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Inhibitory effect of ginsenoside Rb_1 on calcineurin signal pathway in cardiomyocyte hypertrophy induced by prostaglandin $F_{2\alpha}$[1]

Qing-song JIANG[2], Xie-nan HUANG[3], Gui-zhong YANG[4], Xiao-yan JIANG[5], Qi-xin ZHOU[2,6]

[2]Department of Pharmacology, Chongqing Medical University, Chongqing 400016, China; [3]Department of Pharmacology, Zunyi Medical College, Zunyi 563003, China; [4]Department of Biochemistry, Zunyi Medical College, Zunyi 563003, China; [5]Chengdu Vocational College of Agricultural Science and Technology, Chengdu 611100, China

Key words

Rb_1; prostaglandin $F_{2\alpha}$; cardiac hypertrophy; calcineurin

[1] Project supported by the Science Foundation of Guizhou Province, China (No 2004-3057).
[6] Correspondence to Prof Qi-xin ZHOU.
Phn 86-23-6848-5038.
E-mail cqzhouqx@yahoo.com.cn

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Abstract

Aim: To examine the antihypertrophic effect of ginsenoside Rb_1 (Rb_1) induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) *in vitro* and to investigate the possible mechanisms involved in the calcineurin (CaN) signal transduction pathway. **Methods:** The cardiomyocyte hypertrophy induced by $PGF_{2\alpha}$ and the antihypertrophic effect of Rb_1 were evaluated in primary culture by measuring the cell diameter, protein content, and atrial natriuretic peptide (ANP) mRNA expression. ANP and CaN mRNA expressions, CaN and its downstream effectors $NFAT_3$ and $GATA_4$ protein expressions, and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) were assayed by RT-PCR, Western blot, and fluorescent determination using Fura 2/AM, respectively. **Results:** $PGF_{2\alpha}$ (100 nmol/L) significantly increased the cardiomyocyte diameter, protein content and $[Ca^{2+}]_i$, and promoted ANP, CaN mRNA, and CaN/$NFAT_3$/$GATA_4$ protein expressions, which were inhibited by either Rb_1 in a concentration-dependent manner (50, 100, and 200 μg/mL) or *L*-arginine (1 mmol/L). N^G-nitro-*L*-arginine-methyl ester, a nitric oxide synthase inhibitor, could abolish the effects of *L*-arginine, but failed to change the effects of Rb_1 in the experiments above. **Conclusion:** The present data implicate that Rb_1 attenuates cardiac hypertrophy, the underlying mechanism may be involved in the inhibition of the Ca^{2+}-CaN signal transduction pathway.

Introduction

Cardiac hypertrophy is recognized as an adaptive response characterized by the growth of individual cardiomyocytes in size rather than the increase in cell number. Initially beneficial, sustaining cardiac hypertrophy eventually leads to decompensation and results in dilated cardiomyopathy, arrhythmia, fibrotic disease, heart failure, and even sudden death[1]. Furthermore, some studies have indicated that hypertrophy may not be required for a successful adaptation to increased workload[2]. Clinical studies have found that several classes of drugs, including angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium channel blockers[3–5], and nitric oxide (NO)[6] could have some beneficial effects in the prevention or treatment of cardiac hypertrophy. However, more studies should be done to provide more therapeutic choices for cardiac hypertrophy.

It has been reported that ginsenosides extracted from the root of the herb *Panax ginseng* CA. Mey have many pharmacological effects, including increasing the activity of superoxide dismutase[7] and protecting the brain and heart from ischemic and reperfusion injuries[8,9]. Notably, ginsenoside Rb_1 (Rb_1), a major component in ginsenosides, has been shown to elevate the release of NO in rat ventricular myocytes[10] and decrease intracellular free Ca^{2+} in cardiac myocytes and other tissues[11–13], which indicates that Rb_1 may be a potential drug for anticardiac hypertrophy.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been shown to induce cardiac myocyte hypertrophy *in vitro* and cardiac growth *in vivo* and is a good candidate to mediate the growth of

cardiac cells[14]. Meanwhile, Ca^{2+} signaling has been reported to play a critical role in the development of cardiac hypertrophy induced by various hypertrophic stimuli[15]. The increased intracellular Ca^{2+} binds to calmodulin and regulates several downstream effectors, such as calcineurin (CaN), which is a key mediator of cardiac hypertrophy[16,17]. CaN dephosphorylates the nuclear factor of activated T cells (NFAT), and then later translocates to the nucleus where it acts with other transcription factors (eg $GATA_4$) for the activation of downstream target genes to induce cardiac hypertrophy[18,19]. A series of studies has shown that the neuroprotective activities and anti-aging function of Rb_1 were related to decreasing intracellular Ca^{2+}[11–13]. However, whether Rb_1 has antihypertrophic effects on cardiac hypertrophy and inhibits the Ca^{2+}–CaN signal pathway has not known as yet.

