PLOS ONE

RESEARCH ARTICLE

# Role of PKCδ in Enhanced Expression of Gqα/PLCβ1 Proteins and VSMC Hypertrophy in Spontaneously Hypertensive Rats

**Mohammed Emehdi Atef, Madhu B. Anand-Srivastava***

Department of Molecular and Integrative Physiology, Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada

* Madhu.anand-srivastava@umontreal.ca



OPEN ACCESS

**Citation:** Atef ME, Anand-Srivastava MB (2016) Role of PKCδ in Enhanced Expression of Gqα/PLCβ1 Proteins and VSMC Hypertrophy in Spontaneously Hypertensive Rats. PLoS ONE 11(7): e0157955. doi:10.1371/journal.pone.0157955

**Editor:** Luis Eduardo M Quintas, Universidade Federal do Rio de Janeiro, BRAZIL

**Received:** December 22, 2015

**Accepted:** June 6, 2016

**Published:** July 5, 2016



**Data Availability Statement:** All relevant data are within the paper.

**Funding:** MBAS was funded by a grant from the Canadian Institutes of Health Research (CIHR) (MOP-53074).

**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Gqα signaling has been implicated in cardiac hypertrophy. In addition, angiotensin II (Ang II) was also shown to induce its hypertrophic effect through Gqα and PKCδ activation. We recently showed the role of enhanced expression of Gqα/PLCβ1 proteins in vascular smooth muscle cell (VSMC) hypertrophy, however, the role of PKCδ in VSMC hypertrophy in animal model is still lacking. The present study was therefore undertaken to examine the role of PKCδ and the associated signaling mechanisms in VSMC hypertrophy using 16-week-old spontaneously hypertensive rats (SHR). VSMC from 16-week-old SHR exhibited enhanced phosphorylation of PKCδ-Tyr$^{311}$ and increased protein synthesis, marker of hypertrophy, as compared to WKY rats which was attenuated by rottlerin, an inhibitor of PKCδ. In addition, knocking down of PKCδ by PKCδ-siRNA also attenuated enhanced protein synthesis in VSMC from SHR. Furthermore, rottlerin attenuated the increased production of superoxide anion, NAD(P)H oxidase activity, increased expression of Gqα, phospholipase C (PLC)β1, insulin like growth factor-1 receptor (IGF-1R) and epidermal growth factor receptor (EGFR) proteins in VSMC from SHR. In addition, the enhanced phosphorylation of c-Src, PKCδ-Tyr$^{311}$, IGF-1R, EGFR and ERK1/2 exhibited by VSMC from SHR was also attenuated by rottlerin. These results suggest that VSMC from SHR exhibit enhanced activity of PKCδ and that PKCδ is the upstream molecule of reactive oxygen species (ROS) and contributes to the enhanced expression of Gqα and PLCβ1 proteins and resultant VSMC hypertrophy involving c-Src, growth factor receptor transactivation and MAP kinase signaling.

## Introduction

Essential hypertension is associated with vascular remodeling characterized by enhanced media to lumen ratio in arteries [1] and is due to increased vascular smooth muscle cell (VSMC) proliferation and hypertrophy. Guanine nucleotide regulatory proteins (G-proteins) and receptor tyrosine kinases (RTKs) play a major role in the regulation of vascular remodeling and aberration in the expression and/or activity of these molecules contribute to vascular remodeling [2–6]. The Gqα, a heterotrimeric G protein, and phospholipase C (PLC) β regulate

phosphatidyl inositol (PI) turnover activated by many GPCR agonists such as angiotensin II (Ang II), endothelin-1 (ET-1) and thrombin, and play an important role in mediating the pro-hypertrophic response by initiating other signaling mechanisms including RTKs transactivation and MAP kinase activation [7–10]. The levels of Ang II and ET-1 are enhanced in VSMC from spontaneously hypertensive rats (SHR) [11,12] and promote VSMC hypertrophy [2] and proliferation [13] in an autocrine and paracrine way.

Postreceptor signaling pathways activated by growth promoting substances involve activation of protein kinase C (PKC) through 1,2-diacylglycerol (DAG) production and/or intracellular calcium [8,14]. PKC is an intracellular serine/threonine protein kinase family of at least 12 isotypes subdivided into three classes, conventional PKCs (cPKCs), novel PKCs (nPKCs) and atypical PKC (aPKCs), which have distinct functions. PKCs isozymes expression pattern vary according to cell type. PKCδ, PKCα and PKCζ are the most abundant isozymes in VSMC [15,16]. The role of PKC isoforms in vascular hypertrophy is still insufficiently characterized and may vary according to cell type [17,18]. These intracellular serine/threonine kinases are rapidly activated and are implicated in the regulation of cell proliferation [19] and growth [20] and likely play an important role in mediating vascular remodeling. PKCδ is one of nPKCs isoforms that do not require $Ca^{2+}$ but is activated by DAG [21–24]. During the last decade, PKCδ associated with tyrosine $(Tyr)^{311}$ phosphorylation has emerged as a potential mediator in response to many stimuli including Ang II and thrombin [7,25]. Furthermore, the involvement of PKCδ in growth factor activation such as EGFR [8,9,26,27] and IGF-1R [28] has also been reported. However, the role of PKCδ in mediating vascular remodeling in essential hypertension and its possible cross-talk with other signaling mechanism implicated in this process has not yet been well characterized. We recently demonstrated the role of endogenous Ang II and ET-1 in enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy in spontaneously hypertensive rats through the activation of MAPK signaling [2]. We also showed that enhanced oxidative stress exhibited by VSMC from SHR through c-Src and growth factor receptor activation increases MAP kinase signaling and enhances the expression of Gqα and PLCβ1 proteins and results in VSMC hypertrophy [29]. However, the role of PKCδ in mediating vascular remodeling in essential hypertension has not yet been well characterized. The present study is therefore undertaken to examine if VSMC from SHR exhibit enhanced activation of PKCδ and its implication in VSMC hypertrophy and to further explore the signaling mechanism responsible for this process.

We showed that the enhanced activation of PKCδ in VSMC from SHR increases oxidative stress, c-Src and growth factor receptor transactivation that through MAP kinase signaling increases the expression of Gqα and PLCβ1 proteins and results in VSMC hypertrophy.

## Materials and Methods

### Materials

Rottlerin and lucigenin were purchased from Sigma-Aldrich Chemical (St-Louis, Missouri, USA). Leucine, L-[4,5-3H(N)] was purchased from Perkin Elmer (Boston, MA). Monoclonal Gqα antibody (10), monoclonal PLC-β1 antibody (D-8), monoclonal (phospho)-ERK1/2 (phosphospecific-tyrosine-204) antibody, polyclonal (phospho)-PKCδ (phosphospecific-tyrosine-311)-R antibody, monoclonal PKCδ, polyclonal ERK1/2 antibody (C-14), and Western blotting reagents were from St Cruz biotech (Santa Cruz, CA, USA). Polyclonal (phospho)-EGFR antibody (phosphospecific-tyrosine-1173) was from Calbiochem. Polyclonal EGFR, IGF-1R, (phospho)-c-Src (phosphospecific-tyrosine-419) and (phospho)-IGF-1R (phosphospecific-tyrosine-1165/1166) antibodies were from St Cruz biotech. Monoclonal anti-β-actin antibody (A5441) and all other chemicals used in these experiments were purchased from

Sigma-Aldrich (St. Louis, MO). Male spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto rats (WKY) were purchased from Charles River (St-Constant, Quebec, Canada).

## Animals, cell culture and incubation

16-week-old spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto (WKY) rats were euthanized by decapitation. The aorta were dissected out and VSMC were cultured as described previously [30]. As reported earlier [31], these cells were found to contain high levels of smooth muscle-specific actin. Cells were plated in 75-$cm^2$ flasks and incubated at 37°C in 95% air-5% $CO_2$ humidified atmosphere in Dulbecco's modified Eagle's medium (DMEM) (with glucose, L-glutamine, and sodium bicarbonate) containing 1% antibiotics (containing penicillin, streptomycin, and amphotrecin B) and 10% heat-inactivated fetal bovine serum (FBS). Cells were passaged upon reaching confluence with 0.5% trypsin and utilized between passages 2 and 6. Confluent cells were starved by incubation for 24 hours in DMEM without FBS at 37°C to have cell quiescence. For the receptor antagonist studies, VSMC from SHR and WKY were incubated for 16 hours in the absence or presence of variou concentrations of rottlerin (1 μM to 10 μM) dissolved in 1% dimethyl sulfoxide (DMSO). After incubation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in a 200 μl buffer containing 25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5 μg/ml leupeptin on ice. The cell lysates were centrifuged at 12,000 *g* for 15 min at 4°C, and the supernatants were used for Western blot analysis. Cell viability was checked by the trypan blue exclusion technique and indicated that >90~95% cells were viable. All the animal procedures used in the present study were approved by the Comité de Déontologie de l'Expérimentation sur les Animaux (CDEA) of the University of Montreal (#99050). The investigation conforms to the 'Guide for the Care and Use of Laboratory.

