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Full paper

PKCζ interacts with STAT3 and promotes its activation in cardiomyocyte hypertrophy

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ABSTRACT

This study was aimed to investigate the crosstalk between protein kinase C ζ (PKCζ) and signal transducer and activator of transcription 3 (STAT3) in cardiomyocyte hypertrophy. In neonatal rat cardiomyocyte hypertrophic model induced by phenylephrine (PE), the levels of phosphorylated PKCζ and phosphorylated STAT3 were significantly increased, suggesting the activation of both PKCζ and STAT3 in cardiomyocyte hypertrophy. Overexpression of PKCζ by adenovirus infection elevated the expressions of hypertrophic markers atrial natriuretic factor (ANF) and brains natriuretic polypeptide (BNP), as well as the cell surface area; while genetic silencing of PKCζ inhibited PE-induced cardiomyocyte hypertrophy. An interaction between PKCζ and STAT3 in cardiomyocytes was shown by co-immunoprecipitation experiments. Overexpression of PKCζ increased the phosphorylated level of STAT3 at both Ser727 and Tyr705, promoted the nuclear translocation of STAT3, and enhanced the expression of STAT3 downstream target genes c-fos and angiotensinogen (aGT); whereas PKCζ knockdown prevented PE-induced STAT3 activation, nuclear shuttling and transcriptional activation. In conclusion, PKCζ interacts with STAT3 and promotes its activation in cardiomyocyte hypertrophy. Strategies targeting inhibition of PKCζ-STAT3 signaling pathway suggest a therapeutic potential for cardiac hypertrophy.

1. Introduction

Cardiac hypertrophy is a common response of the cardiomyocytes to a variety of physiological and pathological stimuli. However, prolonged hypertrophy deteriorates the cardiac function and eventually leads to heart failure. Therefore, it is of great importance to inhibit cardiac hypertrophy as a therapeutic strategy against heart failure. Cardiac hypertrophy is commonly associated with upregulation of fetal genes, such as atrial natriuretic factor (ANF), brain natriuretic polypeptide (BNP), and genes for fetal isoforms of contractile proteins like β-myosin heavy chain (β-MHC). However, signaling mechanisms responsible for the regulation of fetal gene program remain to be elucidated.

Recent studies suggest that activation of protein kinase C ζ (PKCζ), a member of the atypical PKC isozymes, is closely related to the pathogenesis of cardiac hypertrophy. A significant upregulation of PKCζ has been observed in the left ventricles of cardiac hypertrophy animal model induced by volume overload or by pressure overload (1–3). Sustained activation of PKCζ promotes the expression of ANF in rat cardiomyocytes (4). Knockout of PKCζ prevents angiotensin II-induced cardiac hypertrophy *in vivo* (5). These observations show that PKCζ plays an important role in cardiac hypertrophy.

Abbreviations: PKCζ, protein kinase C ζ; STAT3, Signal transducer and activator of transcription 3; PE, phenylephrine; ANF, atrial natriuretic factor; BNP, brain natriuretic polypeptide; aGT, angiotensinogen; Ser727, serine 727; Tyr705, tyrosine 705.

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Signal transducer and activator of transcription 3 (STAT3) is highlighted as a regulator of a wide variety of biological processes, such as cell survival and apoptosis, inflammation and angiogenesis (6). STAT3 has been found to be important for transducing hypertrophic signals in cardiomyocyte hypertrophy (7, 8). Activation of STAT3 has been observed in cardiac hypertrophy induced by a variety of stress conditions, such as pressure overload (9), acute myocardial infarction (10), and exposure to angiotensin II (11) or isoproterenol (12). Transgenic mice with cardiomyocyte-specific overexpression of STAT3 develop cardiac hypertrophy with increased expressions of the hypertrophic markers ANF and β-MHC (7). Moreover, genes involved in cardiomyocyte hypertrophy, such as c-fos, ANF and angiotensinogen (aGT), can be regulated by STAT3 (8, 13, 14).

The interactions between PKCζ and STAT3 have been proposed by several studies. As a serine/threonine kinase (2), PKCζ regulates the Janus kinase (JAK)/STAT pathway in inflammation (15). Additionally, PKCζ regulates STAT3 activation in pancreatic cancer cells both *in vitro* and *in vivo* (16, 17). However, it is still unknown whether PKCζ regulates STAT3 activation in cardiac hypertrophy.

In this study, we assess the role of PKCζ in cardiomyocyte hypertrophy stimulated by phenylephrine (PE), and investigate the interactions between PKCζ and STAT3 in neonatal rat cardiomyocytes (NRCMs).

2. Materials and methods

2.1. Cell culture

Primary culture of NRCMs were isolated from the hearts of 1 to 3-day-old Sprague–Dawley (SD) rats using a protocol previously described (18). The cardiomyocytes were purified and seeded at a density of 1×10^6 cells/well onto 6-well plates, then cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) and 5-bromodeoxyuridine (0.1 mM) at 37 °C. After 48 h, the culture medium was replaced by DMEM containing 0.1% FBS, and the cells were further incubated for 16–18 h. Subsequently, the cardiomyocytes were treated with PE for indicated time.

2.2. Plasmid transfection and adenovirus infection

STAT3 plasmid was constructed with pEGFP-N3, and confirmed by DNA sequencing in Sangon Biotech Co Ltd (Shanghai, China). Cardiomyocytes were transiently transfected with the plasmid using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Adenovirus expressing PKCζ (Ad-PKCζ) and GFP vector (Ad-GFP) were purchased from Vigene Biosciences (China). Cells were infected to Ad-PKCζ or Ad-GFP at the multiplicity of infection (MOI) of 60 for 48 h before they were harvested for RNA or protein extraction.

