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Different Concentrations of Notoginsenoside R_{g1} Attenuate Hypoxic and Hypercapnia Pulmonary Hypertension by Reducing the Expression of ERK in Rat PASMCs

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Abstract

Pulmonary arterial hypertension (PAH) is a serious disease which is characterized by increased vascular resistance and pressure. We have previously hypothesized that panax notoginseng saponins (PNS) might attenuate pulmonary vasoconstriction under hypoxia and hypercapnia condition. This study aims to investigate the effect of notoginsenoside R_{g1}, a main ingredient of PNS, with various concentrations (8, 40, 100 mg/L, respectively) on extracellular signal regulated kinase (ERK1/2) signaling pathway in pulmonary arterial smooth muscle cells (PASMCs). In addition, PASMCs were randomly divided into six groups: SD rat under normoxic condition as control group (N group), hypoxia hypercapnia group (H group), DMSO control group (HD group), R_{g1}-treatment groups ($R_{gL}R_{gM}$ and R_{gH} group). Western-blot and RT-PCR were used to test the expression of p-ERK protein and the expression of ERK1 mRNA and ERK2 mRNA. This study provided the evidence that the expression of p-ERK protein and the expression of ERK1 mRNA and ERK2 mRNA in HD group and H group were obviously higher than that in N group ($P < 0.01$), Whereas the level of ERK1/2 mRNA in R_{g1}-treatment groups was significantly lower than that in HD group and H group ($P < 0.01$), and the proper concentration of R_{g1} is 40 mg/L. These results suggested that notoginsenoside R_{g1} can attenuate pulmonary vasoconstriction which may lead to HHPV through reducing the expression of ERK1/2.

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Keywords

Pulmonary Arterial Smooth Muscle Cells, Hypoxia Hypercapnia, ERK1/2 Signal Pathway, Notoginsenoside R_{g1}, Rats

1. Introduction

Hypoxic pulmonary vasoconstriction (HPV), is a physiological response to alveolar hypoxia which distributes pulmonary capillary blood flow to alveolar areas of high oxygen partial pressure. Whereas, impairment of this mechanism may result in hypoxaemia, and the pulmonary vasculature which was associated with hypoxia-induced vascular remodeling and pulmonary vasoconstriction leads to pulmonary hypertension, the above was the basic mechanism of pulmonary arterial hypertension (PAH) [1]. Recent studies have shown that PAH is a poor prognosis with pulmonary vasoconstriction and pulmonary vascular remodeling [2]-[4]. Therefore, studying the mechanism of hypoxia hypercapnia-induced pulmonary arterial hypertension (HHPH) has a great significance in treating pulmonary disease.

The extracellular signal regulated kinase (ERK) is one of three major members of the MAPK family and it is an important step in signal transduction of several growth factors, mitogens, neurotransmitters, and hormones [5]. ERK1/2 plays a central role in cell proliferation control and also regulates cell growth [6]. Some studies suggest that ERK signal pathway is associated with the mitogenesis and migration of PASMCs through various factors which may lead to pulmonary vascular remodeling [6] [7].

Panax notoginseng Buck F. H. Chen (Araliaceae) is one of the most widely used traditional Chinese herbal medicines for the treatment of vessel dilation, myocardial consumption of oxygen reduction, platelet aggregation inhibition, free radicals removal, and antioxidation, to name a few [8]. The main effective components are ginsenoside R_{g1}, ginsenoside R_{b1}, and notoginsenoside R_1 [9]-[11] (**Figure 1**). Ginsenosides R_{g1} are bioactive compounds which extracted from Cape Jasmine Fruit (Fructus Gardeniae) and Sanchi (Radix Notoginseng) [12]. The effectiveness of PNS in attenuating chronic hypoxic pulmonary hypertension in rats has previously been reported [13]. It has been showed that ginsenoside R_{g1} positively affects myocardial remodeling and pulmonary hemodynamics [14]. Although PNS has effect in attenuating hypoxia hypercapnia-induced pulmonary vasoconstriction (HHPV) [15], the mechanism of how ginsenoside R_{g1} prevents HHPV and the form of PAH has never been reported. In this study, we investigated the effect of different concentrations of notoginsenoside R_{g1} in

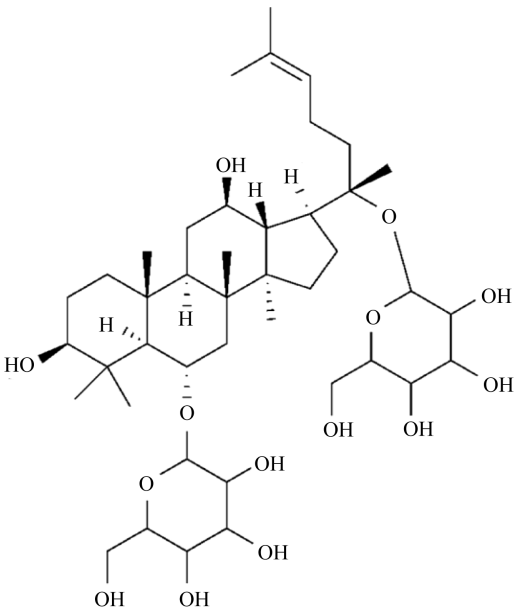


Figure 1. The chemical structure of R_{g1}.