## Western blotting

The levels of protein expression and phosphorylation were determined by Western blotting as described previously [2]. After SDS-PAGE, separated proteins were transferred to a nitrocellulose membrane with a semi-dry transblot apparatus at 15 V for 45 min (Gqα, ERK1/2/p-ERK1/2) or a liquid transfer apparatus at 100 V for 1 h (PLCβ1, EGFR, pEGFR, IGFR, pIGFR, PKCδ, pPKCδ, c-Src and p-Src). Membranes were blocked with 5% dry milk and incubated overnight with specific antibodies. β-actin was used as loading controls. The antibody-antigen complexes were detected by incubating the membranes with horseradish peroxidase-conjugated antibodies for 1 h at room temperature. The blots were washed three times with PBS before reaction with enhanced chemiluminescence (ECL). Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs using the enhanced laser densitometer LKB Ultroscan XL and quantified using the gel-scan XL evaluation software (version 2.1) from Pharmacia (Baie d'Urfé, Québec, Canada).

## Determination of protein synthesis

VSMC from 16 week-old SHR and age-matched WKY were grown to confluence in 12-well culture plates. Protein synthesis (cell hypertrophy) was evaluated by [$^3$H]-leucine incorporation into cells as described previously [32]. Confluent cells were serum deprived for 24 hours to induce cell quiescence and were incubated in the absence or presence of rottlerin (1 to 10 μM) for 24 h. [$^3$H]-leucine (2μCi per well) was also added at the same time as rottlerin. For RNA interference studies, the cells were incubated in the absence or presence of siRNA against

PKCδ. [$^3$H]-leucine was added and further incubated for 24 h before the cells were harvested. The cells were rinsed twice with ice-cold 1X PBS and incubated with 5% TCA for 1 h at 4°C. After being washed twice with ice-cold 1X PBS, the cells were incubated with 0.4 N sodium hydroxide solution for 30 min at room temperature, and radioactivity was determined by liquid scintillation counter and adjusted by protein concentration.

### Transfection of VSMC with siRNA

For siRNA transfection efficiency, the manufacturer's protocol was followed. Briefly, VSMC were seeded in a 12 well plate or petri dishes and cultured in antibiotic free normal growth medium supplemented with 10% FBS until the cells were 60% confluent (~48 hours). On the day of transfection, cells were washed with transfection medium (sc-36868) and incubated with 1ml of transfection reagent (sc-29528) containing 80 pmoles of either scrambled siRNA (sc-37007) or siRNA specific for PKCδ oligonucleotides for 12 hours. The medium was replaced with normal DMEM (containing 10% FBS and 1% antibiotics) for an additional 24 hours (90% confluence).

### Determination of superoxide anion and NADPH oxidase activity

The production of basal superoxide anion ($O_2^-$) was measured using the lucigenin-enhanced chemiluminescence method at a concentration of 5 μM as described previously [33]After 24 hours of starvation, the VSMC were washed with oxygenated Krebs-Hepes buffer (NaCl 50 mM, KCl 2.3 mM, MgSO4 1.2 mM, K2HPO4 0.5 mM, NaHCO3 25 mM, glucose 5.5 mM, EDTA 63.4 μM) and placed in scintillation vials containing lucigenin solution. The emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science, Saint-Laurent, Canada) for 5 min. The NAD (P) H oxidase activity was determined by adding NADH (100 μM). The values were adjusted to the total weight of proteins for each simple.

### Statistical analysis

Results are expressed as means ± SEM. Comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test using GraphPad Prism5. A difference between groups was significant at $P < 0.05$.

## Results

### VSMC from SHR exhibit enhanced expression of PKCδ phosphorylation at Tyr$^{311}$

Several vasoactive peptides such as Ang II and ET-1 activate PKCδ [14, 34]. PKCδ activation associated with phosphorylation at Tyr$^{311}$ has been shown to mediate Ang II–induced VSMC hypertrophy [7]. Since VSMC from SHR exhibit enhanced levels of endogenous Ang II [11] and enhanced protein synthesis [2], it was desirable to investigate if VSMC hypertrophy in SHR is attributed to the overexpression of PKCδ phosphorylated at Tyr$^{311}$. To test this, we determined the levels of PKCδ phosphorylated at Tyr$^{311}$ in VSMC from SHR. The results shown in Fig 1 indicate that the level of PKCδ protein phosphorylated at Tyr$^{311}$ was significantly enhanced by about 100% in VSMC from SHR as compared to VSMC from WKY rats whereas the expression of PKCδ protein was not altered. Rottlerin, an inhibitor of PKCδ, attenuated significantly the enhanced Tyr$^{311}$ phosphorylation in a concentration-dependant manner and at 10 μM, it was completely abolished. In addition, the phosphorylation level of PKCδ at Tyr$^{311}$ in VSMC from WKY rats was also attanuated by rottlerin by about 60%.

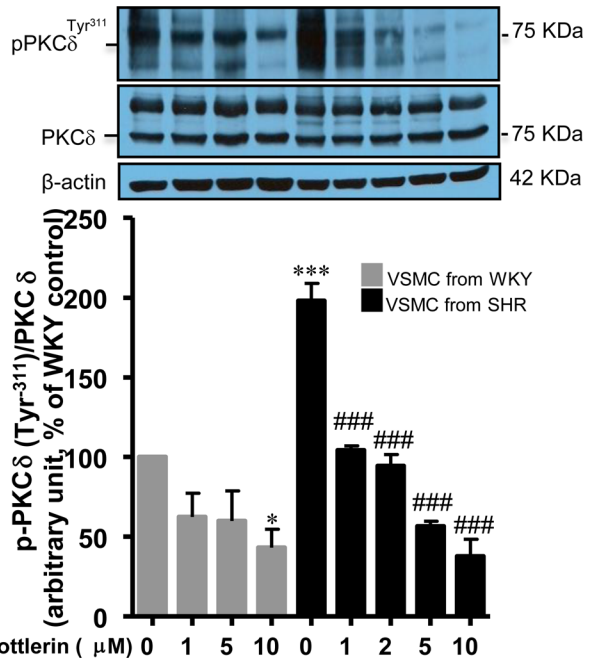


**Fig 1. Effect of rottlerin on PKCδ phosphorylation at Tyr$^{311}$ in VSMC from 16-weeks old SHR and age-matched WKY rats.** VSMC from 16 week-old SHR and age matched WKY rats were incubated with different concentrations of rottlerin for 16 hours. The cell lysates were prepared and subjected to Western blotting using specific antibodies against PKCδ and (phospho)-PKCδ$^{311}$ as described in Materials and Methods. PKCδ phosphorylation level was normalized by total PKCδ and the β-actin was used as a loading control. The proteins were quantified by densitometric scanning. The results are expressed as percentage of control, taken as 100%. Values are means ± SEM of 5 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs. WKY rats; ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g001

## Role of PKCδ in VSMC hypertrophy in SHR

VSMC from SHR have been shown to exhibit hypertrophy (increased protein synthesis) [2]. To investigate the role of PKCδ in enhanced protein synthesis in VSMC from SHR, the effect of rottlerin was tested on protein synthesis in VSMC from SHR and age- matched WKY rats and the results are shown in Fig 2. As reported earlier [2, 35], protein synthesis was significantly augmented in VSMC from SHR by about 50% as compared to WKY rats and rottlerin attenuated the enhanced protein synthesis at all the concentrations used in this study. At 5μM, the inhibition was about 90%. In addition, the protein synthesis in VSMC from WKY rats was also inhibited by rottlerin treatment.

