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Inhibition of farnesylpyrophosphate synthase prevents angiotensin II-induced hypertrophic responses in rat neonatal cardiomyocytes: Involvement of the RhoA/Rho kinase pathway

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ABSTRACT

The RhoA/Rho-kinase (ROCK) pathway is involved in angiotensin (Ang) II-induced cardiac hypertrophy. However, it is still unclear whether inhibition of farnesylpyrophosphate (FPP) synthase can attenuate Ang II-induced hypertrophic responses, and whether it involves the RhoA/ROCK pathway. The anti-hypertrophic effects of inhibition of FPP synthase with alendronate in Ang II-cultured neonatal cardiomyocytes were partially reversed by geranylgeranyol (GGOH) and were mimicked by GGTI-286, a geranylgeranyl transferase-I inhibitor, C3 exoenzyme, an inhibitor of Rho, or Y-27632, an inhibitor of ROCK. Pull-down assay showed alendronate reduced-active RhoA by Ang II was also partially antagonized by GGOH. This study revealed that the inhibition of FPP synthase by alendronate reduces RhoA activation by diminishing geranylgeranylation which prevents Ang II-induced hypertrophic responses in neonatal cardiomyocytes.

Structured summary:
MINT-7260047: *Rhotekin-RBD* (uniprotkb:Q9BST9) *physically interacts* (MI:0915) with *Rhoa* (uniprotkb:P61589) by *pull down* (MI:0096)

1. Introduction

Cardiac hypertrophy is thought to be induced by humoral factors such as angiotensin II (Ang II) [1]. Ventricular hypertrophy is associated with increased cell size and changes in protein content and gene expression, such as brain natriuretic peptide (BNP) [2,3]. Various signal transduction pathways mediate the Ang II-induced hypertrophic response of cardiomyocytes [4–6]. RhoA/ROCK signaling is thought to be activated in the Ang II-induced hypertrophic response of cardiomyocytes, because recent studies have shown that Ang II activates RhoA in cardiomyocytes, and the inhibition of ROCK or Rho suppresses Ang II-induced cardiomyocyte hypertrophy [6–9].

RhoA switches between an inactive guanosine diphosphate (GDP)-bound and an active guanosine triphosphate (GTP)-bound form [10]. RhoA must first be targeted by attachment of 20-carbon geranylgeranyl groups to its C-terminal cysteine residues, known as the geranylgeranylation of RhoA [11]. The enzyme that catalyzes RhoA geranylgeranylation is geranylgeranyl transferase-I [12]. Geranylgeranylation of RhoA is critical for its membrane localization, permitting its interaction with effector molecules such as ROCK, to trigger diverse cellular functions [13].

Experiments have shown that alendronate inhibits farnesyl pyrophosphate (FPP) synthase [14], a key enzyme in the mevalonate pathway, through inhibition of isoprenylation including farnesylation and geranylgeranylation with consecutive decreases in the formation of isoprenoid lipids such as FPP and geranylgeranyl pyrophosphate (GGPP) [15]. The latter is essential for geranylgeranylation and activation of RhoA [16–18].

In this study, we explored that inhibition of FPP synthase by alendronate could interfere with the hypertrophic response induced by Ang II in cultured neonatal ventricular myocytes, and further demonstrated that inhibition of FPP synthase in neonatal cardiomyocytes is, at least in part, via the RhoA/ROCK pathway.

2. Materials and methods

2.1. Materials

Alendronate sodium, angiotensin II, collagenase I, and geranylgeranyol (GGOH) were from Sigma (Sigma–Aldrich Co., USA).

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GGTI-286 was from Calbiochem (San Diego, CA, USA). Y-27632 and C3 exoenzyme were from Alexis (San Diego, USA). All other reagents used in the experiment were of analytical grade.

2.2. Animals

Male, 1–2 day-old Wistar rats were obtained from the Experimental Animal Center, Chinese Academy of Sciences (Shanghai, China). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was approved by the Institutional Animal Care and Use Committee of Zhejiang University.

