Original Research

Atorvastatin prevents rat cardiomyocyte hypertrophy induced by parathyroid hormone 1–34 associated with the Ras-ERK signaling

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Abstract

We investigated the effects of atorvastatin (Ator) on cardiomyocyte hypertrophy (CMH) induced by rat parathyroid hormone 1–34 (PTH1–34) and Ras-extracellular signal regulated protein kinases 1/2 (ERK1/2) signaling. Rat cardiomyocytes were randomly divided into seven groups: normal controls (NC), PTH1–34 (10^{-7} mol/L), Ator (10^{-5} mol/L), farnesyl transferase inhibitors-276 (FTI-276, 4×10^{-5} mol/L), PTH1–34 + Ator, PTH1–34 + FTI-276 and PTH1–34 + Ator + mevalonic acid (MVA, 10^{-4} mol/L). After treatment, the hypertrophic responses of cardiomyocytes were assessed by measuring cell diameter, detecting protein synthesis, and single-cell protein content. The concentrations of hypertrophic markers such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) were measured by ELISA. Protein expressions of ERK1/2, p-ERK1/2 and Ras were detected by western blotting. The results showed that compared with the PTH1–34 group, cellular diameter, 3H-leucine incorporation, single-cell protein content, ANP and BNP concentration decreased by 12.07 μm, 1622 cpm/well, 84.34 pg, 7.13 ng/L and 20.04 μg/L, respectively, and the expressions of Ras and p-ERK1/2 were downregulated in PTH1–34 + Ator group ($P < 0.05$). Compared to the PTH1–34 + Ator group, the corresponding hypertrophic responses and hypertrophic markers increased by 4.95 μm, 750 cpm/well, 49.08 pg, 3.12 ng/L and 9.35 μg/L, respectively, and the expressions of Ras and p-ERK1/2 were upregulated in the PTH1–34 + Ator + MVA group ($P < 0.05$). In conclusion, Ator prevents neonatal rat CMH induced by PTH1–34 and Ras-ERK signaling may be involved in this process.

Keywords: The hypertrophic responses of cardiomyocytes, mevalonic acid, 3H-leucine incorporation, atrial natriuretic peptide, brain natriuretic peptide, atorvastatin

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Introduction

Cardiomyocyte hypertrophy (CMH) is a well-known heart response to increased biomechanical stress, which caused by extrinsic factors, including valvular heart disease and arterial hypertension, or intrinsic factors such as familial hypertrophic cardiomyopathy.[1] Although CMH is an adaptive response to sustain cardiac output under stress, prolonged hypertrophy is an independent risk factor for progressing to heart failure or sudden death.[2,3] Thus, it is essential to determine the molecular mechanisms of the occurrence and development of CMH.

A number of hormones such as angiotensin II,[4] endothelin 1,[5] Urotensin II[6] and parathyroid hormone (PTH)[7] are reported to result in CMH. Hyperparathyroidism (HPT) is characterized by elevated PTH and has been reported to be associated with heart diseases.[8] An increased prevalence of left ventricular hypertrophy (LVH) has been observed in HPT patients.[9] Previous study has confirmed that LVH is related to the high levels of PTH in the patients with end-stage renal failure and PTH may be involved in the pathology of LVH.[9–11] Many signal pathways have been shown to be associated with the progression of cardiovascular diseases. Ciccone et al.[12] have found that interleukin-33/ST2 (suppression of tumorigenicity 2) pathway is involved in cardiovascular disease and plays an important role in protection of cardiac muscle. Furthermore, Wei et al.[13] have demonstrated that small GTP-binding protein (Ras)-extracellular signal regulated kinase (ERK) is associated with the

progression of CMH. However, it remains unknown whether the activation of ERK in CMH induced by PTH[7] depends on Ras.

Recent study has shown that nutraceuticals that are beneficial to vascular health may be able to reduce the overall cardiovascular risk induced by dyslipidemia by acting parallel to statins.[14] Statins, namely hydroxymethyl glutaric acyl coenzyme A reductase inhibitors, have a beneficial role in the improvement of endothelial dysfunction, antioxidant effects, the stabilization of the atherosclerotic plaque and anti-inflammatory responses.[15] More recently, lipid-lowering effect of statins has been demonstrated.[16] Most importantly, some scholars have shown that statins can resist the pathological remodeling of the heart and vessels, as well as prevent cardiac hypertrophy and heart failure by cholesterol-independent mechanisms.[17,18] Takayama et al.[19] have suggested that simvastatin inhibits the aggravation of CMH induced by the large variability of blood pressure through inhibiting the activation of Ras-ERK pathways in a rat model of hypertension. However, the effect of atorvastatin (Ator) on CMH induced by PTH is still unclear.

