Cold-pressed oil from *Citrus aurantifolia* inhibits the proliferation of vascular smooth muscle cells via regulation of PI3K/MAPK signaling pathways

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Abstract. Vascular occlusive disease is a chronic disease with significant morbidity and mortality. Although a variety of therapies and medications have been developed, the likelihood of disease re-emergence is high and this can be life-threatening. Based on a previous screening experiment related to vascular obstructive diseases using 34 types of essential oils, cold-pressed oil (CpO) from *Citrus aurantifolia* (lime) has been demonstrated to have the best effect for the inhibition of vascular smooth muscle cells (VSMCs) proliferation. The aim of the present study was to evaluate the effect of lime CpO on the pathological changes of VSMCs. To determine this, the effect of lime CpO on VSMC proliferation, a major cause of vascular disease, was investigated. To determine the safe concentration interval for toxicity of CpO during VSMC culture, a dilution of $1x10^{-5}$ was determined using Cell Counting Kit-8 assay, which was confirmed to be non-toxic using a lactate dehydrogenase assay. To examine the effect of lime CpO in cellular signaling pathways, changes in phosphorylation of both the PI3K/AKT/mTOR and extracellular signal-regulated MEK/ERK signaling pathways with serum were investigated. Furthermore, lime CpO with FBS also significantly decreased the expression levels of the cell cycle regulators cyclin D1 and proliferating cell nuclear antigen. Additionally, lime CpO with FBS significantly inhibited the sprouting of VSMCs in an *ex vivo* culture system. These results suggested that lime CpO inhibited the abnormal proliferation of VSMCs and can be developed as a nature-based therapeutic agent for obstructive vascular disease.

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Introduction

Vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) are the main cell types that constitute the blood vessels, with their function being to control blood vessel pressure and tension (1). VSMCs maintain homeostasis in a contractile form with little proliferation under physiological conditions (2,3). However, when ECs are damaged, inflammatory factors secreted in response to pathological conditions cause abnormal proliferation of VSMCs, initiating their transformation to the synthetic phenotype due to internal and external changes (3-5). This abnormal proliferation of VSMCs is known to cause arteriosclerosis and restenosis (6,7). Classical surgical therapies for treating these vascular diseases, such as coronary artery bypass grafting or stent-grafted angioplasty, are effective for a short period of time. However, these surgical treatments can cause blood flow disorders due to the abnormal proliferation of VSMCs over time (8). To overcome this problem, a drug-coated stent has been developed to treat vascular diseases more effectively (9). However, the potential for restenosis due to VSMC proliferation remains a major long-term threat (8). Therefore, the development of novel therapeutic agents for the treatment of vascular diseases is still required.

It has been reported that natural compounds have a positive effect on cardiovascular diseases and improve some vascular

functions (9). One of the promising novel treatments of vascular diseases in various natural products is essential oils (EOs). EOs and their constituents are promising as therapeutic agents as they have been demonstrated to functionally improve cardiovascular disease (10,11).

Lime (*Citrus aurantifolia*) is widely cultivated worldwide and is an excellent source of vitamins, particularly vitamin C. Lime EO has previously been used as a fragrance, an antimicrobial agent and for aromatherapy (12,13). Lime EO contains limonene, β-pinene, γ-terpinene, citral and linarul, among other compounds. Lime EO is a volatile complex mixture and has been used as a means to prevent, improve and treat diseases (14). Furthermore, the potential for the use of lime EO as chemotherapy in the prevention and treatment of inflammatory diseases (15,16), cancer (17,18) and oxidative stress (19,20), has been reported in various studies. Regarding the applications in cardiovascular disease, it has been demonstrated that daily intake of lemon (*Citrus limon*), a Citrus species that has a similar nutritional value to lime, lowers systolic blood pressure (21).

To the best of our knowledge, the effect of cold-pressed oil (CpO) from limes on VSMC pathological changes is unclear. It was hypothesized that lime CpO could inhibit the proliferation of VSMC through known vascular signaling pathways (22,23) in cardiovascular disease. The present study investigated whether lime CpO could modulate the MAPK and PI3K signaling pathways to reduce abnormal cell proliferation induced by FBS.

Materials and methods

Lime CpO. Cold pressed lime oil was purchased from Sydney Essential Oil Company. Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed using a Thermo Scientific Model ISQ LT (Thermo Fisher Scientific, Inc.) equipped with both a flame ionization detector (FID) and a mass spectrometer.