To further investigate the implication of PKCδ in increased protein synthesis in VSMC from SHR, we used the siRNA approach to knockdown the PKCδ and examined the effect of knockdown of PKCδ on protein synthesis in VSMC from SHR and WKY rats. Results shown in Fig 3, indicate that the treatment of VSMC with siRNA of PKCδ that attenuated the expression of PKCδ by about 80% (A, B) also inhibited the enhanced protein synthesis by about 70% (D) in VSMC from SHR. However, a small but significant inhibition (≈ 25%) of the protein expression of PKCδ was observed in VSMC from WKY rats (A, B) whereas the protein synthesis was not significantly inhibited in these cells (D). In addition, PKCδ knockdown also attenuated slightly but significantly the PKCδ phosphorylation at Tyr$^{311}$ in VSMC from SHR and age- matched WKY rats (A, C).

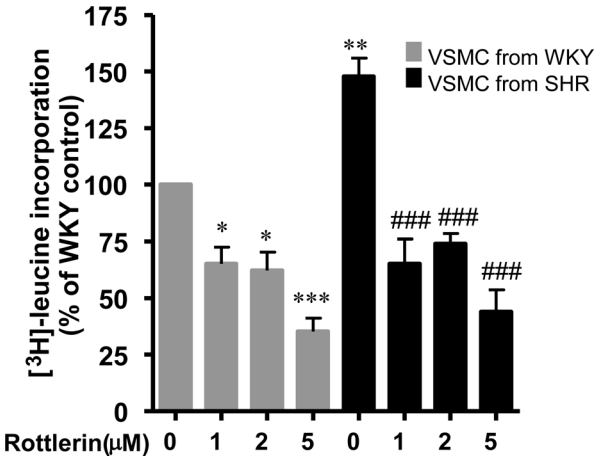


**Fig 2. Effect of rottlerin on protein synthesis in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC from 16 week-old SHR and age matched WKY rats were incubated with different concentration of rottlerin (from 1μM to 5μM). Protein synthesis was determined by [$^3$H]-leucine incorporation as described in Materials and Methods. The results are expressed as a percentage of control, taken as 100%. Values are means ± SEM of 4 separate experiments using different cell cultures. *P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; ### P < 0.001 vs. SH.

doi:10.1371/journal.pone.0157955.g002

## Role of PKCδ in enhanced expression of Gqα and PLCβ1 proteins in VSMC from SHR

We have earlier shown the implication of enhanced expression of Gqα and PLCβ1 in enhanced protein synthesis in VSMC from 16 week-old SHR [2]. Since PKCδ is implicated in enhanced protein synthesis, it was of interest to examine if PKCδ contributes to the enhanced expression of Gqα and PLCβ1 in VSMC from SHR. To test this, the effect of rottlerin on the expression of Gqα and PLCβ1 was investigated in VSMC from SHR and WKY rats and the results are shown in Fig 4. As reported earlier [2], the expression of Gqα (A) and PLCβ1 (B) was significantly augmented by about 165 and 115% respectively in VSMC from SHR as compared to WKY rats and rottlerin significantly inhibited the enhanced expression of Gqα and PLCβ1 proteins and at 10 μM, it was completely attenuated to WKY control level.

## Implication of PKCδ in enhanced production of superoxide anion and NADPH oxidase activity in VSMC from SHR

PKCδ activation has been shown to enhance NADPH oxidase activity and the levels of reactive oxygen species (ROS) [36]. To investigate if enhanced activation (phosphorylation at $Tyr^{311}$) of PKCδ in VSMC from SHR is implicated in enhanced oxidative stress that has been shown to result in enhanced expression of Gqα and PLCβ1 proteins and protein synthesis [29], the effect of rottlerin on production of $O_2^-$ and NADPH oxidase activity was examined in VSMC from SHR and WKY rats. Results shown in Fig 5, indicate that as reported earlier [33], $O_2^-$ production (A) and NADPH oxidase activity (B) were enhanced in VSMC from SHR as compared to WKY rats by about 110% and 100%, respectively and rottlerin attenuated the enhanced levels of $O_2^-$ as well as NADPH oxidase activity in a concentration-dependant manner and at 10 μM these were completely attenuated to control WKY levels. In addition, rottlerin also attenuated the basal $O_2^-$ and NADPH oxidase activity in VSMC from WKY rats.

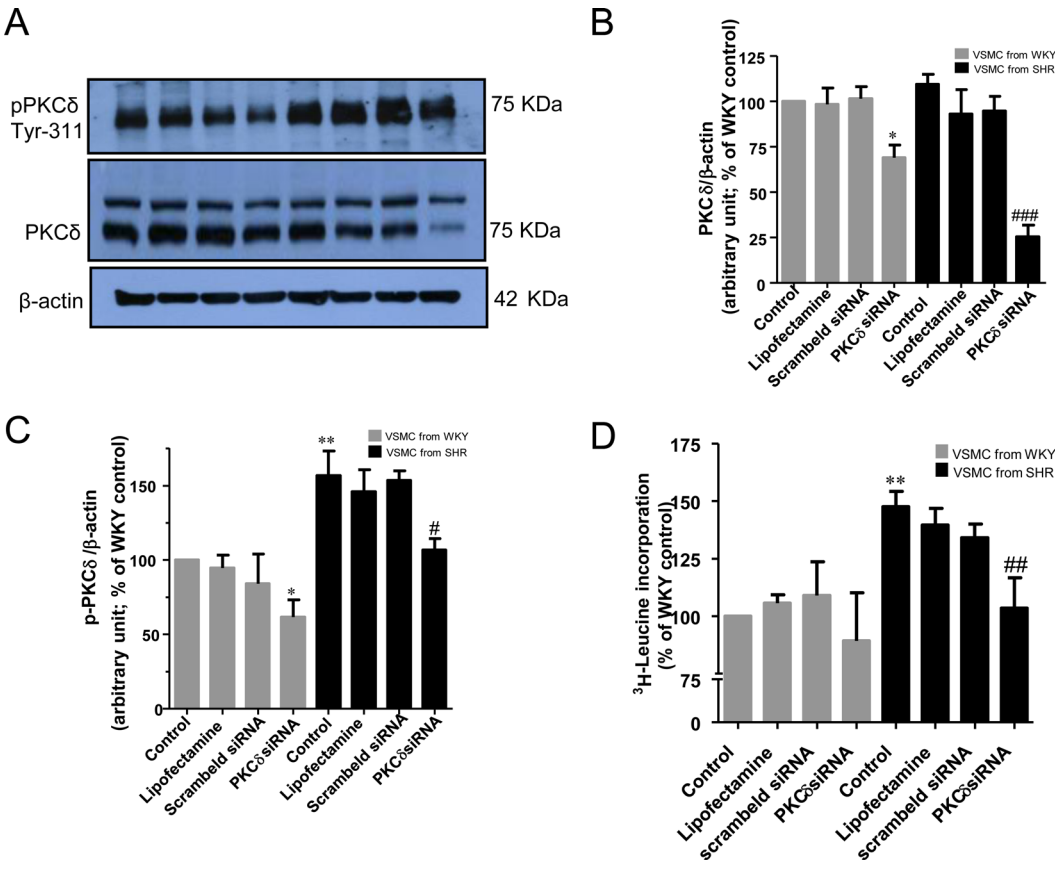


**Fig 3. Effect of the knockdown of PKCδ on protein synthesis in vascular smooth muscle cells (VSMC) from 16-week-old SHR and age-matched WKY rats.** VSMC from 16 week-old SHR and age matched WKY rats were incubated in the absence or presence of PKCδsiRNA for 16 hours as described in Materials and Methods. The cell lysates were prepared and subjected to Western blotting using specific antibodies against PKCδ and (phospho) PKCδ$^{311}$ (A). PKCδ phosphorylation level was normalized by total PKCδ (C) and the levels of PKCδ were normalized by β-actin used as a loading control (B). The proteins were quantified by densitometric scanning (B, C) and the protein synthesis was determined by [$^3$H]-leucine incorporation (D) as described in Materials and Methods. The results are expressed as percentage of control, taken as 100%. Values are means ± SEM of 5 separate experiments using different cell cultures. *P < 0.05, **P < 0.01 vs. WKY rats; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g003

## Implication of PKCδ in c-Src activation in VSMC from SHR

Since PKCδ is implicated in enhanced production of ROS which through c-Src activation was shown to enhance the expression of Gqα and PLCβ1 protein and VSMC hypertrophy in SHR [29], it was of interest to investigate the role of PKCδ in c-Src activation. To test this, the effect of rottlerin on c-Src phosphorylation was examined in VSMC from SHR and WKY rats and the results are shown in Fig 6. The increased phosphorylation of c-Src at Tyr$^{419}$ (70%) was completely attenuated by rottlerin at all the concentrations used (1μM-5μM) and at 10 μM it was completely abolished whereas it did not have any significant effect in VSMC from WKY rats. Furthermore, the expression level of total c-Src was not altered in VSMC from SHR as compared to WKY rats, and rottlerin did not affect the expression level of c-Src.

