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Research Paper

Effects of calcium-activated chloride channels on proliferation of pulmonary artery smooth muscle cells in rats under chronic hypoxic condition

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Abstract

Objective: To investigate the effects of calcium-activated chloride (ClCa) channels on proliferation of pulmonary artery smooth muscle cells(PASMCs) in rats under chronic hypoxic condition. **Methods**: The cultured PASMCs were placed under normoxic and chronic hypoxic conditions: The cells were observed by light and electron microscope; The cell cycles were observed by flow-cytometry; Immunocytochemistry staining was used to detect the expressions of PCNA, c-fos and c-jun of PASMCs; Cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in PASMCs was investigated by fluorescent quantitation using fluorospectrophotometer. **Results**: The PASMCs were contractile phenotype under normoxic conditions. Observation by transmission electron microscope: In kytoplasm of contractile phenotype cells, myofilament bundles were abundant and the content of cell organs such as Golgi's bodies were rare. The PASMCs were synthetic phenotype under chronic hypoxic condition. There were increased free ribosomes, dilated rough endoplasmic reticulums, highly developed Golgi complexes, decreased or disappeared thick filaments and dense body in kytoplasm of synthetic phenotype cells. After NFA and IAA-94, the situations were reversed The number of $S+G_2M$ PASMCs were significantly increased in chronic hypoxic condition; The NFA and IAA-94 were shown to significantly decrease them from $(28.6 \pm 1.0)\%$ to $(16.0 \pm 1.6)\%$ and the number of G_0G_1 PASMCs significantly increased from $(71.4 \pm 1.9)\%$ to $(83.9 \pm 1.6)\%$ ($P < 0.01$). In chronic hypoxic conditions, the expression of proliferating cell nucleus antigen was significantly increased; The NFA and IAA-94 were shown to significantly decrease it from $(81 \pm 6)\%$ to $(27 \pm 7)\%$ ($P < 0.01$). The expression of c-fos and c-jun were significantly increased in chronic hypoxic conditions; The NFA and IAA-94 were shown to significantly decrease them from 0.15 ± 0.02, 0.32 ± 0.05 to 0.05 ± 0.01, 0.12 ± 0.05, respectively ($P < 0.01$); Under chronic hypoxic conditions, $[Ca^{2+}]_i$ was increased; The NFA and IAA-94 decreased it from (281.8 ± 16.5)nmol/L to (117.7 ± 15.4)nmol/L ($P < 0.01$). **Conclusion**: Hypoxia initiated the change of PASMCs from contractile to synthetic phenotype and increased proliferation of PASMCs. NFA and IAA-94 depressed cell proliferation by blocking ClCa channels in hypoxic condition. These may play an important role in proliferation of PASMCs under chronic hypoxic conditions.

Key words: pulmonary artery smooth muscle cells; Ca^{2+}-activated Cl^- channels; niflumic acid; indaryloxyacetic acid; cell proliferation

INTRODUCTION

It was demonstrated that the vascular remodeling of pulmonary was an important contributor to developing hypoxic pulmonary hypertension(HPH). At the same time, proliferation of pulmonary artery smooth muscle cells(PASMCs) is an important contributor to the vascular remodeling that occurs in HPH[1]. Chloridion is the most widely dispersed in the intra-cellular and extracellular anion under physiological conditions[2]. The Cl-channel family of PASMCs has been subdivided into 2 major classes: Ca^{2+}-activated Cl^-(Cl_{Ca}) channels and volume- or swelling-sensitive Cl^-(Cl_{swell}) channels[1]. It has already been well established that[3-4] hypoxia can increase cyto-

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plasmic free Ca^{2+} concentration($[Ca^{2+}]_i$) and contribute to the proliferation of PASMCs and that Cl_{Ca} channels can be activated when $[Ca^{2+}]_i$ increases[5,6]. According to the above, we hypothesised that Cl_{Ca} channels could be activated by $[Ca^{2+}]_i$, and furthermore may play an important role in contributing to the proliferation of PASMCs under hypoxic conditions.