GC-MS analysis. A Durabond-5MS capillary column (60 m x 0.25 mm x 0.25 μm film thickness; Agilent Technologies, Inc.) was used. The carrier gas, helium, had a constant flow rate of 1 ml/min. The injection temperature was 250˚C and 1 μl of the sample was injected with a split ratio of 1:20. The oven temperature was maintained at 50˚C for 5 min, then increased by 10˚C/min to 65˚C and held for 30 min, increased by 5˚C/min to 120˚C and held for 15 min, increased by 1˚C/min to 140˚C and held for 11 min, increased by 10˚C/min to 250˚C and held for 5 min, and finally increased by 20˚C/min to 325˚C and held for 6 min. For the FID, the temperature was set to 300˚C, air flow was set to 350 ml/min, hydrogen flow was set to 35 ml/min and the make-up gas (helium) flow was set to 40 ml/min. The mass interface temperature was 250˚C and the ion source temperature was 250˚C. Mass scan data were acquired in electron ionization (EI) mode at a 0.2 sec scan time rate with a scan range of 35-550 amu. The identification of peaks was performed by comparing the peak average mass spectrum of the peak with an electronic library database (National Institute of Standards and Technology, Environmental Protection Agency and National Institutes of Health Mass Spectral Library; version 2.0 g: https://chemdata.nist.gov/). The identity of the compounds was assigned by comparison of the Kovats retention index, determined in relation to a homologous series of n-alkanes (C7-C30).

Animal care. All animal experiments were approved by the Institutional Animal Care and Use Committee of Catholic Kwandong University, International St. Mary's Hospital (Incheon, South Korea; approval no. CKU 01-2019-008) in cooperation with the Association for Assessment and Accreditation of Laboratory Animal Care and performed in accordance with the Guidelines and Regulations for Animal Care (24). Rats were housed in a room with a stable temperature of 22.5±1.5˚C, 50-60% humidity and 12 h light-dark cycles with *ad libitum* access to food and water. A total of 16 rats were divided into four groups: control (non-treated), lime CpO, FBS (FBS only treated), and FBS and lime CpO. Rats were intramuscularly anesthetized with Zoletil™50 (tiletamine:zolazepam = 1:1; 20 mg/kg; Virbac) and Rompun 2% (xylazine; 5 mg/kg; Bayer AG), and euthanized via an intraperitoneal overdose of sodium pentobarbital (100-200 mg/kg). Euthanasia was confirmed with lack of heartbeat.

Isolation and culture of rat aortic VSMCs. Rat aortic VSMCs were isolated as previously described (25,26). Thoracic aortas from male Sprague-Dawley rats (Orient Bio; n=3; weight, 200-250 g; age, 6-8 weeks) were removed and transferred to incubate in serum-free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) containing 100 U/ml penicillin and 100 μg/ml streptomycin. The aortas were separated from the connective tissue and transferred to a petri dish containing 5 ml of an enzyme dissociation mixture, composed of DMEM with 1 mg/ml collagenase type I (Sigma-Aldrich; Merck KGaA) and 0.5 μg/ml elastase (Thermo Fisher Scientific, Inc.), and incubated for 30 min at 37˚C. The aortas were then transferred to DMEM and the adventitia was stripped off each aorta with forceps under an optical microscope. Subsequently, the aortas were transferred to a conical tube containing 5 ml of enzyme dissociation mixture containing DMEM with 1 mg/ml of collagenase type I (Sigma-Aldrich; Merck KGaA) and 0.5 μg/ml of elastase (Thermo Fisher Scientific, Inc.) and incubated for 2 h at 37˚C. The suspension was centrifuged at 320 x g for 10 min at room temperature and the pellet was re-suspended in DMEM with 10% FBS (Atlas Biologicals, Inc) under room temperature. Rat aortic VSMCs were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin in 75-cm^2 flasks in a 37˚C incubator with 5% CO_2 (Thermo Fisher Scientific, Inc.). Isolated cells from 3 different animals in passages 5-8 were used in the present study.

CpO treatment. The concentration of CpO on VSMCs culture was determined as 10-5 dilution after Cell Counting Kit-8 (CCK-8) assay. For treatment with CpO, VSMCs were seeded into 96-well plates at a density of 5x10^3 cells/well. VSMCs were serum-starved in DMEM containing 0.5% FBS for 24 h and treated with or without 5% FBS in DMEM for the following 24 h to detect the effects of lime CpO under *in vitro* and *ex vivo* conditions in a 37˚C incubator. In order to check the cell signal transduction, MEK1/2 inhibitor U0126 (10 μM; Cell Signaling Technology, Inc.) and mTOR inhibitor

Rapamycin (20 nM; Calbiochem; Merck KGaA) were treated with 5% FBS for 24 h.