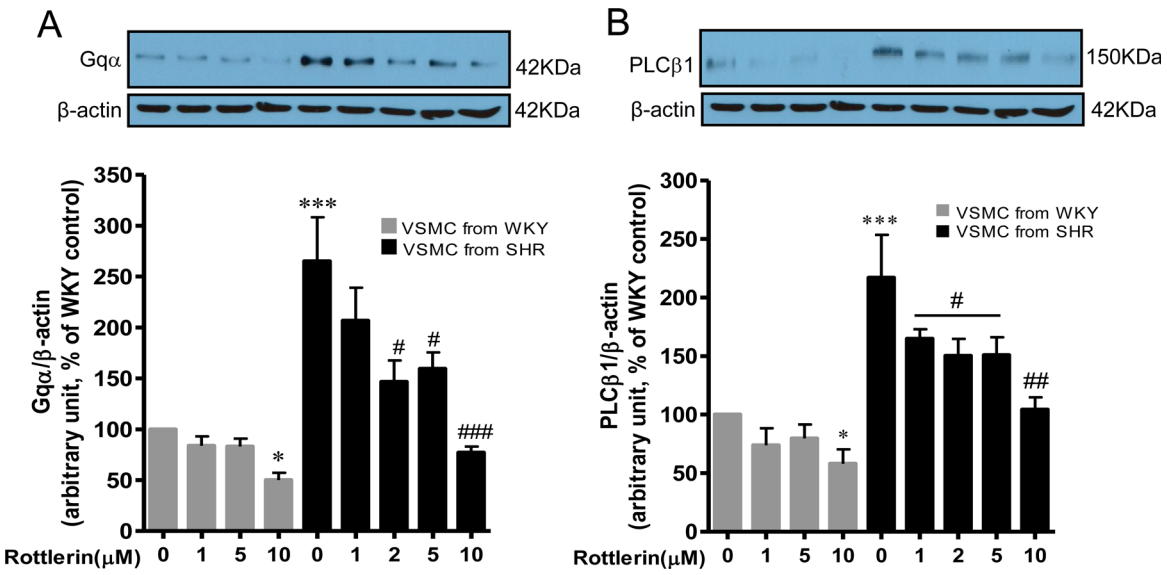


**Fig 4. Effect of rottlerin on enhanced expression of Gqα/PLCβ1 in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC from 16-weeks old SHR and age matched WKY rats were incubated with different concentrations of rottlerin. The cell lysates were prepared and subjected to Western blotting using specific antibodies against Gqα and PLCβ1 (A, B upper panels) as described in Materials and Methods. The β-actin was used as a loading control. The proteins were quantified by densitometric scanning (A, B lower panels). The results are expressed as percentage of control, taken as 100%. Values are means ± SEM of 4 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs. WKY rats; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g004

## Implication of PKCδ in ERK1/2 Phosphorylation

Since enhanced phosphorylation of ERK1/2 was shown to be implicated in enhanced expression of Gqα/PLCβ1 proteins and protein synthesis in VSMC from SHR [2], it was of interest to

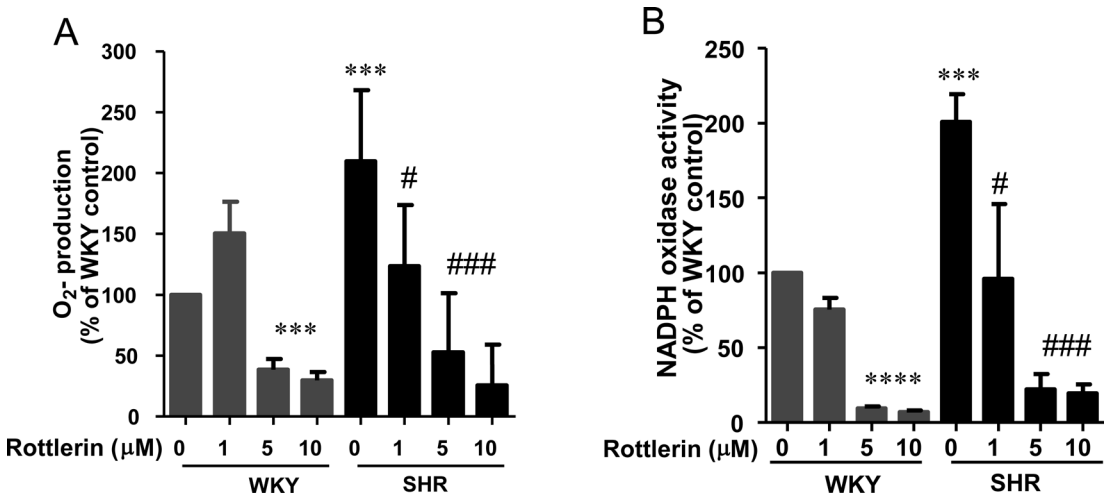


**Fig 5. Effect of rottlerin on NADPH activity and ROS production in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC from 16 week-old SHR and age matched WKY rats were incubated in the absence or presence of different concentration of rottlerin (from 1μM to 10 μM) for 16 hours, and $O_2^{\cdot-}$ production (A) and NADPH activity (B) were determined as described in Materials and Methods. The results are presented as means ± SEM of 4 separate experiments using different cell cultures. ***P < 0.001 vs. WKY rats; # P < 0.05, ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g005

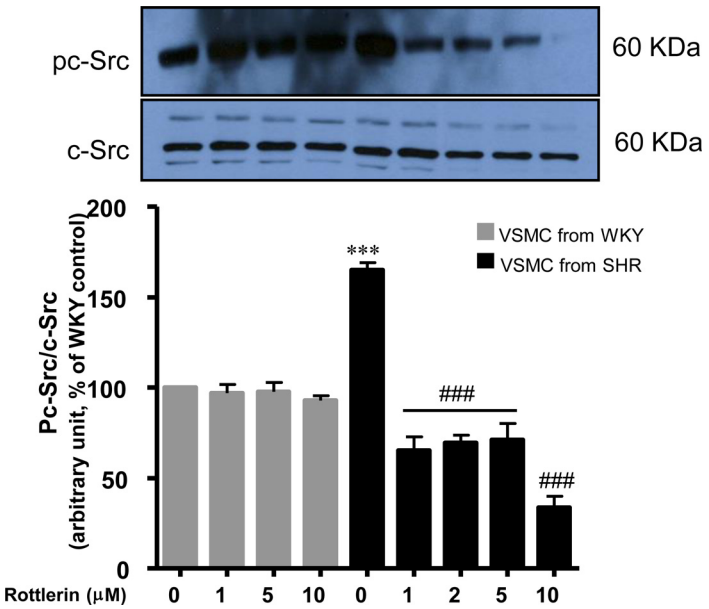


**Fig 6. Effect of PKCδ inhibition on c-Src activity in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC were incubated in the presence or absence of rottlerin (from 1μM to 10μM) for 16 hours. Membranes were prepared and subjected to Western blotting using specific antibodies (phospho)-c-Src and c-Src (upper panels) as described in Materials and Methods. c-Src phosphorylation level was normalized by total c-Src. The proteins were quantified by densitometric scanning (lower panels). The results are expressed as a percentage of control taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. ***P < 0.001 vs. WKY rats; ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g006

examine the contribution of PKCδ, an upstream signaling molecule, in the enhanced phosphorylation of ERK1/2 in VSMC from SHR. To test this, we examined the effects of rottlerin on ERK1/2 phosphorylation in VSMC from SHR and WKY rats and the results are shown in Fig 7. As reported earlier [2], ERK1/2 phosphorylation was significantly enhanced by about 75% in VSMC from SHR as compared to WKY rats. Treatment of cells with rottlerin attenuated significantly the enhanced phosphorylation of ERK1/2 in a concentration-dependant manner in VSMC from SHR and at 5 and 10 μM, it was completely attenuated to control level. In addition, rottlerin at 5 μM and 10 μM inhibited significantly the ERK1/2 phosphorylation in VSMC from WKY rats by about 35% and 50% respectively.

