Original Paper

Down-Regulation of Protein Kinase C- ϵ by Prolonged Incubation with PMA Inhibits the Proliferation of Vascular Smooth Muscle Cells

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Key Words

Protein Kinase C- ϵ • Phorbol myristate acetate (PMA) • Proliferation • Vascular Smooth Muscle Cell (VSMC)

Abstract

Background/Aims: Phorbol myristate acetate (PMA) exerts a pleiotropic effect on the growth and differentiation of various cells. Protein kinase Cs (PKCs) plays a central role in mediating the effects of PMA on cells. The present study investigated whether the down-regulation of protein kinase C- ϵ (PKC- ϵ) is involved in the inhibition of vascular smooth muscle cell (VSMC) proliferation caused by prolonged PMA incubation. **Methods:** Using cell counting, Cell Counting Kit-8 (CCK-8) and EdU incorporation assay on VSMCs, we evaluated the inhibitory effects of prolonged incubation of PMA, of lentiviruses carrying the short-hairpin RNAs (shRNA) of PKC- ϵ and of the PKC- ϵ inhibitor peptide on the proliferation and viability of cells. The effect of PKC- ϵ down-regulation on growth of rat breast cancer SHZ-88 cells was also measured. **Results:** The prolonged incubation of VSMCs with PMA for up to 72 hours resulted in attenuated cell growth rates in a time-dependent manner. The expression of PKC- ϵ , as assessed by Western blotting, was also decreased accordingly. Notably, the number of EdU-positive cells and the cell viability of VSMCs were decreased by shRNA of PKC- ϵ and the PKC- ϵ inhibitor peptide, respectively. The proliferation of rat breast cancer SHZ-88 cells was also attenuated by lentivirus-induced shRNA silencing of PKC- ϵ . **Conclusions:** Prolonged incubation of PMA can inhibit the expression of PKC- ϵ . The effect results in the inhibition of VSMC proliferation. PKC- ϵ silencing can also attenuate breast cancer cell growth, suggesting that PKC- ϵ may be a potential target for anti-cancer drugs.

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Introduction

Vascular smooth muscle cell (VSMC) proliferation and remodelling play a pivotal role in various pathophysiological responses to conditions such as hypotension, atherosclerosis, diabetes mellitus, and injured vessel healing. These processes are regulated by profound but intercommunicated protein kinase signals. Among these signals, protein kinase C (PKC) is a key kinase that is variably expressed in VSMCs to regulate cell functions, including smooth muscle contraction, gene expression, cell growth, and proliferation [1-3]. A signalling pathway between PKCs and mitogen-activated protein kinase (MAPK) seems crucial for the initiation and progression of VSMC proliferation. For example, mitogens can activate MAPK via inducing the kinase translocation from the cytosol to the nucleus where it promotes gene expression and cell growth in undifferentiated cultured VSMCs as well as in differentiated contractile VSM [4], and attenuation of the MAPK pathway by Labeledipinedilol-A can inhibit cell proliferation, which is regulated by Ca^{2+} and PKC [5]. The PKC family is generally divided into three groups, differing in the enzymes' cofactor requirements: conventional (c) PKCs (including α , β I, II and γ isoforms) require calcium and diacylglycerol (DAG) for activation; novel (n) PKCs (comprising δ , θ , ϵ and η isoforms) are activated by DAG; and atypical (a) PKCs include ζ and λ/ι isoforms and are dependent on phosphatidylserine but are not affected by DAG, phorbol ester, or Ca^{2+} . Among PKC kinase family members expressed in VSMCs, PKC- α , - β , - δ , and - ζ are involved in stimuli-induced cell proliferation [6-8]. Although Ca^{2+} -independent PKC- ϵ has been demonstrated to enhance the myofilament force sensitivity to $[\text{Ca}^{2+}]_i$ in VSM and to promote the vasoconstriction associated with hypertension, little is known about the effect of PKC- ϵ on cell proliferation in VSMCs. Phorbol-12-myristate-13-acetate (PMA), a potent tumour promoter, can activate PKC both *in vivo* and *in vitro* [9-12] and modulate macrophage differentiation [13]. However, prolonged incubation with PMA can, in turn, down-regulate the expression of PKCs in various tissues and cells, including VSMCs [14-16]. Using prolonged incubation with PMA we down-regulated the expression of PKCs in rat aortic vascular smooth muscle cells. Experimental validation revealed that the expression of PKC- ϵ is down-regulated, with an accompanying inhibition of cell proliferation. Subsequently, specific suppression of PKC- ϵ results in the inhibition of cell proliferation *in vitro* and *in vivo*. In addition, results from rat mammary tumour cells provided evidence that the PKC- ϵ pathway may be implicated in tumour progression. These findings suggest a novel role of PKC- ϵ in vascular smooth muscle cell proliferation as well as in tumour progression.