PASMCs under hypoxia and hypercapnia condition and intended to explore the mechanism of R_{g1} which had effect on PASMCs through observing the expression of p-ERK protein and the expression of ERK1 mRNA and ERK2 mRNA.

2. Materials and Methods

2.1. Animal Preparation

Adult male Sprague Dawley (SD) rats, weighing 210 g to 300 g, were provided by the Experimental Animal Center of Wenzhou Medical University, China, Animal license NO: SYXK (Zhejiang 2009-0129).

2.2. Medicine and Reagents

Notoginsenoside R_{g1} (>98% pure) was provided by the Department of Organic Chemistry, College of Preclinical Medicine, Jilin University (Jilin, China), and it was preserved in DMSO at 4˚C. High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovinserum (FBS) from Gibco (US). BCA protein assay kit and ECL assay kit was purchase from Pierce (US). SABC-FITC (POD) kit, RT-PCR kit DAB staining kit, monoclonal antibody to SM-alpha-actin were purchase from Takara Biotechnology (Dalian, China). P-ERK, Anti-ERK monoclonal antibodies and goat anti-rabbit IgG/HRP were purchased from Cell Signal Technology (Genetimes Technology Inc. Shanghai, China). RT-PCR kit from TAKARA (Japan).

2.3. Cell Culture

Rat primary PASMCs were cultured from micro-dissected segments of pulmonary arterial as described elsewhere [16]. Cells were grown in DMEM with 10% FBS, 100 units/ml penicillin, 100 units/ml streptomycin at 37˚C in a 5% CO_2 atmosphere and split 1:2 at 80% confluence. PASMCs after reaching 70% - 80% confluence were used in the test.

Cells were starved at least day and night in serum-free DEME before the medicine treatment. This step aims to stop the growth of cells, and make sure that all the cells were at the same starting line before the intervention. The cells were randomly divided into six groups: 1) normoxia group (N group, 5% CO_2, 21% O_2, 24 h). 2) DESO control group with hypoxia and hypercapnia (HD group, 0.05% DMSO with 6% CO_2, 1% O_2, 24 h). 3) hypoxia and hypercapnia (H group, 6% CO_2, 1% O_2, 24 h). 4) different concentrations of notoginsenoside R_{g1} group (R_{gL}, R_{gM}, R_{gH}, represent the concentrations of 8, 40, 100 mg/L, respectively, 6% CO_2, 1% O_2, 24 h).

2.4. Immunoblotting

Cultured cells were incubated in ice-cold RAPI lysate (containing 1 mM PMSF and 10 mM NaF) after washing three times with cold PBS for 30 min on ice to make sure the fully lysating. Then the lysates were centrifuged for 5 min at 12000 rpm to distinguish and remove uncracked cells. The protein concentration was assessed with the BCA as the standard. Protein samples were separated on a 10% SDS-polyacrylamidegel, and then the proteins were transferred into PVDF membranes. The cells were incubating with a phosphorylation of ERK1/2 antibody (1:2000) for 24 hrs after blocking the blots with 5% skim milk. Then washing cells with TBST thrice, incubating cells with anti-rabbit IgG (1:4000) for 1 hrs. The immunereactive bands were detected with the ECL.

2.5. Real-Time PCR

After collecting the cells, RNA was isolated on ice and reverse transcription for compounding cDNA, yielding predicted products of 373 and 394 bp, then PCR was performed as previously described [17]. Primer sequences are presented in **Table 1**. Cycling conditions were as follows: denaturation at 94˚C for 2 min, and then 30 cycles of reactions of reactions of denaturation at 98˚C for 10 s, annealing at 54˚C for 15 s, and elongation at 72˚C for 1 min, followed by a 10 min extension stage at 72˚C.

2.6. Statistical Analyses

Data were expressed as the mean + SEM, and statistical analysis was performed by SPSS17.0. For more than two groups, data were performing with the one-way ANOVA test. $P < 0.05$ was considered significant.

3. Results

Hypoxia and hypercapnia activate the phosphorylation of ERK-1/2, and notoginsenoside R_{g1} attenuates this effect.