In this study, a model of rat CMH was established by the stimulation of PTH,[7,20] and then treated with Ator, mevalonic acid (MVA) and farnesyl transferase inhibitors-276 (FTI-276). MVA has been proved to promote the activity of Ras.[21] FTI-276 was designed as a tet-rapeptide mimetic of the carboxyl terminus of K-Ras4B, which could selectively inhibit Ras farnesylation, thereby, inhibiting the activity of Ras.[22] We aimed to investigate the role and mechanism of Ator in CMH induced by PTH.

Materials and methods

Isolation and culture of cardiomyocyte

The healthy newborn Wistar rats, approximately 1–3-day olds, were provided by the Animal Laboratory Center of Harbin Medical University. Approval from the Animal Ethics Committee of the Animal Laboratory Center of Harbin Medical University was obtained prior to using the animals for research. Rats were sacrificed by cervical dislocation. Cardiomyocytes were isolated according to previously reported procedures.[23] In brief, cardiac apex from the rat was rinsed with phosphate-buffered saline (PBS, pH 7.4) on ice for two times and then finely chopped. Fragments of the tissue were digested with 0.25% trypsin (Gibco, CA, USA) for 5 min at 37 °C in a water bath, which was repeated for five to six times. Next, cardiomyocytes were harvested by filtration, resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, CA, USA) and then centrifuged with percoll separating medium (Gibco, CA, USA). Subsequently, cardiomyocytes were cultured with DMEM containing 5′-bromodeoxyuridine (10^{-4} mol/L, Sigma, St. Louis, MO, USA) to prevent the growth of fibroblast for the first 48 h. On day 3, the cells were washed with PBS to remove the growth medium and then incubated with serum-free DMEM for the next experiments.

Study design

Rat PTH1–34 (Sigma, Louis, MO, USA), Ator (Pfizer, Dalian, China), MVA (Sigma, Louis, MO, USA) and FTI-276 (Santa Cruz, Santa Cruz, USA) were used for the cell treatments. Cardiomyocytes were randomly divided into seven groups and then treated with different drugs: normal control (NC) group, the cells were treated with PBS; PTH group, the cells were treated with PTH1–34 (10^{-7} mol/L);[7] Ator group, the cells were treated with Ator (10^{-5} mol/L);[24] FTI group, the cells were treated with FTI-276 (4×10^{-5} mol/L);[25] PTH + Ator group, the cells were pre-treated with Ator for 2 h and then treated with PTH1–34 for 24 h; PTH + FTI group, the cells were pre-treated with FTI-276 for 2 h and then treated with PTH1–34 for 24 h; PTH + Ator + MVA group, the cells were treated with Ator for 2 h and then co-treated with PTH1–34 and MVA (10^{-4} mol/L)[26] for 24 h.

Hypertrophic response

The hypertrophic responses of cardiomyocytes after the various treatments were assessed by measuring cell diameter, detecting protein synthesis, single-cell protein content, concentrations of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP).

Measurement of cell diameter

After treatment, the cells grew on coverslips were fixed with 95% alcohol for 20 min, and then stained with hematoxylin (Beyotime, Beijing, China) for 3 min. Subsequently, the cells were differentiated in 1% hydrochloric acid alcohol for 2 s, incubated in ammonia water for 2 min and then stained with eosin (Beyotime, Beijing, China) for 1 min. Finally, the cells were dried and mounted with neutral resin. Five fields were randomly selected and observed at a magnification of ×400, and 20 cells were counted in each field. The minimum diameter through the nucleus was detected using Motic Images Advanced 3.0 software (MicroOptic Industrial Group Co., Ltd, Canada).

Detection of protein synthesis and protein content

Protein synthesis in cardiomyocytes was assessed by incorporation of ^{3}H-leucine as previous study.[27] Briefly, after treatment, the cells were co-incubated with 37 kBq ^{3}H-leucine (Beijing Atomic Energy Institute, Beijing, China) for 24 h, rinsed twice with ice-cold PBS and then digested with 0.25% trypsin. Subsequently, cells were fixed in nitrocellulose filter by filtering and treated with 10% trichloroacetic acid (TCA) 4 °C for 30 min. Precipitates were rinsed in 95% ethanol, solubilized in 1 M NaOH for 30 min and neutralized. Finally, radioactivity was determined using a LS-6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA). Protein content was detected by the BCA Protein Quantitative Assay (Beyotime, Beijing, China). Protein content of individual cell = protein concentration × supernatant volume/total cells number of each hole.