## Implication of PKCδ in growth factor receptor expression and activation

We earlier showed that growth factor receptor transactivation and MAP Kinase signaling plays a role in the overexpression of Gqα and PLCβ1 proteins and VSMC hypertrophy in VSMC from SHR [29]. To further investigate the role of PKCδ in growth factor receptor transactivation and/or expression, the effect of rottlerin on the phosphorylation of EGFR and IGF-1R using specific phospho-tyrosine antibodies was examined in VSMC from SHR and the results are shown in Fig 8. Phospho-specific-$Tyr^{1173}$-EGFR detected a single band at 160 kDa (A) and phospho-specific-$Tyr^{1165/1166}$-IGF-1R detected a single band at 90 kDa corresponding to IGF-1R (B), in VSMC from both SHR and WKY rats. However, as reported earlier [29], the extent of growth factor receptor phosphorylation was greater in VSMC from SHR compared to VSMC from WKY rats. The phosphorylation of IGF-1R was increased by approximately 110% in VSMC from SHR compared to WKY rats, whereas the phosphorylation of EGFR was

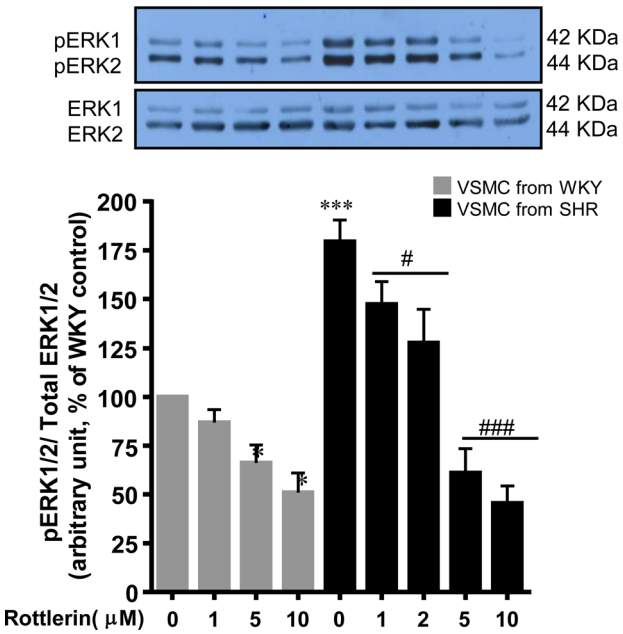


**Fig 7. Effect of PKCδ inhibition on p42/44MAPK signaling in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC were incubated in the presence or absence of rottlerin (from 1μM to 10μM) for 16 hours. Membranes were prepared and subjected to Western blotting using specific antibodies against pERK1/2 and ERK1/2 (upper panels) as described in Materials and Methods. ERK1/2 phosphorylation level was normalized by total ERK1/2. The proteins were quantified by densitometric scanning (lower panels). The results are expressed as a percentage of control taken as 100%. Values are means ± SEM of 3 separate experiments using different cell cultures. *P < 0.05, ***P < 0.001 vs. WKY rats; # P < 0.05, ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g007

augmented by about 80%. The enhanced phosphorylation of EGF-R at $Tyr^{1173}$ was attenuated completely by rottlerin (from 1μM to 5μM), whereas these concentrations of rottlerin were ineffective in reducing significantly the phosphorylation level of EGFR in VSMC from WKY. At a concentration of 10μM, rottlerin completely abolished the enhanced phosphorylation of EGFR in VSMC from SHR and attenuated the phosphorylation level of EGFR by about 50% in VSMC from WKY rats. Rottlerin inhibited significantly the increased phosphorylation of IGF-1R (B) in a concentration dependant manner. Furthermore, these treatments also decreased the phosphorylation level in VSMC from WKY. In addition, the expression levels of EGFR (A) and IGF-1R (B) were also augmented by about 200% and 100% respectively in VSMC from SHR as compared to WKY rats, which was attenuated significantly by rottlerin. Furthermore, rottlerin also attenuated significantly the protein expression of EGFR in VSMC from WKY rats.

## Discussion

Ang II-induced VSMC hypertrophy has been shown to involve PKCδ [7]. In addition, this serine-threonine kinase was also reported to be involved in the processes of cardiac hypertrophy [37,38]. We earlier showed the role of endogenous Ang II and ET-1 in the enhanced expression of Gqα and PLCβ1 proteins and the enhanced protein synthesis in VSMC from SHR through MAPKs singling [2]. In the present study, we demonstrate the implication of enhanced PKCδ activation (associated with PKCδ phosphorylation at $Tyr^{311}$) in the enhanced expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMC form spontaneously hypertensive rats (SHR).

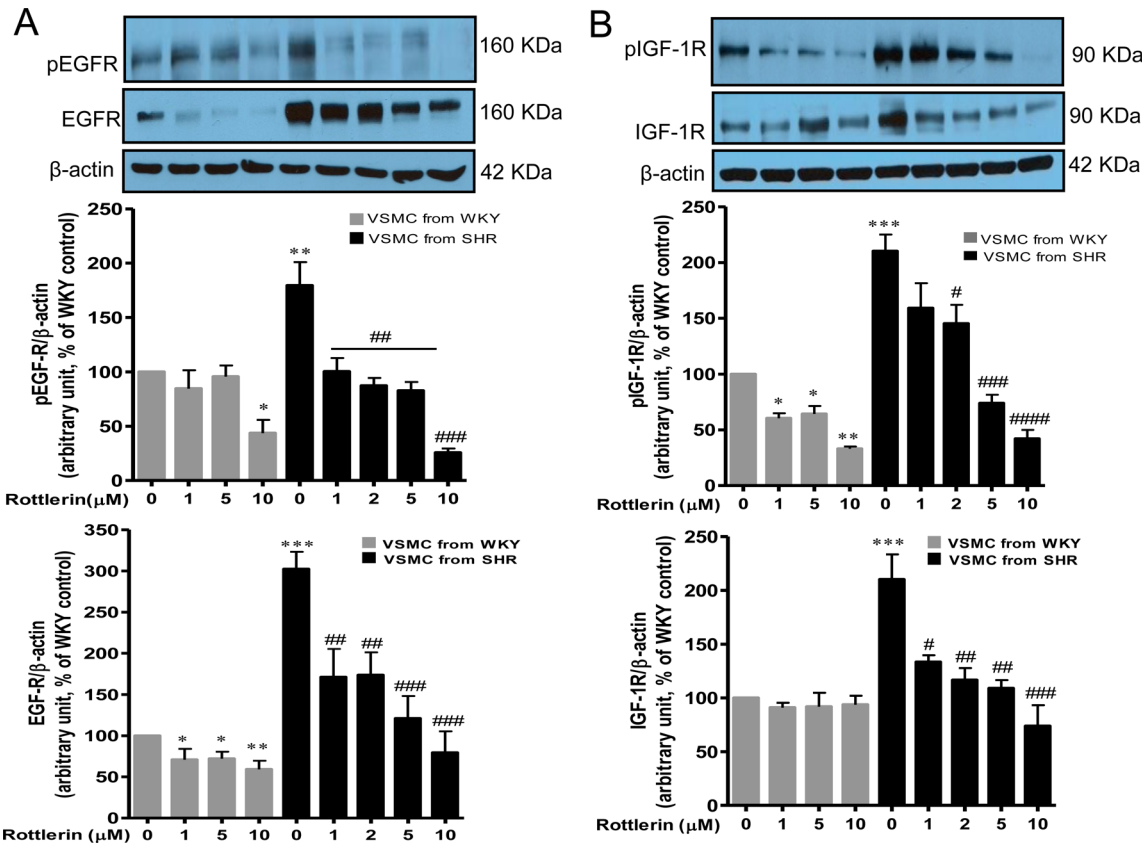


**Fig 8. Effect of rottlerin on EGF-R and IGF-1R phosphorylation and expression in vascular smooth muscle cells (VSMC) from 16-weeks old SHR and age-matched WKY rats.** VSMC were treated with different concentration of rottlerin for 16 hours. The cell lysates were prepared and subjected to Western blotting using specific antibodies against EGF-R/pEGF-R and IGF-1R/p-IGF-1R (A, B upper panels) as described in Materials and methods. The proteins were quantified by densitometric scanning (A, B lower panels). The results are expressed as percentage of control, taken as 100%. Values are means ± SEM of 5 separate experiments using different cell cultures. * P < 0.05, **P < 0.01, ***P < 0.001 vs. WKY rats; # P < 0.05, ## P < 0.01, ### P < 0.001 vs. SHR.

