

# Effect of parathyroid hormone-related protein on intracellular calcium ion and cyclic adenosine monophosphate concentrations in cardiac fibroblasts

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## Abstract

**Objective:** This study aimed to determine the effect of parathyroid hormone-related protein (PTHrP) on proliferation of cardiac fibroblasts (CFs) in primary cultures of neonatal Wistar rats.

**Methods:** Different PTHrP concentrations were added to CFs of neonatal Wistar rats and the cells were grouped according to the concentrations added. A verapamil (VPL) group and a calcitriol (CAL) group were also established. Changes in cell proliferation and in cyclic adenosine monophosphate and calcium ion levels were identified and recorded.

**Results:** We found that as the concentration of PTHrP increased, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, a tetrazolium salt) colorimetric absorbance values (A values) decreased. These values in the PTHrP groups were significantly lower than those in the control group. MTT colorimetric A values and <sup>3</sup>H-thymidine deoxyribose intake were lower in the VPL group, low-dose CAL group, and the PTHrP 10<sup>-7</sup> mol/L group compared with the

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control group. However, MTT colorimetric A values and  $^3\text{H}$ -thymidine deoxyribose intake were higher in the high-dose CAL group than in the PTHrP  $10^{-7}$  mol/L group. As PTHrP concentrations increased, intracellular cyclic adenosine monophosphate concentrations also increased. **Conclusion:** PTHrP, VPL, and low-dose CAL inhibit proliferation of CFs, while high-dose CAL promotes proliferation of CFs.

### Keywords

Parathyroid hormone-related protein, cardiac muscle, fibroblasts, cyclic adenosine monophosphate (cAMP), calcium ion, verapamil, calcitriol

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### Introduction

In 1987, parathyroid hormone-related protein (PTHrP) was found to be a tumor factor that induced hypercalcemia in malignant tumors.<sup>1</sup> The first few amino acids of PTHrP have the same homology sequence as parathyroid hormone (PTH) at the same position and bind with the same PTH/PTHrP receptor. PTHrP is considered to be the primary cause of hypercalcemia in patients suffering from cancer. However, PTHrP mRNA also widely occurs under normal conditions. PTHrP, which is composed of 141 amino acids, has the same receptor as PTH, through which both cause an equal biological response. PTHrP also regulates salt ions, but it is hard to distinguish the difference in effect on the dynamic balance of salt ions between PTHrP and PTH. PTHrP and PTH also have fundamentally different biological effects. PTHrP acts as a local regulator of cell proliferation, cell differentiation, and cell death. Many tissues are able to synthesize and secrete PTHrP.

When PTHrP binds to cell surface receptors, it exerts autocrine, paracrine, and endocrine effects mediated by the signal transduction cascade. Excessive levels of serum PTH/PTHrP not only affect bone metabolism, but also have negative effects

on the cardiovascular system, immune system, kidneys, and endocrine tissues. Studies have shown that when the glomerular filtration rate decreases, serum PTHrP levels exponentially increase and induce side effects in multiple organs and tissues.<sup>2</sup> Therefore, PTHrP is associated with a variety of cardiovascular diseases.<sup>2,3</sup> PTHrP and PTHrP receptor are mainly expressed in cardiomyocytes of vascular smooth muscle cells, endothelial cells, and atrial and ventricular cardiomyocytes. Recent studies have shown that PTHrP regulates blood pressure and growth of blood vessels in the heart.<sup>4,5</sup>

PTH can also promote proliferation of cardiac fibroblasts (CFs).<sup>6</sup> There is a typical vitamin D receptor in parathyroid cells. When 1, 25 (OH) 2D3 binds to vitamin D receptor, it effectively binds to the vitamin D response element in the PTH gene promoter under the action of some co-factors in the nucleus. This inhibits transcription of the preproparathyroid hormone (preproPTH) gene, and therefore, affects synthesis and secretion of PTH. However, how PTHrP affects CFs is unclear. Therefore, this study aimed to investigate the effects of different concentrations of PTHrP on CFs, as well as their intracellular calcium ion and cyclic

adenosine monophosphate (cAMP) concentrations.