Cell proliferation assay. A CCK-8 (DoGenBio Co., Ltd.) assay was used according to the manufacturer's protocol. To each well, 10% (v/v) CCK-8 reagent was added and incubated at 37˚C for 2 h to allow for the formation of water-soluble tetrazolium salts (WST)-8 formazan. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Cytotoxicity assay. The Cytotoxicity Lactate Dehydrogenase (LDH) Assay Kit-WST (cat. no. MK401; Takara Bio, Inc.) was used according to the manufacturer's protocol. Cell suspension and 50 μl DMEM were sequentially added into a 96-well culture plate ($7x10^3$ cells/well) and samples were incubated at 37˚C for 24 h. Samples were mixed with 100 μl working solution at room temperature for 30 min in the dark. The absorbance at 490 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Western blotting. VSMCs were washed once with PBS and lysed using RIPA lysis buffer (Thermo Fisher Scientific, Inc.). Total protein concentration was quantified using an albumin standard (bioPLUS™; BioWORLD). Proteins were then separated by 10% SDS-PAGE by 15-20 mg/ml of protein loading per lane and transferred to a PVDF membrane (MilliporeSigma). After blocking of the membrane with TBS with 0.1% Tween-20 (TBS-T; BioPLUS Chemicals) and 5% (w/v) BSA (bioPLUS™; BioWORLD) in 0.1% TBS-T for 1 h at room temperature, the membrane was washed twice with TBS-T and incubated with the primary antibodies diluted in blocking buffer a ratio of 1:1,000 to 1:2,000 overnight at 4˚C. The membrane was washed three times with TBS-T for 5 min/wash and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted in blocking buffer a ratio of 1:4,000. The membrane was subsequently washed six times with TBS-T for 5 min/wash and bands were detected with an enhanced chemiluminescence reagent (Abclon, Inc.). Band intensities were semi-quantified using a Davinch-Western Imaging System (Davinch-K Co., Ltd.) and ImageJ version 1.44p software (National Institutes of Health). The following antibodies were used in these experiments: anti-PI3K (cat. no. sc-7189; Santa Cruz Biotechnology, Inc.), anti-phosphorylated (p)-PI3K (cat. no. 4228S; Cell Signaling Technology, Inc.), anti-AKT (cat. no. 9272S; Cell Signaling Technology, Inc.), anti-p-AKT (cat. no. 9271S; Cell Signaling Technology, Inc.), anti-mTOR (cat. no. 2972S; Cell Signaling Technology, Inc.), anti-p-mTOR (cat. no. 2971S; Cell Signaling Technology, Inc.), anti-MEK (cat. no. 9122; Cell Signaling Technology, Inc.), anti-p-MEK (cat. no. 9121S; Cell Signaling Technology, Inc.), anti-ERK (cat. no. 9102S; Cell Signaling Technology, Inc.), anti-p-ERK (cat. no. sc-7383; Santa Cruz Biotechnology, Inc.), anti-cyclin D1 (cat. no. 29785; Cell Signaling Technology, Inc.), anti-proliferating cell nuclear antigen (PCNA; cat. no. sc-56; Santa Cruz Biotechnology, Inc.), anti-β-actin (cat. no. ab8227-50; Abcam), anti-caspase-3 (cat. no. ab13847; Abcam) and anti-cleaved caspase-3 (cat. no. ab49822; Abcam). Secondary antibodies were used in these experiments as follows: Goat anti-mouse IgG (H+L)-HRP (cat. no. SA001-500) and goat anti-rabbit IgG (H+L)-HRP (cat. no. SA002-500) from GenDEPOT, LLC. The amount of phosphorylation was calculated by dividing the amount by the total expression amount. This was clarified using magnification of phosphorylation/expression ratio.

Immunocytochemistry. VSMCs were seeded into four-well plastic cell culture dishes ($1x10^5$ cells/well) and were treated with CpO for 24 h in a 37˚C incubator. Subsequently, each well was washed with PBS. Cells were then fixed with 4% paraformaldehyde diluted in PBS for 10 min at room temperature, after which they were washed twice with PBS and permeabilized for 10 min at room temperature with 0.2% Triton X-100 diluted in PBS. After washing with PBS, the cells were blocked in blocking solution (2% BSA and 10% horse serum (Vector Laboratories, Inc.; Maravai LifeSciences) in PBS) for 30 min at room temperature and stained for Ki-67 (Dako; cat. no. M7240; 1:200 dilution) for 1 h at 37˚C. The cells were incubated with a FITC-conjugated anti-mouse (cat. no. 115-095-003; 1:500 dilution; Jackson ImmunoResearch Laboratories, Inc.) secondary antibody at room temperature for 1 h and then stained with a DAPI solution (0.1 μl/ml; Thermo Fisher Scientific, Inc.) for 5 min. Immunofluorescence was detected via confocal microscopy (LSM700; Zeiss GmbH) and analyzed using ZEN 2.5 Blue Edition software (Zeiss GmbH) 1.0 for analysis.

Ex vivo aortic ring assay. *Ex vivo* sprouting of VSMCs was measured via an aortic ring assay using Matrigel (BD Biosciences). The thoracic aortas from the aforementioned 8-week-old Sprague-Dawley rats were removed and transferred into serum-free DMEM. The endothelial lining was removed using a 2-Fr Fogarty balloon catheter (Baxter Healthcare) to minimize the possibility of EC sprouting during the ring assay. The aorta was washed by gradually passing PBS through the aorta three times. After removing perivascular adipose tissue, the aorta was cut into 1-mm-thick segments of aortic ring and placed in Matrigel. The aortic rings were washed with serum-free DMEM twice and starved in DMEM supplemented with 0.5% FBS for 24 h at 37˚C. For the lime CpO group, the medium was changed to DMEM supplemented with 5% FBS containing $1x10^{-5}$ diluted lime CpO. The cells were cultured at 37˚C and the media were changed every 3 days and the aortic rings were monitored daily for up to 7 days for sprouting VSMCs. On the 7th day the results were analyzed.