doi:10.1371/journal.pone.0157955.g008

We show that VSMC from 16-week-old SHR exhibit enhanced PKCδ phosphorylation at $Tyr^{311}$ which may contribute to the enhanced expression of Gqα and PLCβ1 proteins as well as enhanced protein synthesis because PKCδ inhibition with rottlerin, as quantified by the phosphorylation level at $Tyr^{311}$, attenuated significantly the enhanced expression of Gqα and PLCβ1 proteins and the enhanced protein synthesis in VSMC from SHR. This was further supported by our study showing that knockdown of PKCδ with specific siRNA attenuated significantly the enhanced protein synthesis in VSMC from SHR. Our results are in agreement with the study of Nakashima et al who have shown the implication of PKCδ associated with enhanced phosphorylation at $Tyr^{311}$ in Ang II-induced VSMC hypertrophy [7]. Furthermore, the fact that rottlerin also inhibits the PKCδ phosphorylation, the expression of Gqα/PLCβ1 as well as protein synthesis in control cells, suggests the implication of endogenous PKCδ in eliciting these responses. It should be noted that rottlerin has been used as a putative inhibitor of PKCδ in several studies during the last 20 years in order to block the activity of PKCδ [39,40], that was correlated with increased PKCδ phosphorylation [41]. In addition, rottlerin was also shown to inhibit PKCδ activity through mitochondria uncoupling mechanism [42].

PKCδ has been shown to be activated by hyperglycemia-induced oxidative stress [43]. Furthermore, PKCδ activation was also associated with the process of atherosclerosis implicating

LDL oxidation [44]. Our results showing that the inhibition of PKCδ activity with rottlerin results in a significant attenuation of NADPH oxidase activity and $O_2^-$ production and suggests the implication of PKCδ in ROS production in VSMC. Our results are consistent with other studies demonstrating the inhibition of ROS production by rottlerin in many cell types including VSMCs and adipocytes [45,46]. A role of PKCδ in NADPH oxidase activation has been reported in various cells [47]. The mechanisms by which PKCδ activates NADPH oxidase is not clear but may involve the phosphorylation of the cytoplasmic subunits of the oxidase, such as p47*phox*, and initiates its translocation to the membrane [48,49]. In this regard, a role of PKCδ in $p47^{phox}$ activation of NADPH oxidase in Ang II–induced ROS production and VSMC hypertrophy has been shown [36]. In addition, PKCδ activation has also been shown to mediate its effects through increasing ROS production and NADPH subunits expression [26, 50]. We earlier showed that enhanced oxidative stress exhibited by VSMC from SHR contributes to the overexpression of Gqα and PLCβ1 proteins as well as to enhanced protein synthesis [29]. Taken together, it may be suggested that PKCδ-induced enhanced expression of Gqα and PLCβ1 proteins as well as enhanced protein synthesis in VSMC from SHR may also be mediated through its ability to increase oxidative stress.

We earlier showed a role of c-Src in enhanced expression of Gqα and PLCβ1 proteins as well as in enhanced protein synthesis in VSMC from SHR [29]. In this study, we show that the augmented phosphorylation of c-Src in VSMC from SHR is attributed to the enhanced activation of PKCδ because the inhibition of PKCδ by rottlerin attenuated the enhanced phosphorylation of c-Src. Furthermore, the oxidative stress has been demonstrated to be the upstream signaling molecule of c-Src [2]. Taken together, it may be suggested that the enhanced activation of PKCδ through oxidative stress and activation of c-Src contributes to the overexpression of Gqα and PLCβ1 proteins as well as to the enhanced protein synthesis in VSMC from SHR.

As demonstrated in this study, VSMC from SHR exhibit enhanced expression of EGFR and IGF-1R which may be associated with cell dedifferentiation to a synthetic profile characterized by enhanced protein synthesis. The implication of growth factor receptors in enhanced expression of Gqα and PLCβ1 proteins and enhanced protein synthesis in VSMC from SHR has been shown in an earlier study[29]. These results suggest that the enhanced protein expression of Gqα/PLCβ1 as well as EGFR and IGF-1R may reflect a phenotype switch of VSMC from a contractile state to a synthetic state in SHR. In fact, VSMC profile conversion is associated with the modulation of protein expression of certain membrane molecules implicated in VSMC hypertrophy, proliferation and contractility [51,52]. In addition, we also showed that c-Src is the upstream of growth factor receptor activation [29]. The transactivation of several receptor tyrosine kinases (RTKs) has also been reported to involve c-Src [31,53,54]. Our results showing that rottlerin inhibited the increased phosphorylation of IGF-1R and EGFR, suggest a role of PKCδ in enhanced activation of growth factor receptors in VSMC from SHR. In this regard, Hsieh et al have shown a pivotal role of PKCδ in thrombin- induced EGFR expression in VSMC [8].

A role of MAPK in enhanced expression of Gqα/PLCβ1 proteins and enhanced protein synthesis in VSMC from SHR has been shown [2]. Growth factor receptors have also been reported to signal through MAP kinase pathways [31,55]. The fact that rottlerin inhibited the enhanced phosphorylation of ERK1/2 in VSMC from SHR suggests the implication of PKCδ in enhanced activation of MAP kinase. In this context, the inhibition of PKCδ with rottlerin has been reported to inhibit the ET-1- induced MAPK activity in VSMC [56]. Taken together, it may be suggested that enhanced activity of PKCδ in VSMC from SHR through the transactivation of growth factor receptors and MAP kinase signaling increase the expression of Gqα and PLCβ1 and results in VSMC hypertrophy.

In summary, we provide evidence that the enhanced activation of PKCδ at $Tyr^{311}$ phosphorylation in VSMC from SHR through oxidative stress and c-Src, transactivate growth factor receptor which increases the activity of MAP kinase and contributes to the enhanced expression of Gqα and PLCβ1 proteins and VSMC hypertrophy (Fig 9). In addition, we also show for the first time that rottlerin that has been shown to exert neuro-protective effect in both cell culture and preclinical animal models of Parkinson disease [57], anticancer effects in a variety of tumor cell types [58] and induces apoptosis in chronic lymphocytic leukaemia (CLL) cells [59] also inhibits VSMC hypertrophy in SHR model of hypertension. From these studies, it may be suggested that PKCδ protein may serve as a potential target for the development of new therapies for the treatment of cardiovascular diseases.

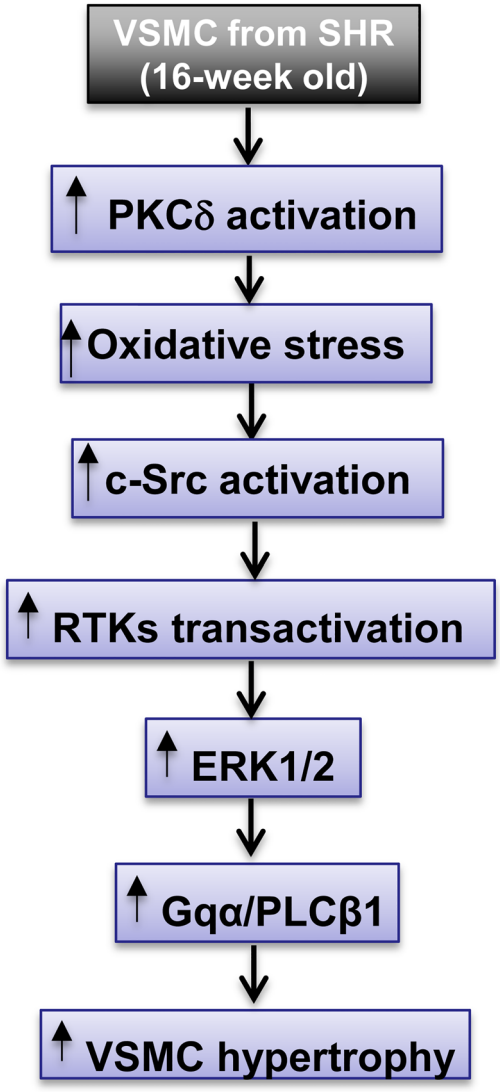


**Fig 9. The possible intracellular signaling mechanisms implicated in PKCδ-induced enhanced expression of Gqα/PLC-β1 proteins and VSMC hypertrophy in SHR.** PKCδ through the activation of ROS production and c-Src, trans-activate RTKs and MAPK that increases the protein expression of Gqα/PLC-β1 and results in enhanced protein synthesis.

doi:10.1371/journal.pone.0157955.g009

## Author Contributions

Conceived and designed the experiments: MEA MBAS. Performed the experiments: MEA. Analyzed the data: MEA MBAS. Contributed reagents/materials/analysis tools: MEA MBAS. Wrote the paper: MEA MBAS.