Materials and Methods

Reagents and Materials

The antibodies used in the present study and their commercial sources were as follows: anti-PKC ϵ (Abcam, Cambridge, MA, USA), anti-Erk1/2 and anti-phospho-Erk1/2 (Cell Signaling Technology, Danvers, MA, USA), and anti-GAPDH (Huaan, Hangzhou, China). Anti-PKC ϵ and anti-phospho-Erk1/2 were diluted with PBS by 1:2000. Anti-Erk1/2 was diluted with PBS by 1:1000. Anti-GAPDH was diluted with PBS by 1:5000. Phorbol-12-myristate-13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The PKC ϵ inhibitor peptide was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Psi-RACK was from Anaspec (Fremont, CA, USA). The CCK-8 kit was from Dojindo Molecular Technologies (Kumamoto, Japan). The EdU kit was obtained from Ribobio (Guangzhou, China).

Primary VSMC culture

All animal experiments were approved by the Institutional Animal Care and Use Committee of Shanghai Jiaotong University and were in compliance with the Guide for the Care and Use of Laboratory Animals (8th edition) published by the National Research Council (United States). Adult male Wistar rats weighing 250–350 g were killed by CO_2 inhalation. Primary culture of aortic VSMCs were prepared following the same protocol as described previously [16]. Cells within passages 6 to 10 from 10 different batches were used in all experiments.

Cell growth curve

The growth of cells was examined by cell counting. Cells first underwent mitogenic quiescence by serum starvation. The cell number under these experimental conditions was used as the baseline. To examine the status of the cell proliferation of VSMCs and SHZ-88, *in vitro* cell numbers were determined at the indicated times after PMA treatment (10 μ M except in Fig. 2A and Fig. 7B) or transfection of PKC- ϵ shRNA (MOI = 20). Triplicate plates were used for each time point, and each data point was the average of three experiments.

CCK-8 assay

The viability of VSMCs, SHZ-88 cells, and MDA-MB-231 cells were evaluated using the CCK-8 assay. Briefly, cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. Cells were treated with the indicated drugs for various time periods (PMA 10 μ M, Psi-RACK 5 μ M, PKC- ϵ inhibitor peptide 10 μ M), or they were transfected with PKC- ϵ shRNA (MOI = 20) and then incubated with CCK-8 solution for 3 h. The absorbance was then measured at 450 nm using a microplate reader (Bio-Rad, Richmond, CA, USA).

EdU assay

According to the manual of a 5-ethynyl-2'-deoxyuridine (EdU) labelling/detection kit, after seeding on a 96-well plate for 24 h, 50 μ M of EdU labelling medium was added to the cell culture to allow incubation for 4 h at 37°C under 5% CO₂. Thereafter, cultured VSMCs were fixed with 4% paraformaldehyde (pH 7.4) for 30 min and incubated with glycine for 5 min. After washing with PBS and permeabilization with 0.5% TritonX-100, staining with 1 $\times$ Apollo reaction cocktail was performed at room temperature for 30 min. Following a wash with 0.5% TritonX-100 in PBS, the cells were incubated with 5 μ g/ml Hoechst 33342 dye at room temperature for 30 min, followed by observation under a fluorescence microscope. The percentage of EdU-positive cells represents the VSMCs proliferation level, and was calculated from six random fields in three independent experiments.