Statistical analysis. Data are presented as the mean ± SEM of at least three independent experiments. For statistical analysis, one-way analysis of variance with Bonferroni's correction was performed for comparisons among more than two groups. All analyses were performed using Prism software (version 5.0; GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Suppression of VSMC proliferation by lime CpO treatment. To evaluate the effect of lime CpO on VSMC proliferation, cells were treated with lime CpO for 24 h. In the group treated with FBS only, the cell proliferation rate was

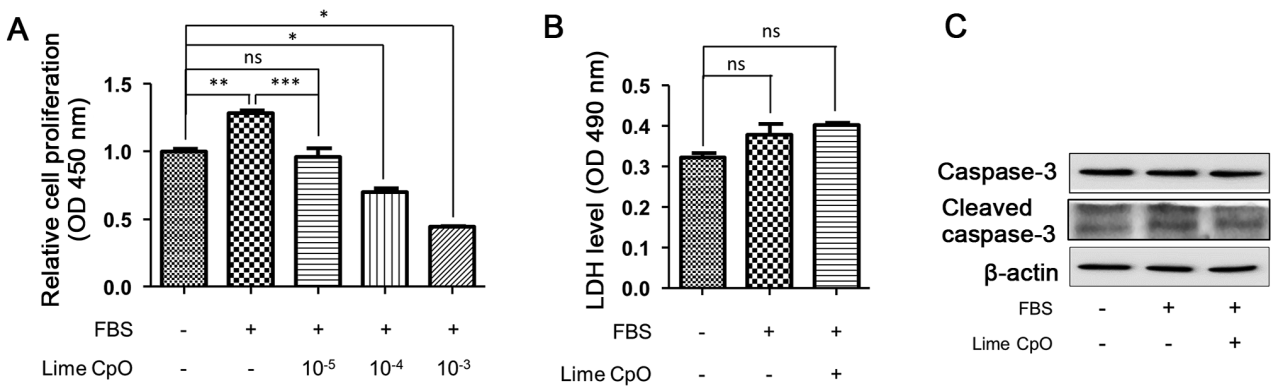

Figure 1. Effects on VSMC proliferation treated with lime CpO. To examine the effect of lime CpO on VSMC proliferation, concentration of 10^{-3} to 10^{-5} of lime CpO was added to 5% FBS-containing DMEM and VSMCs, and cells were cultured for 24 h. (A) Cell proliferation was determined using a CCK-8 assay. (B) To measure cytotoxicity the LDH assay was performed. (C) Following lime CpO treatment, the protein expression levels of caspase-3 and cleaved caspase-3 were analyzed via western blotting. Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001. CCK-8, Cell Counting Kit-8; CpO, cold-pressed oil; LDH, lactate dehydrogenase; ns, not significant; OD, optical density; VSMC, vascular smooth muscle cell.

