

## Original Article

# RELM $\alpha$ can cause contraction of rat aortic smooth muscle cells

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**Abstract:** Objective: This study aims to observe the contraction role of RELM $\alpha$  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:  $1 \times 10^{-7}$  mol/L ANGII group; C:  $1 \times 10^{-8}$  mol/L RELM $\alpha$  group; D:  $2 \times 10^{-8}$  mol/L RELM $\alpha$  group; E:  $4 \times 10^{-8}$  mol/L RELM $\alpha$  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 ( $72 \pm 2.98\%$ ,  $76.65 \pm 2.73\%$ ,  $85.07 \pm 3.06\%$  respectively) were higher than that of group A and B ( $6.35 \pm 0.75\%$ ,  $61.47 \pm 4.47\%$ ) 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: RELM $\alpha$  can cause contraction of rat aortic smooth muscle cells, its mechanism may be via Ca<sup>2+</sup>-CaM-MLCK pathway.

**Keywords:** Aortas, smooth muscle cells, vascular ring, RELM $\alpha$ , cell contraction

## Introduction

Resistin-like molecules  $\alpha$  (RELM $\alpha$ ), formerly known as found in inflammatory zone 1 (FIZZ1), is a hypoxia induced mitogenic factor (HIMF) related with inflammation. Firstly, RELM $\alpha$  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. RELM $\alpha$  has been found in hypoxia pulmonary vascular wall, macrophages and in the stromal vascular fraction of adipose tissue recently. RELM $\alpha$  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 RELM $\alpha$  protein to stimulate isolated artery ring to observe the contraction role of RELM $\alpha$  in rat aortic

smooth muscle cells and explore its mechanism.

## Materials and methods

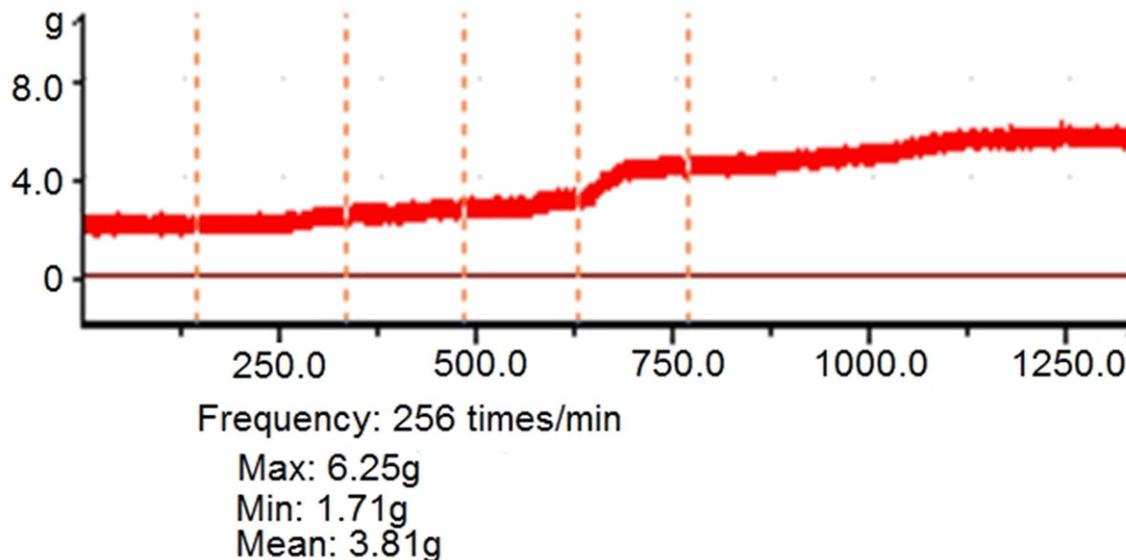
### Cell culture

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  $\mu\text{g}/\text{ul}$  of penicillin and streptomycin. They were cultured at 37°C with 5% CO<sub>2</sub>. They were divided into 5 groups. A: Control group, culture for 48 h; B: intervened culture with  $1 \times 10^{-7}$  mol/L ANGII for 48 h; C: intervened culture with  $1 \times 10^{-8}$  mol/L RELM $\alpha$  for 48 h; D: intervened culture with  $2 \times 10^{-8}$  mol/L RELM $\alpha$  for 48 h; E: intervened culture with  $4 \times 10^{-8}$  mol/L RELM $\alpha$  for 48 h.