2.3. RNA interference

Three different siRNAs for PKCζ (S1, S2, and S3) and negative control siRNA were obtained from Genema (China). The sequences of siRNAs were shown in Supplemental Material Table S1. NRCMs were transfected with either PKCζ siRNAs or negative control siRNA using Lipofectamine 2000 according to the manufacturer's instructions. At 48 h after the transfection, cells were harvested for RNA or protein extraction to compare the silencing efficacy of different duplex siRNAs.

2.4. Western blotting and co-immunoprecipitation (co-IP)

Rabbit anti-phospho-PKCζ polyclonal antibody (diluted 1:500 for WB, Cell Signaling Technology, USA), rabbit anti-PKCζ (C-20) polyclonal antibody (WB: diluted 1:500, IP: diluted 1:10, Santa Cruz, CA, USA), rabbit anti-phospho-STAT3 (Ser727) polyclonal antibody (diluted 1:1000, Cell Signaling Technology), rabbit anti-phospho-STAT3 (Tyr705) polyclonal antibody (diluted 1:1000, Cell Signaling Technology), and mouse anti-STAT3 polyclonal antibody (diluted 1:1000, Cell Signaling Technology) were used in the experiments. Mouse anti-α-tubulin monoclonal antibody (diluted 1:10,000, Sigma–Aldrich, MO, USA) was served as a loading control.

30 μg protein extracts was separated by electrophoresis in SDS–PAGE gel, transferred to PVDF membranes (Millipore, USA), and incubated with primary antibodies overnight at 4 °C, followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h and later by Super Signal Chemiluminescent Substrate (Pierce, USA). The intensity of protein bands was analyzed using a Gel Doc XR System (Bio-Rad, Hercules, CA, USA).

For co-IP, 200 μg proteins were incubated with 1 μg anti-STAT3 or anti-PKCζ antibodies overnight (normal IgG was used as a control), followed by incubation with protein G-agarose beads (Pierce, Rockford, IL, USA) at 4 °C for 4 h. The immunoprecipitated proteins were detected by Western blotting.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Trizol reagent (Takara Biotechnology, Dalian, China) according to manufacturer's instruction. 1 μg of total RNA was reverse transcribed using Thermo RevertAid First Strand cDNA Synthesis Kit. The mRNA expression levels were determined using SYBR-Green Quantitative PCR kit (TOYOBO, Japan) by iCycler iQ system (iCycler, Bio-Rad, Hercules, CA, USA). Rat-specific primers for ANF, BNP, c-fos, and aGT (listed in Supporting Information Table S2) were synthesized by Sangon (China). β-Actin was served as an endogenous control.

2.6. Measurement of cell surface area

Cardiomyocytes grown in 48-well plates were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and incubated with 0.3% Triton-X 100 for 30 min, and further incubated with 0.1% rhodamine-phalloidin for 1 h. After washed with PBS, the cells were incubated with DAPI (dye for the nuclei, Invitrogen) and detected by high Content Analysis System (Thermo, ArrayScanVTI, USA).

2.7. Immunofluorescence staining

Cardiomyocytes grown on confocal dishes were treated with the corresponding stimuli. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and permeabilized with PBS containing 0.1% Triton X-100 for another 10 min at room temperature. The cells were then blocked with 10% goat serum for 30 min at room temperature and incubated with mouse antibody against STAT3 (for STAT3 subcellular detection) in 10% goat serum overnight at 4 °C. Then the dishes were washed and incubated in the dark with Alexa Fluor 488-conjugated secondary antibody at room temperature for 1 h. The nuclei were detected by DAPI solution for 10 min in the dark at room temperature. Finally, the dishes were washed and the images were collected using a Zeiss LSM 510 laser confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

