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Rat Parathyroid Hormone 1 – 34 Signals through the MEK/ERK Pathway to Induce Cardiac Hypertrophy

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This study aimed to characterize the role of the mitogen-activated protein kinase (MAPK) kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway in cardiac hypertrophy induced by parathyroid hormone (PTH). Various concentrations of rat PTH1–34 were used to induce hypertrophy in neonatal rat ventricular cardiomyocytes, and the effects were compared with control cells and those treated with PD98059, a selective inhibitor of MEK1. Hypertrophy was assessed in terms of cell diameter, atrial natriuretic peptide (ANP) mRNA expression and protein synthesis; the MEK/ERK pathway was assessed by measuring levels of phosphorylated ERK1/2. Treatment with PTH1–34 at 100 nM for 24 h effectively induced cardiac hypertrophy (increased cell diameter, protein synthesis and ANP mRNA expression) and also increased levels of phosphorylated ERK1/2 compared with normal control cells. Treatment with PTH1–34 plus PD98059 significantly attenuated these changes. These results demonstrate that inhibition of the MEK/ERK pathway blocks PTH1–34-induced cardiac hypertrophy, suggesting that PTH1–34 might signal through the MAPK pathway to induce hypertrophy in cardiomyocytes.

KEY WORDS: Parathyroid hormone; Mitogen-activated protein kinase kinase (MEK); Extracellular signal-regulated protein kinase (ERK); MEK/ERK pathway; Cardiomyocyte; Hypertrophy; Kidney disease

Introduction

Secondary hyperparathyroidism (SHPT) is a common and serious complication of chronic kidney disease (CKD) resulting from disturbances in the regulation of parathyroid hormone (PTH), calcium, phosphorus and vitamin D.[1] SHPT arises in most patients early in stage 3 of kidney failure.[2,3] Chronic SHPT is associated with several comorbidities, a major one being left ventricular hypertrophy (LVH), which is the most prevalent cardiac complication observed in patients with CKD.[4,5] LVH is also a potent risk factor for the development of cardiac arrhythmias, diastolic dysfunction, congestive heart failure and death.[6,7] A large amount of evidence confirms that LVH in patients with chronic renal failure is related to high blood levels of PTH and PTH is an independent predictor of LVH in patients with CKD.[8,9]

Cardiomyocytes have been identified as target cells for PTH.[10,11] Studies have demonstrated that PTH exerts a hypertrophic effect on adult cardiomyocytes, characterized by an increase in protein synthesis and selective induction of cytosolic creatine kinase.[12] However, the signalling pathways of PTH-induced cardiac hypertrophy remain unknown.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that play a critical role in cellular processes, such as proliferation, differentiation and development. Extracellular signal-regulated kinases 1 and 2 (ERK1/2) are the major isoforms of MAPK that have been well characterized to date and their activation requires phosphorylation by an upstream kinase known as MAPK kinase (MEK). ERK1/2 are activated when cardiomyocytes are exposed to neurohormones, such as endothelin-1 and angiotensin II or mechanical loading.[13 – 15] When activated, ERK may integrate signals from multiple receptor systems and common distal signalling pathways, leading to cardiac hypertrophy.[16] However, it remains unknown whether the ERK1/2 pathway participates in PTH-induced cardiac ventricular hypertrophy.

To study the role of ERK1/2 in the hypertrophic responses induced by PTH in ventricular myocytes, PD98059, an inhibitor of MEK1, was used to inhibit the MEK/ERK pathway in cultured neonatal rat cardiomyocytes.

Materials and methods

Cardiomyocytes were prepared from 3-day-old neonatal Wistar rats supplied by the animal experiment centre of Harbin Medical University. This study conformed with Harbin Medical University's guidelines for the care and use of laboratory animals.