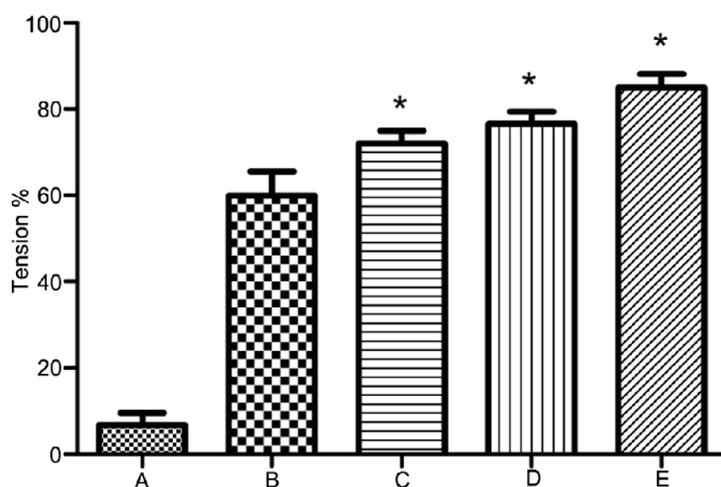
### Tension determination of vascular ring

SD rat was anesthetized with 10% chloral hydrate, the chest is open after execution and

## RELM $\alpha$ and cellular contraction



**Figure 1.** The graph of electro-record of muscle cell contraction.



**Figure 2.** The vascular tension changes in different groups. A: Control group; B:  $1 \times 10^{-7}$  mol/LANGII group; C:  $1 \times 10^{-8}$  mol/L RELM $\alpha$  group; D:  $2 \times 10^{-8}$  mol/L RELM $\alpha$  group; E:  $4 \times 10^{-8}$  mol/L RELM $\alpha$  group. \* $P < 0.01$  vs control.

thoracic aorta was isolated. The isolated thoracic aorta was placed into pre-saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> 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. RELM $\alpha$  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  $\times 100\%$ .

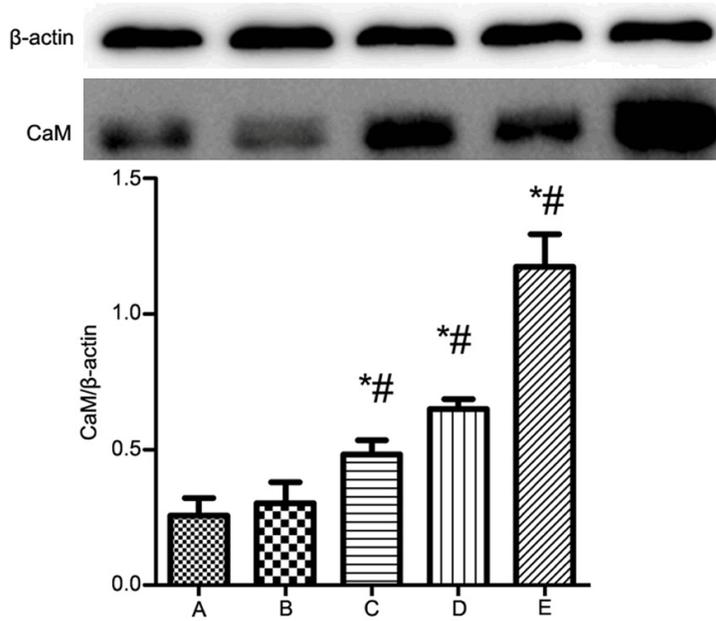
*The expression levels of calmodulin (CaM) and myosin light chain kinase (MLCK) mRNA in different groups detected by RT-PCR*

Total RNA of cells was extracted using RNeasy Mini Kit according to the manufacturer's protocol. 1  $\mu$ g 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

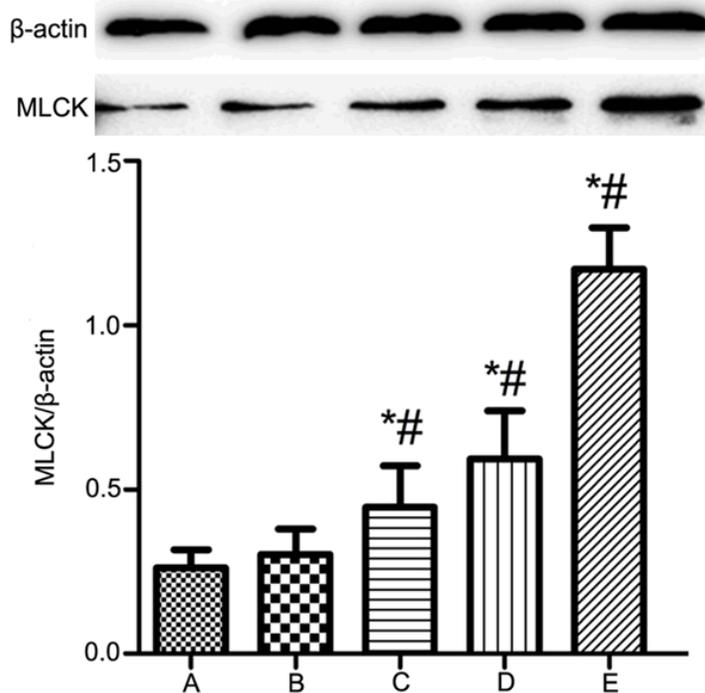
was used as interior reference. The reaction conditions: 95°C pre-denatured for 3 min, 95°C denatured for 20 s, annealing temperature is 58°C for 20 s, 72°C extends 20 s, 35 cycles.

