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CAFFEIC ACID PHENETHYL ESTER INHIBITS ARTERIAL SMOOTH MUSCLE CELL PROLIFERATION AND MIGRATION IN VITRO AND IN VIVO USING A LOCAL DELIVERY SYSTEM

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Abstract – Over the last two decades, significant advances have been made in percutaneous coronary intervention (PCI) for the treatment of atherosclerotic plaques. However, restenosis after PCI still challenges both vascular biologists and interventional cardiologists. In this study, we found that caffeic acid phenethyl ester **(**CAPE) displayed an inhibitory effect on human coronary smooth muscle cell (HCSMC) growth and migration. Flow cytometry analysis showed that the ratio of S phase increased after exposing cells to CAPE for 48-72 h. Pretreatment of cells with CAPE significantly suppressed Cyclin E, CDK2, Cyclin A, and proliferating-cell nuclear antibody expression. We demonstrated that CAPE inhibited AKT 1 and MEK1/2 activation. Using a local infusion system, CAPE was able to regress the intima thickening of the iliac artery in rabbits after balloon injury. The percentage of intimal thickening decreased significantly to 55.0 ± 0.12 in the group after local CAPE infusion compared to the group after saline infusion (98.3 ± 0.41%). In conclusion, CAPE can inhibit the proliferation and migration of HCSMCs by inducing cell cycle arrest. Decreased cell cycle genes and associated signaling pathway target gene expression may mediate anti-proliferative and anti-migration effects of CAPE. Furthermore, CAPE prevents intima thickening in rabbits after balloon angioplasty. These results indicate that CAPE may have therapeutic relevance for the prevention of restenosis during PCI in the treatment of coronary artery diseases.

Key words: caffeic acid phenethyl ester, human coronary smooth muscle cells, proliferation, migration**,** restenosis, local delivery, cell cycle.

INTRODUCTION

Smooth muscle cells (SMCs) constitute a major cell type in restenotic lesions during balloon angioplasty. SMCs in developing intima thickening express a synthetic phenotype and cells become able to migrate, proliferate, and secrete extracellular matrix to increase in mass of the intimal thickenings (5, 18). Caffeic acid phenethyl ester (CAPE) is the major active element of propolis and has a preferential anti-proliferative effect on tumor cells, e.g., colorectal tumors, leukemia, colon carcinoma, and glioma (10, 11, 17, 23). The antioxidative activities of CAPE have been demonstrated in vitro and in different biological systems (9, 21). It has been reported that treatment with CAPE may lead to a reduction of neointima formation by inhibition of nuclear transcription factor Kappa-B (NF-κB) activation after balloon angioplasty in the rat model (9). The underlying mechanism, however, is still not fully understood. Uptake of CAPE can inhibit SMC proliferation in spontaneously hypertensive rats (12). Large doses of oral CAPE ingestion may increase the potential risk of gastric cancer (6). Even at low dietary levels, phenolic compounds can exert additive /synergistic effect on carcinogenesis of forestomach or glandular stomach (7). Therefore,

Abbreviations: **AKT**, serine/threonine-specific protein kinase; **CAPE**, caffeic acid phenethyl ester; **ERK**, extracellular signal-regulated kinases; **HCSMC**, human coronary artery smooth muscle cell; **MEK**, mitogen acticated protein kinase; **NF-κB**, nuclear transcription factor Kappa-B; **PCI**, percutaneous coronary intervention; **PCNA**, proliferating-cell nuclear antibody; **SMC**, smooth muscle cell.

a delivery system to decrease restenosis after angioplasty is urgently required.

Over the last two decades, many advances have been made in percutaneous coronary intervention (PCI) for the treatment of atherosclerotic plaques (16). However, new evidence suggests that the delivery of pharmaceutical agents via drug-eluting stents puts patients at risk for sub-acute thrombosis and restenosis (4, 20). Various pharmacologic tools and implantations have been tried to overcome the restenosis but the problems have not been resolved. Recently a local delivery device, infiltrator angioplasty balloon catheter (IABC), has been developed to deliver fluid-phase substance directly into the vessel wall after conventional angioplasty (2). With angioplasty and a specifically designed InjectionPort, the IABC can deliver agents more precisely and efficiently into the lesion area of the vessel wall without the undesired systematic effect.