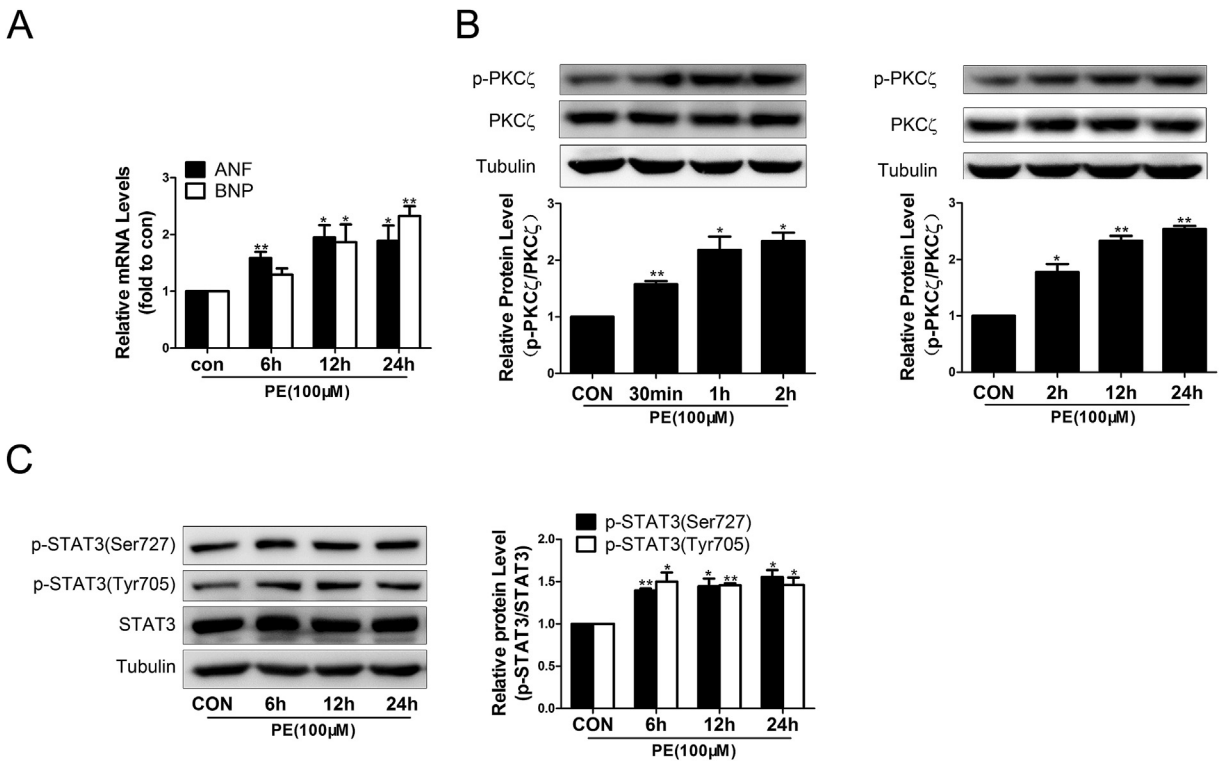

Fig. 1. Expressions of p-PKCζ and p-STAT3 were increased in phenylephrine (PE)-induced cardiomyocyte hypertrophy. (A) Real-time PCR analysis showing the expressions of fetal genes atrial natriuretic factor (ANF) and brain natriuretic polypeptide (BNP) in rat neonatal cardiomyocytes (RNCMs) treated with phenylephrine (PE) 100 μM for indicated times. Western blotting analysis showing the expressions of (B) total PKCζ and phosphorylated PKCζ, and (C) total STAT3 and phosphorylated STAT3 at serine 727 (p-STAT3-Ser727) and tyrosine 705 (p-STAT3-Tyr705) in RNCMs treated with 100 μM PE for indicated times. Data were presented as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control, n = 3.

2.8. Statistical analysis

Data were presented as mean ± SE. Statistical analysis was performed by SPSS statistic software 13.0. Statistical analyses between two groups were performed by unpaired Student's t-test. Differences among groups were tested by one-way analysis of variance (ANOVA) with Tukey's post hoctest. In all cases, value of $P < 0.05$ was considered statistically significant.

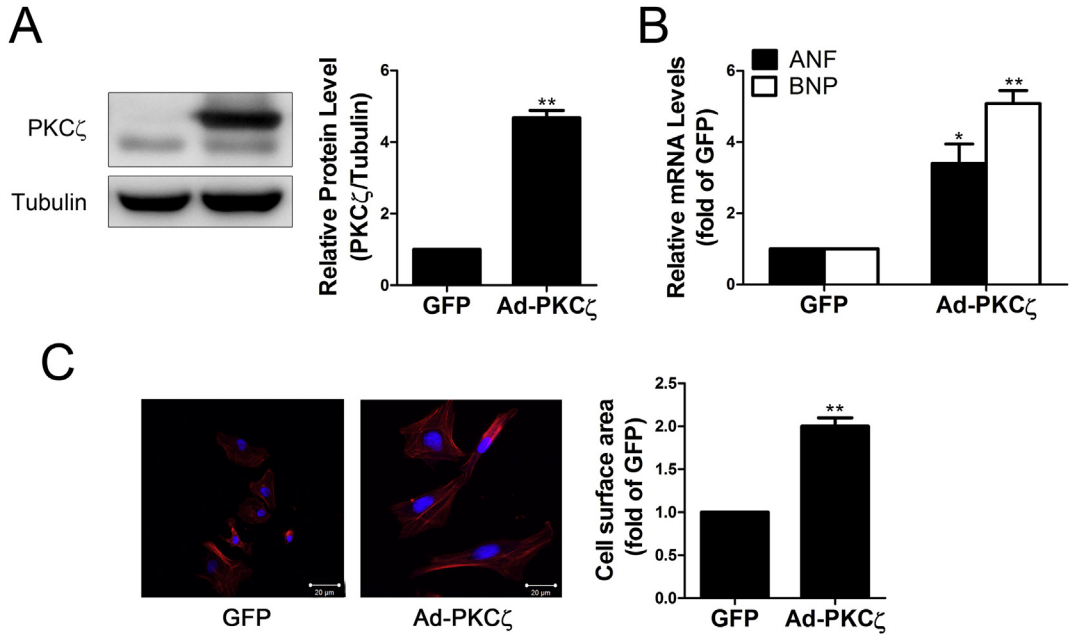

Fig. 2. PKCζ overexpression enhanced hypertrophic responses in primary neonatal rat cardiomyocytes. Cardiomyocytes were infected with Ad-PKCζ or an empty vector for 48 h. (A) Western blotting analysis showing the protein expression of PKCζ; (B) Real-time PCR analysis showing the expressions of fetal genes atrial natriuretic factor (ANF) and brain natriuretic polypeptide (BNP); (C) Cell surface area measured by phalloidin staining. Data were presented as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs GFP, n = 3.

