Cudratricusxanthone A Isolated from the Root Bark of *Cudrania tricuspidata* Inhibits the Proliferation of Vascular Smooth Muscle Cells through the Suppression of PDGF-Receptor Beta Tyrosine Kinase

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Platelet derived growth factor (PDGF)-BB is one of the most potent vascular smooth muscle cell (VSMC) proliferative factors, and abnormal VSMC proliferation by PDGF-BB plays an important role in the development and progression of cardiovascular problems, including restenosis after coronary angioplasty and atherosclerosis. Previous phytochemical studies on the stems or root barks of *Cudrania tricuspidata* (Moraceae) resulted in the isolation of various isoprenylated xanthones and flavonoids, some of which have anti-cancer, hepatoprotective, anti-inflammatory and anti-oxidant activities. In the present study, we investigated the antiproliferative effect of cudratricusxanthone A isolated from the root bark of *C. tricuspidata* and its underlying mechanism in VSMCs. Antiproliferative effects of cudratricusxanthone A on VSMCs were examined by direct cell counting and [^{3}H]-thymidine incorporation assays. Cudratricusxanthone A inhibited [^{3}H]-thymidine incorporations into DNA in VSMCs that occurred in response to treatment with 50 ng/ml PDGF-BB. PDGF-BB-stimulated DNA synthesis was significantly reduced to 86.1, 80.2, 64.2 and 25.1% at concentrations of 0.1, 1, 2 and 3 μM, respectively. Moreover, pre-treatment with cudratricusxanthone A (0.1—3 μM) suppressed this PDGF-BB-stimulated cell number in a concentration-dependent manner. The inhibition percentages were 11.1, 22.7, 51.3 and 81.5% at the concentrations of 0.1, 1, 2 and 3 μM, respectively. We also investigated the mechanism of antiproliferative effects by cudratricusxanthone A in PDGF-BB-stimulated VSMCs. In Western blot analysis, 50 ng/ml PDGF-BB-stimulated phospholipase C (PLC)γ1, Ras, and extracellular signal-regulated kinase1/2 (ERK1/2) phosphorylations were inhibited by cudratricusxanthone A (0.1—3 μM). Consisted with these findings, cudratricusxanthone A inhibited PDGF-receptor β chain (PDGF-Rβ) phosphorylation induced by PDGF-BB in a concentration-dependent manner. These findings suggest that the inhibitory effects of cudratricusxanthone A on DNA synthesis and proliferation by PDGF-BB-stimulated VSMCs are mediated by the suppressions of the PDGF-Rβ and its downstream signaling pathways. Our observation may explain in part mechanistic basis for the prevention of cardiovascular diseases, such as atherosclerosis and restenosis after coronary angioplasty by cudratricusxanthone A.

Key words vascular smooth muscle cell; platelet derived growth factor; cardiovascular disease; cudratricusxanthone A; *cudrania tricuspidata*

Cudrania tricuspidata (Moraceae) is a deciduous trees widely distributed in Korea, China, and Japan. The cortex and root bark of this species have been used as a traditional medicine for the treatment of neuritis and inflammation.[1] Moreover, the pharmacological study showed that the crude extract from the roots of *C. tricuspidata* could inhibit the growth of human cancer cell line.[2] However, the antiproliferative effect of cudratricusxanthone A isolated from the root bark of *C. tricuspidata* on vascular smooth muscle cells (VSMCs) is not understood.