In our studies, the cells were observed by light and electron microscope; The cell cycles were observed by flow-cytometry; immunocytochemistry staining was used to detect the expressions of PCNA, c-fos and c-jun of PASMCs; $[Ca^{2+}]_i$ in PASMCs was investigated by fluorescent quantitation using fluorospectrophotometer. In order to elucidate the role of Cl_{Ca} channels in HPH, this study was to investigate the effects of Cl_{Ca} channels blockers Niflumic acid(NFA) and indaryloxyacetic acid (IAA-94) on proliferation of cultured PASMCs in rats under chronic hypoxic condition.

MATERIALS AND METHODS

Solutions and reagents

Twenty healthy male Sprague-Dawley rats(body weight 200-250 g) Dithiothreitol(DTT, Roche, USA) 4-2-hydroxyethyl-1-piperazine-ethanesulfonicacid (HEPES; Roche, USA) Fetal bovine serum, DMEM (Gibco, USA). Ⅰ-collagenase, Triton X-100, Fura-2/AM, NFA, IAA-94(all Sigma, USA), Other chemicals such as AR reagents made in china. Monoclonal antibody of rat's α-actin, monoclonal antibody- PCNA, anti c-fos antibody, anti c-jun antibody, SP immunohistochemistry kit, and DAB were bought from Santa Cruz biotechnology company.

Pulmonary artery smooth muscle cells culture, identification and grouping

Pulmonary artery smooth muscle cells were cultured as described by Wei[7] which were identified by positive immunocytochemical staining with antibodies against α-smooth muscle actin.

Experimental grouping: The cells were synchronized by serum free medium(SFM) for 24 h, and the cells were divided randomly and incubated in normal oxygen(5% CO_2, 21% O_2, 74% N_2) at 37℃ and hypoxic conditions (5% CO_2, 2% O_2, 93% N_2) at 37℃(Galaxy R CO_2 Incubator,England) respectively for 48 h every observation group(by electron microscope) contained 3 samples, whereas other groups contained 6 samples. The control groups(N: normoxic, H: hypoxic, Nc, Hc) were cultured in SFM; NFA groups(N_{nfa}, H_{nfa}) were cultured in SFM containing 10 μmol/L NFA; IAA-94 groups(N_{IAA-94}, H_{IAA-94}) were incubated in SFM which contained 10 μmol/L IAA-94.

Morphologic evaluation of PASMCs

Morphologic and ultra-structure of PASMCs were observed by light microscope and transmission electron microscope techniques(OPTONEM 10C,Germany).

Observation of cell cycle of PASMCs with flow cytometry

The cells were collected and washed with PBS (0.01 mol/L, pH 7.2) twice, and centrifugated at 1 200 r/min for 5 min, then fixed with pre-cooled 70% ethanol at 4℃ for one night. The cells were harvested and washed with PBS, and then treated with 0.1% Triton X-100 and propidium iodide(final concentration 100 mg/L) at 4℃ for 30 min. Afterwards, the DNA contents and cell cycles were analyzed by flow cytometry (FACSort, USA).

Immunocytochemistry

The coverglasses were collected and washed with PBS three times, then fixed with paraformaldehyde for 20 min. After that, they were washed with PBS. Staining was performed using anti-PCNA antibody according to SP kit instructions whose reagent tite was 1:200. The positive staining was observed as a buffy deposition in the cell nucleus. By microscope, we observed 200 cells randomly every coverglass, and then counted the positive rate.

The immunocytochemistry of c-fos and c-jun were similar to PCNA. The first was anti-c-fos and anti-c-jun antibody instead of anti-PCNA antibody. The AIOD value of PASMCs was measured as the relative amount of protein expression by high resolution pathological image analysis system (HPIAS-2000, Qianping Co, Wuhan, China).