Detection of ANP and BNP concentration

The concentrations of ANP and BNP were measured, respectively, by Rat ANP and BNP Enzyme-linked immunosorbent assays (ELISA) Kit (R&D Systems, Inc., MN, USA) according to the manufacturer's protocol. After treatment, the cells were washed with ice-cold PBS and broken by ultrasound. Then cell supernatant was collected by centrifugation at 3000 r/min for 20 min at 4 °C to detect the content of ANP. For BNP, cell culture supernatant was directly used to detect the content of BNP. The absorbances were read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Western blotting

After treatment, the cells were collected and lysed in RIPA lysis buffer. Protein samples were separated on 12% SDS-PAGE gel, transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in 5% nonfat milk for 1 h, and then incubated overnight at 4 °C with rabbit anti-rat β-actin monoclonal antibody (1:5000, Santa Cruz, Santa Cruz, USA), mouse anti-rat K-Ras monoclonal antibody (1:500, Santa Cruz, Santa Cruz, USA), mouse anti-rat ERK1/2 polyclonal antibody (1:500, Santa Cruz, Santa Cruz, USA) and rabbit anti-rat p-ERK1/2 polyclonal antibody (1:500, Proteintech, Chicago, IL, USA), respectively. After washed for three times with PBS, the membranes were incubated with horse anti-mouse IgG (H + L)-HRP (1:1000, Zhongshan Biotech. Co., Beijing, China) or goat anti-rabbit IgG (H + L)-HRP (1:1000, Zhongshan Biotech. Co., Beijing, China) for 2 h at room temperature, respectively. Ultimately, the proteins were detected with BCIP-NBT (ECL, Santa Cruz, Santa Cruz, USA) and then the protein expression was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analysis was performed by SPSS 18.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Data were expressed as the mean ± SD and analyzed by one-way ANOVA. A value of $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

Results

Hypertrophic responses in various treatment groups

Compared with the NC group, cell diameter was significantly increased by 13.24 μm in the PTH group ($P < 0.05$; Figure 1(a) and (b)). Incorporation rate of ^{3}H-leucine and protein content of individual cell, respectively, were also increased by 1652 cpm/well and 84.62 pg in the PTH group compared with the NC group ($P < 0.05$; Figure 1(b)). These results indicated that PTH1–34 could induce hypertrophic responses in cardiomyocytes. In contrast to the PTH group, Ator significantly decreased cell diameter, incorporation rate of ^{3}H-leucine and protein content of individual cell by 12.07 μm, 1622 cpm/well and 84.34 pg, respectively ($P < 0.05$; Figure 1(a) and (b)). Similarly, FIT-276 reduced cell diameter, incorporation rate of ^{3}H-leucine and protein content of individual cell by 12.70 μm, 1579 cpm/well and 66.38 pg compared with the PTH group ($P < 0.05$; Figure 1(a) and (b)). Meanwhile, there were no significant differences in these hypertrophic responses between the NC group and the Ator or FIT group (Figure 1(a) and (b)). The above results suggested that Ator and FIT-276 might prevent hypertrophic responses induced by PTH1–34 in cardiomyocytes. Compared with the PTH + Ator group, combined treatments of PTH + Ator + MVA increased cell diameter, incorporation rate of 3H-leucine and protein content of individual cell by 4.95 μm, 750 cpm/well, and 49.08 pg, respectively ($P < 0.05$; Figure 1(a) and (b)).

Contents of hypertrophic markers in various treatment groups

ELISA results showed that hypertrophic markers ANP and BNP contents, respectively, were remarkably increased by 9.19 ng/L and 22.82 μg/L in the PTH group compared with the NC group ($P < 0.05$; Figure 2). In contrast to the PTH group, Ator significantly decreased ANP and BNP contents by 7.13 ng/L and 20.04 μg/L, respectively ($P < 0.05$). In addition, compared with the PTH + Ator group, combined treatments of PTH + Ator + MVA increased ANP and BNP contents by 3.12 ng/L and 9.35 μg/L, respectively ($P < 0.05$; Figure 2). All these results were consistent with the results of hypertrophic responses.