Western blotting

Proteins were extracted with the cell lysis solution. Equal amounts of total protein were separated by SDS-PAGE (10% polyacrylamide gel) and then transferred onto a polyvinylidene difluoride membrane by electroblotting for 2 h at 250 mA. The membrane was blocked in 5% non-fat milk in Tris-buffered saline along with 0.5% Tween 20. The membrane was incubated with primary antibodies, followed by the appropriate secondary antibodies, and the detection of specific proteins was performed by enhanced chemiluminescence following the manufacturer's instructions. Densitometric signals were quantified by Image J software. In Fig. 2A and Fig. 7B, cells were treated with different doses of PMA for 4h to study the dosage-effect relationship.

Transfection of PKC- ϵ shRNA

To knock down PKC ϵ expression in VSMCs, we used PKC- ϵ shRNAs designed and synthesized by Genomeditech Co Ltd (Shanghai, China) according to PKC- ϵ cDNA sequences (Gen-Bank accession NM_017171.1). PKC ϵ shRNA sequences were as follows: 5'-GTAACCCTGGAGACTTCAC-3' for shRNA 1, 5'-GATTCAGAACACTGGTGAT-3' for shRNA 2, and 5'-CGCTAAGAGAACTTAACTTT-3' for shRNA3. The sequence 5'-TTCTCCGAACGTGTACGT-3', which had no significant homology to any known human or mouse genes, was used as a negative control. These oligonucleotides were then subcloned into a lentiviral vector (pGMLV-SC5 RNAi; Shanghai Genomeditech Co Ltd, Shanghai, China), and the lentiviruses were produced in 293T cells. After estimating the multiplicity of infection using a standard procedure, the viruses were used to infect VSMCs or the SHZ-88 cell line. Cells were divided into 3 groups-control (infection with PBS alone), mock (infection with the negative control lentiviral vector), and PKC ϵ -RNAi (PKC ϵ -shRNA1, 2, or 3-lentiviral vector). They were subcultured at a density of 2×10^5 cells per well into 6-well cell culture plates. The cells were infected for 72 h with PKC- ϵ shRNA1, 2, and 3 or a negative control lentiviral vector (MOI = 20) at a multiplicity of infection according to the pre-experimental data. In this study, we refer to the PKC ϵ -shRNA1, 2, 3-lentiviral vectors and the negative control lentiviral vector as lentivirus1, 2, 3 and vector, respectively. Thereafter, PKC- ϵ knockdown was confirmed by Western blotting.

Tumour xenograft model

Adult female nude mice (NU/NU, 6 to 8 weeks old), obtained from Shanghai Slack Laboratory Animal Co., LTD, were used as recipients in the tumour xenograft model. The animals were housed under specific pathogen-free conditions. SHZ-88 cells (5×10^6 cells/200 μ L of PBS), obtained from the American Type Culture Collection were injected into the right mammary fat pad of each mouse. The tumour weight and size were assessed four weeks after injection. Mice were anesthetized through the inhalation of sevoflurane. The tumour volume was calculated using digital calipers according to the following formula: volume = length $\times$ width $\times$ height.

Statistical analysis

The results are expressed as the means $\pm$ SEM of multiple experiments for the molecular experiments. Statistical analysis was performed using GraphPad Prism Version 5 by using either one-way or two-way ANOVA with Bonferroni post hoc tests. The number of experiments or animals used in each study is specified for each figure legend. P values less than 0.05 were regarded as statistically significant.