2.3. Cell culture

Neonatal cardiomyocytes were prepared from the ventricles of 1- to 2-day-old Wistar rats as previously described [19] with some modification. Minced ventricular myocardium was dissociated by 0.125% trypsin (Gibco, USA) and 0.05% collagen-I (Sigma–Aldrich) mixture digestion in D-Hanks salt solution (Gibco). The cell suspension was centrifuged at 900 rpm for 6 min and the dissociated cells were enriched in cardiomyocytes by differential adhesion for 60 min and plated at 5×10^5 to 1×10^6 cells/ml. The cardiomyocytes were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Bromodeoxyuridine (0.1 mM) (Amersco, USA) was added to the medium to inhibit proliferation of non-myocytes. This procedure yielded cultures with 90–95% myocytes, as assessed by microscopic observation of cellular contractions. The cardiomyocytes were cultured for 48 h and then in serum-free medium (SFM) for 24 h before initiating the study, when the cells were treated with various agents for the indicated times.

2.4. Measurement of cell surface area

To determine cell surface area, cardiomyocyte images captured by a Cannon camera under an Olympus inverted microscope (Japan) were measured by the method of Simpson and Savion [20]

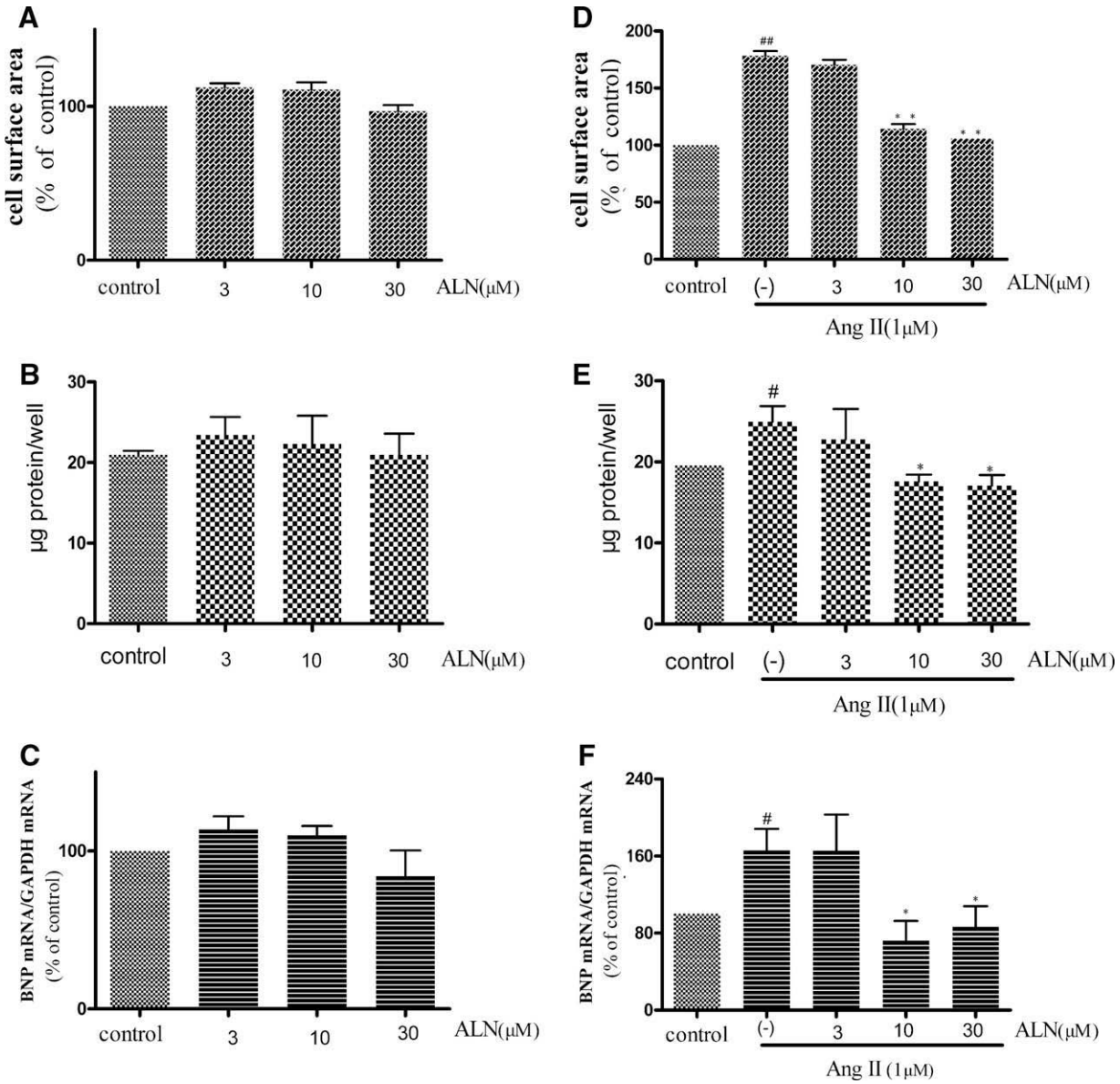

Fig. 1. Effects of alendronate on Ang II-induced hypertrophic response of cardiomyocytes. Myocytes were pre-incubated with alendronate (ALN) (3, 10, or 30 μM) for 30 min and then incubated with or without Ang II (1 μM) for 48 h. Effects of alendronate on (A) cell surface area; (B) protein content; (C) BNP mRNA expression; (D) Ang II-stimulated cell surface area; (E) Ang II-stimulated protein content; (F) Ang II-stimulated BNP mRNA expression. ALN: alendronate. Assays for BNP mRNA expression and protein content, $n = 4$ wells each time, and each experiment was carried out in triplicate. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs Ang II group.

using NIH Image J software. All cells from randomly selected fields in 2 or 3 wells were examined for each condition. We measured 100 cells in each condition. The cell surface area in control cells was normalized to 100% and all of the results were expressed as the percentage of control values. This process was replicated in three independent experiments.