In this study, we examined the effects and the underlying mechanism of CAPE on the growth and migration of human coronary artery smooth muscle cells (HCSMCs) in vitro. Expression of cell cycle target genes and phosphorylated serine/threonine-specific protein kinase (AKT1), mitogen-activated protein kinase kinase (MEK1/2), and extracellular signal-regulated kinase 1/2 (ERK1/2) in cells were examined by using Western blot analysis. By applying the IABC local infusion method, we have also evaluated whether CAPE is able to regress intima thickening in an in vivo experimental rabbit model.

MATERIALS AND METHODS

Cell culture

HCSMCs (PromoCell GmbH, Heidelberg, Germany) were cultured at 37 °C in Medium 199 (GIBCO, New York, U.S.A.) supplemented with 10% fetal bovine serum and penicillin-streptomycin (50 U/ml, Sigma, St. Louis, MO) in a 5% CO_2/95% air atmosphere. The culture medium was replaced every 2 days. The passage of HCSMCs used in the experiment was 3-9.

Cell growth assay

SMCs were seeded on 6-well plastic dishes at a concentration of 1×10^5 per well. Prior to the experiment, 60-70% confluence of SMCs were washed twice with phosphate buffer saline (PBS), and incubated with serum medium supplemented with various concentrations (0, 3, 10, 30, 90 μM) of CAPE for 0, 6, 12, and 24 h respectively. Cells were then stained with trypan blue and cell numbers were counted under an inverted microscope. Data are presented as the mean ± SD of three replicates from six separate experiments.

Cell migration assay

Migration assay was assessed using a method previously described (8) with some modifications. Briefly, SMCs were plated at an initial density of 1x 10^5 cells/ml to form a monolayer. During the last 4 h, cytosine β-D-arabinofuranoside (Ara-C, Sigma, St. Louis, MO, U.S.A.) was added at a final concentration of 4 μM to prevent SMCs proliferation. Cells were washed twice with PBS and incubated with serum medium containing CAPE for 0, 6, 12, 24 h. Then SMCs were wounded by scraping with a pipette tip to make an approximately 400-μm gap in the cell monolayer. The images of cell migration were observed at different time interval and photographed at five marked locations on each dish using a phase contrast microscope. The number of migrated cells were counted and averaged. All experiments were carried out in triplicate and repeated at least 6 times.

Flow cytometry analysis

Cell density was adjusted to 1×10^5 cells/ml then treated with 30 μg/ml CAPE for 24, 48, and 72 h. Cells were trypsinized, centrifuged, collected, washed with PBS and fixed with ice-cold 70% ethanol at 4°C overnight. Cells were then washed with PBS, incubated with propidium iodide (40 μg/ml) (Molecular Probes, Eugene, Oregon, U.S.A.) and DNase-free RNase A (100 μg/ml) for 1 h at 37°C in the dark. The DNA content of the nuclei was analyzed by a flow cytometer (Partec GmbH, Münster, Germany). The percentage of cells in different phases of the cell cycle was analyzed by using Cell-FIT software (Becton Dickinson Instruments).

Western blot analysis

Proteins (50 μg) were separated by 12% SDS-PAGE and electrotransfered to a polyvinylidene fluoride membrane. Blots were probed with mouse anti-human proliferating-cell nuclear antibody (PCNA) (1:5000, v/v), mouse anti-human cyclin E, mouse anti-human CDK2 (1:1000, v/v), mouse anti-human cyclin A (1:1000, v/v), rabbit anti-human AKT1 (1:1000, v/v), rabbit anti-human phosphorylated AKT1(1:1000, v/v), rabbit anti-human ERK1/2 (1:1000, v/v), mouse anti-phosphorylated ERK1/2 (1:1000, v/v), mouse anti-human MEK1/2 (1:1000, v/v), rabbit anti-human phosphorylated MEK1/2 (1:1000, v/v) (Abcam, MA, U.S.A.), followed by HRP-conjugated goat anti-rabbit IgG (1: 5000, v/v) (Zymed, South San Francisco, CA, U.S.A.) or goat anti-mouse IgG (1:5000, v/v) (Zymed, South San Francisco, CA, U.S.A.). After washing with PBS containing 0.5% Tween-20, peroxidase activity was assessed using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, U.S.A.). For an internal control, the same membrane was re-probed with a monoclonal antibody directed against β-actin (1:10000, v/v) (Sigma, St. Louis, MO, U.S.A.). The intensities of the reaction bands were analyzed with the Image Gauge System (Fuji, Tokyo, Japan).