3. Result

3.1. Phospho-PKCζ and Phospho-STAT3 levels were increased by PE in cultured NRCMs

PE has been widely used as a stimulus of cardiac hypertrophy *in vivo* and *in vitro* (19). In this study, primary cultured NRCMs were incubated with 100 μM PE for indicated time points. The mRNA expressions of the hypertrophic markers, including ANF and BNP, were significantly increased after PE treatment for 24 h (Fig. 1A), indicating that the model of PE-induced cardiomyocyte hypertrophy was successfully established. In these hypertrophic cardiomyocytes, the expression of phosphorylated PKCζ was elevated in a time-dependent manner (Fig. 1B). Since phosphorylation of STAT3 at serine residue at amino acid position 727 and at tyrosine 705 are responsible for its activation (20), the effect of PE treatment on STAT3 phosphorylation was investigated. As shown in Fig. 1C, the phosphorylation levels of STAT3 at serine 727 and tyrosine 705 were time-dependently augmented by PE. These observations suggest that both PKCζ and STAT3 were activated in PE-induced cardiomyocyte hypertrophy.

3.2. Effect of PKCζ on cardiomyocyte hypertrophy

NRCMs were infected with Ad-PKCζ or the empty vector Ad-GFP for 48 h. The expression of PKCζ was significantly increased by Ad-PKCζ infection (Fig. 2A). The expressions of hypertrophic markers ANF and BNP, as well as the cell surface area, were investigated. As shown in Fig. 2B and C, the mRNA levels of ANF and BNP, as well as the cell surface area were significantly enhanced by Ad-PKCζ infection. These observations indicate that overexpression of PKCζ might trigger cardiomyocyte hypertrophy.

To investigate the effect of endogenous PKCζ on PE-induced cardiomyocyte hypertrophy, PKCζ was knocked down by RNA interference in NRCMs. After comparing the silencing efficacy of three different siRNA for PKCζ (S1, S2, and S3), S3 was chosen for the following experiments since it showed the best silencing efficacy (Supplementary Fig. S1). The result in Fig. 3A confirmed that PKCζ silencing using S3 inhibited the expression of PKCζ in the presence or absence of PE. Knockdown of PKCζ prevented cardiomyocyte from PE-stimulated hypertrophy, as revealed by the decrease of mRNA levels of ANF and BNP (Fig. 3B), as well as the cell surface area (Fig. 3C). These results suggest that endogenous PKCζ is involved in PE-induced cardiomyocyte hypertrophy.

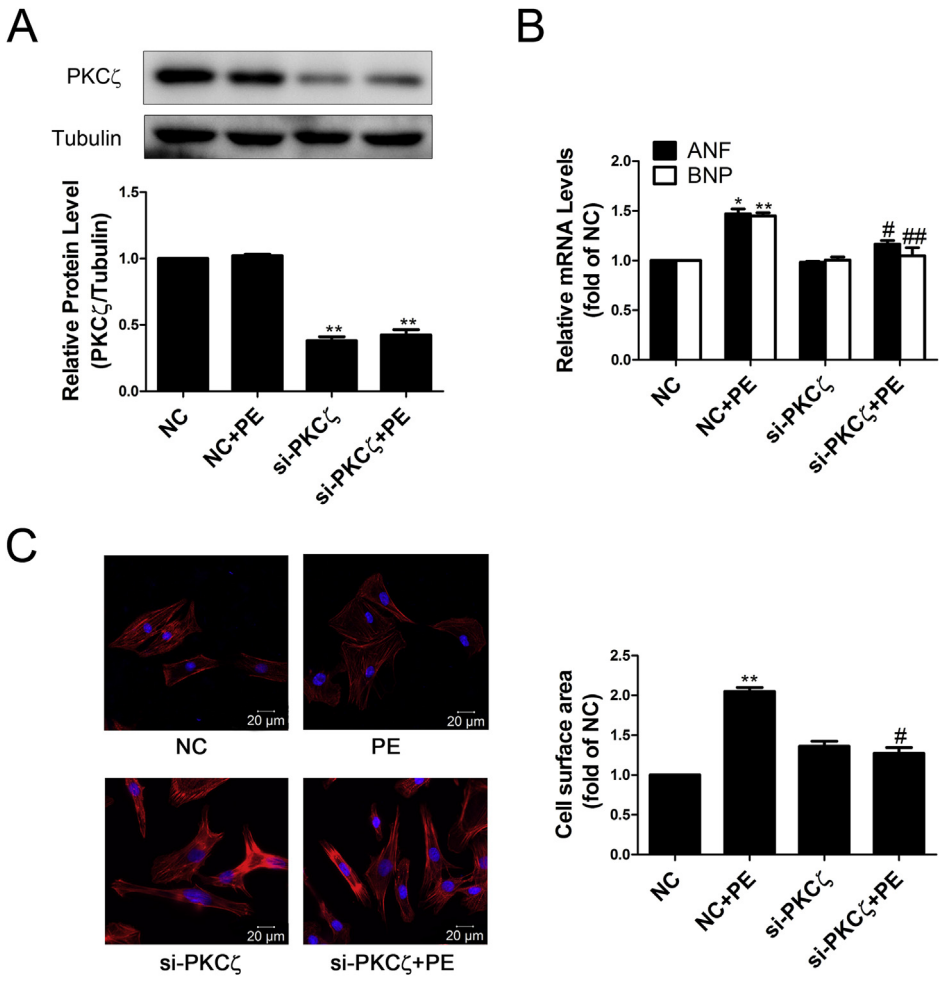

Fig. 3. PKCζ knockdown attenuated hypertrophic responses induced by phenylephrine (PE) treatment. Primary neonatal rat cardiomyocytes were transfected with PKCζ siRNA (si-PKCζ) and negative control (NC) for 24 h, and were treated with or without 100 μM PE for another 24 h. (A) Western blotting analysis showing the protein expression of PKCζ; (B) Real-time PCR analysis showing the expressions of fetal genes atrial natriuretic factor (ANF) and brain natriuretic polypeptide (BNP); (C) Cell surface area measured by phalloidin staining. Data were presented as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs NC; $\#p < 0.05$, $\#\#p < 0.01$ vs NC + PE. n = 3.