Materials and methods

Primary culture of myocytes Ventricular myocytes from 1–3-d-old rats (Animal Center of Institute of Surgery Research of the Third Military Medical University, Chongqing, China) were prepared and cultured for 48 h in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 0.1 mmol/L bromodeoxyuridine (Sigma, St Louis, Missouri, USA) as described previously[20]. The cells were adjusted to 1.5×10^6–3×10^6 cells/mL for measuring intracellular free calcium concentration ($[Ca^{2+}]_i$), or to 0.5×10^6–1×10^5 cells/mL for measuring cell diameter and protein content. The medium was replaced by serum-free DMEM for a further 48 h before the treatment of drugs. 100 nmol/L $PGF_{2\alpha}$ (Cayman Chemical, Ann Arbor, Michigan, USA) was used to stimulate the cardiomyocytes, Rb_1 with 99% purity and final concentrations of 50, 100, and 200 μg/mL (Division of Chinese Material Medical and Natural Products, National Institute for the Control of Pharmaceutical and Biological Products, Ministry of Public Health, Beijing, China) and *L*-arginine with final concentration of 1 mmol/L (Alexis, Lausen, Switzerland) were used to investigate their antihypertrophic effects. N^G-nitro-*L*-arginine-methyl ester (*L*-NAME; Alexis, Lausen, Switzerland) 1 mmol/L was used to investigate the relationship between the antihypertrophic effects of Rb_1 and NO.

Measurement of cardiomyocytic diameters The cardiomyocytes were fixed in 4% polyformaldehyde solution and stained with HE. The diameter of single cells was measured by the BI2000 Imaging Analytic System (Chengdu Taimeng Sci-Tec, Chengdu, China). Five random fields (10–15 cells per field) from each slide were analyzed. The experiments were repeated 3 times.

Measurement of cardiomyocytic protein contents The cardiomyocytes were digested by trypsinase and counted. The cells were then washed 3 times with Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (D-HBSS; in mmol/L: NaCl 137.0, KCl 5.0, Na_2HPO_4 0.6, KH_2PO_4 0.4, $NaHCO_3$ 3.0, glucose 5.6, pH 7.2) by centrifuging at 400×*g* for 2 min. The cardiomyocytes were homogenized with RIPA lysis buffer (Upstate, Lake Placid, New York, USA) and centrifuged at 12 000×*g* for 20 min at 4 °C. The protein concentration in the supernatant was determined by the Bradford assay using bovine γ-globulin as the standard, then the protein concentration per cell was calculated.

Measurement of $[Ca^{2+}]_i$ The $[Ca^{2+}]_i$ was measured by the method described before[21]. Briefly, the cells (1×10^6) were incubated in the medium with Fura 2/AM (5 μmol/L; Sigma, St Louis, USA) for 50 min at 37 °C, then washed 3 times with HBSS (D-HBSS plus 1.30 mmol/L $CaCl_2$ and 0.5 mmol/L $MgCl_2$) containing 0.2% bovine serum albumin by centrifuging at 500×*g* for 2 min. The fluorescence value from 1 mL cell suspension was measured by a Shimadzu RF-5000 spectrofluorometer (Kyoto, Japan) with dual excitation wavelengths at 340 and 380 nm and emission wavelengths at 510 nm. The $[Ca^{2+}]_i$ was calculated by the following equation: $[Ca^{2+}]_i=K_d\times(F-F_{min})/(F_{max}-F)$. Here, K_d was the dissociation constant of Fura 2/AM for Ca^{2+} (about 224 nmol/L at 37 °C), F was the basal fluorescence value of the cells, F_{max} was the fluorescence value under the presence of excess calcium in the cells due to the lysis of the cellular membrane caused by 0.98 g/L Triton-X 100 (Sigma, St Louis, Missori, USA), F_{min} was the fluorescence value under the presence of minimal calcium using 5 mmol/L ethyleneglycotetraacetic acid (EGTA) to chelate the Ca^{2+} in the cells after the lysis of the cellular membrane by Triton-X 100.

