Free Radical Biology & Medicine 49 (2010) 881–893

Contents lists available at ScienceDirect

Free Radical Biology & Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

Inhibition of vascular smooth muscle cell proliferation by the vitamin E derivative pentamethylhydroxychromane in an in vitro and in vivo study: pivotal role of hydroxyl radical-mediated PLCγ1 and JAK2 phosphorylation

Cheng-Ying Hsieh [a], Chien-Liang Liu [b], Ming-Jen Hsu [c], Thanasekaran Jayakumar [a], Duen-Suey Chou [c], Yi-Hsuan Wang [a,d], George Hsiao [c], Joen-Rong Sheu [a,c,*]

[a] Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan
[b] Department of Surgery, Mackay Memorial Hospital, Taipei 104, Taiwan
[c] Department of Pharmacology, Taipei Medical University, Taipei 110, Taiwan
[d] Institute of Law and Interdiscipline, National Chengchi University, Taipei 116, Taiwan

ARTICLE INFO

Article history:
Received 27 January 2010
Revised 18 May 2010
Accepted 11 June 2010
Available online 19 June 2010

Keywords:
PMC
vitamin E derivative
VSMC
PLCγ1
JAK2
hydroxyl radical
Free radicals

ABSTRACT

Abnormal proliferation of vascular smooth muscle cells (VSMCs) plays an important role in the development of cardiovascular diseases. PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane) is the most potent hydrophilic derivative of vitamin E. In this study, we investigated the mechanisms of PMC inhibition of VSMC proliferation in vitro and in vivo. PMC (20 and 50 μM) obviously suppressed proliferation of PDGF-BB-stimulated cells, but not resting cells, and arrested cell cycle progression at the G_2/M phase. A significant reduction in neointimal formation in carotid arteries was observed in PMC (5 mg/kg/day)-treated rats after balloon angioplasty. Activation of STAT3, JAK2, PLCγ1, PKCδ, and ROS, but not ERK1/2, AKT, or PKCα, was markedly inhibited by PMC in PDGF-BB-stimulated VSMCs. Deferoxamine and PMC significantly inhibited the phosphorylation of PLCγ1 and JAK2 and arrested cell cycle progression at the G_2/M phase. These events, however, were reversed in the presence of Fe^{2+}. Moreover, PMC directly inhibited hydroxyl radical formation in both the Fenton reaction and VSMCs according to an electron spin resonance study. In conclusion, this study demonstrates for the first time that PMC inhibits VSMC proliferation in vitro and balloon injury-induced neointimal formation in vivo. The inhibitory mechanism of PMC may involved the inhibition of hydroxyl radical-mediated PLCγ1–PKCδ and JAK2–STAT3 activation and causes cell cycle arrest at the G_2/M phase. PMC treatment may represent a novel approach for lowering the risk of or improving function in abnormal VSMC proliferation-related vascular diseases.

Abnormal proliferation of vascular smooth muscle cells (VSMCs)[1] is implicated in the pathogenesis of several diseases including atherosclerosis, restenosis after angioplasty, transplant vasculopathy, and failure of vein graft bypass [1]. Numerous growth factors and cytokines are reported to be released in human vascular lesions by dysfunctional endothelial cells, inflammatory cells, platelets, and VSMCs, and these mediate chemoattraction, cell migration, proliferation, apoptosis, and matrix modulation [2]. Basic fibroblast growth factor initiates medial proliferation of VSMCs, whereas platelet-derived growth factor (PDGF) induces subsequent migration of VSMCs toward the intima. Intimal proliferation and matrix accumulation were reported to occur under the influence of PDGF, transforming growth factor-β, angiotensin II, epidermal growth factor, and insulin-like growth factor 1 [3]. Although all of these factors may play roles in driving cellular events that lead to vascular proliferative diseases, it is generally considered that PDGF is the main cause [4].

PDGF is a peptide growth factor that provides signals for the proliferation of target cells. PDGF isoforms consist of various combinations of two polypeptide chains (the A- and B-chain), e.g., PDGF-AA, -AB, and -BB. The α- and β-receptors of PDGF were reported to have specific affinities for their isoforms, e.g., the β-receptor interacts only with the PDGF B-chain [5]. PDGF receptor (PDGFR)-β

Abbreviations: CCA, common carotid artery; DCF-DA, 2,7-dichlorofluorescin diacetate; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; EEL, external elastic lamina; ERK, extracellular-regulated kinase; ESR, electron spin resonance; FBS, fetal bovine serum; IEL, internal elastic lamina; IP_3, inositol-(1,4,5)-trisphosphate; JAK, Janus kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, nicotinamide adenine dinucleotide phosphate; NOX, NADPH oxidase; PDGFR, platelet-derived growth factor receptor; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLCγ, phospholipase Cγ; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STAT, signal transducers and activators of transcription; VSMC, vascular smooth muscle cell.

* Corresponding author. Graduate Institute of Medical Sciences, Taipei Medical University, Taipei 110, Taiwan. Fax: +886 2 27390450.
E-mail address: sheujr@tmu.edu.tw (J.-R. Sheu).

0891-5849/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2010.06.014

increases in atherosclerotic lesions, and its expression is primarily limited to VSMCs [6]. PDGF-BB propagates mitogenic signals through the autophosphorylation of its PDGFR-β on tyrosine residues. Tyrosine-phosphorylated PDGFR-β interacts with several other cytoplasmic proteins that constitute Src homology 2 (SH2) domains, including phospholipase Cγ (PLCγ), *ras* guanine 5′-triphosphatase-activating protein, phosphatidylinositol 3-kinase (PI3K), tyrosine phosphatase SHP-2, and members of the signal transducers and activators of transcription (STAT) family. Secondary messengers include inositol-(1,4,5)-trisphosphate (IP_3) and diacylglycerol, intracellular calcium release, and protein kinase C (PKC) activation [7]. Furthermore, the PDGFR transmits its signal into the intracellular space by producing reactive oxygen species (ROS), particularly superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). H_2O_2 plays a pivotal role in PDGF-BB-induced signals because it freely diffuses across cellular membranes and is more stable than $O_2^{\bullet-}$ [8]. H_2O_2 was reported to stimulate the phosphorylation of extracellular-regulated kinase (ERK) 1/2 in PDGF-BB-stimulated VSMC proliferation [9].

PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane), the most potent derivative of vitamin E (i.e., α-tocopherols), in which a phytyl chain is replaced by a methyl group (Figs. 1A and B), can act as a potent antioxidant and inhibit nuclear factor-κB activation [10,11]. It is more hydrophilic than other α-tocopherol derivatives and has potent antiplatelet and free radical-scavenging activities [11]. Recently, we also found that PMC treatment may represent a novel approach to lowering the risk of or improving the function in ischemia–reperfusion brain injury-related disorders [12].

By considering the pivotal roles of VSMC proliferation in the development of atherosclerosis and restenosis, this study was designed to examine the inhibitory mechanisms of PMC in PDGF-BB-stimulated VSMC proliferation in vitro and balloon injury-induced neointimal formation in vivo.

Materials and methods

Materials

Male Wistar rats were purchased from BioLASCO (Taipei, Taiwan). Dulbecco's modified Eagle's medium (DMEM), trypsin (0.25%), L-glutamine, penicillin/streptomycin, and fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). PMC, α-tocopherol, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7-dichlorofluorescin diacetate (DCF-DA), deferoxamine, and dimethyl sulfoxide (DMSO) were from Sigma–Aldrich (St. Louis, MO, USA). Recombinant PDGF-BB was from PeproTech (Rocky Hill, NJ, USA); propidium iodide (PI) was from Calbiochem (Darmstadt, Germany). The RNase A and anti-PKCα and anti-PKCδ monoclonal antibodies (mAbs) were from BD Bioscience (San Jose, CA, USA); the anti-PLCγ1 and anti-phospho-PLCγ1 (Tyr^{783}) polyclonal antibodies (pAbs) were from Signalway Antibody (Pearland, TX, USA). The anti-ERK1/2, anti-phospho-ERK1/2 (Thr^{202}/Tyr^{204}), anti-phospho-AKT (Ser^{473}), anti-Janus kinase 2 (JAK2), and anti-phospho-JAK2 ($Tyr^{1007/1008}$) pAbs and the anti-AKT mAb were purchased from Cell Signaling (Beverly, MA, USA). The anti-phospho-STAT3 (Tyr^{705}) pAb was from Biovision (Mountain View, CA, USA); the anti-α-tubulin mAb was from NeoMarkers (Fremont, CA, USA). The Hybond-P polyvinylidene difluoride (PVDF) membrane, enhanced chemiluminescence (ECL) Western blotting detection reagent and analysis system, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit immunoglobulin G (IgG), and sheep anti-mouse IgG were from Amersham (Buckinghamshire, UK). PMC was dissolved in DMSO and stored at 4 °C until used.

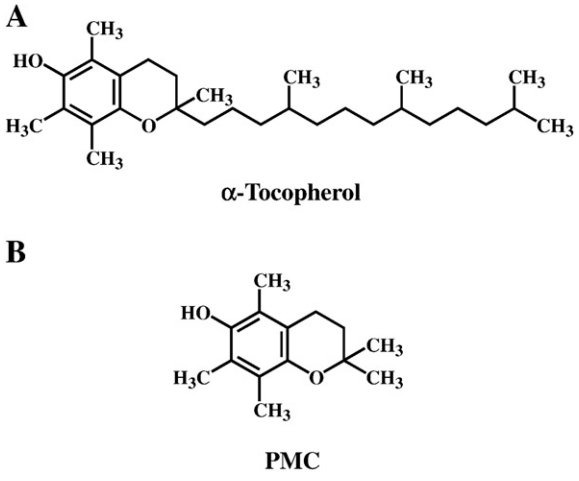

Fig. 1. Chemical structures of (A) α-tocopherol and (B) PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane).

