERK1,2 and AKT in different groups.

The total ERK1,2 was equivalent in the different groups. There was only weak expression of phosphorylated ERK1,2 (p-ERK1,2) in the 21\% O₂ group. When the cells were exposed to hypoxia, the p-ERK1,2 expression was up-regulated (\(p < 0.05\) versus 21\% O₂). GdCl₃ further enhanced the increase of p-ERK1,2 expression induced by hypoxia, but NPS-2390 reduced this up-regulation induced by hypoxia (\(p < 0.05\) versus 2.5\% O₂). During pre-treatment with the combination of GdCl₃ and PD98059 (an inhibitor of MEK1), the increased phosphorylation of ERK1,2 was inhibited (\(p < 0.05\) versus 2.5\% O₂⁺ GdCl₃), but the phosphorylation of ERK1,2 was not changed in the 2.5\% O₂⁺ PD98059⁺ GdCl₃ group (\(p > 0.05\) versus GdCl₃ group) (fig. 4A).

In addition, in the 2.5\% O₂, 2.5\% O₂⁺ GdCl₃ and 2.5\% O₂⁺ NPS2390 groups, the phosphorylation level of Akt was altered similar to the phosphorylation levels of ERK1,2. However, the phosphorylation of AKT in the 2.5\% O₂⁺ LY294002⁺ GdCl₃ group was decreased (\(p < 0.05\) versus 2.5\% O₂⁺ GdCl₃). These changes were not apparent in the 2.5\% O₂⁺ PD98059⁺ GdCl₃ group (\(p > 0.05\) versus 2.5\% O₂⁺ GdCl₃ group) (fig. 4B).

The cell viability of PASMCs in different groups.

The viability of 21\% O₂ group cells was considered to be 100\%, and viability was expressed as percentages of 21\% O₂ group for the other groups. In the 2.5\% O₂ group, the survival rate of PASMCs was significantly increased compared to the 21\% O₂ group (\(p < 0.05\)). In the group treated with 2.5\% O₂ and GdCl₃, the viability was higher than in the 2.5\% O₂ group (\(p < 0.05\)). In comparison with the 2.5\% O₂ group, the group treated with 2.5\% O₂ and NPS2390 had lower cell activity (\(p < 0.05\)). However, compared to the 2.5\% O₂⁺ GdCl₃ group, the survival rate of PASMCs was significantly decreased in the 2.5\% O₂⁺ PD98059⁺ GdCl₃ and 2.5\% O₂⁺ LY294002⁺ GdCl₃ group (\(p < 0.05\)) (fig. 5).

The BrdU incorporation of PASMCs in different groups.

The BrdU incorporation of 21\% O₂ group cells was considered 100\%, and the BrdU incorporation of cells in the other groups was expressed as percentages of the 21\% O₂ group. In the 2.5\% O₂ group, the BrdU incorporation rate was significantly increased (\(p < 0.05\) versus 21\% O₂ group). In the group treated with 2.5\% O₂ and GdCl₃, it was higher than in the 2.5\% O₂ group (\(p < 0.05\)). In comparison with the 2.5\% O₂ group, the group treated with 2.5\% O₂ and NPS2390, it was markedly decreased (\(p < 0.05\)). However, compared to the 2.5\% O₂⁺ GdCl₃ group, it was significantly decreased in the 2.5\% O₂⁺ PD98059⁺ GdCl₃ and 2.5\% O₂⁺ LY294002⁺ GdCl₃ group (\(p < 0.05\)) (fig. 6).

Cell cycle analysis of PASMCs in different groups.

Lowering of oxygen from 21\% to 2.5\% could enhance the number of PASMCs in the S and G₂/M phase, decrease the number of PASMCs in the G₀/G₁ phases and increase the proliferation index \([\text{PI} = (\text{S} + \text{G₂/M})/(\text{S} + \text{G₂/M} + \text{G₀/G₁})]\) compared to the 21\% O₂ group (\(p < 0.05\)). GdCl₃ amplified the effect of hypoxia (\(p < 0.05\) versus 2.5\% O₂ group). In contrast, NPS2390 weakened this effect (\(p < 0.05\) versus 2.5\% O₂ group). At the same time, the proliferation indices were significantly decreased in the 2.5\% O₂⁺
Discussion

Although some investigations have demonstrated that the CaSR is expressed in the cardiovascular system, it remains unclear whether the CaSR is expressed in PAMSCs. To confirm the expression of CaSRs in PAMSCs, RT-PCR, western blotting and immunofluorescence staining were used in this study. A cDNA fragment of 234 bp, which sequence shared 100% identity with the rat CaSR sequence, was found in cultured PAMSCs, indicating the presence of CaSR mRNA in rat PAMSCs. Western blotting analysis showed that CaSR protein lysates from rat PAMSCs and from whole PAs extracts produced bands of 130 kDa, and there were no bands seen in the negative control groups. Our experimental results were similar to those previously described by Ziegelstein in human aortic endothelial cells (100–130 kDa) [24]. Immunofluorescence staining showed that CaSR proteins were located in the cytoplasm and membrane of the PAMSCs, as has been shown in other cell types [25,26]. Based on these data, we confirmed the expression of CaSRs in PAMSCs at the mRNA and protein levels.

The development of pulmonary arterial hypertension (PAH) involves a complex constellation of multiple genes and molecules, which interact with each other and subsequently activate intracellular signalling pathways that eventually result in pulmonary remodelling. Vascular remodelling is characterized largely by medial hypertrophy because of enhanced proliferation of VSMCs or attenuated apoptosis, which can result in lumen obliteration. In addition to other factors, cytoplasmic Ca²⁺ seems to play a central role in pulmonary remodelling [27,28]. Several studies have shown that the activation of CaSRs mediated the increase of [Ca²⁺] via the G-PLC-IP₃ pathway in some cells [10,29].