2.5. Measurement of protein content

Cultured myocytes were treated with various agents for the indicated times. After washing with phosphate buffered saline, the cells were treated with 10% trichloroacetic acid (Sigma–Aldrich) as described by Yamamoto et al. [21]. Thereafter, the precipitates were dissolved in 0.15 N NaOH. The protein content was measured using a BCA protein assay (Beyotime, China).

2.6. Quantitative real-time polymerase chain reaction

Total RNA extraction and RT-PCR were performed as previously described [22]. After 48 h of incubation with various agents, the cultured cardiac myocytes were submitted to RNA extraction. Total RNA was isolated from cultured cells with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Real-time quantitative RT-PCR was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) with SYBR Green PCR master mix (Applied Biosystems) in a total volume of 25 μl. The relative amount of BNP mRNA expression was normalized using the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, as internal control. The primer sequences for each gene (Sangon, Shanghai, China) were: BNP forward, 5′CTGTGACGGGCTGAGGTTGT3′; reverse, 5′TGGCAAGTTTGTGCT GGAAG3′; and GAPDH forward, 5′AAGAAGGTGGTGAAGCAGGC3′; reverse, 5′TCCACCACCCTGT TGCTGTA3′. Data were analyzed with Sequence Detection System software (Applied Biosystems). Each run was completed with melting curve analysis to confirm the specificity of amplification. In addition, products were controlled with gel electrophoresis. Quantification was performed using the standard curve method [23]. In brief, standard curves using six points with serial dilution of the known starting copy number of the appropriate cDNA, leading to a high linear relationship [Correlation coefficient (r) > 0.99)] between the threshold cycle (CT) and the logarithm of the cDNA concentration for both BNP and GAPDH genes. The amount of target and control genes was quantified by measuring CT and determined from the appropriate standard curve. The relative amount of BNP mRNA expression was normalized by GAPDH mRNA to obtain a normalized target value. Each of the experimental normalized sample values was divided by one normalized control sample value (calibrator) to generate the relative expression level.