3.3. Effect of STAT3 on cardiomyocyte hypertrophy

NRCMs were transfected with pEGFP-STAT3 plasmid or an empty vector N3 for 48 h. Fig. 4A showed that STAT3 was overexpressed by transfection of STAT3 plasmid. As shown in Fig. 4B and C, the mRNA expressions of the hypertrophic markers ANF and BNP, as well as the cell surface area were significantly increased by STAT3 overexpression, suggesting that STAT3 deteriorates cardiomyocyte hypertrophy.

3.4. PKCζ interacted with STAT3 in NRCMs

To explore whether PKCζ interacts with STAT3 in NRCMs, co-IP experiments were conducted in NRCMs treated with or without PE, or in cells overexpressed PKCζ or STAT3. As shown in Fig. 5A and B, there are interactions between PKCζ and STAT3, no matter in control cells, PE-treated cells, or cells with PKCζ or STAT3 overexpression.

3.5. PKCζ enhanced activation and nuclear translocation of STAT3

Since PKCζ regulates a series of transcription factors and protein kinases by phosphorylating their serine or threonine residues (21), it is hypothesized that PKCζ phosphorylates STAT3 to evoke STAT3 activation. To investigate the exact role of PKCζ in STAT3 activation, NRCMs were infected with Ad-PKCζ, or transfected with PKCζ siRNA. As shown in Fig. 6A, Ad-PKCζ significantly augmented the phosphorylated level of STAT3 both at Ser727 and Tyr705 without affecting its total protein level. The effect of PKCζ overexpression on STAT3 activation was resembled as that of PE treatment. In contrary, PKCζ knockdown reversed PE-induced upregulation of phosphorylated STAT3 (Fig. 6B).

After activation, STAT3 is transported from the cytoplasm to the nucleus to regulate the transcription of its target genes (22). Thus, the nuclear translocation of STAT3 was investigated by determining the expression of STAT3 in the nuclear fractions of NRCMs and by detecting the subcellular distribution of STAT3 under confocal microscope. A marked increase of phosphorylated STAT3 (at both

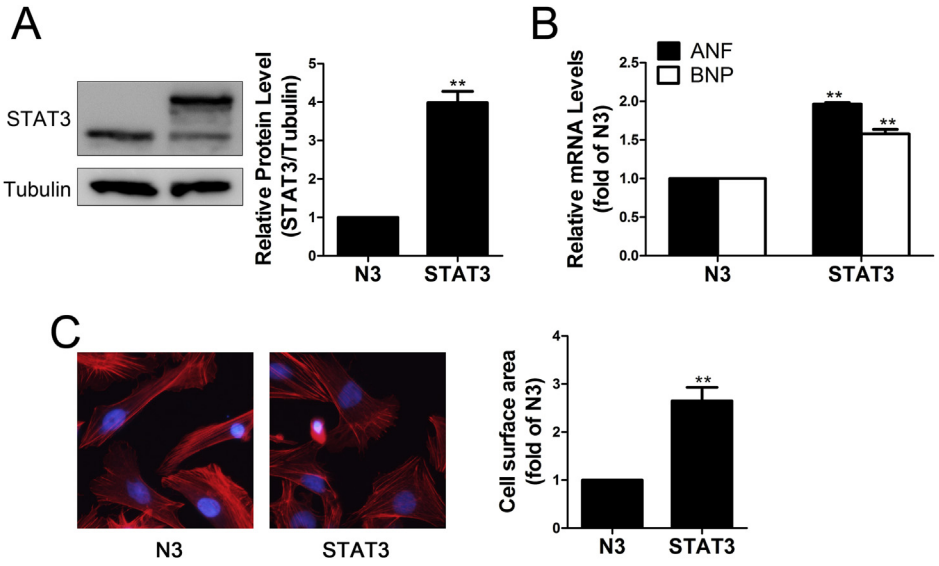

Fig. 4. STAT3 overexpression enhanced hypertrophic responses in primary neonatal rat cardiomyocytes. STAT3 was transiently overexpression in neonatal rat cardiomyocytes with pEGFP-STAT3 plasmid for 48 h. (A) Western blotting analysis showing the protein expression of STAT3; (B) Real-time PCR analysis showing the expressions of fetal genes atrial natriuretic factor (ANF) and brain natriuretic polypeptide (BNP); (C) Cell surface area measured by phalloidin staining. Data were presented as means ± SE. $^{**}p < 0.01$ vs GFP, n = 3.