PREPARATION OF CARDIOMYOCYTES

Cardiac ventricles were isolated from the rats, perfused twice with phosphate buffered saline (PBS, 0.01 mM, pH 7.2) on ice and finely chopped. Slivers of tissue were digested with 0.08% trypsin (Invitrogen, Carlsbad, CA, USA) and gently shaken at 37 °C for 5 min in a water bath; this procedure was repeated eight times. Single cells were harvested by filtration, centrifuged, resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS; Invitrogen) and 1% non-essential amino acids, and plated onto cell culture dishes for 1.5 h in a humidified incubator (95% air and 5% CO_2 at 37 °C) selectively to remove contaminating non-myocytes. Non-adherent cells were collected and replated onto another cell culture dish in DMEM supplemented with 10% FBS. To prevent growth of non-myocytes, the medium was supplemented with 5′-bromodeoxyuridine at a final concentration of 0.1 mM for the first 48 h. On day 3, the cells were rinsed twice with Hank's balanced saline solution to remove the growth media and incubated in serum-free medium. Spontaneously contracting and confluent cells were used for the experiments on day 4 after isolation.

CELL TREATMENTS

Rat PTH1–34 (Sigma, St Louis, MO, USA) was used for the cell treatments. The cells were randomly divided into six groups: (i) normal controls treated with PBS for 24 h; (ii) cells treated with 10 nM PTH1–34 for 24 h; (iii) cells treated with 100 nM PTH1–34 for 24 h; (iv) cells treated with 1 μM PTH1–34 for 24 h; (v) cells treated with 20 μM PD98059 (Promega, Madison, WI, USA) for 1 h and then with 100 nM PTH1–34 for 24 h; and (vi) cells treated with 20 μM PD98059 alone for 25 h.

HYPERTROPHIC RESPONSE

The hypertrophic response of the cultured myocytes after the various treatments was assessed by measuring cell diameter, protein synthesis and atrial natriuretic peptide (ANP) mRNA expression.

For cell diameter, cells were plated on coverslips in a 24-well plate at a density of 1×10^4 cells/cm^2. After treatment, the diameter of the cardiomyocytes in each well was measured with an ocular micrometer in 10 randomly chosen fields.

Protein synthesis was assessed by measuring [^{3}H]leucine incorporation into acid-insoluble cellular material. Cells were plated in 24-well dishes at a density of 1×10^5 cells/well, with six wells for each group. After treatment, they were pulsed with 2.5 μCi/ml [^{3}H]leucine (Beijing Atomic Energy Institute, Beijing, China) for 4 h, washed twice with cold PBS and once with 10% trichloroacetic acid (TCA), and extracted with 10% TCA at 4 °C for 30 min. Cell residues were rinsed in 95% ethanol, solubilized in 0.25 M NaOH at 4 °C for 2 h and then neutralized with 2.5 M HCl plus 1 M Tris–HCl (pH 7.5). Radioactivity was determined using a LS-6500 liquid scintillation counter (Beckmann Coulter, Fullerton, CA, USA).

Expression of ANP was measured by reverse transcription-polymerase chain reaction (RT-PCR). Cells were grown in 60 mm culture dishes at a density of 1×10^6 cells/cm^2. After treatment, total RNA was extracted using RNA TRIzol® reagent (Invitrogen) and quantified spectrophotometrically. It was then reverse transcribed to synthesize cDNA. β-Actin and ANP were amplified with a RT-PCR kit (Promega), using 10 μg total RNA per sample and specific primers (Invitrogen): ANP sense 5′-ATACAGTGCGGTGTCCAACA-3′, antisense 5-TGCTTTTCAAGAGGGCAGAT-3′, product size 257 base pairs (bp); β-actin sense 5′-CATCTGCTGGAAGGTGGACA-3′, antisense 5′-GAGAGGGAAATCGTGCGTGAC-3′, product size 452 bp. β-Actin and ANP were both amplified as follows, β-actin: 30 cycles of 94 °C for 2 min (heat start), 94 °C for 30 s (heat denaturation), 57 °C for 30 s (reannealing) and 72 °C for 30 s (progradation), followed by 72 °C for 2 min (further progradation); ANP: 30 cycles of 94 °C for 2 min (heat start), 94 °C for 30 s (heat denaturation), 48 °C for 45 s (reannealing) and 72 °C for 40 s (progradation), followed by 72 °C 2 min (further progradation). DNA amplifications were detected using 1.2% agarose formaldehyde gel with a PCR molecular weight marker for comparison. The densities of the ANP and internal control β-actin bands were determined by DNA scanner and expressed as the density ratio (ANP/β-actin). Each experiment was repeated three times.