The abnormal migration and proliferation of VSMCs in arterial walls are important pathogenetic factors of vascular disorders such as atherosclerosis and restenosis after angioplasty.[3] Although several growth factors and cytokines are involved in the development of atherosclerotic lesions, one of the principal regulators of mitogenesis in VSMCs is platelet derived growth factor (PDGF)-BB, and that the expression of PDGF-BB is increased in atherosclerotic lesions. In addition, PDGF-BB-induced mitogenesis and proliferation are also known to be prerequisites of intimal thickening, which is invariably observed after angioplasty.[4]

The PDGF-BB-induced mitogenesis signaling pathway has already been relatively well characterized. The binding of PDGF-BB to PDGF-receptor (PDGF-R) leads to phosphorylation of PDGF-R beta chain (PDGF-Rβ) tyrosine residues. This activated PDGF-Rβ is associated with a number of SH2 domain-containing proteins, including the phospholipase C (PLC)γ1.[5] PDGF-BB activates the extracellular regulated kinases 1 and 2 (ERK1/2) by triggering the activation of Ras-Raf-MEK, and its activation is known to be associated with the development and progression of proliferative cardiovascular diseases, such as hypertension and atherosclerosis.[6,7] Therefore, an understanding of its inhibition on PDGF-BB-stimulated VSMC proliferation is important in terms of developing methods of treating cardiovascular disease.

In the present study, we aimed to elucidate the antiproliferative effect and the molecular mechanism of cudratricusxanthone A isolated from the root bark of *C. tricuspidata* in PDGF-BB-stimulated signaling pathways.

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MATERIALS AND METHODS

Materials Cell culture materials were purchased from Gibco-BRL (MD, U.S.A.). The phospho-ERK1/2, phospho-Ras, phospho-PLCγ1, phospho-PDGF-Rβ (Tyr716), and actin antibodies were purchased from New England Biolabs (MA, U.S.A.). PDGF-BB was obtained from Upstate Biotechnology (NY, U.S.A.). The other chemicals used were of the highest analytical grade commercially available.

Extraction and Identification of Cudratricusxanthone A: The root barks of *Cudrania tricuspidata* were collected from the herb garden at Chungbuk National University, Cheongju, Korea, in September 2004 and identified by Emeritus Prof. Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen (CBNU 040915) has been deposited at the Herbarium of College of Pharmacy, Chungbuk National University, Korea. The air-dried root barks of *C. tricuspidata* (1.6 kg) were pulverized and extracted with MeOH (15 l) at room temperature (3×24 h). The extract was concentrated *in vacuo*, and suitably diluted with water, then partitioned with CH_2Cl_2 and EtOAc. The CH_2Cl_2 extract (61 g) was subjected to column chromatography on silica gel eluting with CH_2Cl_2–MeOH (100 : 0) in increasing proportion of MeOH, to yield four fractions (CTC-A—D). CTC-A (12.3 g) was further applied to a silica gel column chromatography eluting with *n*-hexane–acetone (from 100 : 0 to 1 : 1) to give four fractions (CTC-A1—CTC-A4). Fraction CTC-A4 was subjected to RP-18 flash column chromatography (50% MeCN) to give cudratricusxanthone A (71.2 mg). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 500 NMR spectrometer (Karlsruhe, Germany) using acetone-d_6 as a solvent. EI-MS spectrum was obtained on a Hewlett-Packard MS 5988 mass spectrometer.

Cudratricusxanthone A: ^{1}H-NMR (500 MHz, acetone-d_6) δ=13.78 (1H, s, OH-1), 6.84 (1H, s, H-5), 6.34 (1H, dd, J=10.6, 17.4 Hz, H-14), 6.23 (1H, s, H-2), 5.30 (1H, m, H-17), 5.02 (1H, dd, J=1.2, 17.4 Hz, H-15a), 4.89 (1H, dd, J=1.2, 10.6 Hz, H-15b), 4.17 (2H, br d, J=6.8 Hz, H_2-16), 1.82 (3H, s, H_3-20), 1.65 (6H, s, H_3-12, H_3-13), 1.62 (3H, s, H_3-19). ^{13}C-NMR (125 MHz, acetone-d_6) δ=183.3 (C-9), 163.4 (C-3), 162.2 (C-1), 155.5 (C-4a), 153.0 (C-6), 152.3 (C-4b), 151.3 (C-14), 141.1 (C-7), 131.1 (C-18), 128.5 (C-8), 124.2 (C-17), 111.3 (C-8a), 110.6 (C-4), 107.6 (C-15), 104.2 (C-9a), 100.6 (C-5), 99.3 (C-2), 41.4 (C-11), 30.3 (C-12, C-13), 26.0 (C-16), 25.8 (C-19), 18.0 (C-20). EI-MS *m/z* 396 $[M]^+$, 381, 353, 325, 285.[18] The structure of cudratricusxanthone A is presented in Fig. 1A. Cudratricusxanthone A was dissolved in dimethylsulfoxide (DMSO) and added to Dulbecco's modified Eagle's medium (DMEM) with a maximum final DMSO concentration of 0.05%.