RT-PCR analysis of mRNA Total RNA was extracted from the cardiomyocytes by use of an RNeasy mini kit (Qiagen, Valencia, California, USA). RT-PCR was performed with an RT-PCR kit (Promega, San Jose, California, USA) according to the manufacturer's instructions. The nucleotide sequence of the primers were as follows[22,23]: (i) atrial natriuretic peptide (ANP): sense 5'-GCC CTG AGC GAG CAG ACC GA-3', antisense 5'-CGG AAG CTG TTG CAG CCT A-3'; (ii) CaN: sense 5'-ACT GGC ATG CTC CCC AGC GGA-3', antisense 5'-GTG CCG TTA GTC TCT GAG GCG-3'; and (iii) β-actin: sense 5'-GAC TAC CTC ATG AAG ATC CTG ACC-3', antisense 5'-TGA TCT TCA TGG TGC TAG GAG CC-3'. The predicted products in size were 202, 244, and 423 bp, respectively. These primers were synthesized by Beijing Dingguo Biotech (Beijing, China). The following conditions of the RT-PCR reactions were met: (i) 1 cycle at 48 °C for 45

min, 94 °C for 2 min; (ii) 35 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min; and (iii) 72 °C for 7 min. The products were separated by electrophoresis on 1% agarose gel containing ethidium bromide, and photographed. The integral optical density values for each band of ANP, CaN, and β-actin on the gel were assayed by the BI2000 Imaging Analysis System (Chengdu Taimeng Sci-Tec, China). β-actin was used as an internal control for the semiquantitative assay.

Western blotting The protein (30 μg) from cardiomyocytes separated by 10% SDS-PAGE was transferred onto polyvinylidene difluoride nylon membranes. The blots were probed with mouse anti-CaNA-α (1:200 dilution) or anti-$NFAT_3$ (1:100 dilution) or anti-$GATA_4$ antibodies (1:100 dilution; (Santa Cruz Biotechnology, Santa Cruz, California, USA), and then with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (1:2500 dilution) antibodies. Immunodetection was carried out using the BI2000 Imaging Analysis System.

Statistical analysis All of the data were expressed as mean±SD and analyzed by either ANOVA or Student's *t*-test with SPSS 11.5 software (SPSS Inc, Chicago, Illinois, USA. Differences were considered statistically significant at $P<0.05$.

Results

Effects of Rb_1 on $PGF_{2\alpha}$-induced cardiomyocyte hypertrophy Light microscopic findings of the cardiomyocytes showed that the cardiomyocytes treated with $PGF_{2\alpha}$ (100 nmol/L) became swollen and enlarged with undistinguishable borders among the cells (Figure 1B). Rb_1 (200 μg/mL) markedly alleviated the morphological changes induced by $PGF_{2\alpha}$ (Figure 1C). The addition of *L*-NAME (1 mmol/L) could not antagonize the effect of Rb_1 on the hypertrophic myocyte (data not shown).

Table 1 showed that the diameters and protein contents of the cardiomyocytes treated with $PGF_{2\alpha}$ significantly increased, compared with that of the control ($P<0.01$). The treatment of Rb_1 with a variety of concentrations (50, 100, and 200 μg/mL) significantly relieved the changes induced by $PGF_{2\alpha}$ in a concentration-dependent manner ($P<0.05$). *L*-arginine (1 mmol/L) also lowered these changes induced by PGF_2a ($P<0.01$). *L*-NAME (1 mmol/L) abolished the effects of *L*-arginine, but failed to abolish the effects of Rb_1 (200 μg/mL) on the cardiomyocyte diameter and protein content.

There was a low fundamental expression of ANP mRNA in the cardiac myocytes (0.005±0.002, $n=3$). $PGF_{2\alpha}$ treatment obviously increased the ANP mRNA expression, which was significantly antagonized by Rb_1 (200 μg/mL) treatment (Figure 2A).