Results

Prolonged incubation with PMA inhibits the proliferation of VSMCs

PMA has been shown to promote multiple types of cell proliferation, mediated through the activation of PKC isoforms [17, 18]. Given that the expression of PKCs in VSMCs is significantly down-regulated after prolonged PMA treatment [16], we next investigated whether prolonged PMA treatment can inhibit VSMC proliferation. VSMCs were treated

with PMA (10 μ M) for up to 72 hours. As shown in Fig. 1A, prolonged PMA treatment caused a time-dependent decrease in the cell numbers that was evident within 24 hours, a reduction to $\sim$ 67% of the control value after 48 hours, and maintenance below the control level for at least 72 hours during exposure to PMA. The PMA-induced inhibition of cell growth was accompanied by a corresponding decrease in cell viability (Fig. 1B).

Down-regulation of PKC- ϵ expression is involved in the growth inhibition of VSMCs associated with prolonged PMA treatment

We have previously demonstrated that VSMCs with prolonged PMA treatment for up to 24 hours showed decreased PKC ($-\alpha$, $-\epsilon$, $-\delta$, and $-\theta$) expression [16]. Among these subtypes, PKC- α , $-\delta$ and $-\zeta$ have been reported to be involved in the regulation of cell proliferation [6-8]. Despite ample studies focusing on cells responding to stimuli-induced PKCs activation [19-21], little is known about the consequences of PKC down-regulation with respect to VSMC proliferation. We then investigated whether the inhibition of VSMC proliferation induced by prolonged incubation with PMA involves PKC- ϵ . VSMCs were incubated with PMA (1, 5, and 10 μ M) for up to 24 hours. As shown

Fig. 1. Prolonged incubation with PMA inhibits the cell growth and viability of VSMCs. (A) Cell number counting for the growth curve of VSMCs treated with prolonged incubation of PMA. (B) The viability of VSMCs treated with prolonged incubation of PMA. * $P < 0.05$, ** $P < 0.01$, compared with the control group at the corresponding time point. The results are shown as the means $\pm$ SEM of three independent experiments, [Two-way ANOVA (A and B)].

Fig. 2. Down-regulation of PKC- ϵ is involved in prolonged incubation with PMA, inducing the inhibition of VSMC proliferation. (A) PKC- ϵ was down-regulated with PMA incubation for 4 hours at 10 μ M. (B) PKC- ϵ was down-regulated with 10 μ M PMA incubation for 4 and 24 hours. (C) Effects of Psi-RACK, a PKC- ϵ activator, on prolonged incubation with PMA, inducing VSMC proliferation. * $P < 0.05$, ** $P < 0.01$, compared with the control group. # $P < 0.05$, ## $P < 0.01$, compared with PMA group. The results are shown as the means $\pm$ SEM of three independent experiments, [One-way ANOVA (A and B), Two-way ANOVA (C)].

Fig. 3. Effects of PKC- ϵ inhibition on VSMC proliferation. Inhibition of PKC- ϵ with the PKC- ϵ inhibitor peptide suppresses VSMC proliferation. ** $P < 0.01$, compared with the control group. The results are shown as the means $\pm$ SEM of three independent experiments, [Two-way ANOVA].

in Fig. 2A, preincubation of cells with 10 μ M PMA for 4 hours was sufficient to cause marked down-regulation of PKC- ϵ . When the PMA treatment was maintained for up to 24 hours, the cellular levels of PKC- ϵ continued to decrease (Fig. 2B), resulting in the significant inhibition of cell growth and viability (Fig. 1A and B). To verify whether the down-regulation of PKC- ϵ is involved in the inhibition of VSMC proliferation by prolonged PMA treatment, we treated the cells with Psi-RACK, a PKC- ϵ activator. As expected, the inhibition of cell viability induced by prolonged PMA treatment was partly reversed by Psi-RACK (Fig. 2C). Collectively, these data suggested a role for PKC- ϵ in the inhibition of VSMC growth and viability by prolonged PMA treatment.