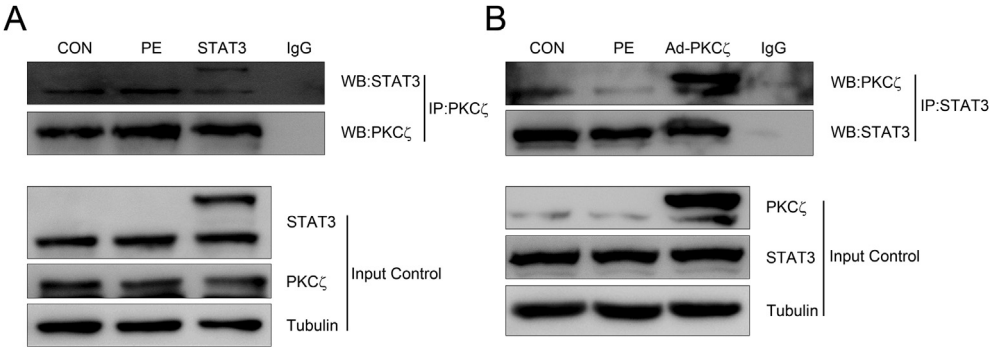

Fig. 5. PKCζ interacted with STAT3 in cardiomyocytes. Co-IP assay was performed to investigate the interaction between PKCζ and STAT3. (A) Primary neonatal rat cardiomyocytes were treated with 100 μM PE for 24 h (PE group) or were transfected STAT3 plasmid (STAT3 group). Total protein extracted from the cardiomyocytes were immunoprecipitated with anti-PKCζ antibody (CON, PE, and STAT3 groups) or anti-IgG antibody (IgG group). Expressions of STAT3 and PKCζ were detected by Western blotting. (B) Primary neonatal rat cardiomyocytes were treated with 100 μM PE for 24 h (PE group) or were infected with PKCζ adenovirus (Ad-PKCζ group). Total protein extracted from the cardiomyocytes were immunoprecipitated with anti-STAT3 antibody (CON, PE, and Ad-PKCζ groups) or anti-IgG antibody (IgG group). Expressions of PKCζ and STAT3 were detected by Western blotting. Expressions of STAT3 and PKCζ in protein extracts without immunoprecipitation were detected by Western blotting and were served as input controls. Representative images out of 3 independent experiments were shown.