Determination of the $[Ca^{2+}]i$ in PASMCs

$[Ca^{2+}]_i$ was determined as Ramafi described[8]. The PASMCs were suspended and then the $[Ca^{2+}]_i$ was determined by fluorescence spectrometer(RF-5301 PC, Shimadiu, Japan) at excitation wavelengths of 340 nm, 380 nm and emission wavelengths of 510 nm, and the triton and EGTA were added to determine the max and minimum. The samples were intervened for 5 min with drugs, then 6 samples parallel detected every time. The control group was added with same volume of normal saline. Moreover, the cells incubated in hypoxia were detected via pre-equilibrium by hypoxia gas.

Data analysis and statistics

Data were presented as mean±*s* and analyzed with SPSS(software version 12.0). Differences between different groups were analyzed by Student's *t* test or *q* test. Multiple comparisons were analyzed by One-way ANOVA. Rates were analyzed by χ^2. $P < 0.05$ was

considered to be statistically significant.

RESULTS

Effects of block agents Cl_{Ca} on morphologic of PASMCs

The PASMCs were contractile phenotype under 21% O^2 conditions. They displayed the characteristic "hill and valley" appearance by high microscope. Observation by transmission electron microscope: In kytoplasm of contractile phenotype cells, myofilament bundles were abundant and content of cell organs such as Golgi's body were rare. The PASMCs were synthetic phenotype under 2% O_2 conditions abolishing the characteristic "hill and valley" appearance. There were increased free ribosomes, dilated rough endoplasmic reticulums, highly developed Golgi complexes and decreased or disappeared thick filaments and dense bodies in the kytoplasm(Fig 1, 2). After NFA and IAA-94, the situations were reversed.

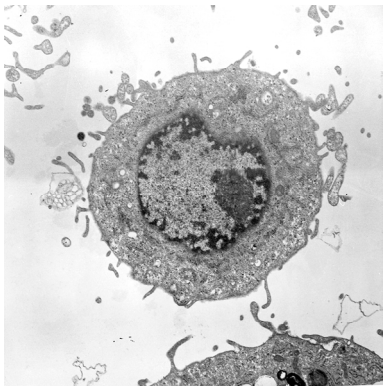

Fig 1 Contractile phenotype cell(Transmission electron microscope, × 6 300)

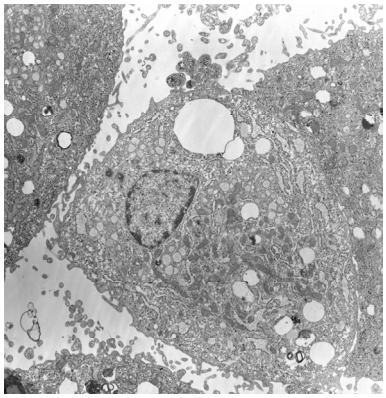

Fig 2 Synthetic phenotype cell(Transmission electron microscope, × 4 000)

Effects of block agents Cl_{Ca} on cell cycle of PASMCs

NFA and IAA-94 had no effect on the cell cycle of PASMCs during normoxia. The number of $S+G_2M$ PASMCs were significantly increased under chronic hypoxic conditions; The NFA and IAA-94 were shown to significantly decrease them, while the number of G_0G_1 PASMCs were significantly increased($P < 0.01$; Tab 1).

Effects of block agents Cl_{Ca} on the expression of PCNA in PASMCs

The positive staining is shown as a buffy deposition in cell nucleus(Fig 3, 4). NFA and IAA-94 had no effect on PCNA expression of PASMCs during normoxia. In the chronic hypoxic condition, the expression of PCNA was significantly increased; whereas the NFA and IAA-94 were shown to significantly decrease it($P < 0.01$; Tab 1).

Tab 1 Effects of Cl_{Ca} channel blockers on cell cycles and expressions of PCNA of PASMCs in normoxic and chronic hypoxic conditions

(n=10, %, $\bar{x} \pm s$)

Group	Number of G_0G_1 stage	Number of $S+G^2M$ stage	Positive frequency of PCNA
Nc	86.3 ± 1.1	13.6 ± 1.1	23 ± 3
N_{NFA}	84.6 ± 1.3	15.4 ± 1.3	25 ± 8
N_{IAA-94}	86.8 ± 1.2	13.3 ± 1.0	22 ± 2
Hc	71.4 ± 1.9*	28.6 ± 1.0*	81 ± 6*
H_{NFA}	83.6 ± 2.6#	16.4 ± 2.6#	29 ± 8#
H_{IAA-94}	83.9 ± 1.6#	16.0 ± 1.6#	27 ± 7#

Compaired with Nc group, *$P < 0.01$; compaired with Hc group, #$P < 0.01$.