Isolation and Culture of Rat Aortic VSMCs Rat aortic vascular smooth muscle cells (VSMCs) were isolated by enzymatic dispersion, as previously described[8] using a modification of the method devised by Chamley *et al.*[9] Cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 8 mM HEPES and 2 mM L-glutamine at 37°C in a humidified 5% CO_2 incubator. The purity of VSMCs cultures were confirmed by the immunocytochemical localization of α-smooth muscle actin. VSMCs between passages 4 and 8 were used in this experiment.

Cell Proliferation and [^{3}H]-Thymidine Incorporation Assays Rat aortic VSMCs were seeded into 12-well culture plates at 1×10^5 cells/ml, and then cultured in DMEM containing 10% FBS at 37°C for 24 h until 70% confluent, and media were then replaced by serum-free medium containing cudratricusxanthone A. Cells were stimulated with 50 ng/ml PDGF-BB, trypsinized with trypsin-EDTA, and counted using a hemocytometer. For [^{3}H]-thymidine incorporation experiments, VSMCs were seeded in 24-well culture plates under the above conditions mentioned, and 2 μCi/ml of [^{3}H]-thymidine was added to medium for 4 h. Reactions were terminated by aspirating medium and washing cultures with phosphate-buffered saline (PBS) containing 10% trichloroacetic acid and ethanol/ether (1 : 1, v/v). The acid-insoluble [^{3}H]-thymidine was extracted into 250 μl of 0.5 M NaOH/well, and this solution was then mixed with 3 ml of scintillation cocktail (Ultimagold, Packard Bioscience, CT, U.S.A.), and quantified using a liquid scintillation counter (LS3801, Beckman, Dusseldorf, Germany). Protein contents were determined in 50 μl aliquots of residual solutions using BCA Protein Assay Reagent Kits (Pierce Biotechnology, IL, U.S.A.).

Cell Morphology Observation VSMCs were pre-cultured in serum-free medium in the presence or absence of cudratricusxanthone A (0.1—3 μM) for 24 h, and then stimulated with 50 ng/ml PDGF-BB for 24 h. Cell morphology was observed under light microscopy (Nikon, Japan).

Western Blot Analysis SDS-PAGE was performed on cell lysates using 7.5—10% acrylamide gels according to the method described by Laemmli.[10] Proteins were transferred to PVDF membranes (Millipore Corp., MA, U.S.A.), which were then blocked overnight at 4°C in Tris-buffered saline containing 0.1% Tween 20 (TBS/T) and 5% skim milk powder. Membranes were then incubated with a 1 : 2000 dilution of phospho-ERK1/2, phospho-Ras, phospho-PLCγ1, phospho-PDGF-Rβ and actin antibodies. Blots were washed with TBS/T, and then incubated with a 1 : 5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody (New England Biolabs, MA, U.S.A.). Proteins were detected using an enhanced chemiluminescence (ECL) Western blotting detect reagent (Amersham Biosciences, Buckinghamshire, UK). The intensities of bands were quantified using a Scion-Image for Window Program.

Statistical Analysis Experimental results are expressed as means±S.D. One-way analysis of variance (ANOVA) followed by Dunnett's test was used for multiple comparisons. p values of <0.05 and <0.01 were considered statistically significant as indicated.