## Materials and methods

This study was conducted in accordance with the declaration of Helsinki. This study was also conducted with approval from the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. All experiments were conducted in accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. Animals were humanely sacrificed under anesthesia.

### Materials

Neonatal Westar rats (1–2 days old) were provided by the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University. Trypsin, bovine serum, bovine serum albumin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Carlsbad, CA, USA). PTHrP, verapamil (VPL), and dimethyl sulfoxide were purchased from Sigma-Aldrich (Shanghai, China).  $^3\text{H}$ -thymidine deoxyribose ( $^3\text{H}$ -TdR) and  $^3\text{H}$ -proline were purchased from the Isotope Laboratory of China Institute of Atomic Energy. An LS-6100 liquid scintillation counter was purchased from Beckman Coulter (Brea, CA, USA). PTHrP was dissolved in dimethyl sulfoxide, and VPL and calcitriol (CAL) were separately packed and stored at  $-20^\circ\text{C}$ . All other reagents were formulated before use.

### Methods

**Groups.** The rats were divided into four groups ( $n=8$  in each group) as follows: (1) control group where only culture medium was added; (2) PTHrP group with different concentrations of  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ , and  $10^{-7}$  mol/L; (3) VPL intervention group where  $10^{-5}$  and  $10^{-7}$  mol/L of VPL

was added to the medium and  $10^{-7}$  mol/L of PTHrP was added after 30 minutes; and (4) CAL intervention group where  $10^{-7}$  and  $10^{-9}$  mol/L of CAL was added to the medium and  $10^{-7}$  mol/L of PTHrP was added after 30 minutes.

**Culture and identification of CFs.** Under aseptic conditions, ventricles of the Wistar rats (2–3 days old) were collected, cut into two to three pieces, and digested with 0.125% trypsin at  $37^\circ\text{C}$  for 20 to 30 minutes. Cells were collected every 5 minutes and collection was conducted six to seven times. The cells were filtered with a sieve, centrifuged ( $89 \times g$  for 3–5 minutes), and washed with a phosphate-buffered saline solution once. The obtained cells were then placed in a 50-mL culture vessel containing DMEM with 10% calf serum. The culture vessel was placed in an incubator with 5%  $\text{CO}_2$  at  $37^\circ\text{C}$ , adherently cultured for 90 minutes, and then adherently cultured a second time. After the bottom of the vessel was fully filled, cells were digested with 0.25% trypsin and passaged to ensure the number and purity of cells. The medium was replaced every 24 hours. When cells grew into a nearly a state of fusion, they were passaged at a ratio of 1: 2, with two or three generations of cells used in the experiment. The percentage of living cells was detected using 1 g/L of trypan blue staining microscopy (a survival rate of 98% was acceptable).

**Tetrazolium salt experiment.** For detection of proliferation of CFs, CFs in the logarithmic phase were obtained and digested with 0.25% trypsin. The concentration of the cell suspension was adjusted with DMEM containing 15% fetal bovine serum to  $1 \times 10^4/\text{mL}$ . The cells were then transferred into 96-well plates ( $200 \mu\text{L}/\text{well}$ ) and cultured under 50 ml/L of  $\text{CO}_2$  with a saturated humidity and a temperature of  $37^\circ\text{C}$  until the cells attached well to the wall. Serum-free DMEM was then added and

cultivation continued for 24 hours to try to induce the cells to synchronously grow into a resting phase. The medium was extracted and discarded, and interventional drugs were added to each well. Each concentration of each drug accounted for six wells, and solution was mixed in each well and then cultivated for 72 hours. Four hours before the end of the incubation period, 20  $\mu$ L of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide ([MTT] a tetrazolium salt) solution (5 g/L) was added to each well. The incubation period was then completed, terminated, and the supernatant of the medium in each well was carefully extracted and discarded. Each well was filled with 150 mL of dimethyl sulfoxide and shaken for 10 minutes to completely dissolve the crystals. The absorbance value (A value) of each well was measured using an enzyme-linked immunoassay analyzer (at a 490-nm wavelength), and the A value of the blank well filled with medium alone was set to zero.