## References


1. Korsgaard N, Aalkjaer C, Heagerty AM, Izzard AS, Mulvany MJ (1993) Histology of subcutaneous small arteries from patients with essential hypertension. Hypertension 22: 523–526. PMID: 8406657
2. Atef ME, Anand-Srivastava MB (2014) Enhanced expression of Gqalpha and PLC-beta1 proteins contributes to vascular smooth muscle cell hypertrophy in SHR: role of endogenous angiotensin II and endothelin-1. Am J Physiol Cell Physiol 307: C97–106. doi: 10.1152/ajpcell.00337.2013 PMID: 24760983
3. Berk BC (2001) Vascular smooth muscle growth: autocrine growth mechanisms. Physiol Rev 81: 999–1030. PMID: 11427690
4. Delafontaine P, Song YH, Li Y (2004) Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels. Arterioscler Thromb Vasc Biol 24: 435–444. PMID: 14604834
5. Dreux AC, Lamb DJ, Modjtahedi H, Ferns GA (2006) The epidermal growth factor receptors and their family of ligands: their putative role in atherogenesis. Atherosclerosis 186: 38–53. PMID: 16076471
6. Zhu B, Zhao G, Witte DP, Hui DY, Fagin JA (2001) Targeted overexpression of IGF-I in smooth muscle cells of transgenic mice enhances neointimal formation through increased proliferation and cell migration after intraarterial injury. Endocrinology 142: 3598–3606. PMID: 11459808
7. Nakashima H, Frank GD, Shirai H, Hinoki A, Higuchi S, Ohtsu H, et al. (2008) Novel role of protein kinase C-delta Tyr 311 phosphorylation in vascular smooth muscle cell hypertrophy by angiotensin II. Hypertension 51: 232–238. doi: 10.1161/HYPERTENSIONAHA.107.101253 PMID: 18180404
8. Hsieh HL, Tung WH, Wu CY, Wang HH, Lin CC, Wang TS, et al. (2009) Thrombin induces EGF receptor expression and cell proliferation via a PKC(delta)/c-Src-dependent pathway in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 29: 1594–1601. doi: 10.1161/ATVBAHA.109.185801 PMID: 19628787
9. Hsieh HL, Sun CC, Wang TS, Yang CM (2008) PKC-delta/c-Src-mediated EGF receptor transactivation regulates thrombin-induced COX-2 expression and PGE(2) production in rat vascular smooth muscle cells. Biochim Biophys Acta 1783: 1563–1575. doi: 10.1016/j.bbamcr.2008.03.016 PMID: 18452714
10. Gavi S, Shumay E, Wang HY, Malbon CC (2006) G-protein-coupled receptors and tyrosine kinases: crossroads in cell signaling and regulation. Trends Endocrinol Metab 17: 48–54. PMID: 16460957
11. Fukuda N, Satoh C, Hu WY, Soma M, Kubo A, Kishioka H, et al. (1999) Production of angiotensin II by homogeneous cultures of vascular smooth muscle cells from spontaneously hypertensive rats. Arterioscler Thromb Vasc Biol 19: 1210–1217. PMID: 10323771
12. Lu MH, Chao CF, Huang CG, Chang LT (2003) Coculture of vascular endothelial cells and smooth muscle cells from spontaneously hypertensive rats. Clin Exp Hypertens 25: 413–425. PMID: 14596366
13. Li Y, Levesque LO, Anand-Srivastava MB (2010) Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. Am J Physiol Heart Circ Physiol 299: H1959–1967. doi: 10.1152/ajpheart.00526.2010 PMID: 20852045
14. Frank GD, Saito S, Motley ED, Sasaki T, Ohba M, Kuroki T, et al. (2002) Requirement of Ca(2+) and PKCdelta for Janus kinase 2 activation by angiotensin II: involvement of PYK2. Mol Endocrinol 16: 367–377. PMID: 11818507
15. Ohanian V, Ohanian J, Shaw L, Scarth S, Parker PJ, Heagerty AM. (1996) Identification of protein kinase C isoforms in rat mesenteric small arteries and their possible role in agonist-induced contraction. Circ Res 78: 806–812. PMID: 8620600
16. Leszczynski D, Joenvaara S, Foegh ML (1996) Protein kinase C-alpha regulates proliferation but not apoptosis in rat coronary vascular smooth muscle cells. Life Sci 58: 599–606. PMID: 8632713
17. Chen C, Mochly-Rosen D (2001) Opposing effects of delta and xi PKC in ethanol-induced cardioprotection. J Mol Cell Cardiol 33: 581–585. PMID: 11181025
18. Dempsey EC, Newton AC, Mochly-Rosen D, Fields AP, Reyland ME, Insel PA, et al. (2000) Protein kinase C isozymes and the regulation of diverse cell responses. Am J Physiol Lung Cell Mol Physiol 279: L429–438. PMID: 10956616

19. Sasaguri T, Kosaka C, Hirata M, Masuda J, Shimokado K, Fujishima M, et al. (1993) Protein kinase C-mediated inhibition of vascular smooth muscle cell proliferation: the isoforms that may mediate G1/S inhibition. Exp Cell Res 208: 311–320. PMID: 8359225

20. Miyamoto A, Nakayama K, Imaki H, Hirose S, Jiang Y, Abe M, et al. (2002) Increased proliferation of B cells and auto-immunity in mice lacking protein kinase Cdelta. Nature 416: 865–869. PMID: 11976687

21. Andrea JE, Walsh MP (1992) Protein kinase C of smooth muscle. Hypertension 20: 585–595. PMID: 1428108

22. Kikkawa U, Matsuzaki H, Yamamoto T (2002) Protein kinase C delta (PKC delta): activation mechanisms and functions. J Biochem 132: 831–839. PMID: 12473183

23. Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607–614. PMID: 1411571

24. Ron D, Kazanietz MG (1999) New insights into the regulation of protein kinase C and novel phorbol ester receptors. FASEB J 13: 1658–1676. PMID: 10506570

25. Hall KJ, Jones ML, Poole AW (2007) Coincident regulation of PKCdelta in human platelets by phosphorylation of Tyr311 and Tyr565 and phospholipase C signalling. Biochem J 406: 501–509. PMID: 17570831

26. Fan CY, Katsuyama M, Yabe-Nishimura C (2005) PKCdelta mediates up-regulation of NOX1, a catalytic subunit of NADPH oxidase, via transactivation of the EGF receptor: possible involvement of PKCdelta in vascular hypertrophy. Biochem J 390: 761–767. PMID: 15913451

27. Uchiyama K, Saito M, Sasaki M, Obara Y, Higashiyama S, Nakahata N (2009) Thromboxane A2 receptor-mediated epidermal growth factor receptor transactivation: involvement of PKC-delta and PKC-epsilon in the shedding of epidermal growth factor receptor ligands. Eur J Pharm Sci 38: 504–511. doi: 10.1016/j.ejps.2009.09.016 PMID: 19804825

28. Li W, Jiang YX, Zhang J, Soon L, Flechner L, Kapoor V, et al. (1998) Protein kinase C-delta is an important signaling molecule in insulin-like growth factor I receptor-mediated cell transformation. Mol Cell Biol 18: 5888–5898. PMID: 9742106

29. Atef ME, Anand-Srivastava MB (2016) Oxidative stress contributes to the enhanced expression of Gqalpha/PLCbeta1 proteins and hypertrophy of VSMC from SHR: role of growth factor receptor transactivation. Am J Physiol Heart Circ Physiol 310: H608–618. doi: 10.1152/ajpheart.00659.2015 PMID: 26747500