VSMC isolation and culture

All animal experiments and care were carried out according to the *Guide for the Care and Use of Laboratory Animals* (NIH, National Academy Press, Washington, DC, 1996). VSMCs were enzymatically dispersed from thoracic aortas of male Wistar rats (250–300 g). The thoracic aorta was removed and stripped of the endothelium and adventitia. VSMCs were obtained by a modified method of a combined collagenase and elastase digestion [13]. Cells were grown in DMEM supplemented with 20 mM Hepes, 10% FBS, 1% penicillin/streptomycin, and 2 mM glutamine at 37 °C in a humidified atmosphere of 5% CO_2. VSMCs from passages 4 to 8 were used in all experiments. Primary cultured rat aortic VSMCs showed the "hills and valleys" pattern, and the expression of α-smooth muscle actin was confirmed (data not shown).

Proliferation assay

VSMCs (2×10^4 cells/well) were seeded on 24-well plates and cultured in DMEM containing 10% FBS for 24 h. The medium was then replaced by serum-free medium for 24 h. Starved VSMCs were pretreated with α-tocopherol and PMC (20 and 50 μM) or an isovolumetric solvent control (0.1% DMSO) for 20 min and then stimulated with PDGF-BB (10 ng/ml) or not for 48 h. The cell number was measured using a colorimetric assay based on the ability of mitochondria in viable cells to reduce MTT as previously described [14]. The cell number index was calculated as the absorbance of treated cells/control cells × 100%.

Cell cycle analysis

For the cell cycle analysis, starved VSMCs (2×10^5 cells/dish) were pretreated with PMC (20 and 50 μM) or a solvent control for 20 min and then stimulated with PDGF-BB (10 ng/ml) or not for 24 h. After 24 h, the cells were detached from the plate using trypsin, washed with PBS, and then fixed in 70% ethanol for 30 min. Cells were then washed with PBS and resuspended in a solution containing RNase (50 μg/ml), PI (80 μg/ml), and Triton X-100 (0.2%). Samples were incubated for 20 min and subjected to a flow cytometric analysis (Beckman Coulter, Ramsey, MN, USA).

Immunoblotting study

Immunoblotting analyses were performed to determine the expression of proteins (ERK1/2, AKT, STAT3, JAK2, PLCγ1, PKCα, and PKCδ) in VSMCs as described previously [15]. Starved VSMCs (2×10^5 cells/dish) were treated with PMC (20 and 50 μM), deferoxamine (0.5 and 1 mM), or a solvent control for 20 min, followed by

the addition of PDGF-BB (10 ng/ml) or H_2O_2 (1 mM) for 10 min. After the experimental period, the proteins were extracted with lysis buffer. Lysates were centrifuged, the supernatant protein (50 μg) was collected and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the separated proteins were electrophoretically transferred onto 0.45-μm PVDF membranes. Blots were blocked with TBST (10 mM Tris-base, 100 mM NaCl, and 0.01% Tween 20) containing 5% bovine serum albumin for 1 h and then probed with various primary antibodies. Membranes were incubated with HRP-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. Immunoreactive bands were detected by an ECL system. Bar graphs depict the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-Profil; Biolight Windows application version 2000.01; Vilber Lourmat, France).

Cell fractionation

Cytosolic and membrane fractions were prepared from VSMCs as described previously with slight modifications to determine PKCα and PKCδ translocation [16]. In brief, starved VSMCs (1×10^6 cells/dish) were pretreated with a solvent control (0.1% DMSO) or PMC (20 and 50 μM) for 20 min, followed by the addition of PDGF-BB (10 ng/ml). After treatment, the cells were washed, scraped into buffer, and centrifuged for 1 h. The supernatant was designated the cytosolic fraction. The pellet was extracted with lysis buffer and centrifuged for 20 min. The supernatant was saved as the membrane fraction. Proteins (15 μg) were determined by SDS–PAGE with immunoblotting.

Measurement of intracellular ROS

Starved VSMCs (2×10^5 cells/dish) were loaded with DCF-DA (20 μM) for 20 min. After treatment with PMC (20 and 50 μM) or a solvent control for 20 min, cells were stimulated with PDGF-BB (10 ng/ml) for 10 min, washed with PBS, and then detached using trypsin. Levels of intracellular ROS were detected by flow cytometry (Beckman Coulter). Data were collected from 10,000 cells per experimental group. All experiments were repeated at least four times to ensure reproducibility.

Measurement of hydroxyl radical ($HO^{\bullet}$) formation by electron spin resonance (ESR) spectrometry

The ESR method used a Bruker EMX ESR spectrometer (Billerica, MA, USA) as described previously [17]. In brief, a Fenton reaction solution (50 μM $FeSO_4$ + 2 mM H_2O_2) or VSMCs (5×10^6/ml) were pretreated with a solvent control (0.1% DMSO) or PMC (50 μM) for 10 min with or without PDGF-BB (10 ng/ml). The rate of hydroxyl radical-scavenging activity was defined by the following equation: inhibition rate = 1 − [signal height (PMC)/signal height (solvent control)] [17].

Balloon angioplasty of the carotid artery in rats

In brief, male Wistar rats (350–400 g) were anesthetized with chloral hydrate (0.4 g/kg, ip), and a 2F embolectomy balloon catheter (Edwards Lifescience, Irvine, CA, USA) was introduced into the right common carotid artery (CCA) via the external artery [18]. The balloon was inflated to distend the CCA and was then withdrawn to the external artery. The procedure was repeated three times, the catheter was removed, and the distal external artery segment was ligated. All animals were divided into five groups: (i) a sham-operated group, (ii) a control (normal saline) group, (iii) a solvent (DMSO) group, and groups treated with PMC at (iv) 5 or (v) 10 mg/kg/day or with (vi) α-tocopherol (10 mg/kg/day). A mini-osmotic pump (Alzet, Cupertino, CA, USA) (0.5 μl/h) containing sufficient solution was subcutaneously implanted at the back of the neck. Fourteen days after the balloon angioplasty, the rats were anesthetized and perfused with normal saline. The right CCA was isolated and then cut into 5-μm sections, stained with hematoxylin and eosin, and photographed using a microscope (Nikon Eclipse TS100, Tokyo, Japan); the neointimal areas were measured using an Image-Pro Express 6.0 system (Bethesda, MD, USA). Areas within the external elastic lamina (EEL), the internal elastic lamina (IEL), and the lumen were measured. The intima area/media area (*I*/*M*) ratio was calculated using the following formula: (IEL area − lumen area)/(EEL area − IEL area) [18].

Statistical analysis

Experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by the method of analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared with the others using the Newman–Keuls method. A P value of <0.05 was considered statistically significant.

Results

Effects of PMC on VSMC proliferation and cell cycle progression stimulated by PDGF-BB

Fig. 2A shows a reduction in the number of resting VSMCs that were treated with α-tocopherol (20 and 50 μM) in a concentration-dependent manner (nontreated group, 100.0 ± 0.0%; 20 μM α-tocopherol, 71.4 ± 5.0%; 50 μM, 58.9 ± 3.9%); however, there were no significant effects observed after PMC (20 and 50 μM) treatment in resting cells. On the other hand, when VSMCs were stimulated by PDGF-BB (10 ng/ml), proliferation increased by approximately 92% (Fig. 2A). Concentration-dependent inhibition was observed in PDGF-BB-stimulated cells during PMC (20 and 50 μM) treatment, at approximately 46.0 and 63.0%, respectively. α-Tocopherol (20 and 50 μM) also exhibited a similar inhibitory effect on cell proliferation of approximately 32.4 and 68.1%, respectively (Fig. 2A). These results suggest that PMC inhibited VSMC proliferation only in the activated state, not in resting cells, whereas α-tocopherol affected both.

Furthermore, the DNA content was analyzed by PI staining to investigate the effect of PMC on cell cycle progression in VSMCs. A flow cytometric study found concentration-dependent accumulation at the G_2/M phase in PMC (20 and 50 μM)-treated VSMCs (Fig. 2B). After stimulation with PDGF-BB (10 ng/ml), increases in the percentages of cells in the S (from 4.4 ± 0.5 to 7.0 ± 0.2%, $P<0.05$; $N=5$) and G_2/M phases (from 17.9 ± 1.9 to 24.8 ± 0.4%, $P<0.001$; $N=5$) were observed (Fig. 2B, graphs a and b), whereas the percentage in the G_0/G_1 phase was reduced (from 76.4 ± 1.2 to 67.0 ± 0.4%, $P<0.001$; $N=5$; Fig. 2B, graphs a and b). PMC (50 μM) treatment resulted in an obvious accumulation of cells at the G_2/M phase, and a reduction was noted in the G_0/G_1 phase compared with the DMSO-treated group (G_2/M phase, 24.8 ± 0.4% vs 33.8 ± 0.6%, $P<0.001$; $N=5$; G_0/G_1 phase, 67.0 ± 0.4% vs 56.4 ± 1.2%, $P<0.001$; $N=5$; Fig. 2B, graph d). These results indicate that PMC was effective in arresting cell cycle progression at the G_2/M phase in VSMCs stimulated by PDGF-BB.