Suppression of PKC- ϵ results in the inhibition of VSMC proliferation

Given that PKC- ϵ is involved in the prolonged PMA treatment-induced inhibition of VSMC growth and viability, we next investigated whether the suppression of PKC- ϵ alone can cause the inhibition of VSMC proliferation. VSMCs were treated with a specific PKC- ϵ inhibitor peptide, which can interact with PKC- ϵ -activated proteins such as Psi-RACK. As shown in Fig. 3, cells treated with the specific PKC- ϵ inhibitor peptide showed decreased cell viability. To define further the involvement of PKC- ϵ in the proliferation of VSMCs, we used PKC- ϵ shRNA to down-regulate specifically the expression of PKC- ϵ . Western blotting revealed that the preincubation of cells with PKC- ϵ shRNA1 and shRNA2 but not shRNA3 resulted in the down-regulation of PKC- ϵ (Fig. 4A). As shown in Fig. 4B and C, the down-regulation of PKC- ϵ , by preincubating cells with PKC- ϵ shRNA1, decreased the cell population and viability. Consistent with this observation, the pretreatment of cells with PKC- ϵ shRNA

Fig. 4. Down-regulation of PKC- ϵ with PKC- ϵ shRNA inhibits VSMC proliferation. (A) Effects of PKC- ϵ silencing by lentivirus1, lentivirus2, and lentivirus3. Protein expression was detected by Western blotting, and densitometric quantitation was performed. (B) VSMC numbers at the indicated time points after seeding on plates in the control group or PKC- ϵ shRNA group. (C) Cell viability of VSMCs at specific time points after seeding on plates in

the control group or PKC- ϵ shRNA group. (D) EdU-positive cells decreased significantly in lentivirus2-transfected group and the percentage of EdU-positive cells was calculated. * $P < 0.05$, ** $P < 0.01$, compared with the control group. The results are shown as the means $\pm$ SEM of three independent experiments, [One-way ANOVA (A and D), Two-way ANOVA (B and C)].

resulted in decreased EdU-positive cells (Fig. 4D), indicating the inhibition of the number of proliferating cells.

The inhibition of cell proliferation caused by PKC- ϵ down-regulation is independent of the ERK signalling pathway

Because phosphorylated ERK expression and subsequent ERK activation are involved in the proliferation of VSMCs in response to many events [22, 23], we investigated whether prolonged PMA treatment affects ERK activity and whether the change in ERK activity is involved in the down-regulation of PKC- ϵ -induced inhibition of cell proliferation. As shown in Fig. 5A, ERK in VSMCs was phosphorylated obviously when VSMCs were incubated with PMA (10 μ M) for 1 hour ($p < 0.01$). However, when the time was expanded to 4 hours, the expression of phosphorylated ERK returned to the base level. Notably, the levels of phosphorylated ERK were continuously decreasing in the presence of PMA, reaching the lowest level at 24 hours, and then slowly returned to the base level after PMA treatment for 72 hours. However, there was no significant change in the total level of ERK.

Because the prolonged incubation with PMA reduced the level of phosphorylated ERK temporally in VSMCs, we next investigated whether ERK is implicated in the inhibition of cell proliferation induced by the down-regulation of PKC- ϵ . As shown in Fig. 5B, the down-regulation of PKC- ϵ itself had no effect on ERK phosphorylation in VSMCs. In addition, VSMCs were pre-infected with lentivirus-PKC- ϵ -shRNA before exposure to PMA for 1 and 4 hours. As shown in Fig. 5C, the delivery of PKC- ϵ shRNA did not impact PMA-induced time-dependent change at the level of phosphorylated ERK expression. Furthermore, Psi-RACK did not change the PMA-induced ERK phosphorylation level (Fig. 5D). These data imply that ERK does not play a key role in PKC- ϵ -silenced VSMCs.