Effects of block agents Cl_{Ca} on the expression of c-fos and c-jun in PASMCs

The positive staining of c-fos was a buffy deposition in kytoplasm, while the c-jun is displayed as a buffy deposition in the cell nucleus(Fig 5, 6). NFA and IAA-94 had no effect on AIOD during normoxia. In chronic hypoxic conditions, the expressions of c-fos and c-jun were significantly increased; However the NFA and IAA-94 were shown to significantly decrease them($P < 0.01$; Tab 2).

Tab 2 Effects of Cl_{Ca} channel blockers on expressions of c-fos and c-jun and $[Ca^{2+}]_i$ of PASMCs in normoxic and chronic hypoxic conditions

(n=10, $\bar{x} \pm s$)

Group	c-fos(A)	c-jun(A)	$[Ca^{2+}]_i$(nmol/L)
Nc	0.05 ± 0.01	0.10 ± 0.04	123.6 ± 18.9
N_{NFA}	0.06 ± 0.01	0.13 ± 0.01	111.1 ± 10.0
N_{IAA-94}	0.07 ± 0.02	0.15 ± 0.05	108.9 ± 19.3
Hc	0.15 ± 0.02*	0.32 ± 0.05*	281.8 ± 16.5*
H_{NFA}	0.06 ± 0.01#	0.16 ± 0.04#	124.4 ± 7.2#
H_{IAA-94}	0.05 ± 0.01#	0.12 ± 0.05#	117.7 ± 15.4#

Compaired with Nc group, *$P < 0.01$; compaired with Hc group, #$P < 0.01$.

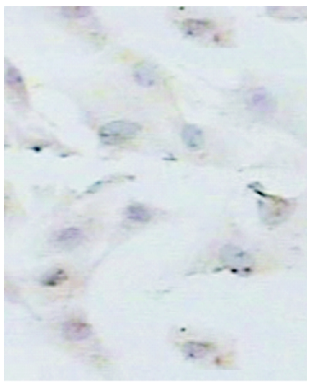

Fig 3 Nc group (SP, × 200)

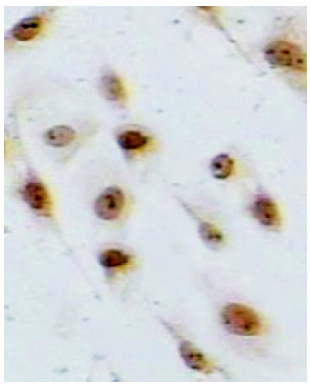

Fig 4 PCNA of Hc group (SP, × 200)

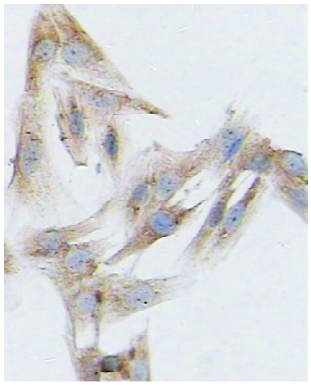

Fig 5 C-fos of Hc group (SP, × 200)

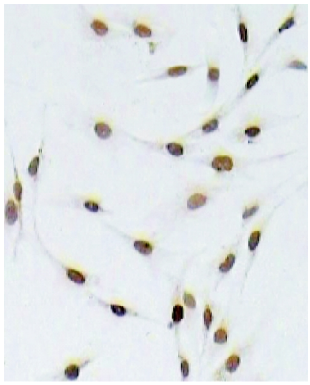

Fig 6 C-jun of Hc group (SP, × 200)

Effects of block agents Cl_{Ca} on $[Ca^{2+}]_i$ of PASMCs

NFA and IAA-94 had no effect on$[Ca^{2+}]_i$ during normoxia. In the chronic hypoxic condition, $[Ca^{2+}]_i$ was increased; The NFA and IAA-94 decreased it($P < 0.01$; Tab 2).