Delivery device

A commercial infiltrator angioplasty balloon catheter (IABC) (Infiltrator®, Boston Scientific Corporation, Boston, MA, U.S.A.) was used in this study (13, 24). The device consists of a conventional noncompliant angioplasty balloon with three InjectorStrips — polyurethane strips with a 0.25-mm high nickel cone (InjectorPort) with a vertical hole — atop an infusate delivery channel mounted longitudinally on the surface. The balloon was 15 mm long

and 3.0–4.0 mm in diameter. The distance covered by the InjectorPorts was 10.7 mm. The catheter shaft was a monorail configuration consisting of three adjacent lumens. The first lumen consisted a guidewire which was inserted through the guide wire lumen as the track for positioning the infiltrator through the other two lumens. The second lumen was used to deliver infusate through the InjectorPorts, and the third lumen contained a support wire and was used to inflate and deflate the balloon. The shaft diameter was 4.2 French, and the total length was 132 cm. Before CAPE delivery, the balloon was flattened and the injector nipples recessed. When the infiltrator was inflated inside the vessel, the balloon was fully apposed against the vessel wall, and the InjectorPort radically extended and penetrated the intimal/medial layers of the artery. In this way, CAPE was delivered directly into the vessel wall with microliter precision.

Denudation and local infusion of CAPE to iliac artery

All animal protocols were approved by the Animal Care and Use Committee of the Veterans General Hospital, Taichung, Taiwan. Twelve male New Zealand white rabbits, each weighing 3.0~3.5 kg, were fed with a 2% high-cholesterol diet for eight weeks. The animals were divided into four groups: (1) without balloon injury, (2) with iliac arterial balloon injury, (3) with arterial balloon injury followed by IABC local normal saline infusion, and (4) with iliac arterial balloon injury followed by IABC local CAPE infusion. The rabbits were anesthetized with intravenous injection of a mixture of ketamine (40 mg/kg) and xylocaine (5 mg/kg). The right femoral artery was exposed and punctured with an 18 G puncture needle, and a standard 0.018-inch guidewire was advanced into the femoral artery. Along the guidewire, a 3.0×20 mm balloon catheter was placed into the femoral artery cephalically to about 6 cm, where the balloon was located around the bifurcation of iliac artery. The balloon catheter was inflated to 8 bars; the balloon catheter was withdrawn to denudate the proximal end of the iliac artery to the puncture orifice for three times. For groups 3 and 4, the balloon catheter was replaced with IABC. The IABC was sent cephalically to 6 cm beyond the puncture orifice, and 0.4 ml of normal saline solution or 100 μM CAPE in 0.4 ml normal saline was infused slowly into the iliac artery after the balloon was inflated to 6 bars. The infusion rate is 0.013 ml/sec. After deflating the balloon, the IABC was pulled back 1 cm, whereupon the infusion procedure as described above was repeated. The femoral artery was then ligated and the skin was closed with a silk suture (size no. 4). Three weeks later, all rabbits were anesthetized and sacrificed. Iliac arteries were isolated, cut, and processed for histochemical study and quantitative analysis.