Attenuation of PKC- ϵ suppresses breast cancer cell proliferation in vitro and in vivo

Prolonged PMA treatment has been shown to suppress the migration and invasion of various cancer cells *in vivo* and *in vitro* [24, 25]; thus, we postulated that the down-regulation

Fig. 5. ERK is not involved in the inhibition of VSMC proliferation caused by the silencing of PKC- ϵ . (A) Biphasic change in ERK phosphorylation by prolonged incubation with PMA in VSMCs. (B) The ERK phosphorylation level was not changed in VSMCs by the silencing of PKC- ϵ . (C) Activation of ERK phosphorylation induced by PMA treatment was not changed when VSMCs were infected with PKC- ϵ shRNA. (D) Inhibition of ERK phosphorylation induced by PMA treatment for 24 or 48 hours was not altered by the PKC- ϵ activator Psi-RACK. **P < 0.01, compared with the control group. Protein expression was detected by Western blotting, and densitometric quantitation was performed. The results are shown as the means $\pm$ SEM of three independent experiments, [One-way ANOVA].

of PKC- ϵ by prolonged PMA incubation may contribute to the suppression of cancer cell proliferation and cancer growth. Indeed, as shown in Fig. 6A and B, the cell growth and cell viability of SHZ-88 were suppressed due to the silencing of PKC- ϵ . To directly test the role of PKC- ϵ in tumour growth *in vivo*, SHZ-88 cells transfected with PKC- ϵ shRNA or the lentivirus vector were implanted into the right side of the mammary fat pad of adult female nude mice, and then the tumours were removed at 4 weeks to measure the weight and volume. As shown in Fig. 6C and D, compared with tumours in mice implanted with normal SHZ-88 cells or cells transfected with negative control shRNA, the tumour weights were reduced in mice implanted with SHZ-88 cells transfected with shRNA against PKC- ϵ . The volumes of the tumour nodules were diminished, with a similar pattern to that of the tumour weights. In addition, we examined the effects of the down-regulation of PKC- ϵ on the proliferation of MB-MDA-231 cells, a human breast cancer cell line. PKC- ϵ was down-regulated in MB-MDA-231 cells with prolonged 10 μ M PMA incubation after 4 hours (Fig. 7A and B). Prolonged PMA treatment resulted in significant inhibition of cell growth and proliferation (Fig. 7C and D). Similar results appeared after using the PKC- ϵ inhibitor peptide (Fig. 7C and D). These findings suggest that PKC- ϵ may be involved in breast cancer progression and is a potential target of cancer therapy.

Discussion

It is now clear that VSMC proliferation contributes to the development of hypertension and atherosclerotic cardiovascular diseases.

Fig. 6. Silencing of PKCε inhibits the growth and viability of SHZ-88 cells and suppresses the growth of breast cancer. (A) SHZ-88 cell numbers were counted after seeding on plates for 1 to 4 days in the negative control shRNA group or PKC-ε shRNA group. (B) The cell viability of SHZ-88 was measured by the CCK8 assay after seeding on plates for 1 to 4 days in the negative control shRNA or PKC-ε shRNA group. (C) Representative images of the gross morphology of nude mice. (a) SHZ-88 cells were injected into nude mice. (b) SHZ-88 cells transfected with

the lentivirus vector were injected into nude mice. (c) SHZ-88 cells transfected with PKC-ε shRNA were injected into nude mice. (d-f) Representative images of tumours isolated from one mouse of each group. (D) Tumour weights of nude mice after injection with normal SHZ-88 cells or cells transfected with the lentivirus vector or PKC-ε shRNA in each group. * $P < 0.05$, ** $P < 0.01$, compared with the control group. Pooled data from six mice are shown; data are expressed as means $\pm$ SEM, [Two-way ANOVA (A and B), ANOVA (D)].

Fig. 7. Prolonged incubation with PMA down-regulates PKCε and inhibits the proliferation of MDA-MB-231 cells. (A) PKC-ε was down-regulated with prolonged incubation of PMA. (B) PKC-ε was down-regulated with 10 μM PMA incubation for 4 hours. (C) Cell number counting for the growth curve of MDA-MB-231 cells treated by prolonged incubation of PMA. (D) Viability of MDA-MB-231 cells treated by prolonged incubation of PMA. * $P < 0.05$, ** $P < 0.01$, compared with the control group at the corresponding time point. ## $P < 0.01$, control group compared with PKC ε inhibitor peptide. The results are shown as the means $\pm$ SEM of three independent experiments, [ANOVA (A and B), Two-way ANOVA (C and D)].