ERK1/2 EXPRESSION

Expression of ERK1/2 was measured using Western blot analysis. Cells were grown in 60 mm culture dishes at a density of 1×10^6 cells/cm^2. After treatment, the cells were lysed and centrifuged, and total protein concentration was determined. Extracts were boiled in sample buffer and proteins were separated on a 10% sodium dodecyl sulphate–polyacrylamide gel. The separated proteins (30 μg/lane) were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and treated with blocking buffer (PBS containing 5% dry milk and 0.2% Tween-20). The membranes were incubated with primary antibody consisting of antiphospho-ERK1/2 (p-ERK1/2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:200 dilution) and anti-actin antibody (1:400

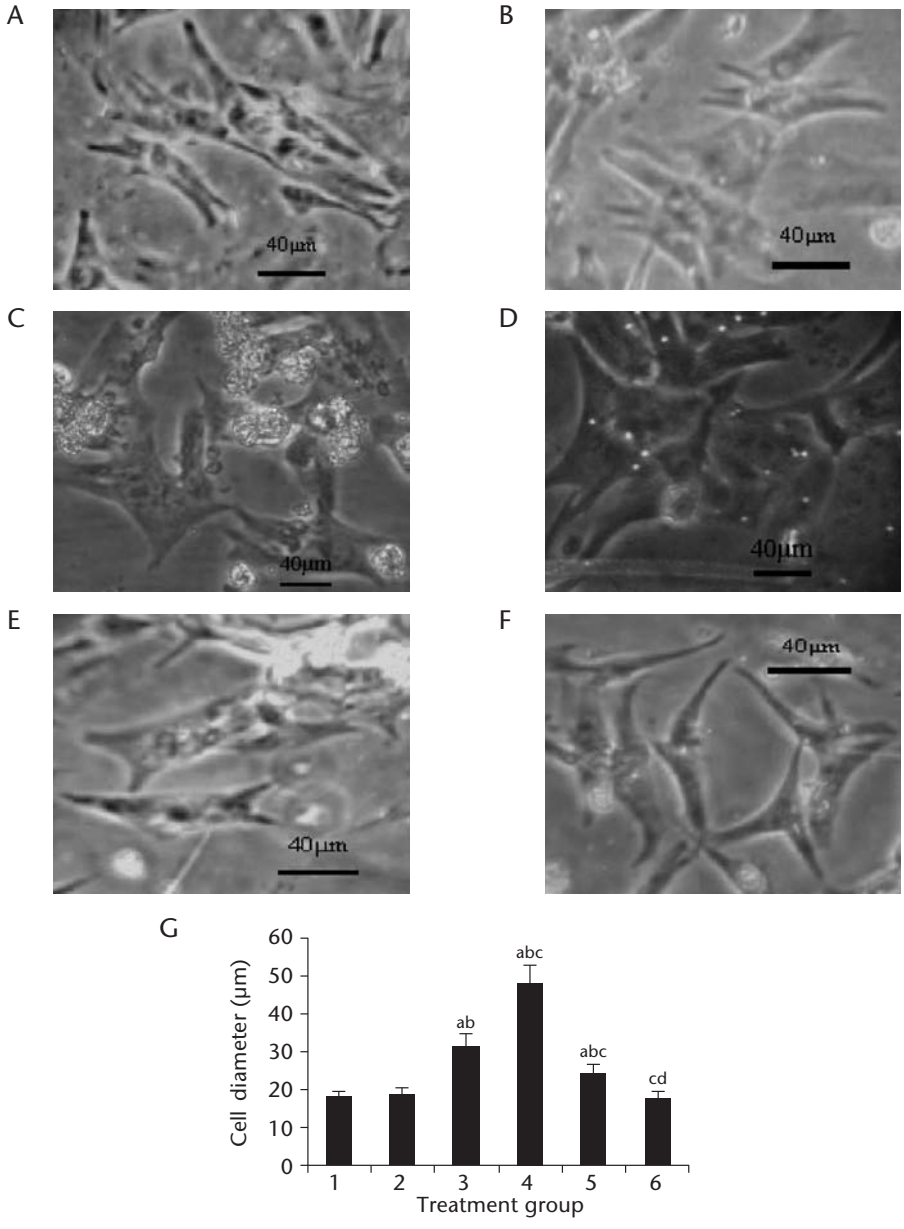

FIGURE 1: Appearance of ventricular cardiomyocytes after different treatments: (A) normal control cells (0 nM parathyroid hormone [PTH] 1–34); (B) 10 nM PTH1–34 for 24 h; (C) 100 nM PTH1–34 for 24 h; (D) 1 μM PTH1–34 for 24 h; (E) 20 μM PD98059 for 1 h then 100 nM PTH1–34 for 24 h; (F) 20 μM PD98059 for 25 h. (G) Graphical representation of mean ± SD diameter of cardiomyocytes as measured with an ocular micrometer (n = 100). Treatment groups 1 – 6 correspond with treatments shown in A – F. [a]$P < 0.05$ vs normal control; [b]$P < 0.05$ vs 10 nM PTH1 – 34; [c]$P < 0.05$ vs 100 nM PTH1–34; [d]$P < 0.05$ vs 100 nM PTH1–34 plus 20 μM PD98059 and not statistically different vs normal control ($P > 0.05$)