Effects of PMC on balloon injury-induced neointimal formation in CCAs

The study showed a remarkable increase in neointimal formation in DMSO-treated balloon-injured rats compared to sham-operated rats (Figs. 3A, images a and b, and 3B). Administration of PMC (5 and 10 mg/kg/day) showed a concentration-dependent reduction in the *I*/*M* ratio compared to the DMSO-treated group (DMSO, 1.8 ± 0.1, $N=7$; 5 mg/kg/day, 0.8 ± 0.2, $N=5$; 10 mg/kg/day, 0.5 ± 0.2, $N=5$; Figs. 3A, images c and d, and 3B). α-Tocopherol (10 mg/kg/day)

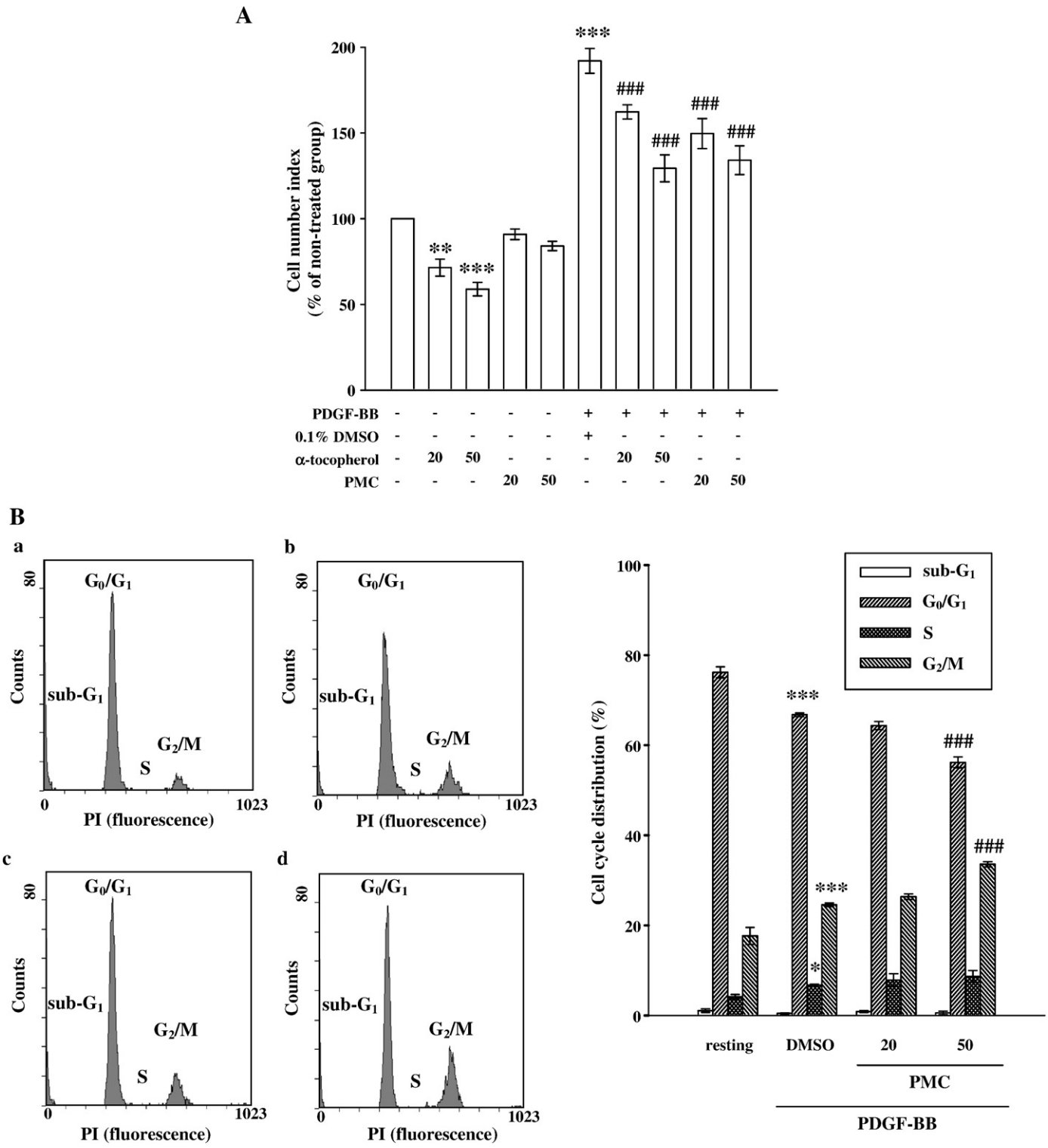

Fig. 2. Effects of PMC on cell proliferation and cell cycle progression in vascular smooth muscle cells (VSMCs) stimulated by PDGF-BB. (A) VSMCs (2×10^4 cells/well) were treated with PBS (resting) or pretreated with α-tocopherol (20 and 50 μM), PMC (20 and 50 μM), or an isovolumetric solvent control (0.1% DMSO), followed by the addition of PDGF-BB (10 ng/ml). Cell numbers were evaluated by an MTT assay as described under Materials and methods. (B) VSMCs (2×10^5 cells/dish) were (graph a) treated with PBS (resting) or pretreated with (graph b) solvent control (0.1% DMSO) or (graph c) 20 μM or (graph d) 50 μM PMC, followed by the addition of PDGF-BB (10 ng/ml). Representative DNA histograms of propidium iodide fluorescence in cells assessed by flow cytometry are shown. Compiled statistical data are shown on the right. $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#\#\#}P<0.001$, compared to the PDGF-BB group. Data are presented as the means ± SEM ($N=5$).

showed no significant effect on neointimal formation (1.5 ± 0.2, $N=5$; Figs. 3A, image e, and 3B). On the other hand, treatment of rats with DMSO did not significantly influence neointimal formation compared to normal saline-treated (control) rats (Fig. 3B).

Effects of PMC on PDGF-BB-induced signaling pathways in VSMCs

PDGF-BB-induced expression of several signaling proteins, including ERK1/2, AKT, STAT3, JAK2, PLCγ1, PKCα, and PKCδ, was

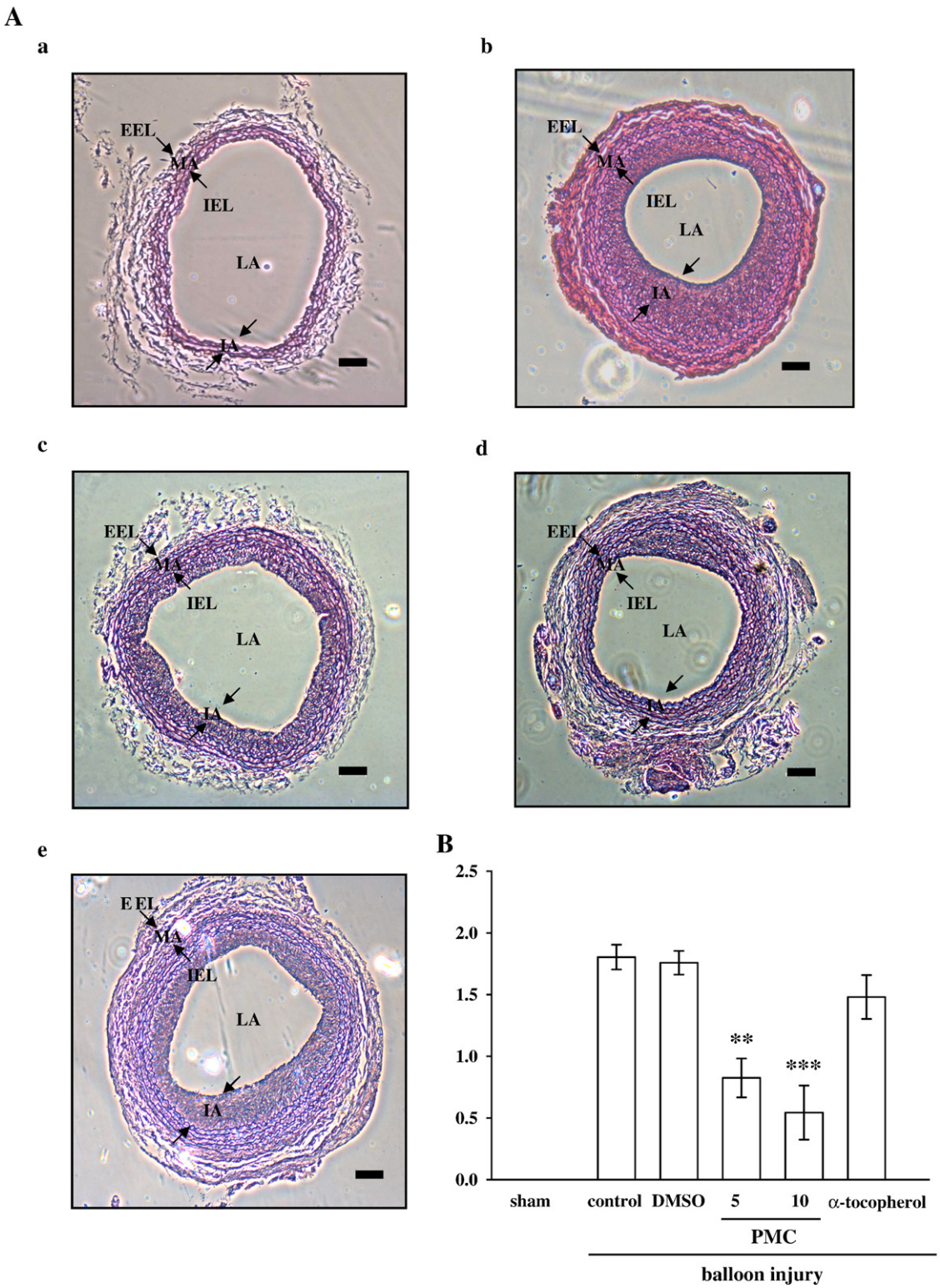

Fig. 3. Effects of PMC and α-tocopherol on balloon injury-induced neointimal formation in rat carotid arteries. (A) Microscopic images were taken in (image a) a sham-operated group and groups administered (image b) an isovolumetric solvent (DMSO), (images c and d) PMC (5 and 10 mg/kg/day), or (image e) α-tocopherol (10 mg/kg/day) 14 days after balloon angioplasty. Compiled statistical data are shown in (B). The black bar indicates 100 μm (original magnification × 400). $^{**}P<0.01$ and $^{***}P<0.001$, compared to the solvent (DMSO) group. Data are presented as the means ± SEM ($N = 5–7$). IA, intimal area; MA, media area; LA, lumen area; IEL, internal elastic lamina; EEL, external elastic lamina.

detected to unravel the mechanisms of PMC in PDGF-BB-induced VSMC proliferation. Phosphorylated JAK2, STAT3, and PLCγ1, but not ERK1/2 or AKT, stimulated by PDGF-BB were markedly inhibited in the presence of PMC (20 and 50 μM; Figs. 4 and 5A). In addition, PDGF-BB induced PKCδ but not PKCα translocation from the cytosol to the membrane (Figs. 5B and C). PMC (20 and 50 μM) concentration-dependently inhibited PKCδ translocation in VSMCs (Fig. 5C).