### *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



**Figure 3.** The expression of CaM in different groups. A: Control group; B: 1×10<sup>-7</sup> mol/LANGII group; C: 1×10<sup>-8</sup> mol/L RELM $\alpha$  group; D: 2×10<sup>-8</sup> mol/L RELM $\alpha$  group; E: 4×10<sup>-8</sup> mol/L RELM $\alpha$  group. \*P<0.05 vs control, #P<0.05 vs ANGII.



**Figure 4.** The expression of MLCK in different groups. A: Control group; B: 1×10<sup>-7</sup> mol/LANGII group; C: 1×10<sup>-8</sup> mol/L RELM $\alpha$  group; D: 2×10<sup>-8</sup> mol/L RELM $\alpha$  group; E: 4×10<sup>-8</sup> mol/L RELM $\alpha$  group. \*P<0.05 vs control, #P<0.05 vs ANGII.

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  $\beta$ -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.

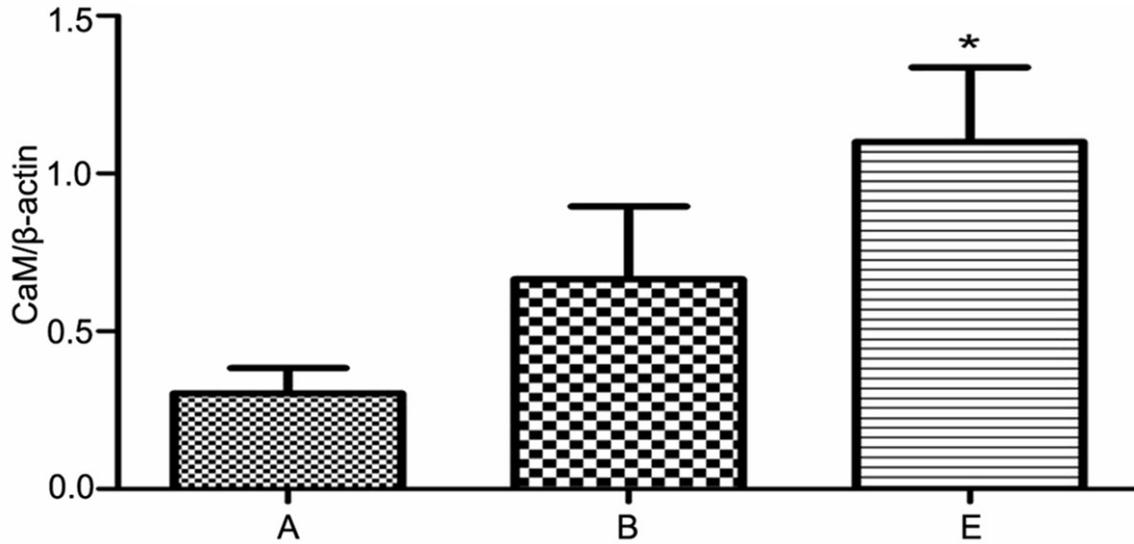
**Results**

*Tension determination of vascular ring*

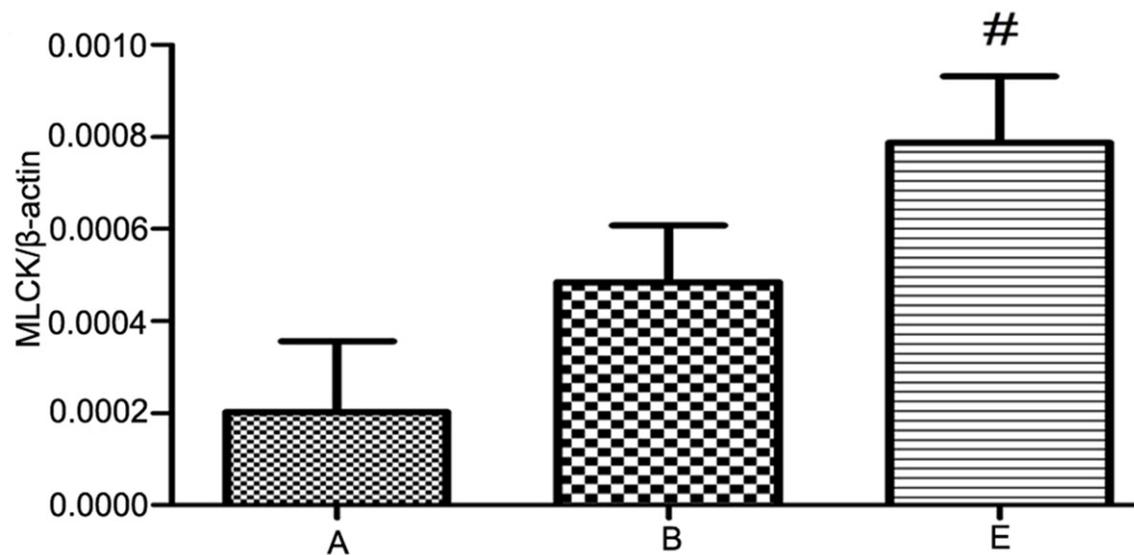
The de-endothelium thoracic aortic ring can obviously be contracted by RELM $\alpha$  under the extracellular fluid containing calcium conditions. The graph of electro-record was shown in **Figure 1**. Vascular tension began to increase after drug administration for 2 min and reached a plateau about 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