dilution) overnight at 4 °C and then for 1 h at 37 °C with antirabbit and antihorse immunoglobulin G conjugated with alkaline phosphatase (1:1000 dilution) as the secondary antibody. Immune complexes were visualized by 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, using a kit from Tian Gen Biotechnology (Beijing, China), according to the manufacturer's instructions. Blots were quantified by UV light scanning densitometry. Each experiment was repeated three times.

STATISTICAL ANALYSIS

Data were expressed as the mean ± SD. Homogeneity of variance was confirmed and differences across multiple conditions were tested by one-way analysis of variance for a completely randomized design. Comparisons between conditions were tested with the Bonferroni test using SPSS® version 10.0 software (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered to be statistically significant.

Results

HYPERTROPHIC RESPONSE

Compared with normal control cardiomyocytes (0 nM PTH1–34), exposure to 100 nM PTH1–34 for 24 h significantly increased cell diameter by 13.6 µm, incorporation of [^{3}H]leucine 0.5-fold and expression of ANP mRNA 0.7-fold ($P < 0.05$); 1 µM PTH1–34 significantly increased cell diameter by 17.4 µm, incorporation of [^{3}H]leucine 0.3-fold and expression of ANP mRNA 0.2-fold compared with 100 nM PTH1–34 ($P < 0.05$); 10 nM PTH1–34 did not induce a hypertrophic response (Figs 1 – 3). These results indicate that the hypertrophic response of cardiomyocytes to PTH1–34 is dose-dependent, with 100 nM PTH1–34 being the minimum effective dose for inducing cardiac hypertrophy.