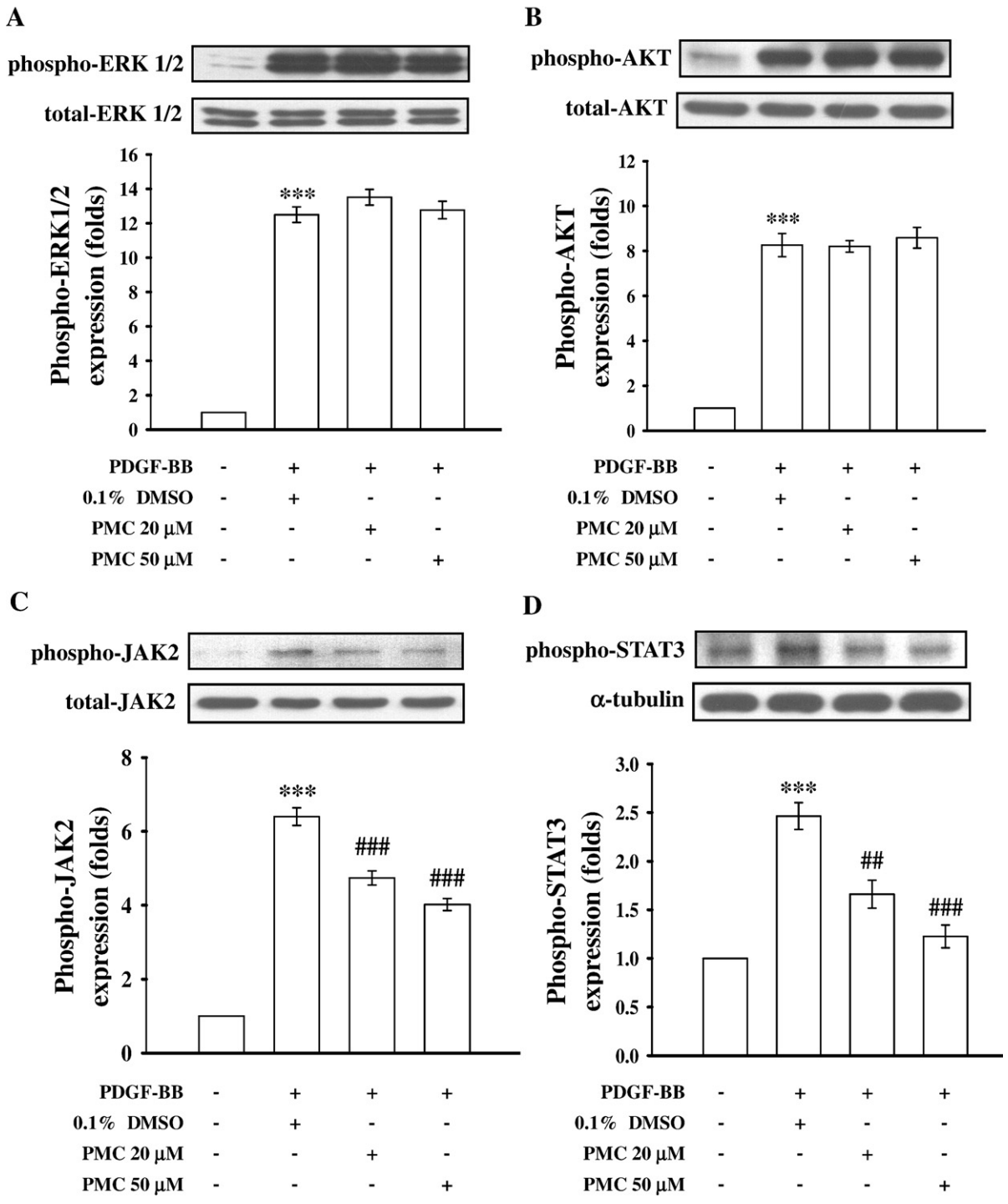

Fig. 4. Effects of PMC on ERK1/2, AKT, JAK2, and STAT3 phosphorylation in VSMCs stimulated by PDGF-BB. VSMCs (2×10^5 cells/dish) were treated with PBS (resting) or pretreated with a solvent control (0.1% DMSO) or PMC (20 and 50 μM), followed by the addition of PDGF-BB (10 ng/ml) to trigger (A) ERK1/2, (B) AKT, (C) JAK2, and (D) STAT3 phosphorylation. $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared to the PDGF-BB group. Data are presented as the means ± SEM ($N=5$).

Roles of ROS and HO• involved in PMC-mediated inhibition of VSMC proliferation

To determine the efficacy of PMC in inhibiting PDGF-BB-induced ROS formation in VSMCs, a cell-permeative ROS-sensitive dye, DCF-DA (nonfluorescent in a reduced state but fluorescent upon oxidation by ROS), was used [19]. In this study, PDGF-BB (10 ng/ml) induced ROS formation by almost twofold compared to resting (untreated) cells, whereas treatment with PMC (20 and 50 μM) markedly inhibited this reaction by approximately 77.4 and 93.4%, respectively (Fig. 6A). Different ROS may influence diverse PDGFR-mediated signaling events [20]. In the following study, we used deferoxamine as a potent ferrous iron chelator to further evaluate the role of hydroxyl radicals in PMC-mediated inhibition of cell proliferation stimulated by PDGF-BB. As shown in Fig. 6B, treatment with deferoxamine (0.5 and 1 mM) significantly inhibited both PLCγ1 and JAK2, but not ERK1/2 or AKT, phosphorylation in VSMCs stimulated by PDGF-BB (Figs. 6B–F). On the other hand, PMC (20

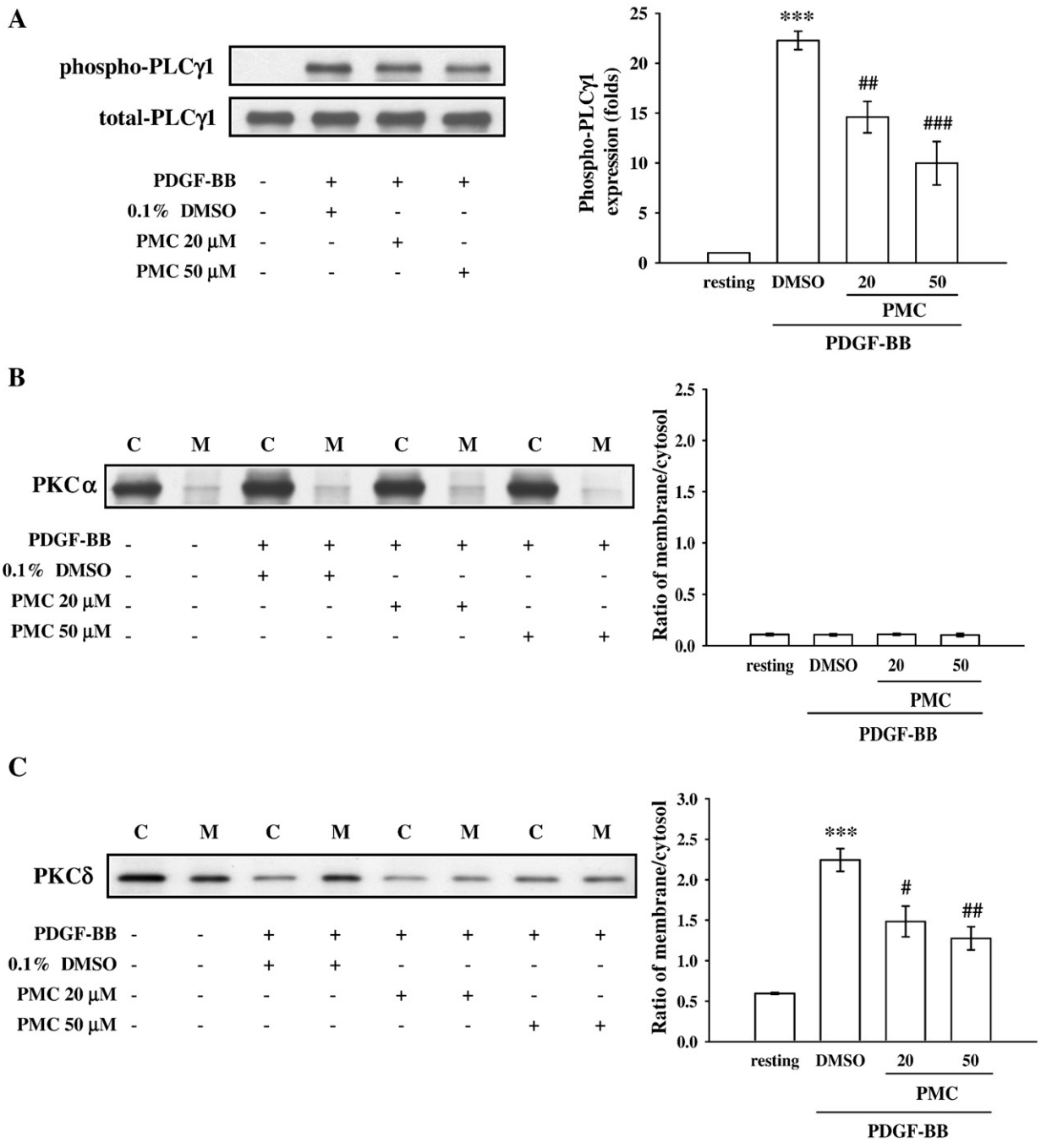

Fig. 5. Effects of PMC on PLCγ1 phosphorylation and PKCα and δ translocation in VSMCs stimulated by PDGF-BB. VSMCs (2×10^5 cells/dish) were treated with PBS (resting) or pretreated with a solvent control (0.1% DMSO) or PMC (20 and 50 μM), followed by the addition of PDGF-BB (10 ng/ml) to trigger (A) PLCγ1 phosphorylation and (B and C) PKCα and δ translocation. $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#}P<0.01$, compared to the PDGF-BB group. Data are presented as the means ± SEM ($N=5$).

and 50 μM) and deferoxamine (DFO; 0.5 and 1 mM) also significantly inhibited both PLCγ1 and JAK2, but not ERK1/2 or AKT, phosphorylation in VSMCs stimulated by H_2O_2 (1 mM; Fig. 7). These results indicate that both PLCγ1 and JAK2 phosphorylation was regulated by hydroxyl radicals in VSMCs.