Expression of Ras and p-ERK1/2 in various treatment groups

After treatment with PTH1–34, the relative expressions of Ras and p-ERK1/2 were increased by 1.30- and 0.72-fold compared with the NC group, respectively ($P < 0.05$). Compared with the PTH group, Ator decreased Ras and p-ERK1/2 expressions by 0.58- and 0.24-fold, respectively ($P < 0.05$). However, the relative expressions of Ras and p-ERK1/2 were increased by 0.37- and 2.26-fold in the PTH + Ator + MVA group compared with the PTH + Ator group ($P < 0.05$; Figure 3).

Discussion

CMH was characterized by the increased cardiomyocyte size and stromal hyperplasia, and the activation of various neuroendocrine factors such as PTH played an important role in CMH.[28,29] In the present study, our results showed that PTH1–34 could induce the hypertrophic responses of cardiomyocytes and elevate contents of hypertrophic markers ANP and BNP. Compared with PTH group, Ator or FIT-276 alone had no effect on hypertrophic responses of cardiomyocytes, while Ator and FIT-276 could prevent these hypertrophic responses induced by PTH1–34. The combination of PTH1–34, Ator, and MVA partly recovered these hypertrophic responses and elevate contents of ANP and BNP compared with the PTH + Ator group. In addition, the expressions of K-Ras, ERK1/2 and p-ERK1/2 had the similar changes in various groups.

The previous study had demonstrated that PTH could regulate the function and proliferation of cardiomyocyte through binding with PTH-1 receptor which located in