Pretreatment of cardiomyocytes with 20

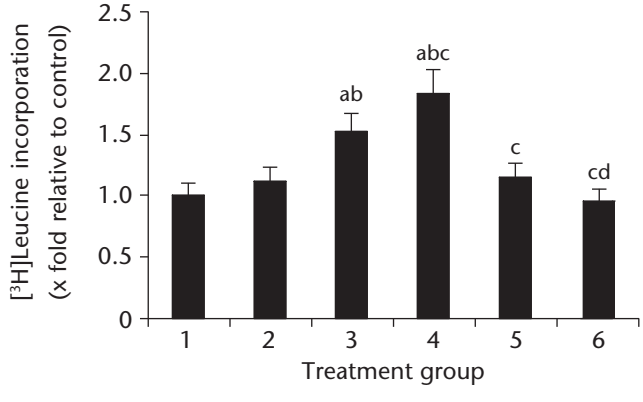

FIGURE 2: Effect of different treatments on [^{3}H]leucine incorporation by ventricular cardiomyocytes, expressed as mean ± SD relative to normal control cells ($n = 6$). Treatment groups: 1, normal control cells (0 nM parathyroid hormone [PTH] 1–34); 2, 10 nM PTH1–34 for 24 h; 3, 100 nM PTH1–34 for 24 h; 4, 1 µM PTH1–34 for 24 h; 5, 20 µM PD98059 for 1 h then 100 nM PTH1–34 for 24 h; 6, 20 mM PD98059 for 25 h. $^aP < 0.05$ vs normal control; $^bP < 0.05$ vs 10 nM PTH1–34; $^cP < 0.05$ vs 100 nM PTH1–34; $^dP < 0.05$ vs 100 nM PTH1–34 plus 20 µM PD98059 and not statistically different vs normal control ($P > 0.05$)

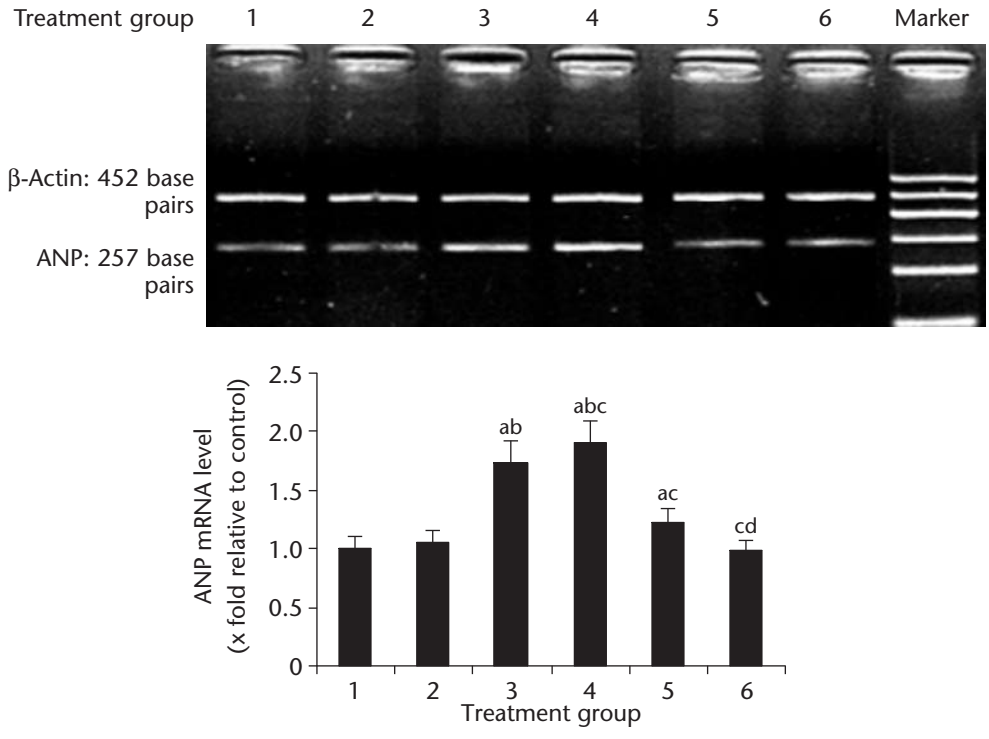

FIGURE 3: Atrial natriuretic peptide (ANP) mRNA in ventricular cardiomyocytes after different treatments, expressed as mean ± SD relative to normal control cells (n = 3). Treatment groups: 1, normal control cells (0 nM parathyroid hormone [PTH] 1–34); 2, 10 nM PTH1–34 for 24 h; 3, 100 nM PTH1–34 for 24 h; 4, 1 μM PTH1–34 for 24 h; 5, 20 μM PD98059 for 1 h then 100 nM PTH1–34 for 24 h; 6, 20 μM PD98059 for 25 h. $^{a}P < 0.05$ vs normal control; $^{b}P < 0.05$ vs 10 nM PTH1–34; $^{c}P < 0.05$ vs 100 nM PTH1–34; $^{d}P < 0.05$ vs 100 nM PTH1–34 plus 20 μM PD98059 and not statistically different vs normal control ($P > 0.05$)

μM PD98059 significantly attenuated the increase in cell diameter, incorporation of [^{3}H]leucine and expression of ANP mRNA in response to 100 nM PTH1 – 34 by 7.1 μm, 0.4-fold and 0.5-fold, respectively, compared with the 100 nM PTH1 – 34 group ($P < 0.05$). PD98059 alone had no effect compared with normal control cells (Figs 1 – 3).