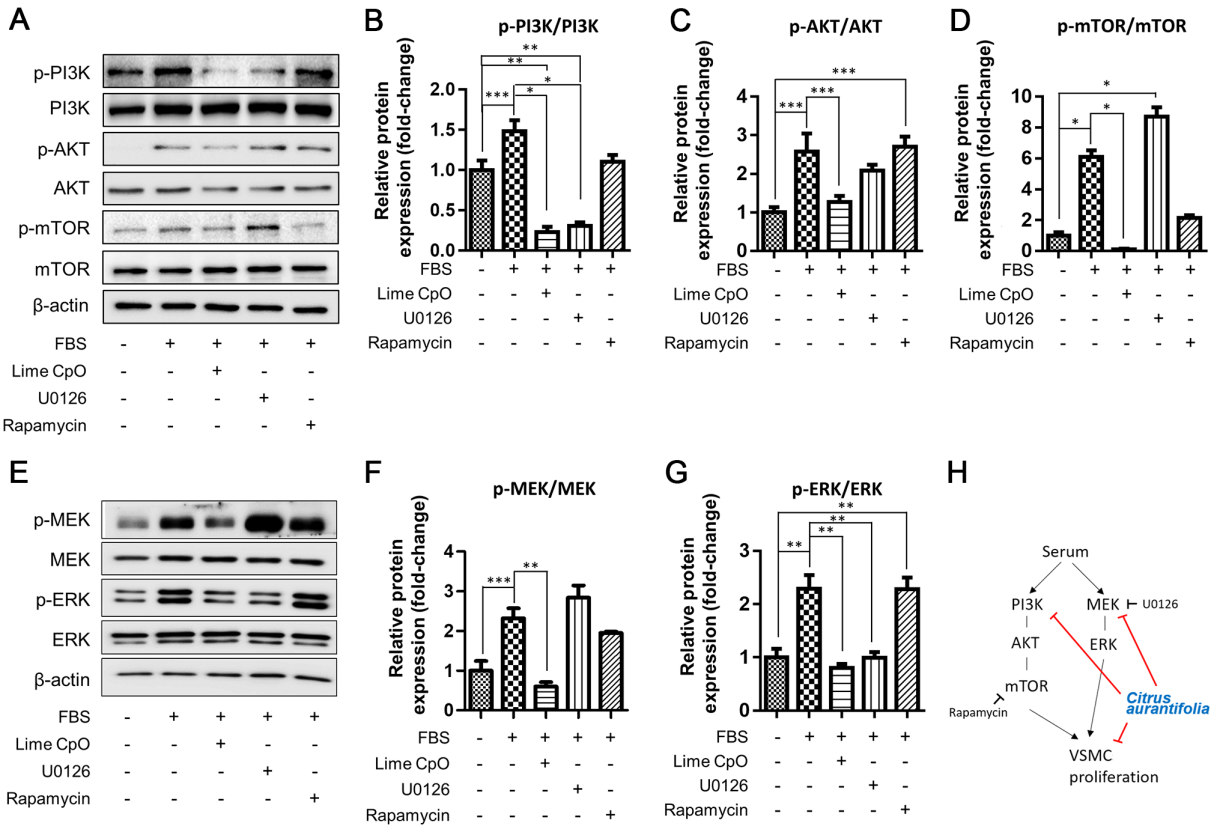

Figure 2. Changes of PI3K and MAPK signaling pathways in VSMC treated with lime CpO. To investigate the effect of lime CpO on cell proliferation, related signaling cascades were analyzed using western blotting. Starved cells were pretreated with 10^{-5} diluted lime CpO for 1 h prior to FBS treatment. (A) PI3K/AKT/mTOR signaling cascade was investigated following 30 min of FBS treatment. (B-D) Protein expression levels and phosphorylation of PI3K, AKT and mTOR were semi-quantified using ImageJ. (E) MEK/ERK signaling cascade was investigated following 30 min of FBS treatment. (F and G) Protein expression levels and phosphorylation of MEK and ERK were semi-quantified using ImageJ. (H) Diagram showing the role of lime CpO in the signaling cascades related to VSMC proliferation. *P<0.001, **P<0.01 and ***P<0.05. CpO, cold-pressed oil; p, phosphorylated; VSMC, vascular smooth muscle cell.

significantly increased by ~30% compared with that of the FBS-negative and lime CpO-negative control groups. Concentration of 10-5 lime CpO was statistically similar to the control, however concentration of 10^{-4} and 10^{-3} lime CpO was significantly lower (Fig. 1A). Based on these results, lime CpO significantly inhibited FBS-stimulated VSMC proliferation at low concentrations (10^{-5}). The effect of lime CpO on cell death was assessed via LDH analysis and it was demonstrated to have no statistically significant effect on cell death compared with the FBS-treated only group (Fig. 1B). Furthermore, caspase-3 and cleaved caspase-3 protein levels were also not affected by lime CpO treatment compared with the FBS-treated or FBS-negative and lime CpO-negative control groups (Fig. 1C). Therefore, the results suggested that