Effects of PMC on hydroxyl radical-mediated PLCγ1 and JAK2 phosphorylation and cell cycle progression in VSMCs stimulated by PDGF-BB

Fig. 8A shows that PMC (50 μM) obviously attenuated hydroxyl radical formation in the Fenton reaction ($Fe^{2+} + H_2O_2$) by approximately 60% compared to the solvent (0.1% DMSO)-treated group according to the ESR study. Moreover, a typical ESR signal of hydroxyl radical formation was shown with PDGF-BB (10 ng/ml) stimulation compared to resting (PBS-treated) cells (Fig. 8B, traces a and b); PMC (50 μM) significantly attenuated hydroxyl radical formation by approximately 40% in VSMCs stimulated by PDGF-BB (10 ng/ml; Fig. 8B, trace c). Furthermore, the addition of Fe^{2+} (10 μM) significantly reversed the PMC-mediated inhibitory effect of PLCγ1 and JAK2 phosphorylation in VSMCs stimulated by PDGF-BB (Fig. 8C).

As shown in Fig. 9, the flow cytometric analysis revealed that deferoxamine (10 and 20 μM) concentration-dependently induced cell accumulation at the G_2/M phase compared to solvent (PBS)-

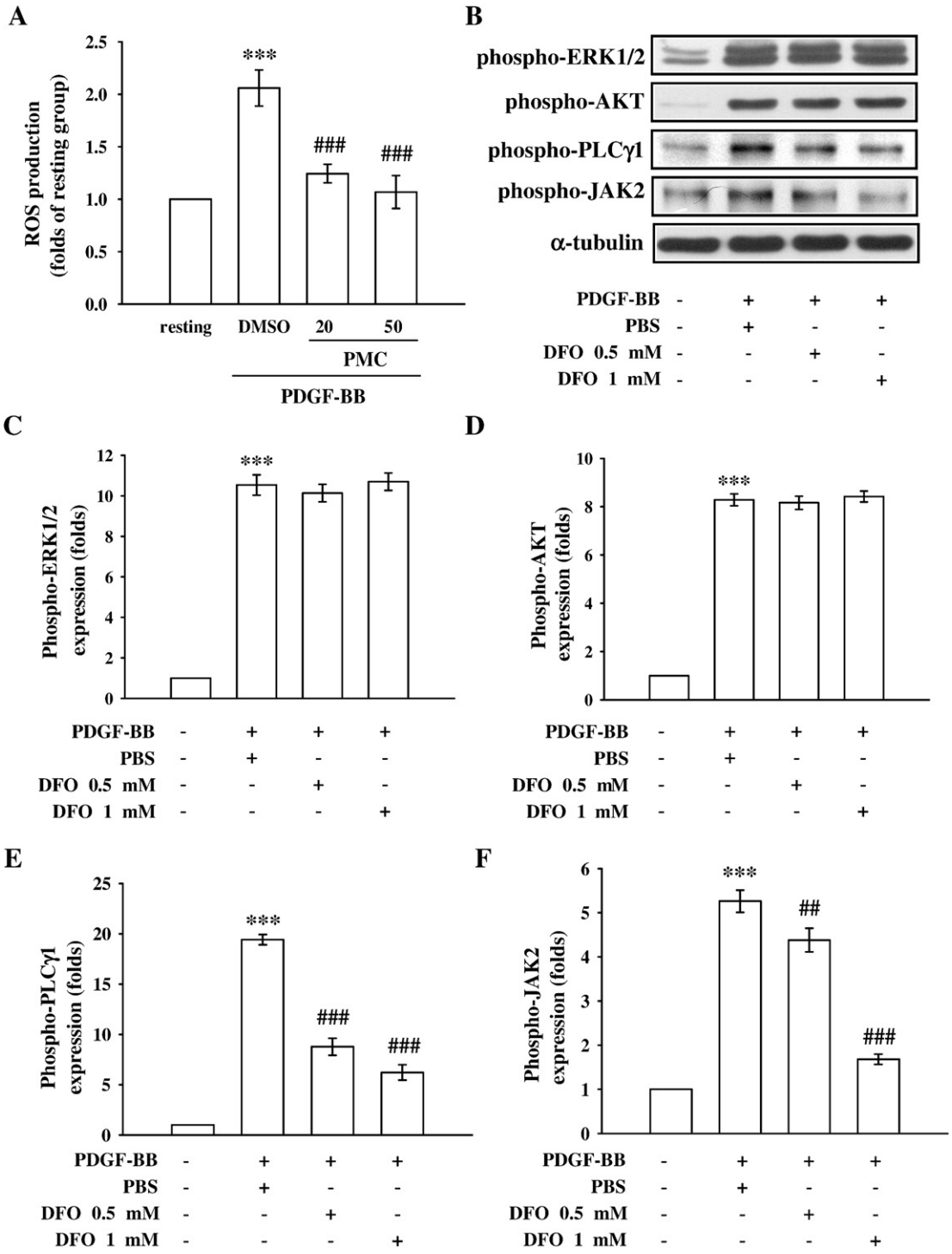

Fig. 6. Effects of PMC and deferoxamine on ROS formation and ERK1/2, AKT, PLCγ1, and JAK2 phosphorylation in VSMCs stimulated by PDGF-BB. VSMCs (2×10^5 cells/dish) were treated with PBS (resting) or pretreated with a solvent control (0.1% DMSO or PBS), PMC (20 and 50 μM), or deferoxamine (DFO; 0.5 and 1 mM), followed by the addition of PDGF-BB (10 ng/ml) to trigger (A) ROS formation and (B) ERK1/2, AKT, PLCγ1, and JAK2 phosphorylation. (C–F) Compiled statistical data are shown. $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared to the PDGF-BB group. Data are presented as the means ± SEM ($N=5$).

treated cells stimulated by PDGF-BB (DFO 10 μM vs PBS, $P<0.05$; $N=5$; 20 μM vs PBS, $P<0.01$; $N=5$; Fig. 9A). On the other hand, Fe^{2+} (10 μM) significantly reversed the PMC (50 μM)-mediated arrest of cell cycle progression at the G_2/M phase stimulated by PDGF-BB (PMC vs PMC + Fe^{2+}, $P<0.01$; $N=5$; Fig. 9B). These results imply that PMC arrest of the cell cycle progression at the G_2/M phase may be related, at least partly, to the scavenging of hydroxyl radicals.

Discussion

This study demonstrates for the first time that PMC, a potent hydrophilic α-tocopherol derivative, inhibits PDGF-BB-stimulated cell proliferation via hydroxyl radical-mediated PLCγ1 and JAK2 phosphorylation, resulting in cell cycle progression being arrested at the G_2/M phase. VSMC proliferation and neointimal formation are