ERK1/2 EXPRESSION

Given that the ERK signalling pathway is involved in endothelin-1- and angiotensin II-induced cardiac hypertrophy,[13,14] changes in ERK protein expression were investigated in PTH1–34-treated cardiomyocytes. Compared with normal control cells (0 nM PTH1–34), exposure to 100 nM and 1 μM PTH1–34 induced p-ERK1/2 by 1.9- and 2.1-fold, respectively ($P < 0.05$). Compared with 100 nM PTH1 – 34, exposure to 1 μM PTH1–34 increased p-ERK1/2 expression by 0.2-fold ($P < 0.05$) (Fig. 4). These results indicate that PTH1–34 activates ERK1/2 in a dose-dependent manner, with 100 nM PTH1–34 being the minimum effective dose.

Pretreatment of cardiomyocytes with 20 μM PD98059 significantly reduced the PTH1–34-stimulated p-ERK1/2 protein level by 1.0-fold

compared with the 100 nM PTH group ($P < 0.05$); 20 μM PD98059 alone had no effect compared with normal control cells (Fig. 4).

Discussion

Intact PTH is encoded on the short arm of chromosome 11 (11p15), comprises 84 amino acids and has a molecular weight of 9225 Da. Amino acids 1 – 34 from the *N*-terminal of PTH is the shortest fragment with complete bioactivity and has been shown to activate protein kinase C and adenylate cyclase in classic PTH target cells.[17] In these cell types, the *N*-terminal region of the hormone including the first two amino acids stimulates adenylate cyclase, and a mid-regional part, including amino acids 28 – 34, stimulates the activity of protein kinase C.[17] This was the basis on which we used commercially available synthetic rat PTH1–34 in the present study, as it contains the two active domains and can induce a hypertrophic effect in adult cardiomyocytes.[1]