RESULTS

Effect of Cudratricusxanthone A on PDGF-BB-Stimulated VSMC Proliferation Figure 1A shows the chemical structure of cudratricusxanthone A isolated from the root bark of *C. tricuspidata*. The morphological observation showed that the cells were gradually reduced in a concentration-dependent manner by the treatment of 0.1—3 μM cudratricusxanthone A (Fig. 1B). To determine whether cudratricusxanthone A inhibits PDGF-BB-stimulated VSMC proliferation, we assessed the effect of cudratricusxanthone A by [^{3}H]-thymidine incorporation assay as an index of cell growth. In the absence of PDGF-BB, VSMCs did not incor-

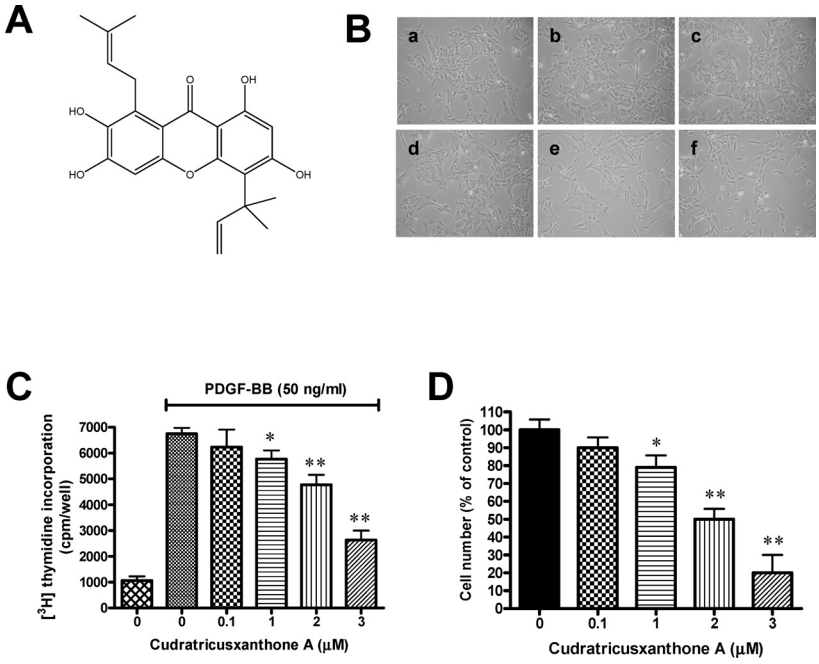

Fig. 1. Effect of Cudratricusxanthone A on PDGF-BB-Stimulated VSMC Morphology, DNA Synthesis and Proliferation

(A) Chemical structure of cudratricusxanthone A. (B) Effect of cudratricusxanthone A on PDGF-BB-stimulated VSMC morphology. Cells were pre-cultured in serum-free medium in the presence or absence of cudratricusxanthone A (0.1—3 μM) for 24 h, and then stimulated with 50 ng/ml PDGF-BB for 24 h. Cell morphology was observed under light microscopy (100× magnification). a, serum free; b, PDGF-BB; c, PDGF-BB+cudratricusxanthone A (0.1 μM); d, PDGF-BB+cudratricusxanthone A (1 μM); e, PDGF-BB+cudratricusxanthone A (2 μM); f, PDGF-BB+cudratricusxanthone A (3 μM). (C) Effect of cudratricusxanthone A on PDGF-BB-stimulated DNA synthesis. Cells were pre-cultured in serum-free medium in the presence or absence of cudratricusxanthone A (0.1—3 μM) for 24 h, and then stimulated with 50 ng/ml PDGF-BB for 20 h. [^{3}H]-Thymidine (2 μCi/ml) was added to medium and cells were incubated for 4 h. Radioactivities were determined using a liquid scintillation counter. (D) Effect of cudratricusxanthone A on the number of VSMCs stimulated by PDGF-BB. Cells were pre-cultured in serum-free medium in the presence or absence of cudratricusxanthone A (0.1—3 μM) for 24 h, and then stimulated with 50 ng/ml PDGF-BB for 24 h. Cells were trypsinized and then counted with a hemocytometer. Values represent means±S.D. from three different assays. $*p<0.05$, $**p<0.01$ compared with PDGF-BB alone.