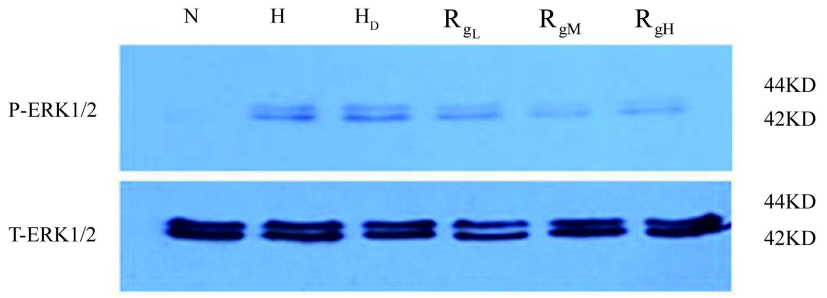
According to western blot analysis (**Figure 2**), the p-ERK1/2 protein expression increased limit in normoxia group. Moreover, the level of p-ERK1/2 protein expression was obviously increased in H group and HD group as compared with N group ($P < 0.05$), whereas notoginsenoside R_{g1} reduced the release of p-ERK1/2 at all three doses ($P < 0.05$). We concluded that notoginsenoside R_{g1} could attenuated the phosphorylation of p-ERK1/2 in PASMCs which under hypoxia and hypercapnia condition, and the optical dose was 40 mg/L.

Notoginsenoside R_{g1} reduced the levels of expression of ERK1 mRNA and ERK2 mRNA in rats PASMCs.

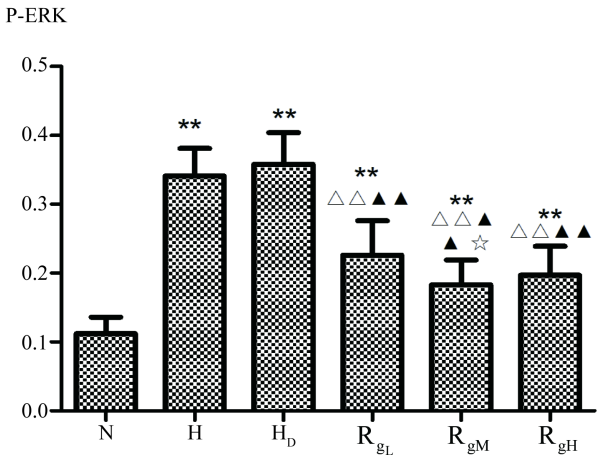
To test whether Notoginsenoside R_{g1} reduced the expression of ERK1 mRNA and ERK2 mRNA, we performed RT-PCR for ERK1 mRNA and ERK2 mRNA (**Figure 3**). We found apparently increased of the level of

Table 1. Primers sequences.

	Primers primers sequences
ERK1	(Forward) 5'-GCTGAATCA CAT CCTGGG TAT-3' (Reverse) 5'-AGA TCTGTATCCTGGCTGGAA-3'
ERK2	(Forward) 5'-GCAGGTGTTCGACGTGGGAAT-3' (Reverse) 5'-GTG CAG AAC ATT AGGTGA ATA-3'
β-actin	(Forward) 5'-GAG ACCTTCAACACCCCAGCC-3' (Reverse) 5'-TCGGGG GAT CGGAACCGCTCA-3'



(a)



(b)

Figure 2. The protein expression of p-ERK in R_{g1} and control groups of rat PVSMCs. (a) The protein expression of p-ERK by the analysis of western blot; (b) Comparison of the protein expression of p-ERK in different groups. Data are expressed as means ± standard error of the mean. $n = 8$ per group. $^{**}P < 0.01$ compared with N group. $^{\triangle\triangle}P < 0.01$ compared with H group. $^{\blacktriangle\blacktriangle}P < 0.01$ compared with HD group. $^{\star}P < 0.05$ compared with R_L group.