Cardiomyocyte hypertrophy produces an

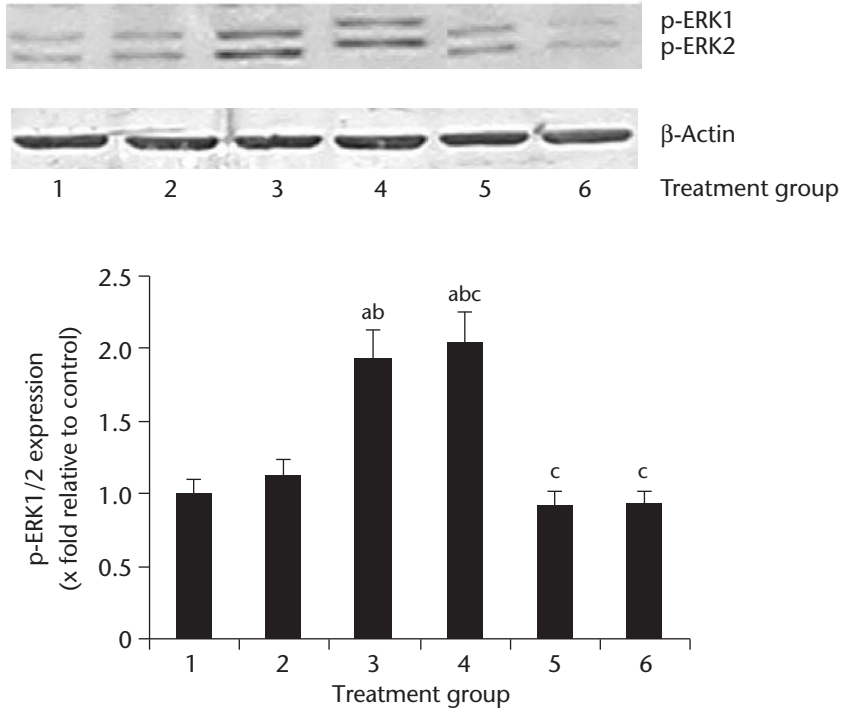

FIGURE 4: Effects of different treatments on phosphorylated extracellular signal-regulated kinases 1 and 2 (p-ERK1/2) detected by Western blot in ventricular cardiomyocytes and expressed as mean ± SD relative to normal control cells ($n = 3$). Treatment groups: 1, normal control cells (0 nM parathyroid hormone [PTH] 1–34); 2, 10 nM PTH1–34 for 24 h; 3, 100 nM PTH1–34 for 24 h; 4, 1 μM PTH1–34 for 24 h; 5, 20 mM PD98059 for 1 h then 100 nM PTH1–34 for 24 h; 6, 20 mM PD98059 for 25 h. $^{a}P < 0.05$ vs normal control; $^{b}P < 0.05$ vs 10 nM PTH1 – 34; $^{c}P < 0.05$ vs 100 nM PTH1 – 34

increase in cell volume and sarcomeres, together with changes in the contractile proteins.[18] At the molecular level, cardiac hypertrophy is characterized by an increase in contractile protein content, induction of the immediate-early genes and re-expression of cardiac embryonic genes (such as ANP, β-myosin heavy chain and skeletal α-actin).[19] These changes depend on the activation of transcription of the corresponding genes and activation of one or more of the intracellular kinase signalling pathways is involved in this.[20,21] The MAPK cascade is one of the important intracellular pathways involved in cardiac hypertrophic responses induced by humoral stimuli and ERK1/2 has been suggested as having a central role.[22] The activated form of ERK1/2, p-ERK1/2, is phosphorylated at specific threosine and tyrosine residue sites. The present study used Western blot to show that cardiomyocytes treated with 100 nM and 1 μM PTH1–34 for 24 h expressed more p-ERK1/2 than normal cells, suggesting that PTH either affects the activity of certain kinases involved in the expression of ERK1/2 or has a direct effect on the expression of ERK1/2.

Under normal conditions, ANP is mainly synthesized, stored and secreted by cardiac atria, with only little secretion from the ventricles. ANP is regarded as a distinctive index that is increased in ventricles in myocardial hypertrophy.[23] The present study showed that 1–34PTH increased the expression of ANP mRNA, while inhibition of the MEK/ERK pathway by PD98059 attenuated the expression of ANP mRNA in ventricular myocytes. Moreover, pretreatment of cardiomyocytes with PD98059 significantly downregulated the increased [^{3}H]leucine incorporation and attenuated the increased cell diameter induced by PTH1–34. These results indicate that ERK1/2 is involved in the pathway producing the PTH-induced hypertrophic response.

In conclusion, the present study showed that the MEK/ERK1/2 pathway plays a critical role in PTH1–34-induced hypertrophic responses in cultured neonatal rat ventricular myocytes.

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Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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References

1 Coladonato JA, Ritz E: Secondary hyperparathyroidism and its therapy as a cardiovascular risk factor among end-stage renal disease patients. *Adv Ren Replace Ther* 2002; **9:** 193 – 199.
2 Slatopolsky E, Delmez JA: Pathogenesis of secondary hyperparathyroidism. *Am J Kidney Dis* 1994; **23:** 229 – 236.
3 Sanchez CP, Goodman WG, Salusky IB: Prevention of renal osteodystrophy in predialysis patients. *Am J Med Sci* 1999; **317:** 398 – 404.
4 Weiner DE, Tighiouart H, Vlagopoulos PT, *et al*: Effects of anemia and left ventricular hypertrophy on cardiovascular disease in patients with chronic kidney disease. *J Am Soc Nephrol* 2005; **16:** 1803 – 1810.
5 United States Renal Data System: *USRDS 2002 Annual Data Report*. Minneapolis: USRDS, 2002 (available at http://www.usrds.org/adr_2002.htm).
6 Hennersdorf MG, Strauer BE: Arterial hypertension and cardiac arrhythmias. *J Hypertens* 2001; **19:** 167 – 177.
7 Vakili BA, Okin PM, Devereux RB: Prognostic implications of left ventricular hypertrophy. *Am Heart J* 2001; **141:** 334 – 341.