porate [^{3}H]-thymidine into DNA, but 50 ng/ml PDGF-BB caused [^{3}H]-thymidine incorporation (6749 cpm/well). The increased [^{3}H]-thymidine incorporation was significantly reduced to 6235, 5773, 4775 and 2135 cpm/well at cudratricusxanthone A concentrations of 0.1, 1, 2 and 3 μM, respectively (Fig. 1C). We also assessed the inhibitory effects of cudratricusxanthone A by direct cell counting. VSMCs were pre-cultured in the presence of cudratricusxanthone A (0.1—3 μM) in serum-depleted medium for 24 h, and then were stimulated with 50 ng/ml PDGF-BB for 24 h. Pre-treatment with cudratricusxanthone A suppressed the PDGF-BB-stimulated proliferation in a concentration-dependent manner. The inhibition percentages were 11.1±8.3, 22.7±8.3, 51.3±7.2 and 81.5±9.3% at the concentrations of 0.1, 1, 2 and 3 μM, respectively (Fig. 1D).

Effect of Cudratricusxanthone A on the PLCγ1, Ras, ERK1/2 and PDGF-Rβ Tyrosine Kinase Phosphorylations of PDGF-BB-Stimulated VSMCs PDGF-BB binding to PDGF receptor leads to the activation of several intracellular signaling cascades, and one of these, the ERK1/2 pathway, plays a central role in regulation of VSMC growth.[6,11] Thus, we hypothesized that the ERK1/2 pathway might be involved in the inhibition induced by cudratricusxanthone A. VSMCs were precultured in the presence or absence of cudratricusxanthone A (0.1—3 μM) in serum-free medium for 24 h, and then stimulated for 20 min with 50 ng/ml PDGF-BB. As shown in Fig. 2A, ERK1/2 phosphorylation was significantly inhibited by cudratricusxanthone A (0.1—3 μM) in a concentration-dependent manner, with the inhibition percentages of 7.7±15.1, 24.6±10.5, 80.0±10.1 and 89.6±7.6% at the concentrations of 0.1, 1, 2 and 3 μM, respectively. We next examined whether the inhibitory effect of cudratricusxanthone A was induced by blocking Ras phosphorylation. Consistent with the inhibitory effect of ERK 1/2, PDGF-BB-stimulated Ras activations were reduced by cudratricusxanthone A (0.1—3 μM) in a concentration-dependent manner. The inhibition percentages were 1.9±8.3, 35.0±5.2, 67.3±12.2 and 79.7±8.3% at the concentrations of 0.1, 1, 2 and 3 μM, respectively. Similarly, PDGF-BB-stimulated PLCγ1 phosphorylation was decreased to 9.7±7.4, 46.8±13.1, 70.8±10.1 and 89.1±7.6% at concentrations of 0.1, 1, 2 and 3 μM, respectively. Therefore, the antiproliferative effect of cudratricusxanthone A might be mediated by the downregulation of ERK1/2, Ras and PLCγ1 signaling pathways in VSMCs.

Because cudratricusxanthone A inhibited the downstream components of PDGF-BB such as ERK1/2, Ras and PLCγ1 phosphorylation with a similar pattern, PDGF-Rβ phosphorylation, an upstream of PDGF-BB signaling may be a direct target for cudratricusxanthone A and lead to the inhibition of VSMCs proliferation. Pre-treatment with cudratricusxanthone A significantly inhibited PDGF-Rβ (Tyr716) phosphorylation induced by PDGF-BB in a concentration-dependent manner, with the inhibition percentages of 10.2±18.8, 27.1±16.5, 74.6±10.5 and 92.4±4.8% at the concentrations of 0.1, 1, 2 and 3 μM, respectively (Fig. 2B).