To investigate the potential role of the CaSR in pulmonary vascular remodelling induced by hypoxia, we observed the expression of CaSR protein in PAMSCs under hypoxic conditions and found that hypoxia markedly increased the expression of CaSR; GdCl₃ (an agonist of CaSR) [30,31] amplified the effect of hypoxia; and NPS2390 (an antagonist...
of CaSR) weakened the effect of hypoxia. Meanwhile, we observed the expression of PCNA under hypoxic conditions and detected similar changes to the expression of the CaSR. PCNA is an essential substance in the DNA synthesis phase of eukaryotic cells and is also an important index for evaluating the status of cell proliferation [14]. These results suggested that hypoxia could cause an increase in the expression of CaSRs and PCNA. Therefore, we speculate that the activation of CaSR induced by hypoxia plays an important role in pulmonary vascular remodelling during PAH through up-regulating the proliferation of PASMCs because of a CaSR-mediated increase of [Ca^{2+}].

To provide support for this hypothesis, we measured the change of [Ca^{2+}] and found that lowering of oxygen from 21% to 2.5% significantly increased [Ca^{2+}]; this increase was up-regulated by GdCl3 and down-regulated by NPS2390. Meanwhile, we investigated the cell viability,
BrdU incorporation rate and the cell cycle changes of PASMCs under hypoxic conditions, to explore the relationship between the increase of CaSR expression and the proliferation of PASMCs. MTT is highly dependent on mitochondrial and reducing activity of the cells which is altered in hypoxic conditions. BrdU incorporation assay is another quantified DNA assay for survival/proliferation. The amount of incorporated BrdU is a measure of the rate of DNA synthesis of the cells, and thus, it can reflect cell proliferation indirectly. MTT results showed that lowering of oxygen from 21% to 2.5% could enhance the survival rate of PASMCs; GdCl₃ or NPS2390 could magnify or attenuate this effect of hypoxia, respectively. In the experiments with BrdU incorporation, the same results were observed. The results of cell cycle analysis in this study showed that hypoxia increased the number of PASMCs in the S and G₂/M phase and decreased the number of PASMCs in the G₀/G₁ phases. GdCl₃ had a synergistic effect with 2.5% O₂.
on stimulation of cell cycle change, while NPS2390 inhibited the process. These results suggest that hypoxia through the CaSR may promote the conversion from the G0/G1 phases to the S phase and stimulate cell cycle progression.

Next, we sought to investigate the mechanism by which the CaSR is involved in the proliferation of PASMCs under hypoxia. The MEK1/ERK1,2 signal pathway is essential for cellular functions in regulating cell proliferation, differentiation and apoptosis. The activation of the CaSR resulting in the proliferation of VSMCs via the MEK1/ERK1,2 pathway has been studied by several laboratories [12,13]. Our results showed that lowering of oxygen from 21% to 2.5% induced the phosphorylation of ERK1,2 protein in PASMCs, which was up-regulated by GdCl3 and down-regulated by NPS2390. In addition, the phospho-ERK1,2 up-regulation that was induced by hypoxia and GdCl3 was markedly reduced in the presence of PD98059 (a MEK1 inhibitor), indicating that their effects were mediated through the classic MEK1/ERK1,2 pathway. In this study, we have demonstrated that the increase of the PCNA protein expression, survival rate of cells, BrdU incorporation rate and the proliferation and apoptosis. The activation of the CaSR resulting in the PI3K pathway in this proliferative response. We found that lowering of oxygen from 21% to 2.5% induced the phosphorylation of the AKT protein, which was up-regulated by GdCl3 and down-regulated by NPS2390. These results indicate that MEK1/ERK1,2 signal pathway is crucial for the proliferation of PASMCs induced by activated CaSR under hypoxia.

The phosphatidylinositol 3-kinase (PI3K) pathway has been shown to be important in the growth, survival and proliferation of a wide range of cell types [34,35]. Specifically, we were interested in determining the sensitivity of PI3K to hypoxia-activated CaSRs and in understanding the role of the PI3K pathway in this proliferative response. We found that lowering of oxygen from 21% to 2.5% induced the phosphorylation of the AKT protein, which was up-regulated by GdCl3 and down-regulated by NPS2390. LY294002 (a PI3K inhibitor) decreased the proliferative response that was induced by hypoxia and GdCl3. These data suggested that PI3K pathways are involved in the proliferative response to CaSR mediated by hypoxia in the PASMCs.

In summary, the present study demonstrated, for the first time, that functional expression of CaSRs exists in rat pulmonary artery smooth muscle cells, and CaSR activation is involved the proliferation of PASMCs under hypoxia. Furthermore, we showed that the MEK1/ERK1,2 and PI3K pathways play an important role in this response. In other experiments, we observed that the activation of CaSR could induce contraction of pulmonary arterial smooth muscle. Both vasoconstriction and vascular remodelling are closely associated with PAH. These findings may contribute to the development of new strategies for the treatment of hypoxia-induced PAH by changing the activity and the expression of CaSR.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (No.30871012, No. 30700288, No. 81070123, No. 81000059), the Special Scientific Research Fund for Doctor Discipline of University (No. 20070226012) and the graduate innovative research projects in Heilongjiang Province (YJSCX 2009-223HLJ).

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