8 Nasri H, Baradaran A, Naderi AS: Close association between parathyroid hormone and left ventricular function and structure in end-stage renal failure patients under maintenance hemodialysis. *Acta Med Austriaca* 2004; **31:** 67 – 72.
9 Randon RB, Rohde LE, Comerlato L, *et al*: The role of secondary hyperparathyroidism in left ventricular hypertrophy of patients under chronic hemodialysis. *Braz J Med Biol Res* 2005; **38:** 1409 – 1416.
10 Bogin E, Massry SG, Harary I: Effect of parathyroid hormone on rat heart cells. *J Clin Invest* 1981; **67:** 1215 – 1227.
11 Schlüter KD, Piper HM: Cardiovascular actions of parathyroid hormone and parathyroid hormone-related peptide. *Cardiovasc Res* 1998; **37:** 34 – 41.
12 Schlüter KD, Wingender E, Tegge W, *et al*: Parathyroid hormone-related protein antagonizes the action of parathyroid hormone on adult cardiomyocytes. *J Biol Chem* 1996; **271:** 3074 – 3078.
13 Takahashi H, Takeishi Y, Arimoto T, *et al*: Pitavastatin inhibits cardiac hypertrophy in a rat model of progressive renal injury. *J Cardiovasc Pharmacol* 2005; **46:** 487 – 493.
14 de Jonge HW, Dekkers DH, Houtsmuller AB, *et al*: Differential signaling and hypertrophic responses in cyclically stretched vs endothelin-1 stimulated neonatal rat cardiomyocytes. *Cell Biochem Biophys* 2007; **47:** 21 – 32.
15 Pan J, Singh US, Takahashi T, *et al*: PKC mediates cyclic stretch-induced cardiac hypertrophy through Rho family GTPases and mitogen-activated protein kinases in cardiomyocytes. *J Cell Physiol* 2005; **202:** 536 – 553.
16 Takahashi H, Takeishi Y, Seidler T, *et al*: Adenovirus-mediated overexpression of diacylglycerol kinase-zeta inhibits endothelin-1-induced cardiomyocyte hypertrophy. *Circulation* 2005; **111:** 1510 – 1516.
17 Schlüter KD, Hellstern H, Wingender E, *et al*: The central part of parathyroid hormone stimulates thymidine incorporation of chondrocytes. *J Biol Chem* 1989; **264:** 11087 – 11092.
18 Yang L, Zou X, Liang Q, *et al*: Sodium tanshinone IIA sulfonate depresses angiotensin II-induced cardiomyocyte hypertrophy through MEK/ERK pathway. *Exp Mol Med* 2007, **39:** 65 – 73.
19 Zhang SQ, Ding B, Guo ZG, *et al*: Inhibitory effect of antisense oligodeoxynucleotide to p44/p42 MAPK on angiotensin II-induced hypertrophic response in cultured neonatal rat cardiac myocyte. *Acta Pharmacol Sin* 2004; **25:** 41 – 46.
20 Kilic A, Velic A, De Windt LJ, *et al*: Enhanced activity of the myocardial Na^+/H^+ exchanger NHE-1 contributes to cardiac remodeling in atrial natriuretic peptide receptor-deficient mice. *Circulation* 2005; **112:** 2307 – 2317.
21 Padmasekar M, Nandigama R, Wartenberg M, *et al*: The acute phase protein α_2-macroglobulin induces rat ventricular cardiomyocyte hypertrophy via ERK1,2 and PI3-kinase/Akt pathways. *Cardiovasc Res* 2005; **75:** 118 – 128.
22 Ueyama T, Kawashima S, Sakoda T, *et al*: Requirement of activation of the extracellular signal-regulated kinase cascade in myocardial cell hypertrophy. *J Mol Cell Cardiol* 2000; **32:** 947 – 960.
23 Silberbach M, Roberts CT Jr: Natriuretic peptide signalling: molecular and cellular pathways to growth regulation. *Cell Signal* 2001; **13:** 221 – 231.

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