DISCUSSION

Pulmonary vascular remodeling caused by PASMCs hyperplasia is the main pathological change of HPH, therefore there it is vital to investigate the proliferation of PASMCs. Vessel smooth muscle cells can be divided into contractile and synthetic phenotypes. The former is dedifferentiated phenotype, the latter is differentiated one which occurs during cell development and with diseases. The two types can transform with each other and are modulated by cytokines and the extracellur matrix[9-10]. There is plenty of myofilament in the cytoplasm of shrink cells, including α-SM-actin. When the synthetic phenotype is found to be abundant in endoplasmic reticulum, mitochondria and Golgi complex(which participates in biosynthesis) and the myofilaments exist dispersedly the contractive ability is lost, but the ability of migration, proliferation and secretion is enhanced[11-12]. In our present study, we found hypoxia initiated the change of PASMCs from contractile to synthetic phenotype. NFA and IAA-94 could hamper this situation, which suggested that Cl_{Ca} may participate in the morphology of PASMCs.

Within some proteins such as calcitonin, the enzymes related to the origination and progress of DNA replication and PCNA correlates significantly with cell proliferation[13-14] and this is necessary for cells to transform from G_0G_1 phase to the S phase in cell cycle. PCNA is a kind of nuclear protein, which participates in DNA synthesis directly. PCNA acts as delta cofactors of DNA polymerase in DNA synthesis. It is a critical index to evaluate cell proliferation[15-16]. We found, in chronic hypoxic condition, the expression of PCNA enhanced, cell cycle was in advance, cell proliferative response enhanced, which was similar to the previous study[17]; NFA and IAA-94 could return the advanced cell cycle of PASMCs in vitro, attenuate the PCNA staining, which suggested Cl_{Ca} may take part in the proliferation of PASMCs.

In our present study:chronic hypoxia increased $[Ca^{2+}]_i$ while NFA and IAA-94 decreased it. This suggested ClCa channels can be activated by the increasing of $[Ca^{2+}]_i$. Ca^{2+} is an important second messenger[18]. In the brain and PASMCs cultured in vitro, the relationship between Ca^{2+} and cell proliferation is exhibited[19-20]: on one hand the increase of$[Ca^{2+}]_i$ can activate spectrin in kytoplasm; on the other hand it also activates P-CREB transcription factor in cell nucleus and increases the expression of c-fos rapidly. All these factors made the cell enter the cell cycle from G_0 stage and lead to cell proliferation. The expression of these Ca^{2+} dependent transcription factors played an important role in the differentiation and proliferation of PASMCs. This study suggested that the expression of PCNA increased and the cell cycle was in advance, and the PASMCs were synthetic phenotype; at the same time$[Ca^{2+}]_i$ increased and the expression of c-fos and c-jun was enhanced in chronic hypoxia(while NFA and IAA-94 could decrease them). The mechanism may be as follows:chronic hypoxia can increase$[Ca^{2+}]_i$, activate Cl_{Ca}, lead cytomembrane to depolarize, open voltage-dependent Ca^{2+} channels, activate the message transmission passageway about cell proliferation, induce rapid transcription and expression, promote the synthesis of DNA synthesis associated protein, and then finally promote cell proliferation; NFA and IAA-94 can inhibit Cl_{Ca}, close voltage-dependent Ca^{2+} channels, decrease the inflow of ecto-Ca^{2+}, degrade $[Ca^{2+}]_i$, and then step down cell proliferation.

In conclusion, in chronic hypoxia, the inhibition of Cl_{Ca} can decrease the abnormal increase of Ca^{2+} and inhibit cell proliferation. All these suggested Cl_{Ca} may participate in PASMCs proliferation, pachynsis and vessel wall remolding, which is important in investigating patho-

physiology changes and therefore provides a new trend for the prevention and treatment of HPH.

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