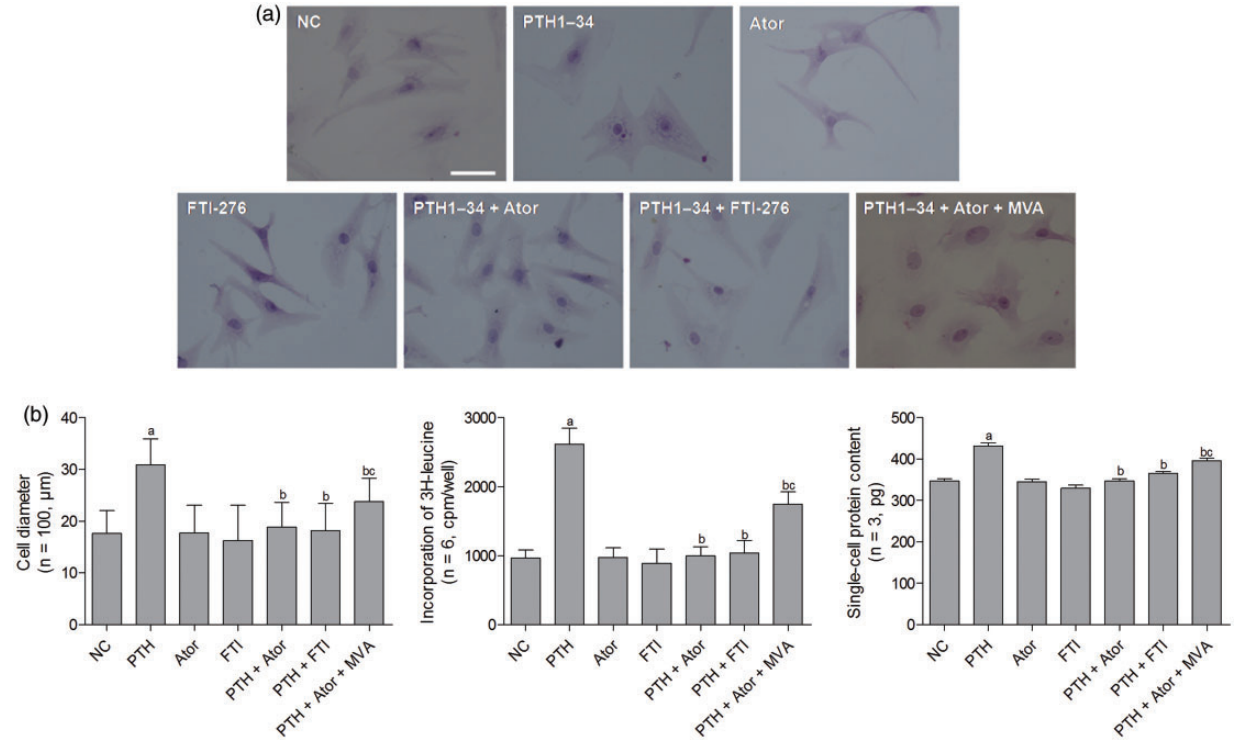

Figure 1 Hypertrophic response, including cell diameter, incorporation of 3H-leucine, and single-cell protein content in cardiomyocytes after the various treatments. (a) Hematoxylin-eosin staining showed that compared with the NC group, cell diameter was significantly increased in the PTH group; however, cell diameter was significantly decreased in the PTH + Ator and the PTH + FTI groups compared with the PTH group. Furthermore, cell diameter was significantly increased in the PTH + Ator + MVA group compared with the PTH + Ator group. Bar = 50 μm. (b) Bar graphs showed the results of incorporation of 3H-leucine and single-cell protein content was consistent with the result of cell diameter after the various treatments. $^{a}P < 0.05$ versus NC group, $^{b}P < 0.05$ versus PTH group, $^{c}P < 0.05$ versus PTH + Ator group. NC, normal control; PTH, parathyroid hormone; MVA, mevalonic acid, the activator of Ras; FTI, farnesyl transferase inhibitors, the inhibitor of Ras.(A color version of this figure is available in the online journal.)

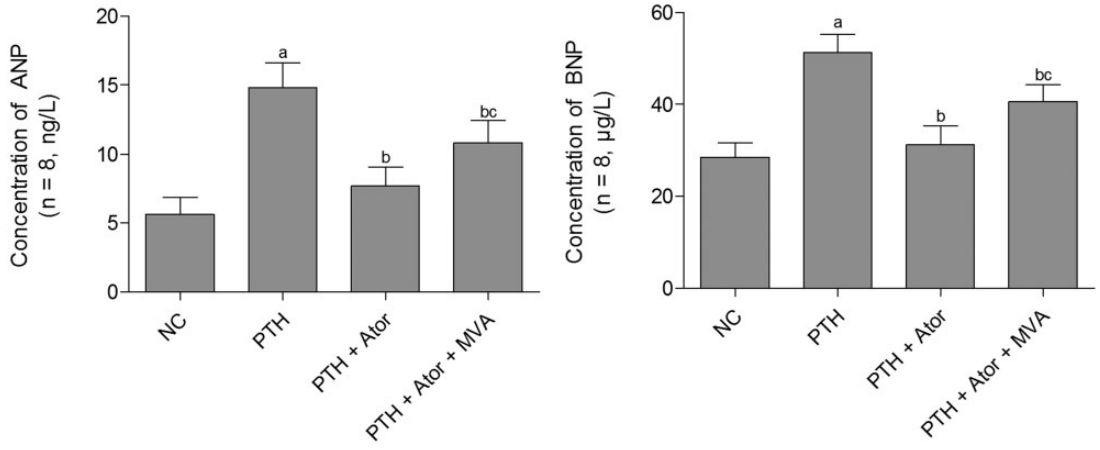

Figure 2 The concentrations of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in cardiomyocytes after the various treatments. Compared with the NC group, ANP and BNP contents were significantly increased in the PTH group; however, ANP and BNP contents were significantly decreased in the PTH + Ator group compared with the PTH group. Furthermore, ANP and BNP contents were significantly increased in the PTH + Ator + MVA group compared with the PTH + Ator group. $^{a}P < 0.05$ versus NC group, $^{b}P < 0.05$ versus PTH group, $^{c}P < 0.05$ versus PTH + Ator group. NC, normal control; PTH, parathyroid hormone; MVA, mevalonic acid, the activator of Ras; FTI, farnesyl transferase inhibitors, the inhibitor of Ras

cardiomyocyte.[30] Further studies had shown that elevated PTH was associated with CMH, and CMH could be induced by PTH.[7,20] Consistently, our study also successfully established the model of rat CMH by the stimulation of PTH1–34. Previous studies had suggested that statins could inhibit CMH in hypertensive rats[19] and apolipoprotein E-deficient mice.[31] Similarly, we found that Ator could effectively prevent the hypertrophic responses of cardiomyocyte induced by PTH1–34, indicating that Ator might prevent CMH induced by PTH1–34. Currently, the specific mechanism of statins in inhibiting CMH was still equivocal. Some researches showed that the suppressive effect of statins on the development of CMH might be mediated through increasing the levels of peroxisome proliferator