Morphology

Iliac arteries were fixed with 4% paraformaldehyde in PBS for 2 h. The bifurcation of the iliac artery was paraffin-embedded, and cross sections, 6 μm thick, were taken every 120 μm from the proximal end to the distal end of the bifurcation. Approximately 50–60 sections of iliac bifurcation were collected for haematoxylin and eosin staining. The sections were photographed under a low-power, video-digitized camera and stored in an image analysis system (Image Gauge, Fuji Film, Tokyo, Japan). The areas of the intima, the inner lumen, and the media were measured for determining the intimal thickening: [(area within the elastic lamina – area within the inner lumen) / (area within the media – area within the inner lumen)] × 100% (3).

Statistical analysis

Data were expressed as mean ± SD. The two groups were compared via a two-tailed *t*-test using SAS software. The cell growth and cell migration were analyzed via ANOVA followed by post-hoc analysis. $P<0.05$ was considered statistically significant.

RESULTS

Effects of CAPE on cell growth inhibition

Using a trypan blue exclusion assay, CAPE induced an inhibitory effect on cell growth (Fig. 1). Cell number of the control group increased to about 2.5 fold after 24 h; however, treatment of CAPE inhibited HSCMCs growth in a dose-dependent manner. Cytotoxicity was observed at a high dose of CAPE (90 μM) after treatment for 24 h. Cell numbers, however, did not fall below those measured at 6 to 24 h after 30 μM CAPE treatment, indicating that CAPE arrested cell growth at this dose. We, therefore, chose 30 μM of CAPE to study cell migration and to elucidate the underlying mechanism in the following experiments. DNA fragmentation assay was not detectable in the cells at various concentrations of CAPE treatment throughout the experiments (data not shown).

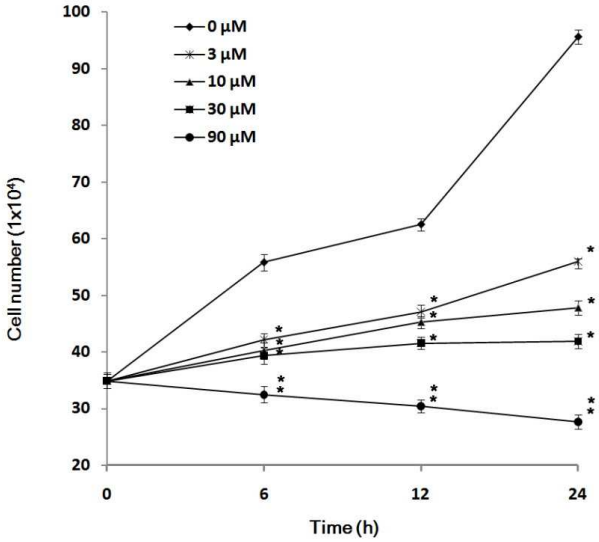

Figure 1. Inhibition of cell growth of HCSMCs by CAPE. The Cells were treated with CAPE at indicated concentrations for the indicated times. The viable cells were determined by trypan blue dye exclusion assay. Data presented as mean ± SD of three independent experiments. *p<0.05, **p<0.01, compared to the control group.

Effects of CAPE on cell migration

To assess the effects of CAPE on HCSMC migration, cell culture images after wounding experiments were photographed and analyzed at different time intervals. The data showed that the number of migrated HCSMCs were significantly

increased at 6 h and beyond without CAPE treatment. This time-dependent migration was strongly inhibited in the cells treated with 30 μM CAPE (Fig. 2A, 2B).