DISCUSSION

PDGF is an important VSMCs mitogen and is thought to

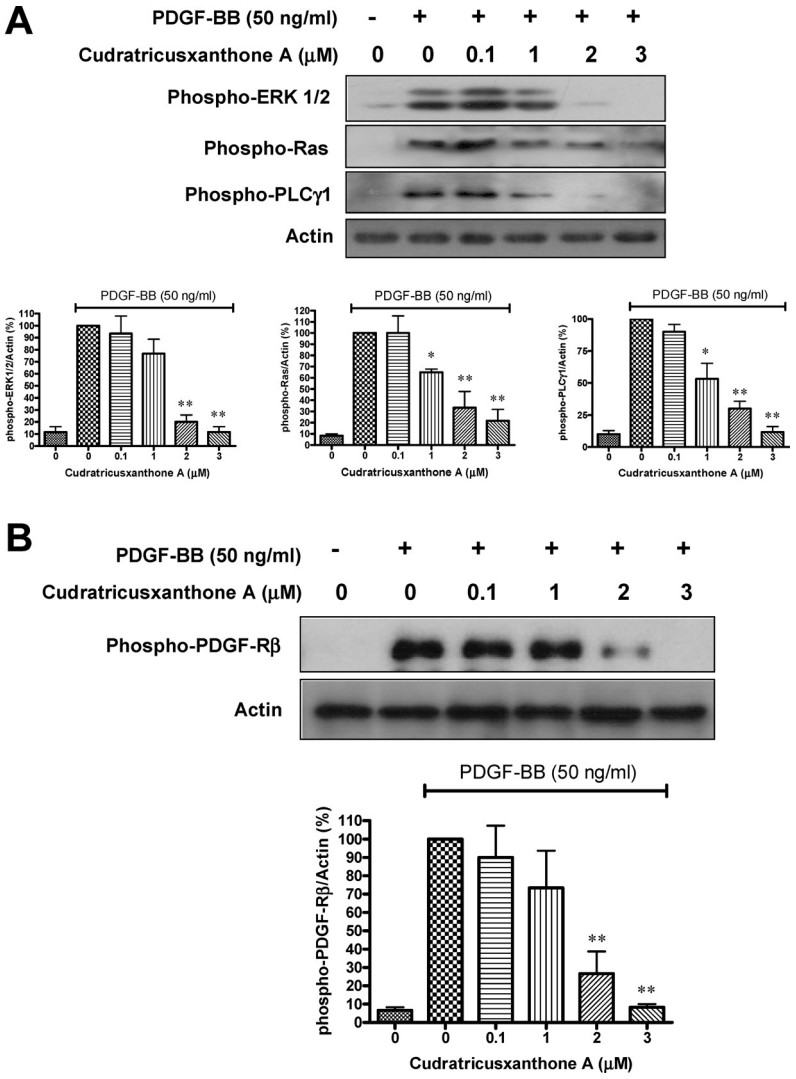

Fig. 2. Effect of Cudratricusxanthone A on ERK1/2, Ras, PLCγ1 and PDGF-Rβ Phosphorylation in PDGF-BB-Stimulated VSMCs

Cells were cultured in 12-well plates until confluent and medium was then replaced with serum-free medium in the presence or absence of cudratricusxanthone A (0.1—3 μM) for 24 h. Cells were stimulated with 50 ng/ml PDGF-BB for 20 (A) or 5 min (B). The cells were then lysed, and proteins were analyzed in lysates by SDS-PAGE. Relative activities were quantified by scanning densitometry and showed the levels of each activity as relative value of the total actin. Western blot was repeated three times. Data are expressed as mean±S.D. (n=3); $*p<0.05$, $**p<0.01$ compared with PDGF-BB alone.

mediate phenotypic modulation of VSMCs proliferation.[4] We now clearly demonstrated that cudratricusxanthone A reduces PDGF-BB-stimulated VSMCs proliferation, acting on early events in DNA synthesis as established both by a decrease in [^{3}H]-thymidine incorporation and by a reduction in the cell counting (Figs. 1C, D). The results suggest that cudratricusxanthone A could be useful as an antiproliferating agent for employing it as a potential preventive/therapeutic agent to treat cardiovascular disease, including atherosclerosis. Therefore, we hyphothesized that the antiproliferative properties of cudratricusxanthone A were causally related to the modulation of the signaling cascade involved in VSMC proliferation.