2.7. RhoA activity assay

Pull-down assay to measure RhoA activity was performed using a Rho activation assay kit, according to the manufacturer's protocol (Cytoskeleton, Denver, CO, USA). Sub-confluent cardiac myocytes were incubated in SFM for 24 h before addition of 30 μM alendronate with or without comparable GGOH for a further 48 h. Cells were then stimulated with 1 μM Ang II for 15 min at 37 °C before addition of lysis buffer as described by Aoki et al. [6]. A protein assay was performed prior to the pull-down assay to equalize total protein concentration in each treatment group. Meanwhile, GAPDH protein was used as the internal control. Whole cell lysates were incubated with agarose-conjugated rhotekin-RBD for 60 min at 4 °C and then washed once with wash buffer. Agarose beads were boiled in SDS–PAGE sample buffer to release active Rho prior to undergoing precipitation with the Rhotekin GTP-Rho binding domain (Cytoskeleton). After precipitation, samples were processed for Western blotting with a specific anti-RhoA antibody (Cytoskeleton). Meanwhile, 20 μg total cell lysate per sample was used to detect total RhoA.

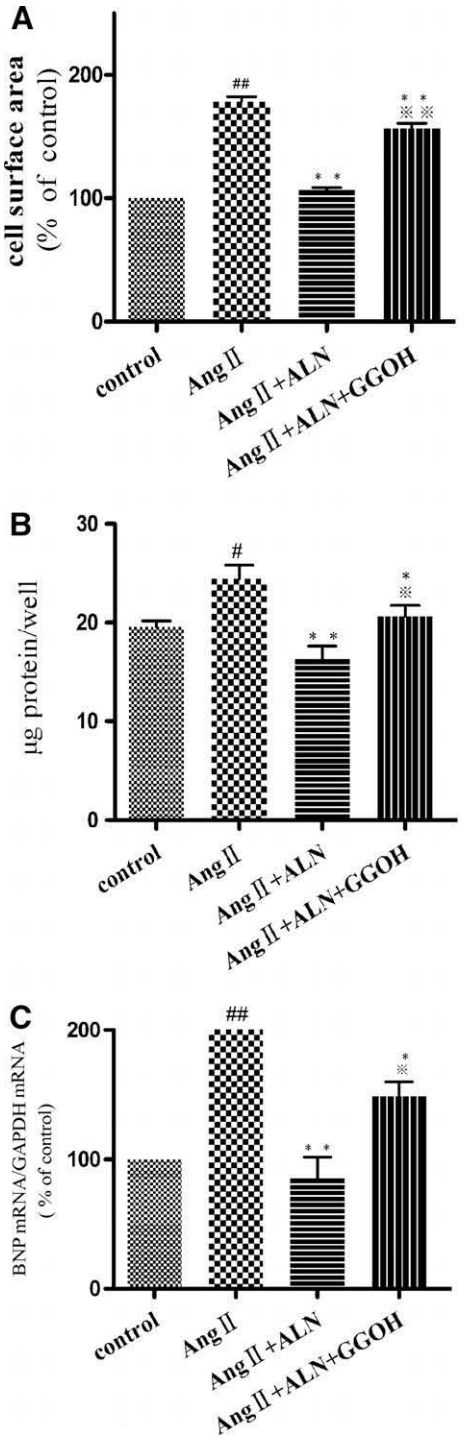

Fig. 2. Influence of GGOH on anti-hypertrophic action of alendronate. Myocytes were pre-incubated with 30 μM ALN alone or in combination with equal GGOH for 30 min and then were stimulated combined with Ang II (1 μM) for 48 h. (A) Cell area surface; (B) protein content; (C) BNP mRNA expression. ALN: alendronate. Assays for BNP mRNA expression and protein content, $n = 4$ wells each time, and each experiment was carried out in triplicate. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs Ang II group; $^{※}P < 0.05$ and $^{※※}P < 0.01$ vs Ang II + ALN group.

2.8. Statistical analysis

Results are expressed as means ± standard error of the mean (S.E.M.) of at least three separate experiments. Statistical significance was determined using one-way ANOVA followed by a post hoc test. The differences were considered statistically significant at a value of $P < 0.05$.