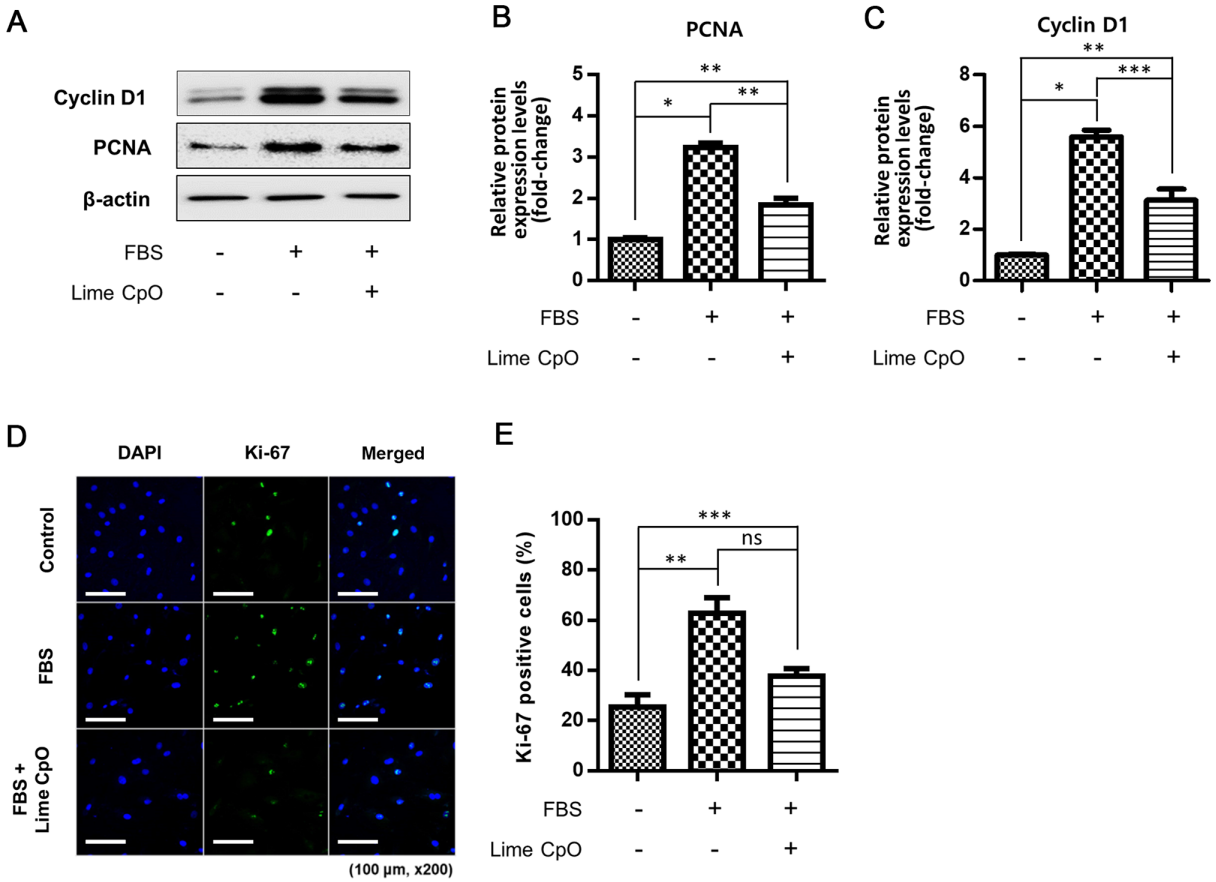

Figure 3. Regulation of the cell cycle in VSMCs by treating lime CpO. (A) Effects of lime CpO on cell cycle-related proteins (cyclin D1 and PCNA) were analyzed using western blotting. Protein expression levels of (B) PCNA and (C) cyclin D1 were semi-quantified using ImageJ. (D) Following lime CpO treatment, Ki-67 was immunostained. Green, Ki-67; blue, DAPI. Scale bar, 100 μm. (E) Percentages of Ki-67-positive VSMCs were quantified by counting the number of Ki-67 (green) labeled cells divided by DAPI (blue) labeled cells (five individual fields/group). *P<0.001, **P<0.01 and ***P<0.05. CpO, cold-pressed oil; Ctl, control; ns, not significant; PCNA, proliferating cell nuclear antigen; VSMC, vascular smooth muscle cell.

lime CpO may inhibit FBS-induced proliferation of VSMCs but does not cause cell death.

Changes in AKT and ERK signaling cascades in VSMC proliferation. The mechanism by which lime CpO regulated proliferation was investigated further. Since phosphorylation of the PI3K/AKT/mTOR signaling cascade is known to regulate VSMC proliferation (27), the phosphorylation of these signaling cascades was analyzed. Phosphorylation of the PI3K/AKT/mTOR (Fig. 2A-D) and MEK/ERK signaling pathways (Fig. 2E-G) was significantly increased by 1.5-6.2-fold in FBS-treated cells compared with the FBS-negative and lime CpO-negative control group. However, the signal intensity of each phosphorylated protein band was significantly reduced in the lime CpO-treated group compared with the FBS-treated cells or the U0126 or Rapamycin-treated cells. These results demonstrated that lime CpO inhibited phosphorylation in a manner similar to that of the MEK and mTOR inhibitors in VSMCs (Fig. 2H).

Regulation of cell cycle regulators in VSMCs. It was subsequently demonstrated that the cell cycle-regulating factors cyclin D1 and PCNA were essential for regulating proliferation in lime CpO-treated cells. The protein expression levels of cyclin D1 and PCNA were significantly increased in FBS-treated cells compared with FBS-negative and lime CpO-negative control cells; however, the lime CpO-treated cells displayed significantly reduced protein expression levels compared with the FBS-treated cells (Fig. 3A-C). Ki-67 was observed in the nucleus during cell division, indicating cell proliferation (Fig. 3D-E). The number of Ki-67-positive cells was significantly increased by ~35% in the FBS-treated group compared with the FBS-negative and lime CpO-negative control group. However, the number of Ki-67-positive cells was significantly reduced in the lime CpO-treated group compared with the FBS-treated group (Fig. 3D and E). This result suggested that lime CpO had the potential to suppress the cell cycle of VSMCs and regulate VSMC proliferation.

Inhibition of VSMC proliferation in endothelium-denuded aortic rings. The effect of lime CpO on the proliferation and migration of VSMCs in tissues was investigated and an *ex vivo* aortic ring analysis was performed (Fig. 4A). EC-denuded vascular tissue was cultured in DMEM containing FBS to observe the proliferation and migration of VSMCs. Intravascular VSMCs spread to the outside of the aortic rings and significantly proliferated in response to FBS compared with the cells in the FBS-negative and lime CpO-negative control group. However, the degree of spread of the lime CpO and FBS-treated VSMCs was significantly decreased