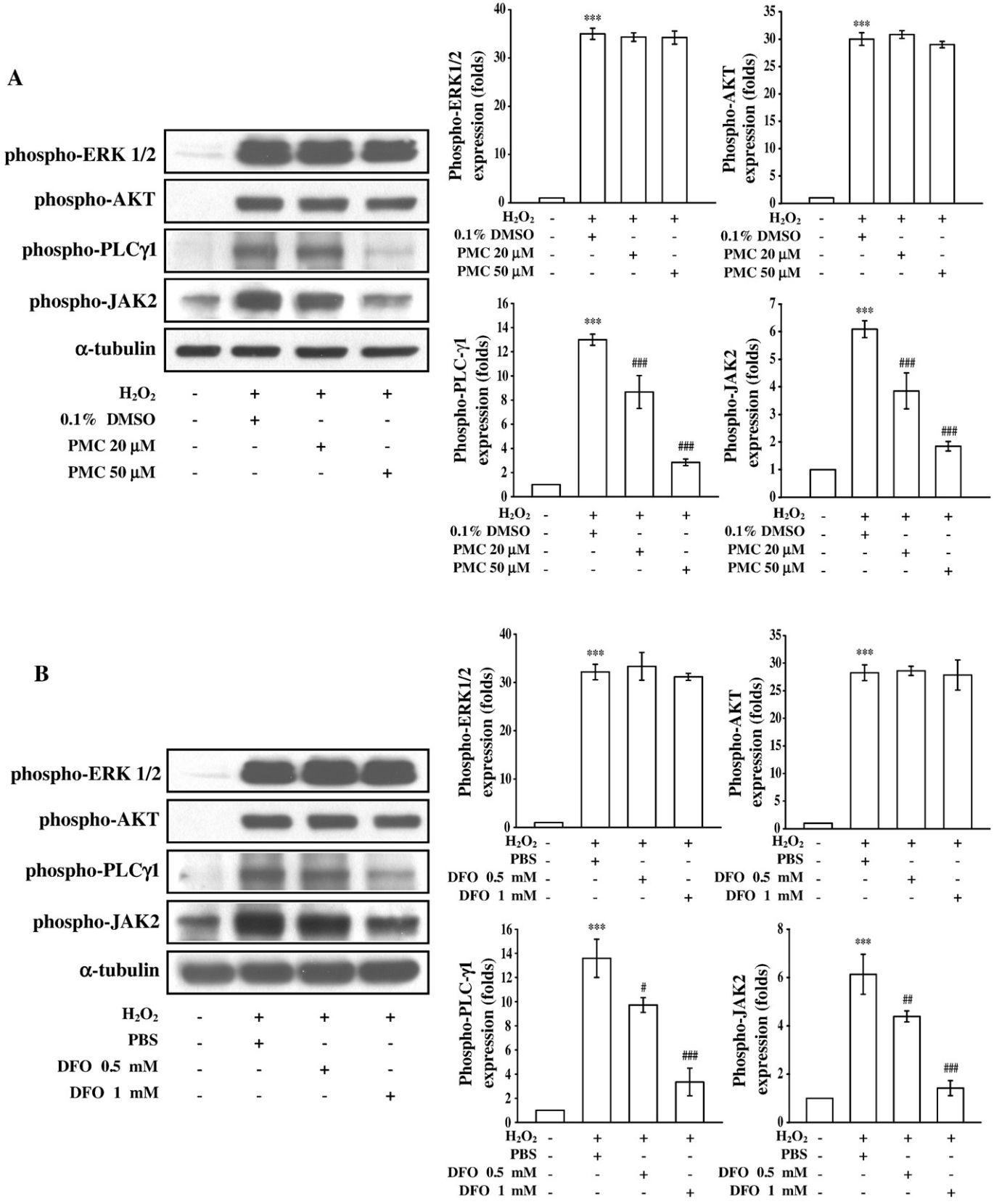

Fig. 7. Effects of PMC and deferoxamine on ERK1/2, AKT, PLCγ1, and JAK2 phosphorylation in VSMCs stimulated by H_2O_2. VSMCs (2×10^5 cells/dish) were treated with PBS (resting) or pretreated with (A) a solvent control (0.1% DMSO) or PMC (20 and 50 μM) or (B) a solvent control (PBS) or deferoxamine (DFO; 0.5 and 1 mM), followed by the addition of H_2O_2 (1 mM) to trigger ERK1/2, AKT, PLCγ1, and JAK2 phosphorylation. $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, and $^{\#\#\#}P<0.001$, compared to the H_2O_2 group. Data are presented as the means ± SEM ($N=5$).

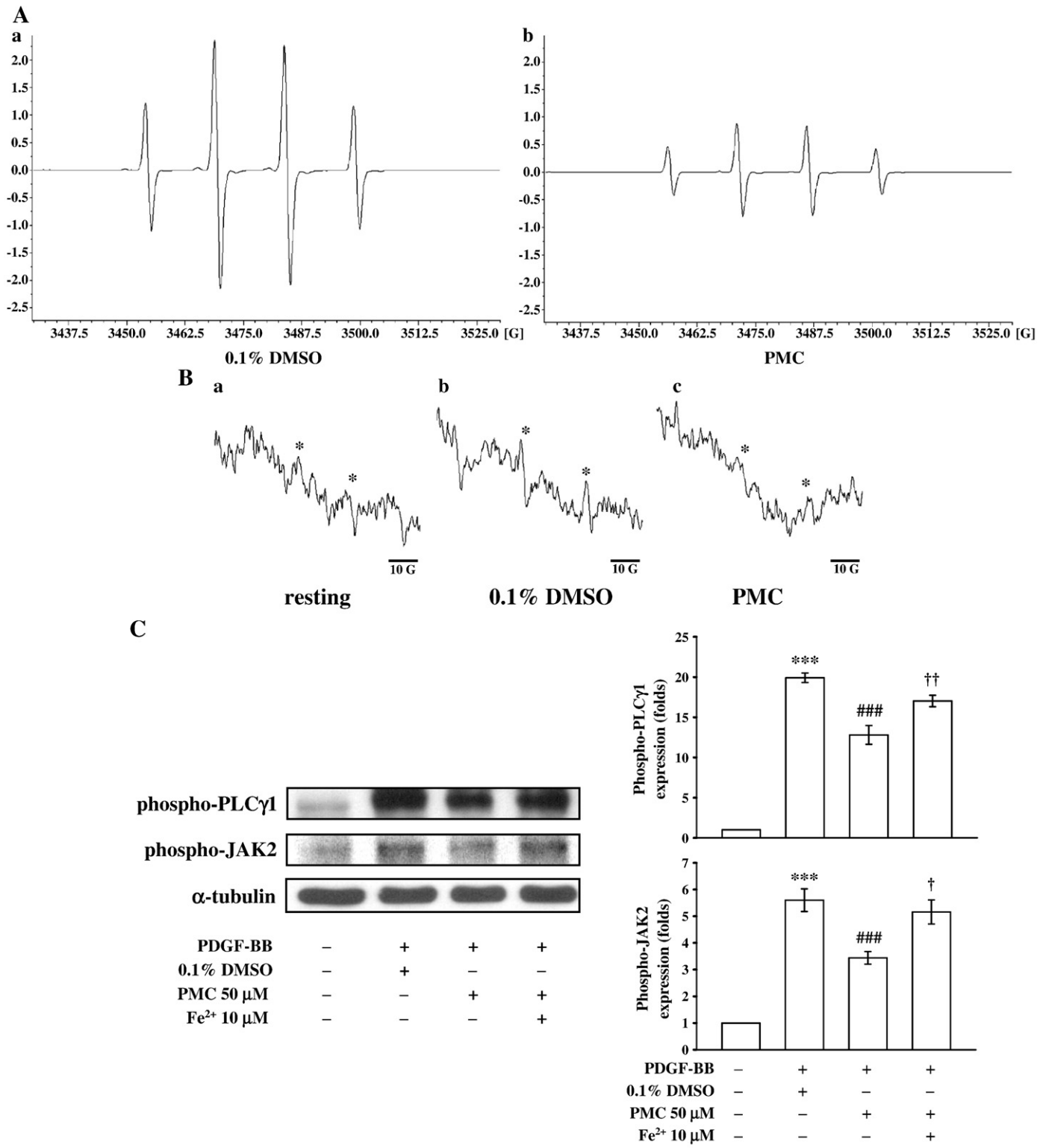

Fig. 8. Effects of PMC in hydroxyl radical-mediated PLCγ1 and JAK2 phosphorylation in VSMCs stimulated by PDGF-BB. (A) ESR spectra show the effects of a solvent control (spectrum a, 0.1% DMSO) and PMC (spectrum b, 50 μM) on hydroxyl radical formation in the Fenton reaction. (B) VSMCs (5×10^6 cells/ml) were treated with PBS (trace a, resting) or pretreated with a solvent control (trace b, 0.1% DMSO) or PMC (trace c, 50 μM), followed by the addition of PDGF-BB (10 ng/ml). Asterisks indicate the formation of hydroxyl radicals. Spectra are representative examples of three similar experiments. (C) VSMCs (2×10^5 cells/dish) were treated with PBS (resting) or pretreated with a solvent control (0.1% DMSO), PMC (50 μM), or PMC with $FeSO_4$ (10 μM), followed by the addition of PDGF-BB (10 ng/ml) to trigger PLCγ1 and JAK2 phosphorylation. $^{***}P<0.001$, compared to the resting (PBS treatment) group; $^{\#\#\#}P<0.001$, compared to the PDGF-BB group; $^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$, compared to the PMC group. Data are presented as the means ± SEM ($N=5$).

important events in the pathophysiological course of atherosclerosis and restenosis after balloon angioplasty. Therefore, modulation of VSMC proliferation has critical therapeutic implications [1]. The high lipophilicity of α-tocopherol and its relatively slow cellular uptake may lead to its clinical usefulness, especially in emergencies [21]. Therefore, development of more-hydrophilic analogues of α-tocopherol is important in exploring its pharmacological function and clinical applications. PMC was reported to be about 18 times more potent