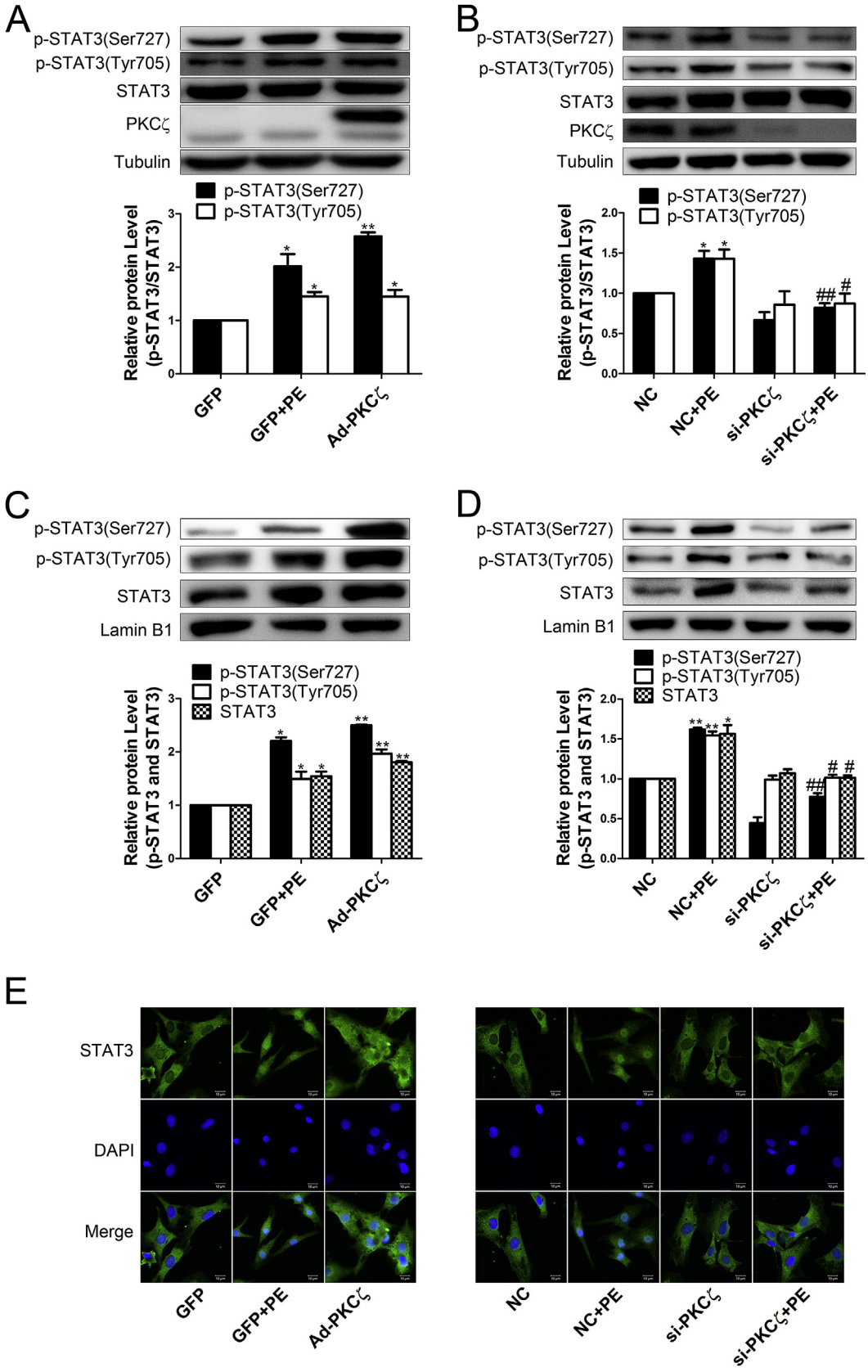

Fig. 6. Effect of PKCζ on the activation and nuclear translocation of STAT3 in primary neonatal rat cardiomyocytes. (A) and (C) Cardiomyocytes were infected with adenovirus overexpressing PKCζ or GFP control for 48 h. Western blotting analysis showing the phosphorylated levels of STAT3 at Ser727 and Tyr705, as well as the total STAT3 expression in (A) total protein extracts and (C) nuclear protein extracts. $^{*}p < 0.05$, $^{**}p < 0.01$ vs GFP, n = 3. (B) and (D) Cardiomyocytes were transfected with PKCζ siRNA (si-PKCζ) or negative control (NC) for 24 h, and were treated with or without 100 μM phenylephrine (PE) for another 24 h. Western blotting analysis showing the phosphorylated levels of STAT3 at Ser727 and Tyr705, as well as total STAT3 expression in (B) total protein extracts and (D) nuclear protein extracts. Data were presented as means ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$ vs NC, n = 3. $\#p < 0.05$, $\#\#p < 0.01$ vs NC + PE, n = 3. (E) Immunofluorescence of STAT3 by confocal microscopy showing the subcellular location of STAT3 in neonatal rat cardiomyocytes infected with Ad-PKCζ or transfected with si-PKCζ or in the presence of PE. Bar = 10 μm.

Ser727 and Tyr705) and total STAT3 in the nuclear fractions was shown in cardiomyocytes treated with PE or infected with Ad-PKCζ (Fig. 6C). By contrast, PE-induced upregulation of nuclear expressions of STAT3, phosphorylated STAT3 at Ser727 and Tyr705, were significantly reversed by si-PKCζ (Fig. 6D). In addition, the fluorescence of STAT3 was assembled in the nucleus of NRCMs with PE treatment or PKCζ overexpression, but remained in the cytoplasm in the control cells or cells silencing PKCζ (Fig. 6E).

Taken together, these results indicate that PKCζ promotes the activation and nuclear shuttling of STAT3 in NRCMs, probably leading to cardiomyocyte hypertrophy.

3.6. *Effect of PKCζ on the expressions of STAT3 target genes*

To confirm the role of PKCζ in the regulation of the transcriptional activity of STAT3, the expressions of STAT3 target genes c-fos and aGT were investigated. Exposure to PE increased the mRNA levels of these target genes (Fig. 7A and B). Similarly, overexpression of PKCζ augmented the mRNA expressions of these genes (Fig. 7A). However, knockdown of PKCζ suppressed the expressions of c-fos and aGT induced by PE (Fig. 7A). These observations indicate that PKCζ enhances the transcriptional activity of STAT3 in cardiomyocyte hypertrophy.

4. Discussion

The present study demonstrates an interaction between PKCζ and STAT3 in cardiomyocytes. PKCζ favors the phosphorylation and activation of STAT3, triggers its nuclear transport, and subsequently elevates the expressions of its downstream target genes. The regulation of STAT3 by PKCζ contributes to the development of cardiomyocyte hypertrophy.

PKC is a family of serine/threonine kinases consisting of 11 different isoenzymes that are divided into three subgroups dependent on the activation mode. The activation of conventional PKC isoforms (α, β1, β2, and γ) relies on Ca^{2+}, phospholipids such as dioleoylglycerol (DAG) and phosphatidylserine (PS). The novel PKC isoforms (δ, ε, η, and θ) are Ca^{2+}-independent, but require DAG and PS for their activation. Both groups can be activated by phorbol esters like phorbol-12,13-myristate-acetate (PMA). However, the atypical subfamily of PKC lacks the sensitivity to Ca^{2+} and DAG/PMA (23).

In cardiac hypertrophy, the conventional PKC isoforms α and β (24–27), and novel PKC isoforms δ and ε (25, 28–30), are generally upregulated and activated. Their increased expression and activation significantly promote the development of cardiac hypertrophy (24–30). PKC isoforms γ and τ is unaltered or decreased in cardiac hypertrophy models, and is not involved in the regulation of cardiac contractility and heart failure (31–33). Since mechanisms underlying the regulation of conventional and novel PKC isoforms in cardiac hypertrophy have been extensively reported, the present study focus on the effect of atypical PKC (PKCζ) on cardiac hypertrophy.

PKCζ, a member of the atypical PKC subfamily, has been reported to present in cardiomyocyte (34) and participate in the regulation of cardiomyocyte myofilament protein phosphorylation (21). In this study, the expression of phosphorylated PKCζ was significantly increased in a model of cardiomyocyte hypertrophy induced by PE (Fig. 1), suggesting that PKCζ was activated in cardiomyocyte hypertrophy. Additionally, PKCζ overexpression enhanced the hypertrophic responses in cultured NRCMs (Fig. 2), while genetic silencing of PKCζ protected NRCMs from PE-induced hypertrophy (Fig. 3). Thus, these observations provide solid evidence for the role of PKCζ in cardiomyocyte hypertrophy. Consistent with our observations, it is reported that PKCζ is upregulated in the left ventricle of hypertrophic hearts induced by volume overload and pressure overload (1–3), and that the phosphorylation level of PKCζ at Thr410 is elevated in aortic clamping hearts (2).

STAT3 has been implicated to be involved cardiac hypertrophy. STAT3 plays an essential role in mediating survival of cardiac myocytes (7–9). Cardiac-specific overexpression of STAT3 in transgenic mice leads to the development of myocardial hypertrophy (7). In line with these studies, this study showed a significant increase of phosphorylated STAT3 in PE-induced cardiomyocyte hypertrophy (Fig. 1). Overexpression of STAT3 mimicked the hypertrophic responses, as viewed by the increase of ANF, BNP and cell surface area (Fig. 4).