**Determination of DNA synthesis in CFs.** DNA synthesis in CFs was reflected by the incorporation rate of  $^3\text{H}$ -TdR. CFs underwent drug interventional cultivation for 72 hours. Four hours before the end of the intervention,  $^3\text{H}$ -TdR ( $18.5 \times 10^4$  Bq/mL) and vitamin C (50 mg/L) were added. After intervention, if the percentage of living cells was confirmed by trypan blue staining to be >95%, the cells were digested with 0.25% trypsin into suspension. The cells were then collected by a multi-head collector, transferred onto glass fiber filter paper, and rinsed with distilled water. Cells were washed with 6% trichloroacetic acid for 5 minutes to break up the cells, and the product was adsorbed on the glass fiber filter paper. After the membrane was washed with anhydrous ethanol, cells were vacuum filtered, dried, and placed in a scintillation flask. A volume of 0.5 mL of 2,5-dibenzoxazole/2,2'-p-phenylenebis (5-

phenyloxazol)/xylene scintillation liquid was then added and left to rest overnight, and radioactivity was then determined using a liquid scintillation counter (Beckman Coulter, Fullerton, CA, US). Results are expressed as a count per million (cpm) value.

**Determination of collagen synthesis in CFs.** Collagen synthesis in CFs was determined by a  $^3\text{H}$ -proline incorporation experiment. Four hours before the end of drug intervention,  $^3\text{H}$ -proline ( $18.5 \times 10^4$  Bq/mL) and vitamin C (50 mg/L) were added. After intervention, 0.25% trypsin was added to digest cells into the suspension. The cells were then collected by a multi-head collector, transferred onto a glass fiber filter paper, and rinsed with distilled water. Cells were washed with 6% trichloroacetic acid for 5 minutes to break up the cells, and the product was adsorbed on the glass fiber filter paper. After the membrane was washed with anhydrous ethanol, cells were vacuum filtered, dried, and placed in a scintillation flask. A volume of 0.5 mL of 2,5-dibenzoxazole/2,2'-p-phenylenebis (5-phenyloxazol)/xylene scintillation liquid was added, left to rest overnight, and radioactivity was then determined using a liquid scintillation counter. Results are expressed as a cpm value.

**Determination of calcium in CFs.** After drug intervention, the CFs were transferred from cell preservation solution (KB solution) to Tyrode's solution, centrifuged at 500 r/min for 1 minute, and the supernatant was removed. Cells were then placed in a 20- $\mu$ mol/L loading solution containing the fluorescent probe Fluo-3/AM (Institute of Isotopes, Chinese Academy of Atomic Energy, Beijing, China), incubated at 37°C for 1 hour, and centrifuged at 500 r/min for 1 minute. After removing the loading solution, cells were washed three times. Cells were diluted to the required

concentration (30–40 cells in the view field of a 10 times objective lens), added to a bath tank, and underwent adherent culture. Those with complete morphology were observed under light microscopy. Under a 40 times optical lens, Fluo-3/AM passively diffused into cells, was hydrolyzed by esterase, released Fluo-3, and formed Fluo-3 and calcium ( $\text{Ca}^{2+}$ ) complexes. These complexes were excited by a laser at a wavelength of 488 nm and fluorescence was produced. The fluorescence value was positively correlated with the calcium ion concentration  $[\text{Ca}^{2+}]_i$ . ( $[\text{Ca}^{2+}]_i$  is expressed as a change in fluorescence value). Under a laser scanning confocal microscope (Axio imager lsm-800; Carl Zeiss, Oberkochen, Germany), a time series procedure was used to scan the XY plane of cells using a time interval of 20 s and scanning for 30 times. The average fluorescent intensity (FI) of the cells' XY plane was calculated and this showed changes in  $[\text{Ca}^{2+}]_i$ .