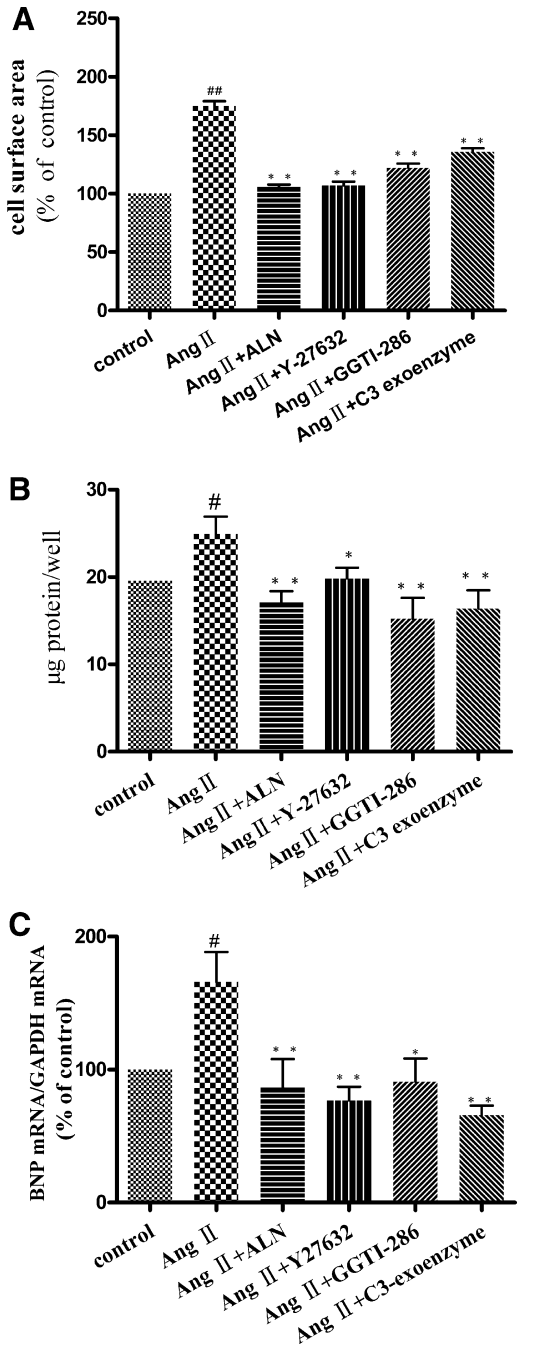

Fig. 3. Effects of Rho/ROCK inhibitors on Ang II-induced cardiomyocyte hypertrophy. Myocytes were pre-incubated with Y-27362 (at 10 μM), GGTI-286 (at 10 μM), C3 exoenzyme (at 30 ng/mL), or ALN (at 30 μM) for 30 min and then stimulated combined with Ang II (1 μM) for 48 h. (A) Cell area surface; (B) protein content; (C) BNP mRNA expression. ALN: alendronate. Assays for BNP mRNA expression and protein content, $n = 4$ wells each time and each experiment was carried out in triplicate. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs Ang II group.

3. Results

3.1. Prevention of cardiomyocyte hypertrophy by inhibition of FPP synthase

Alendronate alone did not affect cell surface area, protein content, or BNP mRNA expression in cultured cardiomyocytes (Fig. 1A–C). However, pretreatment with alendronate significantly attenuated cell surface area, protein content, and BNP mRNA expression in neonatal cardiomyocytes stimulated with Ang II in a dose-dependent manner (Fig. 1D–F).

3.2. Regulation of the anti-hypertrophic effect of alendronate by geranylgeranylated protein

In order to explore whether the reversal of cardiomyocyte hypertrophy by alendronate is associated with the geranylgeranylated protein, we treated cardiomyocytes with alendronate along with GGOH (Sigma–Aldrich Co., USA). The results showed that the effects of alendronate on cell surface area (106.84 ± 1.83 vs 156.80 ± 4.91%, $P < 0.01$), protein content (16.33 ± 1.34 vs 20.66 ± 1.08 μg/well, $P < 0.05$), and BNP mRNA expression (85.65 ± 16.07 vs 148.50 ± 11.62%, $P < 0.05$) were prevented by GGOH treatment (Fig. 2A–C).