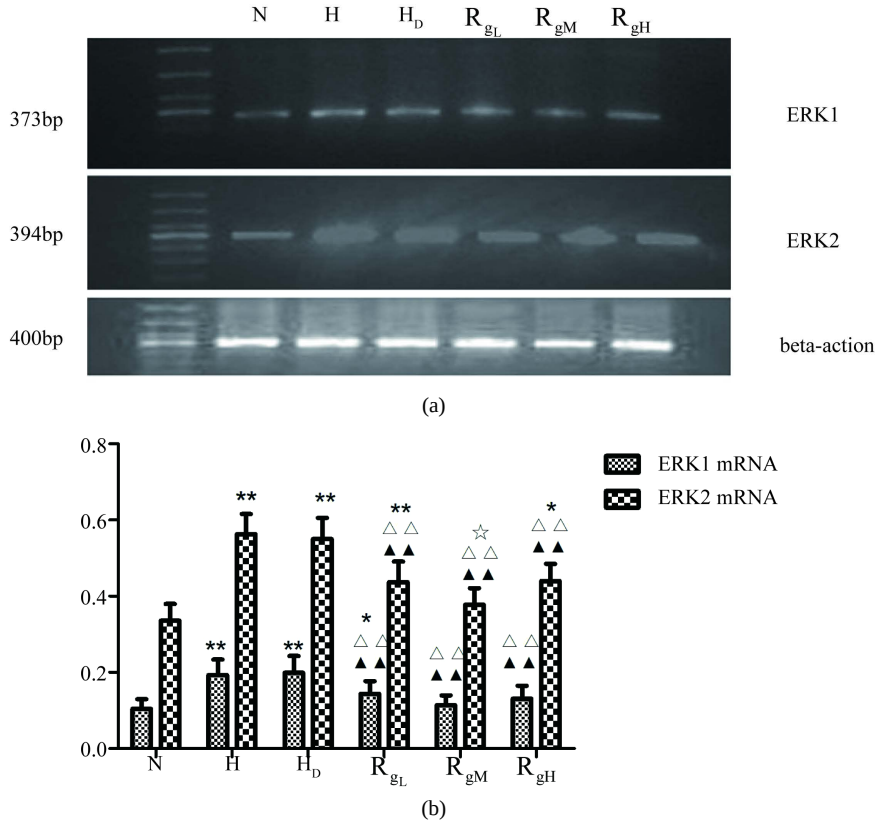


Figure 3. The mRNA expression of ERK1, ERK2 in R_{g1} and control groups of rat PASMCs (a). The mRNA expression of ERK1, ERK2 by the analysis of RT-PCR (b). Comparison of the mRNA expression of ERK1, ERK2 in different groups. Data are expressed as means ± standard error of the mean. $n = 8$ per group. $^{*}P < 0.05$ compared with N group. $^{**}P < 0.01$ compared with N group. $^{\triangle\triangle}P < 0.01$ compared with H group. $^{\blacktriangle\blacktriangle}P < 0.01$ compared with HD group. $^{\star}P < 0.05$ compared with R_L group.

ERK1 mRNA and ERK2 mRNA in H group and HD group compared to N group ($P < 0.05$). RT-PCR showed the evidence that three doses of Notoginsenoside R_{g1} resulted in a significant decrease in the expression of ERK1 mRNA and ERK2 mRNA ($P < 0.01$). We concluded that the invention of Notoginsenoside R_{g1} in rats PASMCs made a obviously decline in the expression of ERK1 mRNA and ERK2 mRNA.

4. Discussion

We have successfully built a cell model of pulmonary hypertension by exposing PASMCs under hypoxia and hypercapnia condition. Some studies have previously demonstrated that ERK signaling pathway, which belongs to MAPK family, played a pivotal role in the pathogenesis of pulmonary vasoconstricton [18]. Furthermore, it has been confirm that ERK signaling pathway was associated with the cell proliferation [19]. A additional study showed that ERK1/2 signaling pathway modulated the proliferation and migration of PASMCs induced by hypoxia [20] [21].

Hypoxic and hypercapnia pulmonary hypertension (HHPH) was a disease with poor prognosis, its clinical symptoms were apparent, such as dyspnea, right-sided heart failure, and it was characterized by an increase resistance in pulmonary artery [22]. A large number of researches have devoted much time and energy into revealing the specific mechanisms; however, the results still remain unknown. Some researchers consider that hypoxia and hypercapnia-induced the release of vasoactive substances such as endothelin, nitrogen monoxidum (NO), and it ultimately leads to the contraction of blood vessels. In our study, we found that the obviously increased expression of ERK1/2 mRNA and phosphorylation of ERK 1/2 under hypoxia and hypercapnia condition. This suggested that ERK1/2 signaling pathway was associated with the mechanism of HHPV in the cellular

level through inhibiting K^+ entry [20] and decreasing Na, K-ATPase activity [23], that may ultimately leads to the constriction of PASMCs.

5. Conclusion

In summary, we have previously showed that PNS can relieve the pulmonary rings in rats under hypoxia and hypercapnia condition [24]. Our study demonstrated that the expression of ERK1/2 mRNA and phosphorylation of ERK 1/2 in rats PASMCs which was culcured by Notoginsenoside R_{g1} had an apparently decline compared with H group and HD group. In addition, we also found hypoxia and hypercapnia activated MAPKs signal pathway in rats PASMCs and this effect could be reduced by culturing with Notoginsenoside R_{g1}. These findings provided clues that Notoginsenoside R_{g1} might be employed as a new strategy to prevent HHPH and open novel perspectives in the cure of other lung disease.

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