**Determination of cAMP.** Levels of cAMP were determined by radioimmunoassay. After CFs were successfully cultivated, the concentration was adjusted to  $10^5$  to  $10^6$  cells/mL. The cells' solution (1 mL) was obtained and centrifuged at 1500 r/min for 5 minutes to cause precipitation of cells. The supernatant was then discarded, and 1 mL of acetate buffer (50 mmol/L, pH 4.75) was added to the cellular precipitation and mixed into the suspension. Cells were broken down using a sonifier cell disrupter (Ningbo Xinzhi Biotechnology Co., Ltd., Ningbo, China). The product was centrifuged at 3000 r/min for 15 minutes, and 100  $\mu\text{L}$  of the supernatant was used for assay, according to the kit's (Wuhan Yilairuite Biotechnology Co., Ltd., Wuhan, China) instructions. A total of 5  $\mu\text{L}$  of acetylation reagents, 100  $\mu\text{L}$  of  $\text{I}^{125}$ , and 100  $\mu\text{L}$  of anti-serum were added to the supernatant, shaken well, and then left to stand overnight at 4°C. Subsequently, 100  $\mu\text{L}$  of

rabbit serum and 100  $\mu\text{L}$  of sheep anti-rabbit immunoglobulin (1: 5000) were added to each tube, left overnight at 4°C to enable a full reaction, and later centrifuged at 3000 r/min for 15 minutes. The supernatant was then extracted under negative pressure and gamma radiation intensity was determined. A standard curve was created and cAMP levels were calculated with results expressed as pmol/L.

**Statistical analysis.** Values are expressed as mean  $\pm$  standard deviation. All data were entered into Excel (Jinshan Software Co., Ltd., Beijing, China) to establish a database and SPSS version 13.0 statistical software (Chicago, IL, USA) was used to process the data. Comparison between groups was conducted using one-way analysis of variance and comparison between two groups was conducted using the SNK-q test.

## Results

### *Effects of different concentrations of PTHrP on proliferation and collagen synthesis of CFs in fetal rats*

We found that as the concentration ( $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ , and  $10^{-7}$  mol/L) of PTHrP increased, MTT colorimetric A values decreased. These mean values were significantly lower in the PTHrP groups than that in the control group (all  $P < 0.01$ ). Additionally,  $^3\text{H}$ -TdR intake decreased as PTHrP concentrations increased. The mean  $^3\text{H}$ -TdR intake was significantly lower in the PTHrP groups than in the control group (all  $P < 0.01$ ). The mean MTT colorimetric A value and  $^3\text{H}$ -TdR intake were significantly lower in the VPL intervention group than in the PTHrP  $10^{-7}$  L group (all  $P < 0.01$ ). The mean MTT colorimetric A value and  $^3\text{H}$ -TdR intake were significantly lower in the low-dose CAL intervention group than in the PTHrP  $10^{-7}$

**Table 1.** Effects of different concentrations of PTHrP on DNA synthesis, proliferation, and collagen synthesis of cardiac fibroblasts in fetal rats (n = 8 in each group).

Groups	PTHrP (mol/L)	Amount of <sup>3</sup> H-TdR every minute (cpm)	A <sub>490</sub>	<sup>3</sup> H-proline (cpm)
Control group	0	1554.06 ± 111.67	0.365 ± 0.013	360.95 ± 47.54
PTHrP groups	10 <sup>-10</sup>	1486.25 ± 129.03*	0.319 ± 0.011*	493.52 ± 69.84*
	10 <sup>-9</sup>	1403.57 ± 108.25*	0.302 ± 0.025*	489.88 ± 63.28*
	10 <sup>-8</sup>	1314.98 ± 131.52*	0.258 ± 0.018*	474.74 ± 97.52*
	10 <sup>-7</sup>	1248.29 ± 115.45*	0.219 ± 0.008*	470.71 ± 85.53*
PTHrP+ver	10 <sup>-7</sup> +10 <sup>-7</sup>	1224.56 ± 123.26#	0.201 ± 0.018#	468.72 ± 89.12#
	10 <sup>-7</sup> +10 <sup>-5</sup>	1219.47 ± 103.67#	0.198 ± 0.025#	468.72 ± 92.53 #
PTHrP+cal	10 <sup>-7</sup> +10 <sup>-9</sup>	1234.61 ± 112.83#	0.215 ± 0.017#	469.73 ± 40.91#
	10 <sup>-7</sup> +10 <sup>-7</sup>	1609.16 ± 110.70#	0.402 ± 0.025#	469.73 ± 52.03#

Note: Values are mean ± standard deviation. \* $P < 0.01$  compared with the control group; # $P < 0.01$  compared with the PTHrP 10<sup>-7</sup> mol/L group. PTHrP, parathyroid hormone-related protein; <sup>3</sup>H-TdR, <sup>3</sup>H-thymidine deoxyribose; cpm, counts per million; ver, verapamil; cal, calcitriol.