3.3. Effects of Rho/ROCK inhibitors on Ang II-induced hypertrophic response in cardiomyocytes

Because GGOH partially reversed the inhibitory effects of alendronate on Ang II-induced hypertrophy, and RhoA is a geranylgeranylated protein [24], we sought to investigate the influence of Rho/ROCK inhibition. We found that GGTI-286 (10 μM), a specific inhibitor of geranylgeranyl transferase-I [25], which regulates the

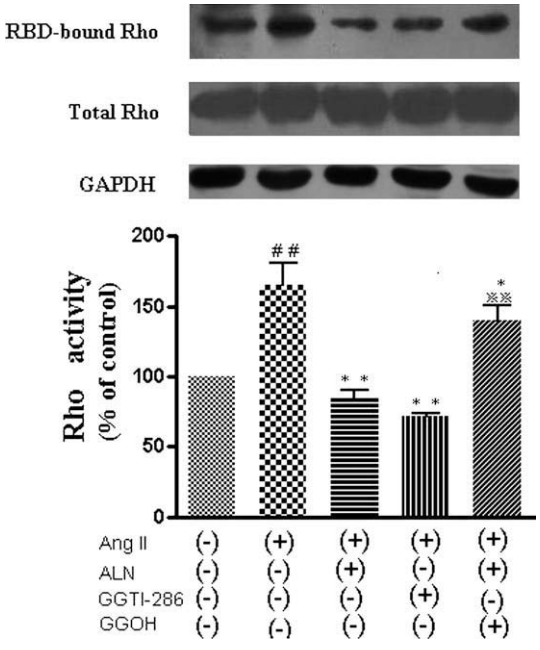

Fig. 4. RhoA expression and activation. Serum-starved cells in the absence or presence of 10 μM GGTI-286 or 30 μM alendronate were combined with or without GGOH before incubation with Ang II for 15 min. Bound RhoA proteins were detected by Western blot using polyclonal antibody against RhoA (upper panel). Western blotting of the total amount of RhoA in cell lysates (middle panel) was also performed in the same lysates. GAPDH protein was the endogenous control for each sample (lower panel). ALN: alendronate. Values shown are representative of four independent experiments. $^{\#\#}P < 0.01$ vs control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs Ang II group; $^{\&\&}P < 0.01$ vs Ang II + ALN group.

geranylgeranylation of Rho, mimicked the inhibitory effect of alendronate on cell surface area, protein content, and BNP mRNA expression (Fig. 3). Furthermore, C3 exoenzyme (30 ng/ml), a specific inhibitor of Rho [26], significantly inhibited increases of cell surface area, protein content, and BNP mRNA expression in the group treated with Ang II (Fig. 3). Similarly, Y-27632 (10 μM), a ROCK inhibitor [27], inhibited Ang II-induced hypertrophy as assessed by cell surface area, protein content, and BNP mRNA expression (Fig. 3).

3.4. Effect of alendronate on RhoA activation

Since inhibition of Rho geranylgeranylation is the most likely mechanism to explain the anti-hypertrophic effects of alendronate on cardiomyocytes, we investigated the effect of alendronate on RhoA activation. We used a pull-down assay with the fusion protein GST-RBD, which specifically recognizes Rho-GTP, the active form of Rho. An increase in Rho-GTP occurred in cardiomyocytes treated with Ang II for 15 min. The active form of Rho (GTP-bound) was elevated to 165.20 ± 15.53% of control after the addition of Ang II (Fig. 4). Pretreatment with 30 μM alendronate markedly reduced RhoA activation to 85.20 ± 4.81% ($P < 0.01$). However, in the presence of 30 μM GGOH, the inhibitory effect of alendronate against the activation of Rho by Ang II was markedly reversed to 139.60 ± 10.76%, but was still significantly lower than the Ang II group (Fig. 4).These results suggest that Rho activation is partially attenuated by alendronate via the inhibition of geranylgeranylation. Furthermore, the inactivation was mimicked by GGTI-286 (10 μM) (Fig. 4), which confirms that alendronate inhibits the hypertrophic response through inhibition of Rho geranylgeranylation.

