Original Article

RELMα can cause contraction of rat aortic smooth muscle cells

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Abstract: Objective: This study aims to observe the contraction role of RELMα in rat aortic smooth muscle cells and explore its mechanism. Methods: Rat aortas smooth muscle cells were cultivated using tissue explants method. They were divided into 5 groups. A: Control group; B: 1×10^{-7} mol/L ANGII group; C: 1×10^{-8} mol/L RELMα group; D: 2×10^{-8} mol/L RELMα group; E: 4×10^{-8} mol/L RELMα group. The thoracic aortic tension signal of rats was recorded by Powerlab system. The expression levels of CaM and MLCK were detected by western blotting and RT-PCR methods. Results: Tension changes of rat thoracic aorta vascular ring in group C, D and E (72±2.98%, 76.65±2.73%, 85.07±3.06% respectively) were higher than that of group A and B (6.35±0.75%, 61.47±4.47%) with dose-dependent (P<0.01). The expression levels of CaM and MLCK in group C were higher than that of group A and B while were lower than that of group D and E (P<0.05). The expression levels of CaM and MLCK in group E were the highest among the groups (P<0.05). Conclusions: RELMα can cause contraction of rat aortic smooth muscle cells, its mechanism may be via Ca^{2+}-CaM-MLCK pathway.

Keywords: Aortas, smooth muscle cells, vascular ring, RELMα, cell contraction

Introduction

Resistin-like molecules α (RELMα), formerly known as found in inflammatory zone 1 (FIZZ1), is a hypoxia induced mitogenic factor (HIMF) related with inflammation. Firstly, RELMα was found to play an important role in pulmonary vascular remodeling in the model of hypoxia pulmonary hypertension. Decreased RELM concentration can reduce the mean pulmonary artery pressure pulmonary vascular resistance and remodeling caused by chronic hypoxia in rats. RELMα has been found in hypoxia pulmonary vascular wall, macrophages and in the stromal vascular fraction of adipose tissue recently. RELMα is a pulmonary vascular agent with more advantage than endothelin-1, angiotensin II and 5-HT, but its function mechanism is still unknown [1-8].

In this study, we used recombinant RELMα protein to stimulate isolated artery ring to observe the contraction role of RELMα in rat aortic smooth muscle cells and explore its mechanism.

Materials and methods

Cell culture

Rat aortic smooth muscle cells (RASMC) were purchased from ScienCell Research Laboratories. The cells were grown in DMEM high glucose medium supplemented with 10% fetal bovine serum, 100 µg/ul of penicillin and streptomycin. They were cultured at 37°C with 5% CO₂. They were divided into 5 groups. A: Control group, culture for 48 h; B: intervened culture with 1×10^{-7} mol/L ANGII for 48 h; C: intervened culture with 1×10^{-8} mol/L RELMα for 48 h; D: intervened culture with 2×10^{-8} mol/L RELMα for 48 h; E: intervened culture with 4×10^{-8} mol/L RELMα for 48 h.

Tension determination of vascular ring

SD rat was anesthetized with 10% chloral hydrate, the chest is open after execution and
The thoracic aorta was isolated. The isolated thoracic aorta was placed into pre-saturated with 95% O₂ and 5% CO₂ mixed gas K-H buffer at 4°C and the connective tissue around the thoracic aorta was eliminated carefully. Finally it was cut into 3-4 mm vascular ring. In this study we used de-endothelium vascular rings and their tension changes were recorded with Powerlab four channel physiological instrument. RELMα and ANGII were added to test their effects on the vascular ring. The calculation formula of the tension changes = (tension values after drug administration-tension values before drug administration)/ tension values before drug administration ×100%.

The expression levels of calmodulin (CaM) and myosin light chain kinase (MLCK) mRNA in different groups detected by RT-PCR

Total RNA of cells was extracted using RNeasy Mini Kit according to the manufacturer’s protocol. 1 μg total RNA was subjected to reverse transcription using reverse transcription kit. Fluorescence quantitative PCR was carried out using SYBR RT-PCR kit according to the manufacturer’s protocol. B-actin was used as interior reference. The reaction conditions: 95°C pre-denatured for 3 min, 95°C denatured for 20 s, annealing temperature is 58°C for 20 s, 72°C extends 20 s, 35 cycles.

Protein extraction and western blotting

Total proteins of cells in different groups were extracted and analyzed with SDS-PAGE electrophoresis. Then it was electrotransferred to the PVDF membrane. After the transmembrane, PVDF membrane was rinsed with TBS for 10 to
RELMα and cellular contraction

15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaked at room temperature for one hour. It was incubated at room temperature for two hours after added with appropriate dilution degree of primary antibody (diluted with TBST containing 1% (w/v) skimmed milk powder). Then the membrane was rinsed with TBST for three times (5 to 10 minutes one time). The membrane was incubated at room temperature for one hour with HRP labeled secondary antibody (1: 10000) diluted with TBST containing 0.05% (w/v) skimmed milk powder and rinsed for three times with TBST (5 to 10 minutes at a time). The protein bands were scanned and quantified as a ratio to β-actin.

**Statistical analysis**

All statistical analyses were performed using SPSS version 17.0 statistical software. Data were expressed as means ± standard deviations (SD). Differences among groups were analyzed using variance analysis. The categorical data were analyzed using chi square test. Values of \( P<0.05 \) were considered statistically significant.