To understand the mechanism of downregulation of PDGF-BB-induced VSMC proliferation, we examined whether the effect of cudratricusxanthone A is mediated by the downregulation of the intracellular signaling pathways. The MAPK signaling pathway is considered to be a critical step in the proliferation of VSMCs[12] and Pyles *et al.* reported that MAPKs were activated in response to balloon overstretch injury in porcine carotid arteries.[7] Using balloon injured carotid artery, it was demonstrated that MAPK signaling, especially ERK1/2, is increased and that medial cell replication following injury is reduced by PD098059.[13] ERK1/2 (a MAPK family member) plays a central role on cell growth regulation, and recently this pathway was found to be involved by triggering Ras-Raf activation, MEK phosphorylation, and ERK1/2 phosphorylations in the proliferative effect of PDGF-BB in VSMCs.[6,7,11,14] As shown in Fig. 2, cudratricusxanthone A decreases ERK1/2, Ras, PLCγ1 and PDGF-Rβ tyrosine kinase phosphorylation. It is, therefore, possible that the cudratricusxanthone A-induced inhibition of VSMC proliferation and DNA synthesis is mediated by the suppression of Ras-Raf-ERK1/2 signaling pathway *via* blocking of PDGF-Rβ tyrosine reside, 716 (Fig. 3). In animal studies using neutralizing antibodies and the PDGF-Rβ tyrosine kinase inhibitor, AG1296, it reduced VSMC proliferation *in vitro* and prevented cardiovascular problems.[15—17] Thus, cudratricusxanthone A may be possible to target the PDGF-Rβ antagonist or its tyrosine kinase in-

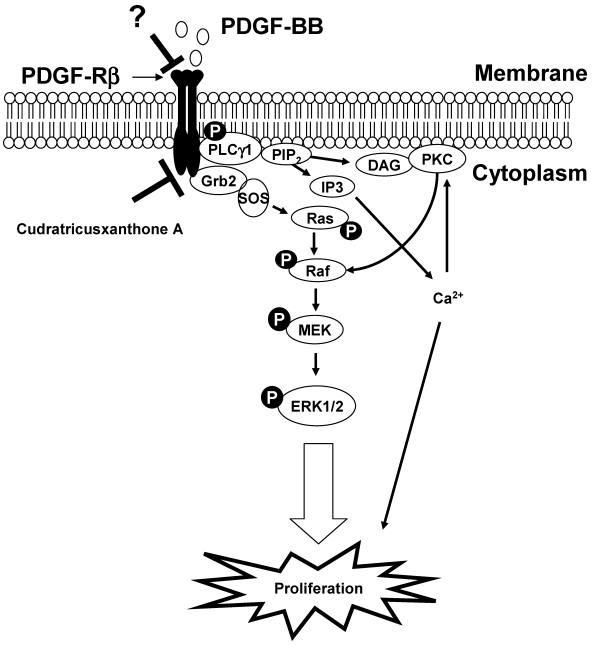

Fig. 3. Proposed Mechanism for the Inhibition of PDGF-BB-Stimulated VSMC Proliferation by Cudratricusxanthone A

Cudratricusxanthone A inhibits VSMC proliferation by suppressing the PDGF-Rβ tyrosine reside leading to downregulation of Ras-Raf-ERK1/2 and PLCγ1 activation.

hibitor, as upstream of the PLCγ1. Studies to elucidate the PDGF-Rβ antagonism of cudratricusxanthone A are currently being progressed in our laboratory.

Taken together, the present study shows evidence that the antiproliferative activity of cudratricusxanthone A is mediated by the downregulation of PLCγ1 and Ras-Raf-MEK-ERK1/2 signaling pathways through the inhibition of PDGF-Rβ tyrosine kinase in VSMCs (Fig. 3), and may serve as a lead compound with development of anti-atherosclerotic drugs.

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