L group (both  $P < 0.01$ ). However, the mean MTT colorimetric A value and <sup>3</sup>H-TdR intake were significantly higher in the high-dose CAL intervention group than in the PTHrP 10<sup>-7</sup> L group (both  $P < 0.01$ , Table 1).

As the concentration of PTHrP increased, the incorporation rate of <sup>3</sup>H-proline significantly decreased. The amount of <sup>3</sup>H-proline incorporation was significantly lower in the VPL 10<sup>-5</sup> or 10<sup>-7</sup> mol/L group than in the PTHrP 10<sup>-7</sup> mol/L group (both  $P < 0.01$ ). The amount of <sup>3</sup>H-proline incorporation was significantly lower in the CAL 10<sup>-9</sup> or 10<sup>-7</sup> mol/L group than in the PTHrP 10<sup>-7</sup> mol/L group (both  $P < 0.01$ , Table 1). PTHrP levels were significantly reduced with addition of 10<sup>-5</sup> mol/L VPL or 10<sup>-7</sup> mol/L VPL (both  $P < 0.01$ ). PTHrP levels were significantly reduced with the addition of 10<sup>-9</sup> mol/L CAL ( $P < 0.01$ ), but PTHrP levels did not change with addition of 10<sup>-7</sup> mol/L CAL.

#### Effects of different concentrations of PTHrP on cAMP concentration in rat CFs

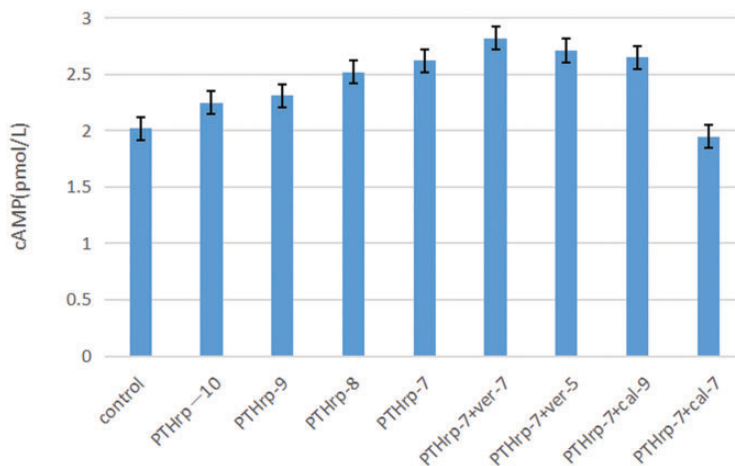
The mean cAMP level in CFs was significantly higher in all of the PTHrP groups than in the control group (all  $P < 0.01$ ).

Furthermore, cAMP levels were slightly higher in the high-dose and low-dose VPL intervention groups compared with the low-dose CAL intervention group and the PTHrP 10<sup>-7</sup> L alone group. However, cAMP levels appeared to lower in the high-dose CAL intervention group compared with the control group ( $P > 0.05$ , Figure 1). As the PTHrP concentration increased, the intracellular cAMP level also increased ( $P < 0.01$ ).

#### Effects of different concentrations of PTHrP on [Ca<sup>2+</sup>]<sub>i</sub> in rat CFs

Peak [Ca<sup>2+</sup>]<sub>i</sub> in CFs was not significantly different compared with the control group. However, peak [Ca<sup>2+</sup>]<sub>i</sub> in the VPL 10<sup>-9</sup> and 10<sup>-7</sup> mol/L groups was significantly lower compared with that in the PTHrP 10<sup>-7</sup> mol/L alone group (both  $P < 0.01$ ). The mean [Ca<sup>2+</sup>]<sub>i</sub> was significantly higher in the CAL 10<sup>-7</sup> mol/L group than in the PTHrP 10<sup>-7</sup> mol/L group ( $P < 0.01$ ), but not in the PTHrP 10<sup>-7</sup> mol/L alone group ( $P < 0.01$ ). Moreover, the difference in mean peak [Ca<sup>2+</sup>]<sub>i</sub> between the CAL 10<sup>-9</sup> mol/L alone group and the control group was not significant (Figure 2).