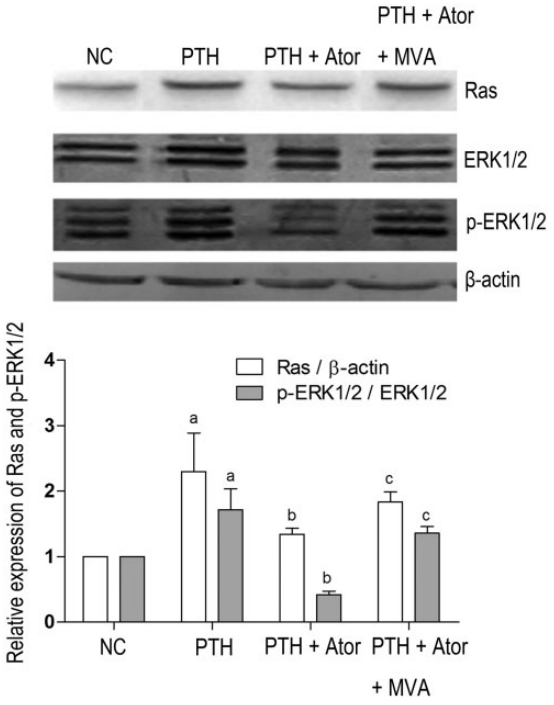

Figure 3 Expressions of K-Ras, ERK1/2 and p-ERK1/2 in cardiomyocytes after the various treatments. The expressions of Ras and p-ERK1/2 were increased in the PTH group compared with the NC group. Compared to the PTH group, the expressions of Ras and p-ERK1/2 were decreased in the PTH + Ator group, while compared to the PTH + Ator group, the expressions of Ras and p-ERK1/2 were increased in the PTH + Ator + MVA group. $^{a}P < 0.05$ versus NC group, $^{b}P < 0.05$ versus PTH group, $^{c}P < 0.05$ versus PTH + Ator group. NC, normal control; PTH, parathyroid hormone; MVA, mevalonic acid, the activator of Ras; FTI, farnesyl transferase inhibitors, the inhibitor of Ras

activated receptor α (PPAR α) and PPAR γ,[31] as well as preventing the activation of nuclear factor (NF)-κB.[32] However, the majority of studies suggested that the suppressive effect of statins on CMH was associated with the inhibition of MVA pathway and small GTPases activity.[33]

In recent years, Ras-ERK signaling was focused on cancer research.[34] Ras family, one member of small GTP binding protein superfamily, regulated various biology events such as cell proliferation, differentiation and apoptosis.[35] Some scholars had shown that Ras-ERK signaling involved in the occurrence and development of CMH;[13,36] however, Duquesnes et al.[37] had suggested that the activation of ERK1/2 pathway was predominantly independent of the activity of Ras during cardiomyocyte stretch. In our study, we found that the expressions of Ras and p-ERK1/2 were increased during CMH induced by PTH1–34; however, Ator inhibited the expressions of Ras and p-ERK1/2. Importantly, we found that FTI-276, inhibitor of Ras activation, had a preventing effect on CMH. Thus, we speculated that Ator might prevent the occurrence of CMH related to the activation of Ras-ERK signaling. Senthil et al.[38] had suggested that Ator prevented the process of CMH by decreasing the expression of Ras in transgene rabbit. Indolfi et al.[39] also had demonstrated that simvastatin prevented CMH induced by pressure overload through inhibiting Ras activation. To further confirm whether Ras-ERK involved in the anti-CMH effect of Ator, we treated cardiomyocyte with the combination of FIT1–34, Ator and MVA. Our result showed that the combination of PTH1–34, Ator and MVA partly recovered these hypertrophic responses compared with the PTH + Ator group, indicating that MVA, as the activator of Ras activity, might partly obstruct anti-CMH effect of Ator. We speculated that anti-CMH effect of Ator might be associated with Ras-ERK signaling. Meanwhile, this result prompted that the other pathways except for Ras might involve in this process.

Unfortunately, our study has several limitations. First, a MVA alone treatment and a PTH + MVA treatment were lacking in this study. Therefore, we cannot confirm that whether PTH + MVA have a synergistic effect on the hypertrophic responses. The expressions of Ras and p-ERK1/2 were only detected after treatment for 24 h, and our study was preliminary. Thus, the investigation of mechanisms will be more complete by studying the activation of Ras-ERK signaling at different time-points after PTH1–34 administration.

Conclusions

In summary, our study indicated that Ator attenuated neonatal rat CMH induced by PTH1–34, which might be associated with the activation of Ras-ERK signaling. However, the further study is essential to determine the association of Ator and Ras-ERK signaling, as well as explore whether other pathways involve in the anti-CMH effect of Ator.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; XL, CZ, CY and RX conducted the experiments, MS, SM, LL and SZ supplied critical reagents and cells, XL and CZ wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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