Although quiescent/contractile VSMCs are the predominant phenotype under normal conditions, VSMCs can switch their phenotype from a normal contractile state to non-contractile, proliferative, and migratory behaviour to adapt to the physiological (e.g., hormones and growth factors) or pathophysiological (e.g., low oxygen tension, mitogenic factors, and acidosis) stimuli, resulting in the structural remodelling of blood vessels. Thus, this phenotypic switch plays an important role in the initiation and progression of common human vascular disorders, such as systemic and pulmonary hypertension, ischaemic heart disease and stroke [26-29]. Although PKCs have emerged as a new target for treating the so-called "plasticity" of VSMCs, the principal finding of the present study is that the down-regulation of PKC- ϵ can mediate the suppression of VSMC proliferation.

We used the PMA-induced down-regulation of PKCs to demonstrate the inhibitory effect of PKC- ϵ on proliferation in VSMCs. Such down-regulation of PKC- ϵ is essential for the inhibition of cell growth, a finding that is further confirmed by the lentiviral delivery of shRNAs designed to down-regulate selectively the expression of the PKC- ϵ isoform. PMA, initial a potent mouse skin tumor promoter, have been widely used as either an activator or repressor of PKCs depending on its exposure time. In general, short time of treatment with PMA activates PKC, whereas the prolonged incubation with the compound causes depletion of the isoforms. Although prolonged incubation with PMA causes down-regulation of PKCs in various cell systems, the dosage ranges vary considerably [15, 16, 30]. The doses of PMA used in this study are based in our previous publication [16]. We used PMA prolonged treatment to demonstrate the down-regulatory effects of PMA on PKC- ϵ expression in VSMCs. Such a down-regulation is essential for the prolonged PMA treatment-induced inhibition of cell proliferation, a result that was confirmed by the reversible cell viability inhibition of PMA in the presence of the PKC- ϵ activator (Fig. 2C). The down-regulatory effect of PMA should not be limited to PKC- ϵ because PMA prolonged treatment can also down-regulate the expression of other PKCs ($-\alpha$, δ , θ) in VSMCs [16]. Notably, the significant decrease in cell viability measured by the CCK-8 assay was observed as early as 24 hours and was sustained until 72 hours after PMA treatment (Fig. 1B). This seems to be at odds with the data obtained from cell counting that showed a significant decrease in cell growth by PMA incubation starting from 48 hours (Fig. 1A). In the longer lasting experiment, the obtained results of the cell viability determined using the CCK-8 assay are strongly dependent on the number of cells that could reproduce and on their time of generation. By contrast, the cell counting methods covers cells capable of reproducing as well as those that are alive but cannot reproduce [31]. This may explain the discrepancy between the data obtained using cell counting and the CCK-8 assay. However, the reason for the indifference in cell viability determined by the CCK-8 assay between cultures after 72 hours of treatment with or without PMA (data not shown) remains unclear. Some changes in the cells, such as cell division, after much longer (> 72 hours) exposure to PMA may be far more complicated than we thought.

A recent study by Jain et al. [32] showed an increase in the proliferation of rat VSMCs in an endogenous interleukin-1 receptor-associated kinase-1 (IRAK)-ERK-PKC- ϵ -dependent manner in a rat balloon injury model. The present data indicate that the phosphorylation of ERK in VSMCs was increased by PMA in a rapid and transient manner. The acute effect of PMA on ERK activation has been documented to be dependent on PKC- ϵ activation [33]. However, the present results indicate that PKC- ϵ is not engaged in the down-regulation of phosphorylation of ERK by the prolonged PMA treatment. The experimental evidence supporting such an argument is that there is no change in the levels of phosphorylation of ERK by PKC- ϵ shRNA in VSMCs. The association between ERK and PKC- ϵ in regulating the proliferation of VSMCs is elusive. D-erythro-N,N-dimethylsphingosine (DMS), a sphingosine kinase inhibitor, could block [3H]-thymidine incorporation and ERK-1/2 activation, independent of PKC, in VSMCs in response to foetal calf serum (FCS), indicating that ERK-1/2, but not PKC, is an essential component of FCS-mediated cell proliferation [34]. However, a later study showed that platelet-derived growth factor (PDGF)-stimulated ERK-1/2 phosphorylation could be inhibited by PKC- ϵ siRNA [35]. The results of the present CCK-8 and EdU cell viability and proliferation assays revealed that the down-regulation of PKC- ϵ