Given that PKCζ and STAT3 are closely associated with cardiomyocyte hypertrophy, it is hypothesized that the crosstalk between these two deleterious factors maybe play an important role in pathological myocardial hypertrophy. The co-IP results clearly demonstrated an interaction between PKCζ and STAT3 in cardiomyocytes with or without PE treatment (Fig. 5). Although it is reported that PKCζ can regulate the phosphorylation of STAT3 at tyrosine and serine residues (15), currently there is no information concerning the effects of PKCζ on STAT3 in cardiac cells. The present study demonstrated that overexpression of PKCζ with adenovirus

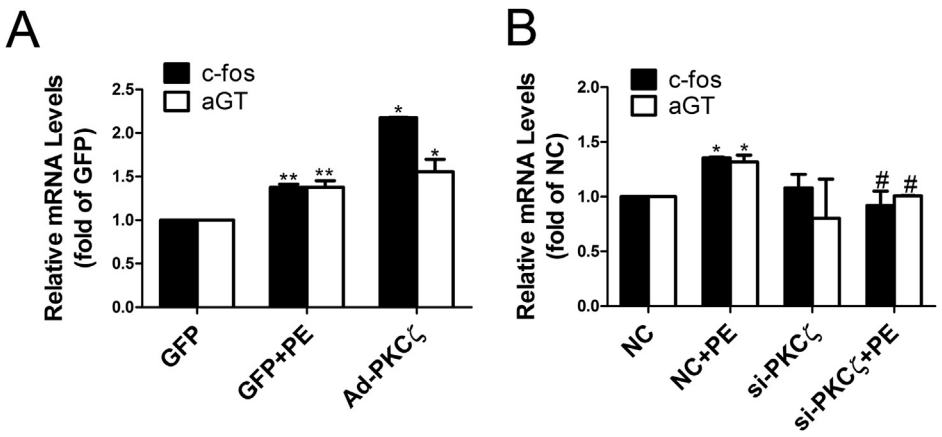

Fig. 7. Effect of PKCζ on the transcriptional activity of STAT3. Real-time PCR analysis showing the mRNA levels of STAT3 target genes c-fos and angiotensinogen (aGT) in primary cultured neonatal rat cardiomyocytes. (A) infected with PKCζ adenovirus or GFP control for 48 h and (B) transfected with PKCζ siRNA (si-PKCζ) and negative control (NC) for 24 h, and were treated with or without 100 μM phenylephrine (PE) for another 24 h. Data were presented as means ± SE. (A) $^{*}p < 0.05$, $^{**}p < 0.01$ vs GFP, n = 3. (B) $^{*}p < 0.05$, $^{**}p < 0.01$ vs NC; $\#p < 0.05$, $\#\#p < 0.01$ vs NC + PE, n = 3.

infection enhanced the phosphorylated levels of STAT3 at both Ser727 and Tyr705 without altering the expression of total STAT3 (Fig. 6A). Moreover, silencing of PKCζ attenuated PE-induced increase of the phosphorylated levels of STAT3 (Fig. 6B). Thus, these results confirm the regulatory role of PKCζ in the activation of STAT3 in cardiomyocyte hypertrophy.

STAT3, as a latent cytoplasmic transcription factor, is activated when its Tyr705 site is phosphorylated. The activated STAT3 forms a dimer and shuttles to the nucleus, where it binds to promoter elements and regulates the expressions of numerous genes (22). Interestingly, the phosphorylation of C-terminal Ser727 residue of STAT3 is also important, since it is essential for maximizing the transcriptional activity of STAT3 (20). Indeed, STAT3 was transported to the nucleus after phosphorylated by PKCζ at both Tyr705 and Ser727, as implied by the Western blotting data detecting the nuclear expressions of STAT3 and phosphorylated STAT3, as well as the immunofluorescent results directly showing the translocation of STAT3 (Fig. 6C, D & E). Moreover, PKCζ enhanced the mRNA expressions of c-fos and aGT (Fig. 7), which are target genes of STAT3 and are associated with cardiac hypertrophy (8, 14, 35). This result further confirms the role of PKCζ in the transcriptional activation of STAT3, which promotes the pathogenesis of cardiac hypertrophy.

The exact mechanism by which PKCζ mediates STAT3 phosphorylation is still not clear. As a serine/threonine kinase, it is not surprising that PKCζ phosphorylates the Ser727 site of STAT3, since the amino acid sequence of STAT3 Ser727 coincides with the consensus phosphorylation site motifs for PKCζ (36) and Ser727 directly serves as a substrate for PKCζ. However, the phosphorylation of STAT3 Tyr705 by PKCζ seems puzzled. One possible explanation is that the phosphorylation of Tyr705 might be a downstream cascade following Ser727 phosphorylation, which it is proved to maximize the transcriptional activity of STAT3 (37). Another explanation is that PKCζ may phosphorylate Tyr705 indirectly, possibly via intermediate factors like Jak activation (38). The exact mechanisms underlying the regulation of PKCζ on STAT3 need to be further elucidated.

As a limitation, the present study only investigated the interaction between PKCζ and STAT3 in PE-induced cardiomyocyte hypertrophic model. Further studies using *in vivo* cardiac hypertrophic animal models, such as abdominal aortic constriction and angiotensin II perfusion, should be elucidated to unmask the exact role of PKCζ and STAT3 in the development of cardiac hypertrophy.

In conclusion, the present study demonstrates that PKCζ interacts with STAT3 in cardiomyocytes. PKCζ might promote the development of cardiomyocyte hypertrophy via activation of STAT3 signaling. Strategies targeting inhibition of PKCζ-STAT3 signaling pathway suggest a therapeutic potential for cardiac hypertrophy.

Conflicts of interest

No.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.03.010.

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