**Figure 1.** Effects of different concentrations of PTHrP on cAMP levels in rat cardiac fibroblasts. PTHrP, parathyroid hormone-related protein; cAMP, cyclic adenosine monophosphate; ver, verapamil; cal, calcitriol.

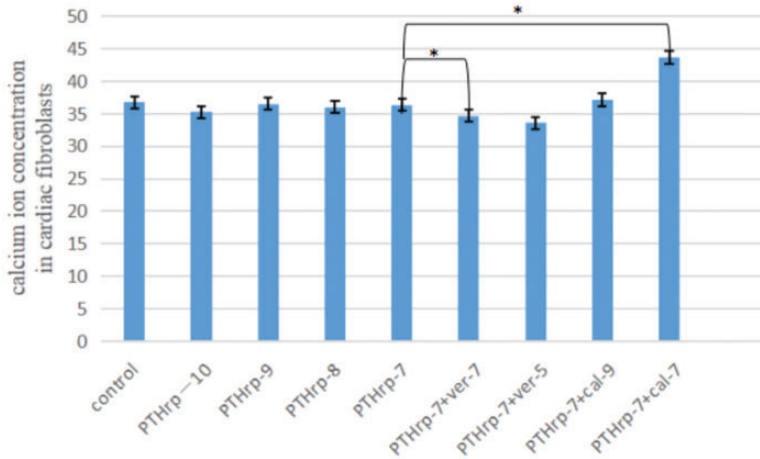
\*Compared with the control group,  $P < 0.01$ ; #compared with the PTHrp+Cal<sup>-9</sup> group,  $P < 0.05$ ; ¥ compared with the control group,  $P > 0.05$ .

## Discussion

The incidence of cardiovascular disease in patients with chronic renal failure is 10 to 30 times that of the normal population, and almost all patients with uremia have cardiovascular complications. These complications are the primary cause of death from chronic renal failure, accounting for 50% of the total mortality, and cardiac complications, such as uremic cardiomyopathy and heart valve calcification, are common.<sup>7,8</sup> Myocardial fibrosis is a pathological feature of uremic cardiomyopathy,<sup>9-12</sup> with CFs as the main cells involved in synthesis and secretion of the extracellular matrix. Myocardial cells lose their ability to proliferate after birth, but non-myocardial cells maintain their proliferation ability. Excessive proliferation of CFs and an increase in collagen synthesis are the main pathological reasons for myocardial fibrosis and left ventricular hypertrophy.

PTHrP is a type of active molecule that is secreted by a variety of tissues and has extensive biological effects. Eight of 13

amino acids in the amino terminus of PTHrP and PTH are the same, and while their primary structures in 13 to 34 areas are different, their space conformations are similar. Furthermore, both can bind with the same PTH/PTHrP receptor.<sup>13</sup> PTH/PTHrP receptors belong to a membrane receptor superfamily that couples with TOG protein. This receptor has three cytoplasmic loops through a number of G proteins, such as Gs, Gi, and Gq. The third cytoplasmic loop couples with two signaling pathways of adenylyl cyclase-cAMP-protein kinase A (PKA) and inositol-1, 4, 5-triphosphate-cytoplasmic Ca<sup>2+</sup>-protein kinase C (PKC). Lys on the 319th site in the second cytoplasmic loop plays a key role in activation of the PKC pathway and the tail of receptor C-terminus may couple with Gi protein. This receptor can bind with PTH and PTHrP with almost identical affinity. When binding with a receptor, PTHrP plays a biological role through activation of two signaling pathways of adenylyl cyclase-adenosine cyclophosphate-PKA and phospholipase



**Figure 2.** Effects of different concentrations of PTHrP on calcium ion concentrations in rat cardiac fibroblasts.

PTHrP, parathyroid hormone-related protein; ver, verapamil; cal, calcitriol.

\* $P < 0.05$ .