15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and

and 85.07±3.06% respectively. Tension changes in group C, D and E were higher than that of



**Figure 5.** RT-PCR of Cam in different groups. A: Control group; B:  $1 \times 10^{-7}$  mol/LANGII group; E:  $4 \times 10^{-8}$  mol/L RELM $\alpha$  group. \*, #P<0.05, compared with control group.



**Figure 6.** RT-PCR of MLCK in different groups. A: Control group; B:  $1 \times 10^{-7}$  mol/LANGII group; E:  $4 \times 10^{-8}$  mol/L RELM $\alpha$  group. \*, #P<0.05, compared with control group.

group A and B with dose-dependent ( $P < 0.01$ ), it was the highest in group E (**Figure 2**).

*The effects of recombinant RELM $\alpha$  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  $0.25 \pm 0.06$ ,  $0.30 \pm 0.08$ ,  $0.48 \pm 0.05$ ,  $0.64 \pm 0.04$  and  $1.17 \pm 0.12$  respectively. The expression levels of CaM increased significantly after

RELM $\alpha$  administration and in a dose-dependent manner. Compared with control group, the differences were significant ( $P < 0.05$ ).

*The effects of recombinant RELM $\alpha$  on the MLCK*

As shown in **Figure 4**, the normalized expression levels of MLCK in group A, B, C, D and E were  $0.26 \pm 0.06$ ,  $0.29 \pm 0.07$ ,  $0.45 \pm 0.12$ ,  $0.59 \pm 0.14$  and  $1.10 \pm 0.12$  respectively. The expression levels of MLCK also increased significantly

after RELM $\alpha$  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  $0.30 \pm 0.08$ ,  $0.66 \pm 0.23$  and  $1.1 \pm 0.23$  respectively, the normalized expression levels of MLCK mRNA in group A, B and E were  $2.01 \times 10^{-4} \pm 1.55 \times 10^{-4}$ ,  $4.85 \times 10^{-4} \pm 1.24 \times 10^{-4}$  and  $7.87 \times 10^{-4} \pm 2.80 \times 10^{-4}$  respectively. RELM $\alpha$  could increase the expression levels of CaM and MLCK significantly ( $P < 0.05$ ).

### Discussion

RELM $\alpha$  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 RELM $\alpha$  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 RELM $\alpha$  has obvious specificity and widely exist in vascular smooth muscle cells and endothelial cells, monocytes, activated macrophages and atherosclerotic plaque. RELM $\alpha$  increased when inducing by IL-4 and IL-13.

In this study, we observed the contraction effect of RELM $\alpha$  on blood vessels using the isolated perfused vascular ring method to elucidate the direct role of RELM $\alpha$  on cardiovascular system. We found that the contraction effect of RELM $\alpha$  was stronger than that of ANGII and in a dose-dependent manner. Fan [21] found that RELM $\alpha$  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<sup>2+</sup> 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<sup>2+</sup>, CaM and MLCK. CaM was activated when the intracellular calcium concentration increased, MLCK was activated after combination of activated CaM and Ca<sup>2+</sup> and MLCK which causing the phosphorylation of 20kD myosin light chain (MLC<sub>20</sub>) 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<sub>20</sub>. 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 RELM $\alpha$ . We found that the expression levels of CaM and MLCK increased significantly after RELM $\alpha$  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 RELM $\alpha$  group. These results suggested that RELM $\alpha$  caused contraction of rat aortic smooth muscle cells may through Ca<sup>2+</sup>-CaM-MLCK pathway.

In summary, we found that RELM $\alpha$  can cause contraction of vascular smooth muscle through Ca<sup>2+</sup>-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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