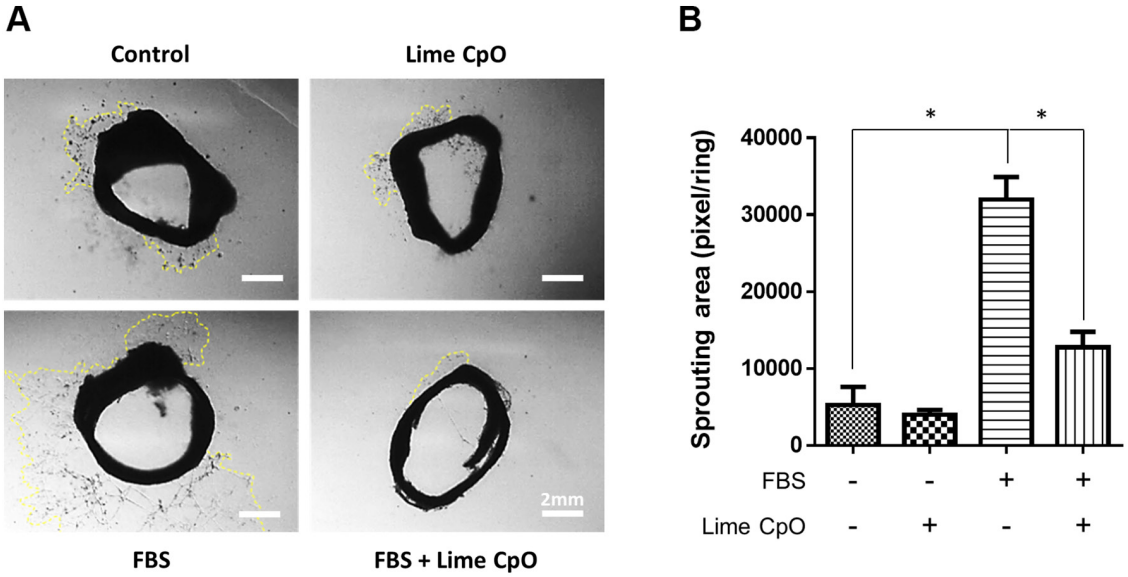

Figure 4. Inhibition of VSMC-sprouting from endothelium-denuded aortic rings treated with lime CpO. To investigate the effects of lime CpO on VSMC increase in blood vessels, a 1-mm-thick aortic ring was built into the Matrigel and cultivated for 7 days with DMEM containing 5% FBS and/or lime CpO. (A) Yellow-dotted lines indicate the sprouting of VSMCs from the aortic rings. Scale bar, 2 mm. (B) VSMC sprouting, as indicated by the area inside the dotted line, was quantified using ImageJ. *P<0.001. CpO, cold-pressed oil; Ctl, control; VSMC, vascular smooth muscle cell.

compared with the FBS-treated group, to a similar level to that of the control cells (treated with nether CpO or FBS) (Fig. 4B). These results suggested that lime CpO inhibited abnormal VSMC proliferation in injured vascular tissues and possibly contributed to tissue homeostasis.

Analysis of lime CpO components. In the present study, GC-MS analysis was performed to evaluate the characteristics and components of lime CpO (Table I). Table I displays the results of the component analysis of lime CpO by GC-MS. In total, ~36 types of volatile compounds were detected as main materials, including 23 monoterpenes, 9 sesquiterpenes and 4 other components. Monoterpenes (M) and sesquiterpenes (S) accounted for 94.6 and 4.6% of EOs, respectively, in summed area % of GC-MS spectrometry (Table I). D-limonene (41.40%), γ-terpinene (13.19%), terpinolene (10.41%) and α-terpineol (8.61%) were the major monoterpenes among the detected volatile components, overall accounting for 73.61% of the total volatile components (Table I).

Discussion

The present study investigated the effect of lime CpO, a natural substance, on VSMC proliferation. To examine whether lime CpO was involved in VSMC regulation, cellular signaling pathways were analyzed using western blotting. Lime CpO was demonstrated to modulate cell cycle regulators in VSMCs. Furthermore, in *ex vivo* conditions, lime CpO negatively regulated VSMC proliferation. Among 36 types of volatile compounds identified in lime CpO, four molecules (D-limonene, γ-terpinene, terpinolene and α-terpineol) were specifically detected as the main components.

In the past decade, natural products have been demonstrated to be mostly non-toxic and have been used successfully as therapeutics for numerous diseases (10,28-30). Among these natural products, EOs are promising pharmaceutical agents as they are clinically applicable and can be properly managed and industrialized for human diseases (31). Depending on the EO extraction method, such as steam distillation, expression (for example cold compression) and solvent extraction, the properties of the active compounds may show different distribution ratio in total ingredients, but the main active substances remain unchanged (32). According to a review by Narang and Jiraungkoorskul (17), lime and its related byproducts have been suggested to have potential therapeutic effects in colon cancer, pancreatic cancer, breast cancer, skin cancer and lymphoma. Lime and its related byproducts have also been demonstrated to inhibit the cell cycle and cell proliferation (17). Patil *et al* (18) reported that lime inhibited cancer cell proliferation by modulating Bax, Bcl-2, caspase-3 and p53 in pancreatic cancer cells. Furthermore, lime has been reported to improve obesity, the atherogenic index and fatty liver disease by inducing antioxidant capacity and hypolipidemic effects (33). Despite the various previous studies on lime CpO, to the best of our knowledge, there are currently no reports on the inhibition of VSMC proliferation, a major cause of occlusive vascular disease.

The present study demonstrated that lime CpO treatment at 10^{-5} concentrations inhibited FBS-induced VSMC proliferation. Furthermore, lime CpO reduced FBS-induced phosphorylation of the MAPK/ERK and PI3K/AKT/mTOR signaling pathways. Lime CpO inhibited the MAPK/ERK and PI3K/AKT/mTOR signaling pathways at comparable or higher degree. However, there was no change in the expression or activity of caspase-3 under the experimental conditions of the present study. A possible reason for why these results differed from previous reports is that the lime CpO dose is modifiable for different cytotoxic effects under various cell types. In the present study, preliminary experiments were conducted to determine concentrations that were not cytotoxic (Fig. 1A). Compared with conventional chemical drugs, lime CpO is a natural compound with little

Table I. Chemical Composition of Lime CpO by MS and KI identification in GC-MS spectrometry.