could inhibit VSMC proliferation. Although the expression of PKC-ε was largely reduced in the PKC-ε shRNA-transfected VSMCs, the change in ERK phosphorylation in the presence of PMA was not significantly different from that in normal cells treated with PMA (Fig. 5C and D). However, others have shown that the silencing of PKC-ε inhibited PDGF-induced ERK 1/2 phosphorylation, indicating that PKC-ε is up-stream of ERK [32]. Regardless of the hierarchy or parallel role of PKC-ε in regulating cell proliferation in relation to ERK, the data presented in Fig. 2 demonstrate that prolonged PMA treatment-caused inhibition of VSMC proliferation is mediated, at least in part, through PKC-ε.

Being one of effectors of the tumor promoter phorbol esters, PKC family proteins have been intensively studied for their contribution to cancer. The roles of PKCs in cell proliferation and the resultant tumor growth in different cancer cell lines have been documented either to be negative or positive [36]. Such contrasting effects may be due to isoform-specific signal transduction of different isoforms in different tumor types and contexts. Results of the present CCK-8 assays revealed that cell viability of SHZ-88 were suppressed due to the silencing of PKC-ε. Consistently, the down-regulation of PKC-ε also attenuated breast cancer growth *in vivo*. These results agree with those by Newton and Messing showing that uncontrolled PKCε activation is associated with cancer development [37]. Given that different PKC isoforms may display a distinct functional role in the control of normal and cancer cell fates [6-8, 38-40], the inhibition of PKC-ε may be an attractive therapeutic strategy not only for treating proliferative VSMC-related diseases, such as atherosclerosis and restenosis, but also for targeting certain types of cancer like breast cancer.

Limitation: Direct role of PKC-ε in mediating cell hypertrophy was not examined in this study. VSMCs hypertrophy and proliferation are interweaved in the clinical course of vascular diseases such as hypertension, atherosclerosis, restenosis. A previous study by Ali et al. [10] showed that PKCs activation and expression of basic fibroblast growth factor (bFGF) are both required to transduce vascular hypertrophic responses to vasoconstricting growth promoters. However, increased bFGF expression alone is not enough to stimulate VSMC growth without the PKC activation. Although PMA increased nFGF of VSMCs in time dependent manner, prolonged incubation with PMA also down-regulated PKCs, which may counteract its stimulating VSMCs hypertrophy. Future studies will be needed to address these important issues clearly related to the role of PKCs activation in mediating cell proliferation and hypertrophy in cardiovascular diseases.

In conclusion, prolonged PMA incubation inhibits cell growth and the cell proliferation of VSMCs and breast cancer cells through inhibiting PKC-ε. This effect is independent of the ERK1/2 signalling pathway. These results suggest that PKC-ε is involved in VSMC and breast cancer cell proliferation. Inhibition of PKC-ε might be a new therapeutic method to delay the development of some cardiovascular diseases, as well as breast cancer.

Abbreviations

PMA (Phorbol myristate acetate); PKCs (Protein kinase Cs); VSMC (vascular smooth muscle cell); CCK-8 (Cell Counting Kit-8); shRNA (short-hairpin RNAs); MAPK (mitogen-activated protein kinase); DAG (diacylglycerol); EdU (5-ethynyl-2'-deoxyuridine); DMS (D-erythro-N,N-dimethylsphingosine); FCS (foetal calf serum); PDGF (platelet-derived growth factor).

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Disclosure Statement

We declare that we have no financial or commercial conflicts of interest.

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