4. Discussion

The results of the present study demonstrated inhibition of FPP synthase by alendronate, which protected against Ang II-mediated hypertrophic responses as measured by increases of cell surface area, protein content, and BNP mRNA expression in neonatal cardiomyocytes. The underlying mechanism involved inhibition of GGPP and RhoA signaling. Previous studies have shown that the primary mechanism of action of the FPP synthase inhibitor alendronate is due to the reduction of isoprenoid intermediates such as GGPP [14,15,28,29], that is essential for the post-translational geranylgeranylation of small Rho GTPase signaling proteins, including RhoA (Fig. 5). GGOH is metabolized to GGPP in cells [30]. In our study, incubation with GGOH partially reversed the alendronate-induced inhibition of the cardiac hypertrophic response evoked by Ang II, suggesting that the anti-hypertrophic effect of alendronate on Ang II activated cardiac hypertrophy may be associated, at least in part, with the suppression of GGPP, and thus might be via limitation of RhoA geranylgeranylation.

In cancer cells and endothelial cells, alendronate exerts its effect by inhibiting FPP synthase by inhibiting RhoA geranylgeranylation and activity [31,32]. However, little is known about whether FPP synthase inhibition modulates the RhoA signaling pathway in neonatal cardiomyocytes.

Ang II evokes a variety of hypertrophic responses such as enlarged cell size [2,20], changes in gene expression [2,3], and