C-cytoplasmic  $\text{Ca}^{2+}$ -PKC. There is an important difference between the PLC/PKC and the cAMP/PKA signaling pathways where some target cells function through activation of the adenylyl cyclase reaction and have no response to the PKC pathway, such as distal renal tubules.<sup>14</sup> PTHrP has many biological functions, including participating in growth and development of a variety of organs, and promoting infiltration and metastasis of breast cancers, prostate cancer, and non-small cell lung cancer.<sup>15-18</sup>

Studies have shown that for different target tissues, different segments of PTHrP inhibit or promote cell proliferation.<sup>19-21</sup> PTH and PTHrP, which has a similar structural function to PTH, can promote cell proliferation of osteoblasts, cartilage cells, pancreatic cells, vascular endothelial cells, digestive tract mucosa cells, and renal cells. Our study showed that as PTHrP concentrations increased, MTT colorimetric A values (representing CF proliferation) decreased. These values in the PTHrP groups were significantly lower than those in the control group and had

certain concentration-dependent features. Additionally,  $^3\text{H}$ -TdR intake (representing DNA synthesis) also decreased as PTHrP concentrations increased.  $^3\text{H}$ -TdR intake was significantly lower in the PTHrP groups than in the control group. Furthermore, as the concentration of PTHrP increased, the incorporation rate of  $^3\text{H}$ -proline significantly decreased. These findings suggested that PTHrP inhibited proliferation of CFs and synthesis of collagen. Further investigation of the effect of CFs on intracellular cAMP showed that the cAMP levels increased as PTHrP levels increased. The mechanism for this finding may be that binding of PTHrP and its receptor activates  $G_s$  protein, which results in higher cAMP levels in cells, and this activates the PKA pathway. An increase in intracellular concentrations of cAMP promotes cell differentiation, but inhibits proliferation, thereby slowing proliferation of myocardial fibrosis. In the current study, intracellular cAMP levels were slightly elevated in the VPL groups and reduced in the low-dose CAL group compared with the PTHrP  $10^{-7}$  L alone group.



Further investigation into the effect of PTHrP on intracellular  $[Ca^{2+}]_i$  in CFs showed that PTHrP did not significantly increase  $Ca^{2+}$  influx in neonatal CFs. These findings suggested that PTHrP inhibited CF proliferation by increasing cAMP levels. We also found that low-dose VPL and CAL affected intracellular  $[Ca^{2+}]_i$  and inhibited proliferation.

When PTH levels increase, intracellular  $Ca^{2+}$  (exogenous and endogenous) increases for a variety of reasons, resulting in impaired mitochondrial oxidative function and decreased generation of ATP. Furthermore, as phospholipid levels of the cell membrane decrease, the activity of  $Ca^{2+}$ -ATPase and  $Na^+$ - $K^+$ -ATPase also decreases, and the exchange of  $Na^+$ - $Ca^{2+}$  decreases. This eventually leads to decreased  $Ca^{2+}$  excretion and sustained increases in intracellular  $[Ca^{2+}]_i$ , forming a vicious circle. An increase in intracellular  $[Ca^{2+}]_i$  is involved in formation of myocardial lesions. However, in our study, interestingly, the effects of PTHrP and PTH on  $[Ca^{2+}]_i$  in CFs had the opposite effect. Therefore, PTHrP might have its own targets in CFs, independent of PTH signaling. Different from the mechanism of PTH, PTHrP increases intracellular cAMP levels, but does not increase  $Ca^{2+}$  influx in neonatal cardiac myocytes. A non-overloaded intracellular  $[Ca^{2+}]_i$  may be the reason why cardiac myocytes do not proliferate, but the specific mechanism requires further research.

PTHrP is involved in many physiological and pathological activities in various types of cells. The function and mechanisms of PTHrP in embryonic development, soluble osseous changes in bone metastasis of malignant tumors, cardiovascular development, and blood pressure regulation are clear. However, to better understand the mechanism of PTHrP in CFs, further experiments and clinical observations are required. Additionally, expression of

alpha-smooth muscle actin and the signaling pathway involved (e.g., MAPK, PKB/Akt, PKC, nuclear factor-kappa B) are unknown and should be researched further.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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