RT	Constituent	Area, %	KI[a]	Classification
24.55	α-Pinene	0.90	927	(M)
27.08	Camphene	0.26	943	(M)
30.36	Linalool-3,7-oxide	0.10	963	(M)
31.89	β-Pinene	1.48	972	(M)
34.15	β-Myrcene	0.36	986	(M)
39.22	1,4-Cineole	1.39	1026	(M)
39.52	α-Terpinene	1.05	1029	(M)
40.61	ϱ-Cymene	4.23	1039	(M)
41.27	D-Limonene	41.40	1045	(M)
41.50	Eucalyptol	1.11	1048	(M)
42.77	Ocimene quintoxide	0.12	1059	(O)
42.98	(Z)-β-Ocimene	0.32	1061	(M)
44.08	γ-Terpinene	13.19	1072	(M)
46.19	Terpinolene	10.41	1092	(M)
46.57	ϱ-Cymenene	0.33	1095	(M)
47.14	Linalool	0.13	1101	(M)
48.49	Fenchyl alcohol	0.48	1121	(M)
49.53	1-Terpineol	0.58	1136	(M)
50.48	β-Terpineol	0.72	1150	(M)
51.79	Decanal	0.25	1169	(O)
52.22	endo-Borneol	0.18	1175	(M)
52.77	Terpinen-4-ol	0.44	1183	(M)
53.24	ϱ-Cymen-8-ol	0.20	1190	(M)
53.92	α-Terpineol	8.61	1200	(M)
54.26	γ-Terpineol	1.63	1204	(M)
68.01	trans-Caryophyllene	0.47	1434	(S)
68.33	α-Bergamotene	0.78	1442	(S)
68.89	β-Farnesene	0.10	1457	(S)
69.39	α-Humulene	0.13	1471	(S)
69.95	α-Selinene	0.14	1486	(S)
70.42	β-Cadinene	0.17	1498	(S)
70.69	α-Farnesene	0.75	1507	(S)
70.95	β-Bisabolene	1.52	1516	(S)
71.94	β-Maaliene	0.26	1548	(S)
89.83	Butyl palmitate	0.24	2188	(O)
91.85	Butyl stearate	0.20	2389	(O)

[a]KI on a DB-5 column in reference to n-alkanes. MS, National Institute of Standards and Technology, Environmental Protection Agency and National Institutes of Health Mass Spectral Library; KI, Kovats retention index; RT, Retention time; M, monoterpenes, S, sesquiterpenes; O, others.

to no toxicity and controllable doses that can be used in the body (34). The use of natural products as therapeutics is advantageous as they are traditionally used by human and therefore less likely to cause an adverse reaction when developed as medicines (9). However, in future work, lime CpO toxicity will be tested in other vascular constituent cells besides VSMCs, such as vascular ECs and vascular fibroblasts. Furthermore, whether the same effect is shown in other batches of lime CpO and identification of the active compounds via selection experiments with various combinations of constituents from lime CpO should be investigated in future studies.

A previous report suggested that neroli (*Citrus aurantium* L.) EO is an endothelium and smooth muscle-dependent vasodilator, which alleviates cardiovascular disease (35). It has been reported that neroli EO modulated intracellular Ca^{2+} concentrations via the inhibition of cation channel-mediated extracellular Ca^{2+} influx and store-operated Ca^{2+} release mediated by the ryanodine receptor signaling pathway (35). Among the proposed constituent compounds of neroli EO,

D-limonene and α-terpineol are included in the data from the present study, suggesting that lime CpO may also act as a smooth muscle-dependent vasodilator (35). Furthermore, neroli EO have also reported to possess 100% singlet oxygen scavenging activity as a strong antioxidant (12,36,37). Therefore, future work may examine the endothelium and/or smooth muscle-mediated vasodilator effect of lime CpO in a cardiovascular disease model. In the present study, lime CpO inhibited excess VSMC proliferation, which causes occlusive vascular disease, via the regulation of MAPK and PI3K signaling pathways. These results suggest that lime CpO may be developed as a potential therapeutic or health supplement for the treatment of cardiovascular disease. Future studies may explore the different effects of lime CpO compared with lime EO and validate its pharmacological effects on classified compounds including D-limonene, γ-terpinene, terpinolene and α-terpineol to investigate the proposed molecules of *in vivo* cardiovascular disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BWS, CYL and IKK were involved in the conceptualization, writing, editing and data analysis. JHP, BK, SLe, SLi, SWK, JWC, MK, JHK, SSL, MJP, HM and KCH performed and analyzed the experiments, and edited the manuscript. BWS and IKK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was performed according to a protocol approved by the Institutional Animal Care and Use Committee of Catholic Kwandong University (approval no. CKU 01-2019-008; Incheon, South Korea).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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