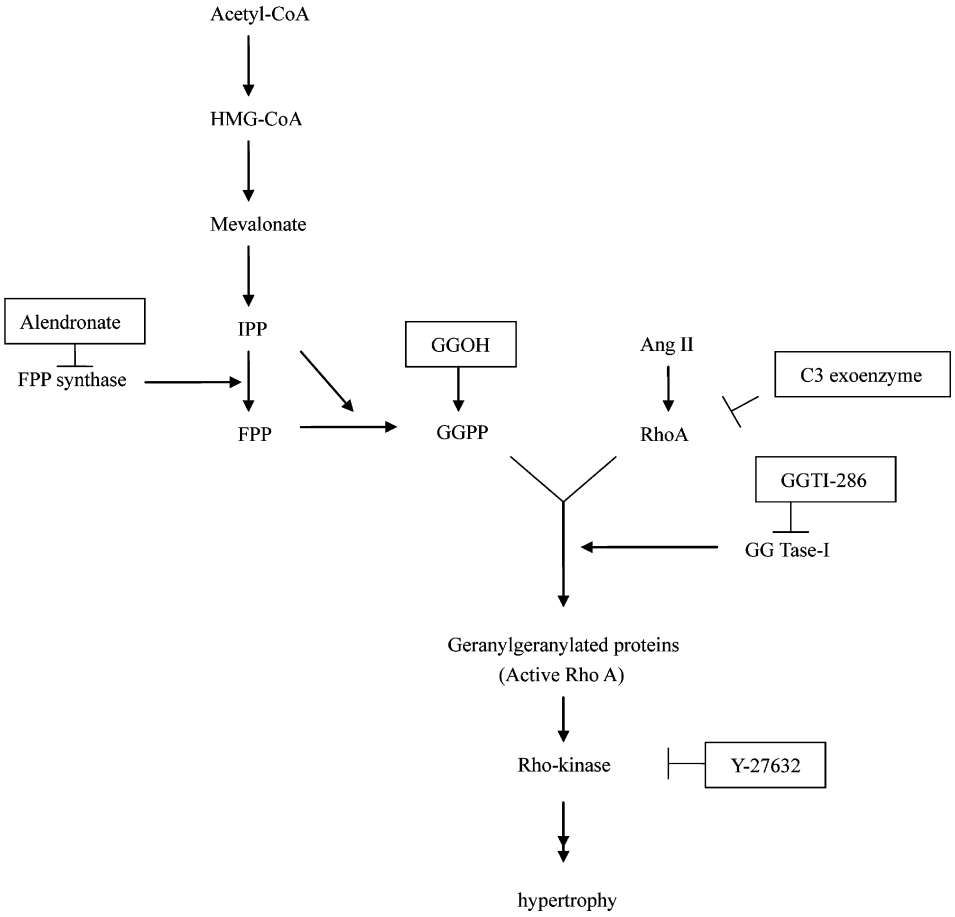

Fig. 5. Schematic representation of the anti-hypertrophic response of alendronate via regulation of isoprenoid products and RhoA/ROCK activation in neonatal cardiomyocytes. Cell-permeable geranylgeranyol (GGOH) is converted to GGPP in cells.

increases of protein content [21] in cardiomyocytes. Notably, evidence indicates that the activated RhoA/ROCK pathway contributes to Ang II-induced cardiac hypertrophy [6–9], though various pathways contribute to the Ang II mediation mechanism.

sGGPP activates RhoA via geranylgeranylation [13]. The active GTP-bound form of RhoA elicits downstream signaling such as ROCK which influences cytoskeleton organization and gene transcription [13,33]. We found a higher level of GTP-bound active RhoA but a similar level of total RhoA in cardiomyocytes in response to Ang II, which is consistent with several other investigations [6,8]. Furthermore, administration of alendronate decreased the level of active RhoA but had no effect on total RhoA expression. Thus, our results showed that the FDS inhibitor alendronate only affects the activated form of RhoA. In addition, we demonstrated that the alendronate-induced decrease in the activated form of RhoA, was reversed by GGOH, as was cardiac hypertrophy, suggesting that the inhibition of FPP synthase reduced the activation of RhoA in cardiomyocytes via depletion of GGPP and consequent suppression of RhoA geranylgeranylation. Studies have demonstrated that inhibition of RhoA by C3 exoenzyme attenuates hypertrophic response of cardiomyocytes induced by Ang II, as indicated by expression of specific genes including atrial natriuretic factor (ANF) [6] and protein synthesis [9]. This was further confirmed by our in vitro study as shown by decreases in cell surface area, protein content, and BNP mRNA expression (Fig. 3). Furthermore, GGTI-286, the specific inhibitor of geranylgeranylation [12], which attenuated the Ang II-induced RhoA activity (Fig. 4), was also found to inhibit cardiac hypertrophy induced by Ang II (Fig. 3).This is supported by the report of Laufs et al. [34] demonstrating that inhibition of geranylgeranyl transferase by GGTI-286 (50 μM) prevented the molecular characteristics of the hypertrophic phenotype ANF expression induced by Ang II. In addition, in our study, inhibition of ROCK, the downstream effector of RhoA, by Y27632 attenuated Ang II-induced cardiomyocyte hypertrophy, consistent with an in vitro report by Morikawa-Futamatsu et al. [8] and in vivo studies showing that long-term inhibition of ROCK suppresses Ang II-induced cardiovascular hypertrophy in rats [7], as well as in apolipoprotein E-deficient mice [35]. All these results further support the notion that inhibition of RhoA activation and RhoA geranylgeranylation is of importance for the protection by alendronate against Ang II-induced hypertrophic responses in neonatal cardiomyocytes.

In conclusion, administration of the GGPP substrate GGOH partially abolishes the protection against Ang II-mediated cardiac hypertrophic responses, and the reduction of Ang II-evoked RhoA activity in vitro, induced by alendronate. This suggests that lowering GGPP formation and subsequent inactivation of RhoA/ROCK signaling may be the primary mechanism underlying the inhibition of FPP synthase by alendronate in neonatal cardiomyocytes, and a link between the FPP synthase and Ang II-mediated RhoA/ROCK signaling in cardiac hypertrophy was demonstrated.

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