**Results**

*Tension determination of vascular ring*

The de-endothelium thoracic aortic ring can obviously be contracted by RELMα under the extracellular fluid containing calcium conditions. The graph of electrorecord was shown in **Figure 1**. Vascular tension began to increase after drug administration for 2 min and reached a plateau about 10 min. The vascular tension changes of group A, B, C, D and E were 6.35±0.75\%, 61.47±4.47\%, 72±2.98\%, 76.65±2.73\% and 85.07±3.06\% respectively. Tension changes in group C, D and E were higher than that of
The effects of recombinant RELMα on the CaM

The results of Western blotting were shown in Figure 3. It showed that the normalized expression levels of CaM in group A, B, C, D and E were 0.25±0.06, 0.30±0.08, 0.48±0.05, 0.64±0.04 and 1.17±0.12 respectively. The expression levels of CaM increased significantly after RELMα administration and in a dose-dependent manner. Compared with control group, the differences were significant (P<0.05).

The effects of recombinant RELMα on the MLCK

As shown in Figure 4, the normalized expression levels of MLCK in group A, B, C, D and E were 0.26±0.06, 0.29±0.07, 0.45±0.12, 0.59±0.14 and 1.10±0.12 respectively. The expression levels of MLCK also increased significantly after RELMα and cellular contraction

Figure 5. RT-PCR of CaM in different groups. A: Control group; B: 1×10⁻⁷ mol/L ANGII group; E: 4×10⁻⁸ mol/L RELMα group. *, #P<0.05, compared with control group.

Figure 6. RT-PCR of MLCK in different groups. A: Control group; B: 1×10⁻⁷ mol/L ANGII group; E: 4×10⁻⁸ mol/L RELMα group. *, #P<0.05, compared with control group.
after RELMα administration and also in a dose-dependent manner. Compared with control group, the differences were significant (P<0.05).

**RT-PCR results**

RT-PCR results were shown in Figures 5 and 6. We found that the normalized expression levels of CaM mRNA in group A, B and E were 0.30±0.08, 0.66±0.23 and 1.1±0.23 respectively, the normalized expression levels of MLCK mRNA in group A, B and E were 2.01×10^{-4}±1.55×10^{-4}, 4.85×10^{-4}±1.24×10^{-4} and 7.87×10^{-4}±2.80×10^{-4} respectively. RELMα could increase the expression levels of CaM and MLCK significantly (P<0.05).

**Discussion**

RELMα is a kind of cytokines belongs to secreted protein family. There is a highly conserved cysteine sequence at the C-end [9, 10]. It increased in adipose stromal vascular and lung tissue with inflammatory reaction mediated by chronic hypoxia and Th2 [11-14]. Previous studies found that RELMα has the functions of promoting proliferation, angiogenesis, vascular contraction and chemokine [7, 15, 16]. It plays important roles in pulmonary arterial hypertension [17], bronchial asthma [18], pulmonary fibrosis [19], silicosis and atherosclerosis [20]. The distribution of RELMα has obvious specificity and widely exist in vascular smooth muscle cells and endothelial cells, monocytes, activated macrophages and atherosclerotic plaque. RELMα increased when inducing by IL-4 and IL-13.

In this study, we observed the contraction effect of RELMα on blood vessels using the isolated perfused vascular ring method to elucidate the direct role of RELMα on cardiovascular system. We found that the contraction effect of RELMα was stronger than that of ANGII and in a dose-dependent manner. Fan [21] found that RELMα can induce the increase of intracellular calcium concentration after stimulating human pulmonary artery smooth muscle cells, which was regulated by PLC-IP3 pathway. This process is continuous and dynamic, while the process of the increase of intracellular calcium concentration inducing by ANGII was rapid and transient. Ca^{2+} is an important second messenger in cells and an important molecular basis of vascular contraction [22]. The regulatory mechanism of vascular smooth muscle contraction mainly includes Ca^{2+}, CaM and MLCK. CaM was activated when the intracellular calcium concentration increased, MLCK was activated after combination of activated CaM and Ca^{2+} and MLCK which causing the phosphorylation of 20kD myosin light chain (MLC_{20}) and leading to vascular smooth muscle contraction. Chen [23] found that FIZZ1 can enhance the response to airway smooth muscle contraction by up-regulating the expression levels of MLCK and MLC_{20}. FIZZ1 may cause tracheal epithelial injury after treatment of airway by FIZZ1 and activate c-Raf-ERK1/2-p38MAPK signal transduction pathway to contract airway smooth muscle.

In this study, we explored the mechanism of contraction in rat aortic smooth muscle cells caused by RELMα. We found that the expression levels of CaM and MLCK increased significantly after RELMα administration and in a dose-dependent manner. Compared with control and ANGII group, the differences were significant (P<0.05). They were the highest in 40 nmol/L RELMα group. These results suggested that RELMα caused contraction of rat aortic smooth muscle cells may through Ca^{2+}-CaM-MLCK pathway.

In summary, we found that RELMα can cause contraction of vascular smooth muscle through Ca^{2+}-CaM-MLCK pathway, which may play an important role in the occurrence and development of vascular spasm in patients with coronary heart disease. It provides new targets for clinical prevention and treatment of coronary heart disease.

**Disclosure of conflict of interest**

None.

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