Effects of Rb_1 on $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ in cardiomyocytes The resting $[Ca^{2+}]_i$ was 149.7±26.2 nmol/L ($n=6$), and it increased by 83% after the cardiomyocytes were treated with $PGF_{2\alpha}$ (100 nmol/L) for 48 h ($P<0.01$). Treatments with either Rb_1 at the concentrations of 50, 100, and 200 μg/mL or *L*-arginine (1 mmol/L) strongly blocked the $[Ca^{2+}]_i$ increase caused by $PGF_{2\alpha}$. Once again, *L*-NAME (1 mmol/L) treatment abolished the effect of *L*-arginine ($P<0.01$), but failed to antagonize the effect of Rb_1 ($P>0.05$; Figure 3).

Effects of Rb_1 on transcription of CaN and expressions of CaN, $NFAT_3$, and $GATA_4$ proteins from cardiomyocytes treated by $PGF_{2\alpha}$ The relative CaN mRNA expression was 0.225±0.023 in the control and increased by 52% in the $PGF_{2\alpha}$-treated cardiomyocytes (Figure 2B; $n=3$, $P<0.01$). Similar treatments with $PGF_{2\alpha}$ also significantly increased the expres-

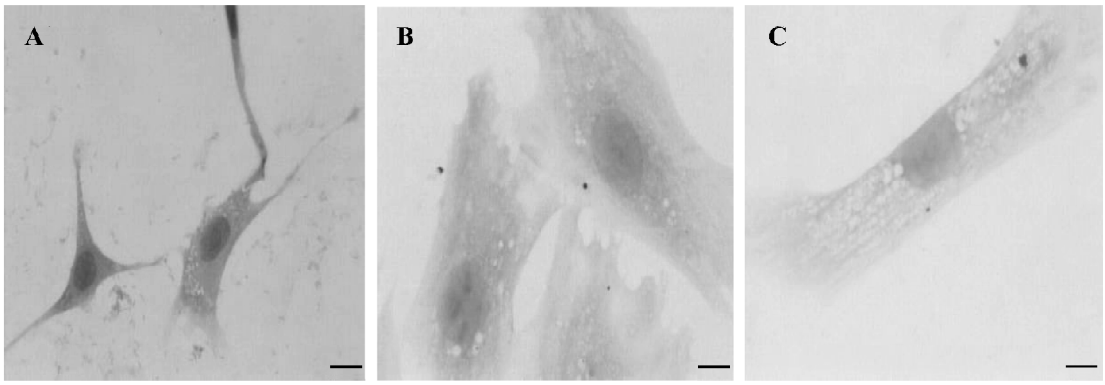

Figure 1. Effects of Rb_1 on cultured cardiomyocytes (HE×400). (A) control; (B) $PGF_{2\alpha}$ (100 nmol/L)-pretreated group; (C) $PGF_{2\alpha}$ (100 nmol/L) plus Rb_1 (200 μg/mL)-pretreated group. Cardiomyocytes treated with $PGF_{2\alpha}$ became swollen and enlarged with undistinguishable borders among the cells. Rb_1 markedly alleviated the morphological changes induced by $PGF_{2\alpha}$.

Table 1. Effects of Rb_1 on the changes of cardiomyocyte diameter (n=3, mean±SD) and protein level (n=6, mean±SD) induced by $PGF_{2\alpha}$. $^cP<0.01$ *vs* control; $^dP>0.05$, $^eP<0.05$, $^fP<0.01$ *vs* $PGF_{2\alpha}$ (100 nmol/L); $^iP<0.01$ *vs* *L*-NAME (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L); $^lP<0.01$ *vs* *L*-arginine (1 mmol/L) +$PGF_{2\alpha}$ (100 nmol/L).