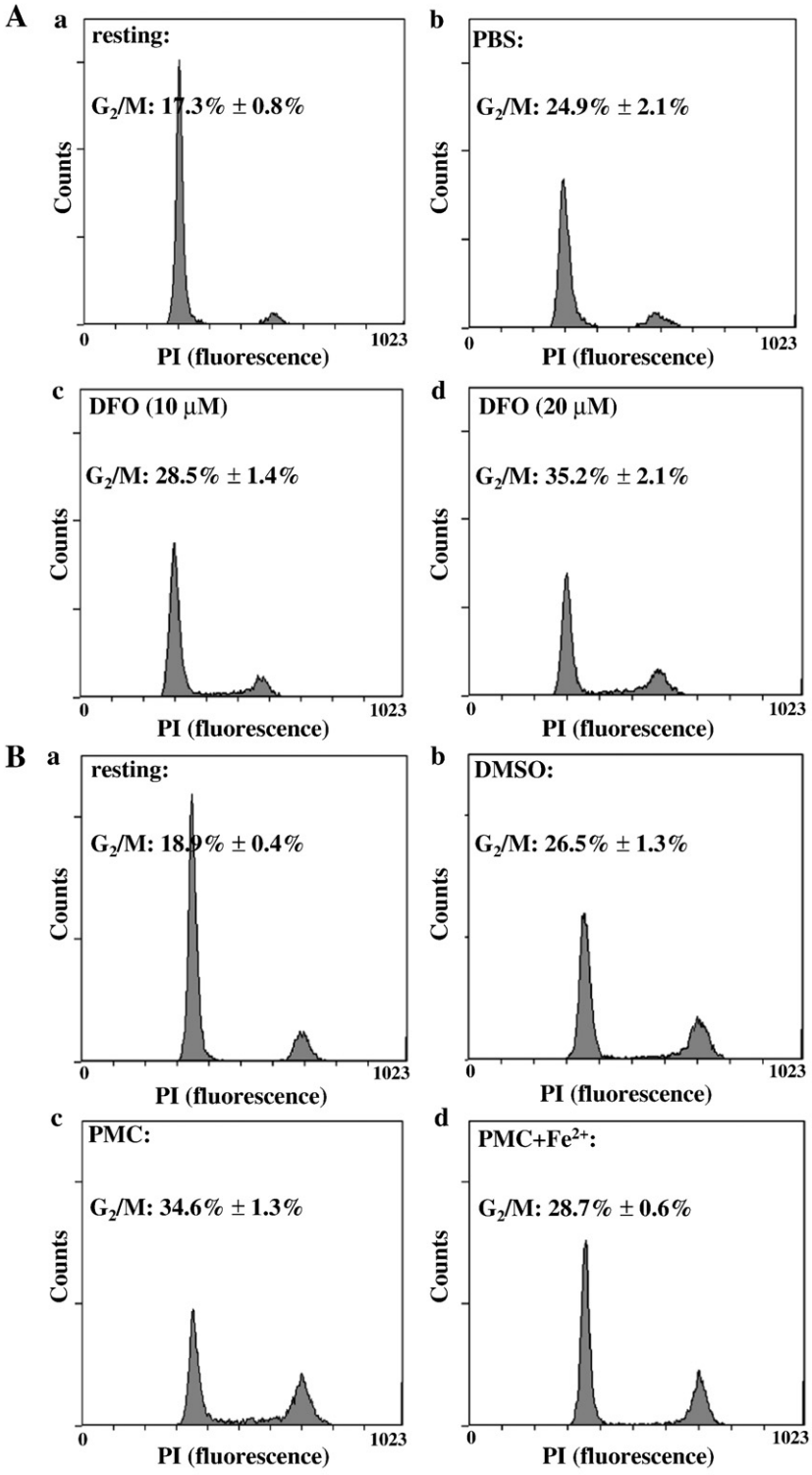

Fig. 9. Effects of deferoxamine and PMC on cell cycle progression in VSMCs stimulated by PDGF-BB. (A) VSMCs (2×10^5 cells/dish) were (graph a) treated with PBS (resting) or pretreated with (graph b) a solvent control (PBS) or (graph c) 10 μM or (graph d) 20 μM deferoxamine (DFO). (B) VSMCs (2×10^5 cells/dish) were (graph a) treated with PBS (resting) or pretreated with (graph b) a solvent control (0.1% DMSO), (graph c) PMC (50 μM), or (graph d) PMC (50 μM) plus $FeSO_4$ (10 μM), followed by the addition of PDGF-BB (10 ng/ml) to trigger cell cycle progression. Representative DNA histograms of propidium iodide fluorescence in cells assessed by flow cytometry are shown. The profiles are representative examples of five similar experiments.

than α-tocopherol in inhibiting lipid peroxidation by thiobarbituric acid-reactive substances [11]. In this study, we found that PMC inhibited only PDGF-BB-stimulated VSMC proliferation and showed no effects in resting cells, indicating that PMC does not interfere with the growth of normal VSMCs.

The cell cycle can be divided into distinct phases including a synthesis (S) phase, in which DNA is replicated, and a mitosis (M) phase, in which cell division occurs. In animal cells, the growth and synthesis of components required for these phases are regulated by extracellular growth factors, and they occur mainly in two gap phases,

G_1 (between M and S) and G_2 (between S and M) [22]. In this study, we found that loss of the proliferative capacity of VSMCs treated with PMC was associated with G_2/M phase arrest. PDGF released from platelets after adhesion to an injured vessel wall may be a key stimulus for VSMC proliferation, and it is a major contributor to neointimal formation [5]. In the in vivo study, we further demonstrated that administration of PMC led to a significant reduction in neointimal formation in the CCA induced by balloon angioplasty, which implies that the inhibitory activity of PMC may result from suppression of PDGF-BB-stimulated VSMC proliferation. On the other hand, in the early stage of the inflammatory process after vascular injury, activated leukocytes, neutrophils as well as monocytes, and platelets play an important role. These inflammatory processes are followed by the proliferation of vascular components, such as VSMCs and extracellular matrix, leading to neointimal thickening [23]. In our previous studies [11,12], we have demonstrated that PMC possesses potent inhibitory effects against neutrophil activation, inflammatory responses, and platelet aggregation. Therefore, these inhibitory effects of PMC may also be involved in reduction of neointimal formation.

PDGFR-β engages several well-characterized signaling pathways, e.g., Ras–ERK1/2, PI3K–AKT, PLCγ1–PKC, and JAK–STAT, which are known to be involved in multiple cellular and developmental responses [24]. In this study, PMC suppressed PDGF-BB-stimulated PLCγ1 phosphorylation and PKCδ translocation in VSMCs. Phosphoinositide-specific PLC is a key enzyme in signal transduction, and there are six major families of PLC enzymes, which consist of at least 13 PLC isoforms. PLCγ is the only isoform of PLC that is activated by tyrosine phosphorylation [25]. PLCγ1 was reported to play an important role in mitogenic responses in VSMCs, including cell migration and proliferation [26]. Activated PLCγ1 hydrolyzes its substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), to produce two secondary messengers: IP_3 and diacylglycerol. The former provokes the release of intracellularly stored Ca^{2+} to elevate cytoplasmic free Ca^{2+} levels, and the latter serves as an endogenous activator of PKC [27] (Fig. 10). We examined the effects of PMC on the activation of the two most abundant PKC isozymes, PKCα and PKCδ, in VSMCs and found that PMC suppressed PKCδ but not PKCα translocation in VSMCs. A study conducted by Ha and Exton [28] also demonstrated that PDGF can induce PKCε translocation but not that of PKCα or PKCζ. PKCδ was reported to have important roles in VSMC proliferation and survival [29]. This therefore suggests that PMC suppresses cell proliferation through inhibition of the PLCγ1–PKCδ cascade in VSMCs (Fig. 10).

On the other hand, the JAK–STAT cascade is also known to play an important role in the PDGF-induced signaling pathway. JAKs form a family of intracellular tyrosine kinases comprising four members: JAK1, JAK2, JAK3, and Tyk2. With the exception of JAK3, which is primarily expressed in hematopoietic cells, the others are ubiquitously expressed [30]. Activated JAKs, in turn, phosphorylate STAT proteins (which are recruited to the tyrosine-phosphorylated receptor through their SH2 domain) on a single tyrosine residue, inducing the translocation of STATs to the nucleus [30]. The JAK–STAT cascade was initially recognized as the primary mediator of intracellular signaling induced by interferon in hematopoietic and immune cells. PDGF-BB can induce JAK–STAT activation, which is known to be involved in cell proliferation and migration in VSMCs [31]. This study showed that PMC can suppress PDGF-BB-induced JAK2 and STAT3 phosphorylation, indicating that the JAK2–STAT3 cascade is involved in the inhibitory mechanisms of PMC in cell proliferation by VSMCs (Fig. 10).

In 1995, Sundaresan et al. [9] reported that PDGF can induce the intracellular production of ROS in VSMCs. They also provided evidence that the introduction of catalase, a scavenger of H_2O_2, into cells leads to a reduction in PDGF-induced tyrosine phosphorylation of ERK1/2. Recently, NADPH oxidase (NOX) was implicated as a possible ROS-producing system, which in turn responds to PDGF stimulation. NOX produces $O_2^{\bullet-}$ via a single-electron reduction (Fig. 10). $O_2^{\bullet-}$ is highly reactive and short-lived and can spontaneously or enzymatically dismutate to a second signaling intermediate, H_2O_2, via superoxide dismutase [8] (Fig. 10). Moreover, we also demonstrated that diphenylene iodonium chloride (10 μM), an inhibitor of NOX, obviously inhibited hydroxyl radical formation, which is approximated at 70% in VSMCs stimulated by PDGF-BB (10 ng/ml) in the ESR study ($N=3$; data not shown). Although production of $O_2^{\bullet-}$ contributes to the main biological activity of NOX, much of the signaling is mediated by the dismutation product H_2O_2. H_2O_2 is more stable than $O_2^{\bullet-}$ and is capable of crossing biological membranes and then interacting with ferrous ion (in the Fenton reaction) to cause the formation of the very aggressive and short-lived hydroxyl radicals [8] (Fig. 10). H_2O_2 is known to be involved in PDGF-BB-induced ERK1/2 and PLCγ1 activation in VSMCs [9,32]. It was also reported that activation of the JAK–STAT cascade responds to PDGF-BB-induced H_2O_2 formation in fibroblasts [33]. Moreover, Wedgwood and Black [34] confirmed that EUK-134, a superoxide dismutase/catalase mimetic, completely abolished VSMC proliferation and revealed an increase in cells at the G_2/M phase due to the scavenging of ROS, indicating that ROS play an important role in VSMC proliferation. In the present study, the ESR study provided direct evidence that PMC obviously inhibited hydroxyl radical formation in both the Fenton reaction and the PDGF-BB-stimulated VSMCs. PMC and deferoxamine

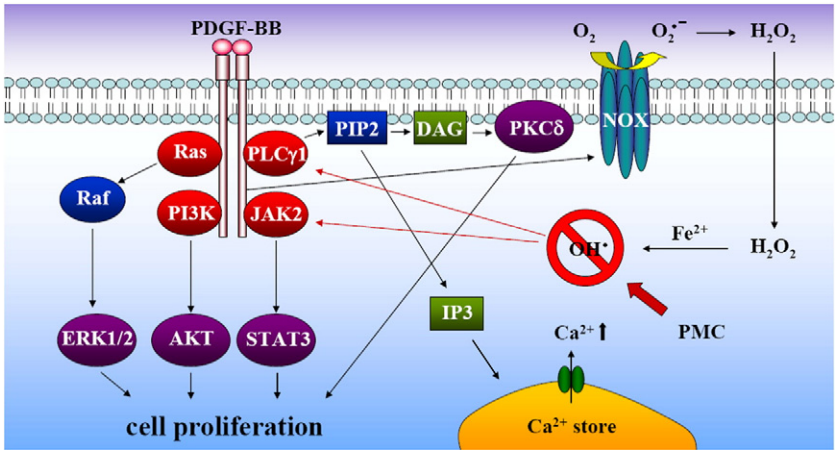

Fig. 10. Hypothetical scheme of the inhibitory mechanism of PMC in VSMC proliferation stimulated by PDGF-BB. PDGF-BB propagates mitogenic signals through PDGFR-β, which engages several well-characterized signaling pathways, i.e., Ras–Raf–ERK1/2, PI3K–AKT, PLCγ1–PKCδ, and JAK2–STAT3. Activated PLCγ1 hydrolyzes PIP_2 to produce two secondary messengers, IP_3 and diacylglycerol (DAG). Nicotinamide adenine dinucleotide phosphate oxidase (NOX) produces superoxide ($O_2^{\bullet-}$), followed by the induction of H_2O_2. H_2O_2 is capable of interacting with Fe^{2+} to cause hydroxyl radical ($HO^{\bullet}$) formation, which can activate PLCγ1 and JAK2 phosphorylation.

selectively suppressed both JAK2 and PLCγ1 phosphorylation and arrested cell cycle progression at the G_2/M phase, and both effects were significantly reversed in the presence of Fe^{2+}. These results suggest that activation of both JAK2–STAT3 and PLCγ1–PKCδ as well as the accumulation of cells at the G_2/M phase may be regulated by hydroxyl radicals in VSMCs (Fig. 10).