30. Anand-Srivastava MB, Franks DJ, Cantin M, Genest J (1982) Presence of "Ra" and "P"-site receptors for adenosine coupled to adenylate cyclase in cultured vascular smooth muscle cells. Biochem Biophys Res Commun 108: 213–219. PMID: 6293481

31. Gomez Sandoval YH, Anand-Srivastava MB (2011) Enhanced levels of endogenous endothelin-1 contribute to the over expression of Gialpha protein in vascular smooth muscle cells from SHR: Role of growth factor receptor activation. Cell Signal 23: 354–362. doi: 10.1016/j.cellsig.2010.10.005 PMID: 20959139

32. Li Y, Hashim S, Anand-Srivastava MB (2006) Intracellular peptides of natriuretic peptide receptor-C inhibit vascular hypertrophy via Gqalpha/MAP kinase signaling pathways. Cardiovasc Res 72: 464–472. PMID: 17007826

33. Saha S, Li Y, Anand-Srivastava MB (2008) Reduced levels of cyclic AMP contribute to the enhanced oxidative stress in vascular smooth muscle cells from spontaneously hypertensive rats. Can J Physiol Pharmacol 86: 190–198. doi: 10.1139/Y08-012 PMID: 18418428

34. Clerk A, Bogoyevitch MA, Anderson MB, Sugden PH (1994) Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. J Biol Chem 269: 32848–32857. PMID: 7806510

35. Atef ME, Anand-Srivastava MB (2011) Enhanced Expression of G(alpha)q and PLC{beta}1 in Aortic Vascular Smooth Muscle Cells from Spontaneously Hypertensive Rats: Role of Endogenous Angiotensine II and Endothelin-1 (Abstract). FASEB J 25: 1009.1009.

36. Lv P, Miao SB, Shu YN, Dong LH, Liu G, Xie XL et al. (2012) Phosphorylation of smooth muscle 22alpha facilitates angiotensin II-induced ROS production via activation of the PKCdelta-P47phox axis through release of PKCdelta and actin dynamics and is associated with hypertrophy and hyperplasia of vascular smooth muscle cells in vitro and in vivo. Circ Res 111: 697–707. doi: 10.1161/CIRCRESAHA.112.272013 PMID: 22798525

37. Braun MU, LaRosee P, Simonis G, Borst MM, Strasser RH (2004) Regulation of protein kinase C isozymes in volume overload cardiac hypertrophy. Mol Cell Biochem 262: 135–143. PMID: 15532718

38. Koide Y, Tamura K, Suzuki A, Kitamura K, Yokoyama K, Hashimoto T, et al. (2003) Differential induction of protein kinase C isoforms at the cardiac hypertrophy stage and congestive heart failure stage in Dahl salt-sensitive rats. Hypertens Res 26: 421–426. PMID: 12887134

39. Frasch SC, Henson PM, Kailey JM, Richter DA, Janes MS, Fadok VA et al. (2000) Regulation of phospholipid scramblase activity during apoptosis and cell activation by protein kinase Cdelta. J Biol Chem 275: 23065–23073. PMID: 10770950

40. Li C, Wernig F, Leitges M, Hu Y, Xu Q (2003) Mechanical stress-activated PKCdelta regulates smooth muscle cell migration. FASEB J 17: 2106–2108. PMID: 12958154

41. Steinberg SF (2004) Distinctive activation mechanisms and functions for protein kinase Cdelta. Biochem J 384: 449–459. PMID: 15491280

42. Soltoff SP (2001) Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. J Biol Chem 276: 37986–37992. PMID: 11498535

43. Ha H, Yu MR, Choi YJ, Lee HB (2001) Activation of protein kinase c-delta and c-epsilon by oxidative stress in early diabetic rat kidney. Am J Kidney Dis 38: S204–207. PMID: 11576956

44. Larroque-Cardoso P, Swiader A, Ingueneau C, Negre-Salvayre A, Elbaz M, Reyland ME, et al. (2013) Role of protein kinase C delta in ER stress and apoptosis induced by oxidized LDL in human vascular smooth muscle cells. Cell Death Dis 4: e520. doi: 10.1038/cddis.2013.47 PMID: 23449456

45. Lee IT, Lin CC, Wang CH, Cherng WJ, Wang JS, Yang CM. (2013) ATP stimulates PGE(2)/cyclin D1-dependent VSMCs proliferation via STAT3 activation: role of PKCs-dependent NADPH oxidase/ROS generation. Biochem Pharmacol 85: 954–964. doi: 10.1016/j.bcp.2012.12.016 PMID: 23318226

46. Talior I, Tennenbaum T, Kuroki T, Eldar-Finkelman H (2005) PKC-delta-dependent activation of oxidative stress in adipocytes of obese and insulin-resistant mice: role for NADPH oxidase. Am J Physiol Endocrinol Metab 288: E405–411. PMID: 15507533

47. Bankers-Fulbright JL, Kita H, Gleich GJ, O'Grady SM (2001) Regulation of human eosinophil NADPH oxidase activity: a central role for PKCdelta. J Cell Physiol 189: 306–315. PMID: 11748588

48. Fontayne A, Dang PM, Gougerot-Pocidalo MA, El-Benna J (2002) Phosphorylation of p47phox sites by PKC alpha, beta II, delta, and zeta: effect on binding to p22phox and on NADPH oxidase activation. Biochemistry 41: 7743–7750. PMID: 12056906

49. Reeves EP, Dekker LV, Forbes LV, Wientjes FB, Grogan A, Pappin DJ, et al. (1999) Direct interaction between p47phox and protein kinase C: evidence for targeting of protein kinase C by p47phox in neutrophils. Biochem J 344 Pt 3: 859–866. PMID: 10585874

50. Greene MW, Burrington CM, Lynch DT, Davenport SK, Johnson AK, Horsman MJ, et al. (2014) Lipid metabolism, oxidative stress and cell death are regulated by PKC delta in a dietary model of nonalcoholic steatohepatitis. PLoS One 9: e85848. doi: 10.1371/journal.pone.0085848 PMID: 24454937

51. Zhang MJ, Zhou Y, Chen L, Wang YQ, Wang X, Pi Y1, et al. (2016) An overview of potential molecular mechanisms involved in VSMC phenotypic modulation. Histochem Cell Biol 145: 119–130. doi: 10.1007/s00418-015-1386-3 PMID: 26708152

52. Berra-Romani R, Mazzocco-Spezzia A, Pulina MV, Golovina VA (2008) Ca2+ handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. Am J Physiol Cell Physiol 295: C779–790. doi: 10.1152/ajpcell.00173.2008 PMID: 18596214

53. Liebmann C (2001) Regulation of MAP kinase activity by peptide receptor signalling pathway: paradigms of multiplicity. Cell Signal 13: 777–785. PMID: 11583913

54. Luttrell LM, Della Rocca GJ, van Biesen T, Luttrell DK, Lefkowitz RJ (1997) Gbetagamma subunits mediate Src-dependent phosphorylation of the epidermal growth factor receptor. A scaffold for G protein-coupled receptor-mediated Ras activation. J Biol Chem 272: 4637–4644. PMID: 9020193

55. Katz M, Amit I, Yarden Y (2007) Regulation of MAPKs by growth factors and receptor tyrosine kinases. Biochim Biophys Acta 1773: 1161–1176. PMID: 17306385

56. Chen QW, Edvinsson L, Xu CB (2009) Role of ERK/MAPK in endothelin receptor signaling in human aortic smooth muscle cells. BMC Cell Biol 10: 52. doi: 10.1186/1471-2121-10-52 PMID: 19575782

57. Zhang D, Anantharam V, Kanthasamy A, Kanthasamy AG (2007) Neuroprotective effect of protein kinase C delta inhibitor rottlerin in cell culture and animal models of Parkinson's disease. J Pharmacol Exp Ther 322: 913–922. PMID: 17565007

58. Maioli E, Torricelli C, Valacchi G (2012) Rottlerin and cancer: novel evidence and mechanisms. ScientificWorldJournal 2012: 350826. doi: 10.1100/2012/350826 PMID: 22272173

59. Ringshausen I, Oelsner M, Weick K, Bogner C, Peschel C, Decker T (2006) Mechanisms of apoptosis-induction by rottlerin: therapeutic implications for B-CLL. Leukemia 20: 514–520. PMID: 16437144