Group	Cell diameter (μm)	Protein level (pg/cell)
Control	36±11	419.2±41.6
$PGF_{2\alpha}$ (100 nmol/L)	115±23^c	548.5±59.2^c
Rb_1 50 (μg/mL)+$PGF_{2\alpha}$ (100 nmol/L)	94±23^f	466.9±56.9^e
Rb_1 100 (μg/mL)+$PGF_{2\alpha}$ (100 nmol/L)	68±18^f	424.1±44.8^f
Rb_1 200 (μg/mL)+$PGF_{2\alpha}$ (100 nmol/L)	59±19^f	408.7±47.4^f
L-arginine (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L)	58±17^f	397.9±45.8^f
L-NAME (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L)	112±22^d	523.8±75.9^d
Rb_1 200 (μg/mL)+*L*-NAME (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L)	68±17^i	431.7±36.2^i
L-arginine (1 mmol/L)+*L*-NAME (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L)	108±14^l	539.7±36.3^l

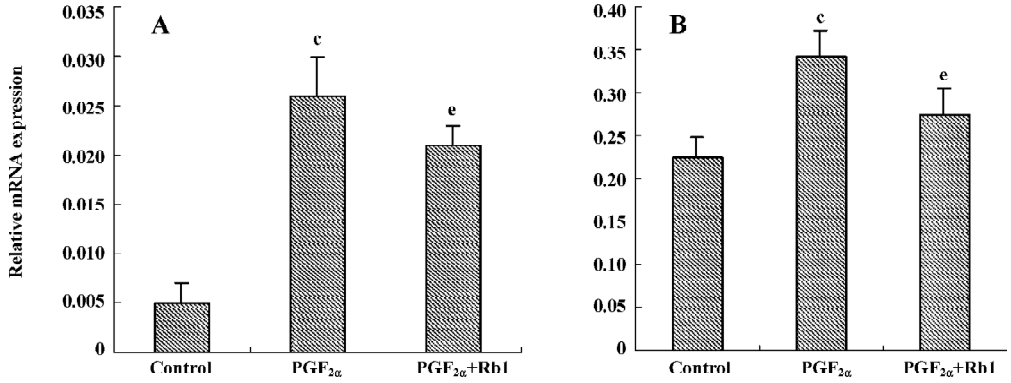

Figure 2. Effects of Rb_1 (200 μg/mL) on the upregu-lated transcription of ANP and CaN in cardiomyocytes treated with $PGF_{2\alpha}$ (100 nmol/L). (A) transcription of ANP relative to β-actin; (B) transcription of CaN relative to β-actin. n=3. Mean±SD. $^cP<0.01$ *vs* control; $^eP<0.05$ *vs* $PGF_{2\alpha}$-treated group.

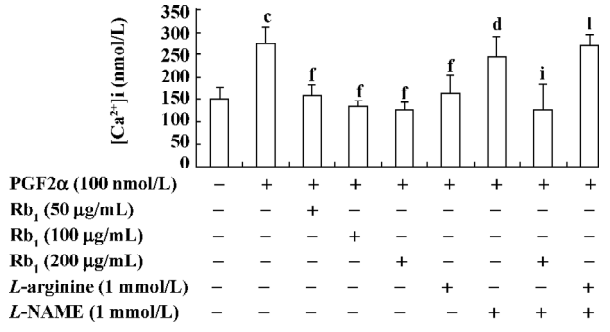

Figure 3. Effects of Rb_1 on the $PGF_{2\alpha}$-induced $[Ca^{2+}]_i$ elevation from cardiomyocytes. Cultured cardiomyocytes were treated with $PGF_{2\alpha}$ (100 nmol/L) in the presence of a variety of Rb_1 concentrations (50, 100, and 200 μg/mL), *L*-arginine (1 mmol/L), and *L*-NAME (1 mmol/L). $[Ca^{2+}]_i$ changes of cardiomyocytes were calculated by the fluorescence method. n=6. Mean±SD. $^cP<0.01$ *vs* control; $^dP>0.05$, $^fP<0.01$ *vs* $PGF_{2\alpha}$ (100 nmol/L); $^iP<0.01$ *vs* *L*-NAME (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L); $^lP<0.01$ *vs* *L*-arginine (1 mmol/L)+$PGF_{2\alpha}$ (100 nmol/L).

sions of the CaN, $NFAT_3$, and $GATA_4$ proteins of cardiomyocytes. The treatment of Rb_1 (200 μg/mL) markedly decreased the mRNA expression of CaN and the protein expressions of CaN, $NFAT_3$, and $GATA_4$ from cardiomyocytes treated by $PGF_{2\alpha}$. *L*-arginine (1 mmol/L) also inhibited the protein expressions of CaN and its downstream factors ($P<0.05$; Figure 4, n=3).