In conclusion, this study demonstrates for the first time that PMC, a potent hydrophilic α-tocopherol derivative, inhibits PDGF-BB-stimulated VSMC proliferation and neointimal formation after angioplasty in carotid arteries of rats. The mechanisms of PMC inhibition of VSMC proliferation may involve the inhibition of hydroxyl radical-mediated PLCγ1–PKCδ and JAK2–STAT3 activation and cause cell cycle arrest at the G_2/M phase. PMC is more hydrophilic than α-tocopherol, and thus there should be fewer limitations in its clinical application compared to α-tocopherol. Therefore, PMC may be a potential therapeutic agent for treating VSMC proliferation-related vascular diseases.

Acknowledgment

This work was supported by grants from the National Science Council of Taiwan (NSC93-2321-B-038-001, 94-2321-B-038-001, and 97-2320-B-038-016-MY3).

References

[1] Hedin, U.; Roy, J.; Tran, P. K. Control of smooth muscle cell proliferation in vascular disease. *Curr. Opin. Lipidol.* **15:**559–565; 2004.
[2] Ross, R. Cell biology of atherosclerosis. *Annu. Rev. Physiol.* **57:**791–804; 1995.
[3] Dzau, V. J.; Braun-Dullaeus, R. C.; Sedding, D. G. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat. Med.* **8:** 1249–1256; 2002.
[4] Ferns, G. A.; Raines, E. W.; Sprugel, K. H.; Motani, A. S.; Reidy, M. A.; Ross, R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* **253:**1129–1132; 1991.
[5] Raines, E. W. PDGF and cardiovascular disease. *Cytokine Growth Factor Rev.* **15:** 237–254; 2004.
[6] Wilcox, J. N.; Smith, K. M.; Williams, L. T.; Schwartz, S. M.; Gordon, D. Platelet-derived growth factor mRNA detection in human atherosclerotic plaques by in situ hybridization. *J. Clin. Invest.* **82:**1134–1143; 1988.
[7] Heldin, C. H.; Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. *Physiol. Rev.* **79:**1283–1316; 1999.
[8] Brown, D. I.; Griendling, K. K. Nox proteins in signal transduction. *Free Radic. Biol. Med.* **47:**1239–1253; 2009.
[9] Sundaresan, M.; Yu, Z. X.; Ferrans, V. J.; Irani, K.; Finkel, T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* **270:**296–299; 1995.
[10] Suzuki, Y. J.; Packer, L. Inhibition of NF-kappa B activation by vitamin E derivatives. *Biochem. Biophys. Res. Commun.* **193:**277–283; 1993.
[11] Hsiao, G.; Yen, M. H.; Lee, Y. M.; Sheu, J. R. Antithrombotic effect of PMC, a potent alpha-tocopherol analogue on platelet plug formation in vivo. *Br. J. Haematol.* **117:** 699–704; 2002.
[12] Hsiao, G.; Lee, J. J.; Chen, Y. C.; Lin, J. H.; Shen, M. Y.; Lin, K. H.; Chou, D. S.; Sheu, J. R. Neuroprotective effects of PMC, a potent alpha-tocopherol derivative, in brain ischemia–reperfusion: reduced neutrophil activation and anti-oxidant actions. *Biochem. Pharmacol.* **73:**682–693; 2007.
[13] Pauly, R. R.; Bilato, C.; Cheng, L.; Monticone, R.; Crow, M. T. Vascular smooth muscle cell cultures. *Methods Cell Biol.* **52:**133–154; 1997.
[14] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:**55–63; 1983.
[15] Hsiao, G.; Lin, K. H.; Chang, Y.; Chen, T. L.; Tzu, N. H.; Chou, D. S.; Sheu, J. R. Protective mechanisms of inosine in platelet activation and cerebral ischemic damage. *Arterioscler. Thromb. Vasc. Biol.* **25:**1998–2004; 2005.
[16] Yano, K.; Bauchat, J. R.; Liimatta, M. B.; Clemmons, D. R.; Duan, C. Down-regulation of protein kinase C inhibits insulin-like growth factor I-induced vascular smooth muscle cell proliferation, migration, and gene expression. *Endocrinology* **140:** 4622–4632; 1999.
[17] Chou, D. S.; Hsiao, G.; Shen, M. Y.; Tsai, Y. J.; Chen, T. F.; Sheu, J. R. ESR spin trapping of a carbon-centered free radical from agonist-stimulated human platelets. *Free Radic. Biol. Med.* **39:**237–248; 2005.
[18] Yoshida, T.; Azuma, H.; Aihara, K.; Fujimura, M.; Akaike, M.; Mitsui, T.; Matsumoto, T. Vascular smooth muscle cell proliferation is dependent upon upregulation of mitochondrial transcription factor A (mtTFA) expression in injured rat carotid artery. *Atherosclerosis* **178:**39–47; 2005.
[19] Wang, Q. S.; Zheng, Y. M.; Dong, L.; Ho, Y. S.; Guo, Z.; Wang, Y. X. Role of mitochondrial reactive oxygen species in hypoxia-dependent increase in intracellular calcium in pulmonary artery myocytes. *Free Radic. Biol. Med.* **42:** 642–653; 2007.
[20] Kang, S. W. Two axes in platelet-derived growth factor signaling: tyrosine phosphorylation and reactive oxygen species. *Cell Mol. Life Sci.* **64:**533–541; 2007.
[21] Ingold, K. U.; Burton, G. W.; Foster, D. O.; Hughes, L.; Lindsay, D. A.; Webb, A. Biokinetics of and discrimination between dietary RRR- and SRR-alpha-tocopherols in the male rat. *Lipids* **22:**163–172; 1987.
[22] Bjorklund, M.; Taipale, M.; Varjosalo, M.; Saharinen, J.; Lahdenpera, J.; Taipale, J. Identification of pathways regulating cell size and cell-cycle progression by RNAi. *Nature* **439:**1009–1013; 2006.
[23] Inoue, T.; Node, K. Molecular basis of restenosis and novel issues of drug-eluting stents. *Circ. J.* **73:**615–621; 2009.
[24] Andrae, J.; Gallini, R.; Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes Dev.* **22:**1276–1312; 2008.
[25] Schmitz, U.; Ishida, M.; Berk, B. C. Angiotensin II stimulates tyrosine phosphorylation of phospholipase C-gamma-associated proteins: characterization of a c-Src-dependent 97-kD protein in vascular smooth muscle cells. *Circ. Res.* **81:** 550–557; 1997.
[26] Tallquist, M. D.; Klinghoffer, R. A.; Heuchel, R.; Mueting-Nelsen, P. F.; Corrin, P. D.; Heldin, C. H.; Johnson, R. J.; Soriano, P. Retention of PDGFR-beta function in mice in the absence of phosphatidylinositol 3′-kinase and phospholipase Cgamma signaling pathways. *Genes Dev.* **14:**3179–3190; 2000.
[27] Liao, H. J.; de Los, S. J.; Carpenter, G. Contrasting role of phospholipase C-γ1 in the expression of immediate early genes induced by epidermal or platelet-derived growth factors. *Exp. Cell Res.* **312:**807–816; 2006.
[28] Ha, K. S.; Exton, J. H. Differential translocation of protein kinase C isozymes by thrombin and platelet-derived growth factor: a possible function for phosphatidylcholine-derived diacylglycerol. *J. Biol. Chem.* **268:**10534–10539; 1993.
[29] Jackson, D. N.; Foster, D. A. The enigmatic protein kinase Cdelta: complex roles in cell proliferation and survival. *FASEB J.* **18:**627–636; 2004.
[30] Yeh, T. C.; Pellegrini, S. The Janus kinase family of protein tyrosine kinases and their role in signaling. *Cell Mol. Life Sci.* **55:**1523–1534; 1999.
[31] Yellaturu, C. R.; Rao, G. N. Cytosolic phospholipase A2 is an effector of Jak/STAT signaling and is involved in platelet-derived growth factor BB-induced growth in vascular smooth muscle cells. *J. Biol. Chem.* **278:**9986–9992; 2003.
[32] Choi, M. H.; Lee, I. K.; Kim, G. W.; Kim, B. U.; Han, Y. H.; Yu, D. Y.; Park, H. S.; Kim, K. Y.; Lee, J. S.; Choi, C.; Bae, Y. S.; Lee, B. I.; Rhee, S. G.; Kang, S. W. Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* **435:**347–353; 2005.
[33] Simon, A. R.; Rai, U.; Fanburg, B. L.; Cochran, B. H. Activation of the JAK–STAT pathway by reactive oxygen species. *Am. J. Physiol.* **275:**C1640–C1652; 1998.
[34] Wedgwood, S.; Black, S. M. Combined superoxide dismutase/catalase mimetics alter fetal pulmonary arterial smooth muscle cell growth. *Antioxid. Redox Signaling* **6:**191–197; 2004.