Discussion

It has been reported that the morphological changes of cardiomyocyte hypertrophy can be induced *in vitro* by stimulating cultured neonatal cardiomyocytes with various growth factors and cytokines, such as angiotensin II, endothelin-1, and $PGF_{2\alpha}$, which was similar to those induced by pressure or volume load[14,24–26]. The characteristic phenotype of hypertrophy following growth factor stimulation includes an increase of cell volume and protein synthesis

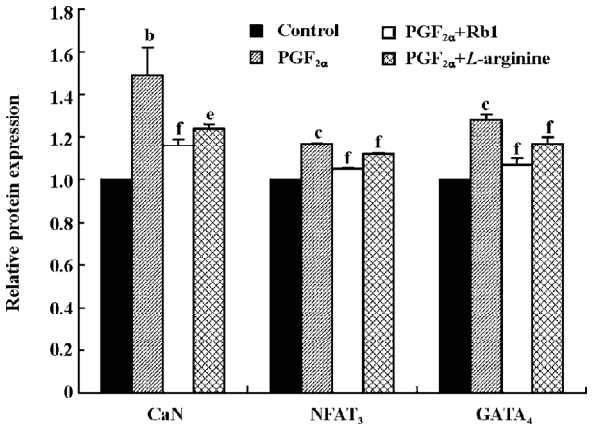

Figure 4. Effects of Rb_1 (200 μg/mL) on the increased expressions of CaN, $NFAT_3$, and $GATA_4$ in cardiomyocytes pretreated with $PGF_{2\alpha}$ (100 nmol/L). n=3. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* control; $^eP<0.05$, $^fP<0.01$ *vs* $PGF_{2\alpha}$ 100 nmol/L.

with an accumulation of contractile proteins, organization of the contractile proteins into sarcomeric units, as well as the re-expression of fetal cardiac genes, including ANP[27]. In this study, the findings from measuring the diameter, protein content, and ANP mRNA expression of the cardiac myocytes suggested that $PGF_{2\alpha}$ could induce cardiomyocyte hypertrophy resembling that described by Lai *et al*[14], and that Rb_1 could significantly decrease the elevated cardiomyocyte volume, protein content, and ANP mRNA expression caused by $PGF_{2\alpha}$.

Our results showed that $PGF_{2\alpha}$ induced cardiomyocyte hypertrophy with the elevating $[Ca^{2+}]_i$ level. Meanwhile, the antihypertrophic effects of Rb_1 were accompanied simultaneously with the alleviating $[Ca^{2+}]_i$ level. *L*-NAME, an NO synthase inhibitor, did not antagonize both effects of Rb_1 on hypertrophy and $[Ca^{2+}]_i$ of cardiomyocytes; on the contrary, it abolished the antihypertrophic and decreasing $[Ca^{2+}]_i$ effects of *L*-arginine. These results suggested that the directly decreasing $[Ca^{2+}]_i$ effect rather than the NO release may be responsible for the antihypertrophic effects of Rb_1.

In the past several years, a number of experiments have implicated that the CaN signal transduction pathway may play an important role in the cardiomyocyte hypertrophy process[28]. We have previously reported that cardiac hypertrophy by $PGF_{2\alpha}$ may be mediated by the CaN signal pathway in rats[29]. In the present paper, the fact that the transcription and expression of CaN, as well as the expression of the CaN downstream factors increased with elevating $[Ca^{2+}]_i$ under the stimulation of $PGF_{2\alpha}$ to the cardiomyocytes, which were blunted by Rb_1, suggested that the interference of the CaN signaling pathway might be involved in the antihyper-trophic mechanisms of Rb_1.

L-arginine, an NO donor, was observed to inhibit cardiomyocyte hypertrophy[30], which could be abolished by *L*-NAME. Surprisingly, *L*-NAME had no influence on either the antihypertrophic effect or on the inhibiting $[Ca^{2+}]_i$ rise from Rb_1. The results suggested that the antihypertrophic effect of Rb_1 might be different from *L*-arginine, but the relationship between the Rb_1 effects and NO still needs much investigation.

In conclusion, our study demonstrates that Rb_1 can alleviate cardiac hypertrophy *in vitro*, which may be mediated in part by an inhibitive effect on elevated $[Ca^{2+}]_i$ due to the inactivation of the CaN transduction pathway.

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