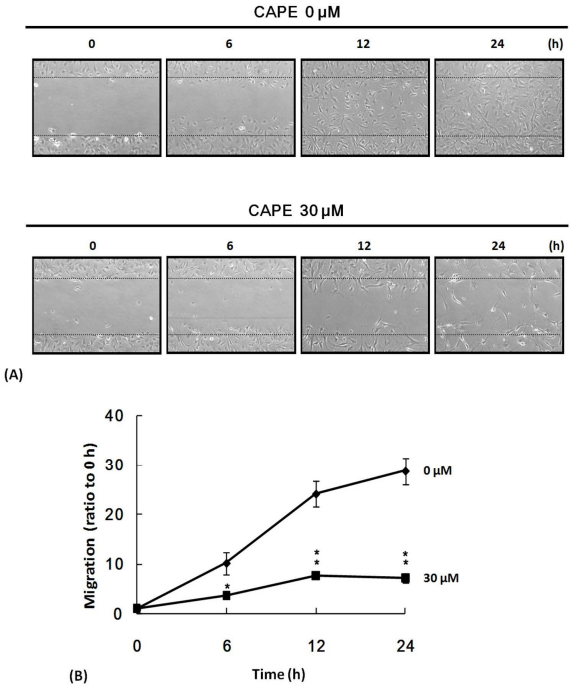

Figure 2. Inhibition of cell migration of HCSMCs by CAPE. Migration assay was assessed by mechanical wound healing assay. (A) SMC migration was evaluated at 6, 12, and 24 h after 30 μM CAPE treatment. The data showed that SMCs started to migrate at 6 h without CAPE treatment. The migration was strongly inhibited from 6 to 24 h after CAPE treatment. (B) Quantitative data showed that cell migration was significantly repressed in the presence of CAPE. *p<0.05, **p<0.01, compared to the control group.

Effects of CAPE on cell cycle progression

In order to examine the effects of CAPE on cell cycle progression, HCSMCs were treated with 30 μM of CAPE for 12, 24, 48, and 72 h. The distributions of cells in each of the cell cycle were determined by flow cytometry. The results showed that the percentage of the cell population in the S phase increased significantly while those in G2/M phase and G0/G1 phase decreased significantly after treatment with CAPE for 48 and 72 h (Fig. 3A, 3B). These results indicated that CAPE caused cell cycle arrest in the S phase.

Effects of CAPE on the expression of cell cycle genes

To conclusively illustrate the effects of CAPE on the progression of the cell cycle, we analyzed the expressions of PCNA, Cyclin E, CDK2, and Cyclin A using Western blot analysis. PCNA is a marker of proliferation at the late G1 phase, exhibiting maximal levels at S phase (15). Our results showed that the expression of PCNA was decreased after the treatment of CAPE for 12-72 h. Moreover, the levels of CDK2, Cyclin E, and Cyclin A were decreased markedly compared with those in the non-CAPE treated group (Fig. 4A). The quantitative results on the level of specific protein were presented in figure 4B.

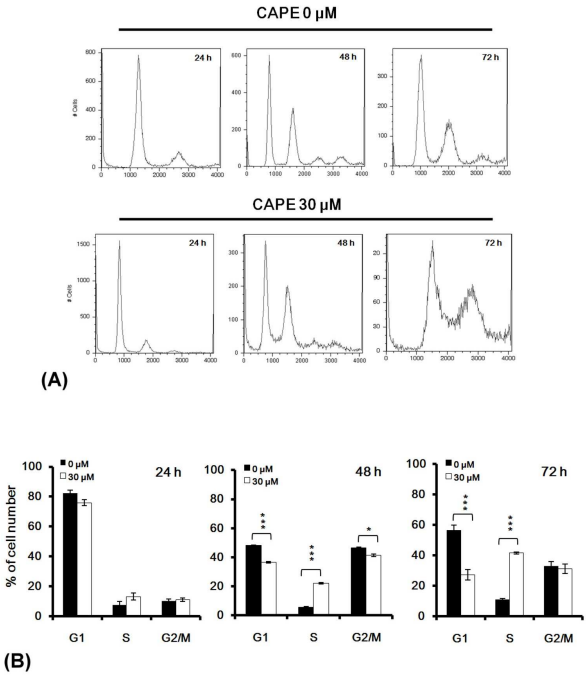

Figure 3. Effect of CAPE on cell cycle distribution_in HCSMCs. (A) After treatment with CAPE for 24, 48 and 72 h, cells were fixed and stained with propidium iodide, and DNA content was analyzed by flow cytometry. The data showed that the percentage of cell number of S phase increased and the percentage of cell number of G1 and G2 phases decreased after treatment with CAPE for 48 and 72 h. These results indicate that CAPE inhibited cell cycle progression by causing cell cycle arrest at the S phase. (B) The cell number percentage in each phase (G0/G1, S, and G2/M) of cell cycle was calculated and expressed. *p<0.05, ***p<0.001, compared to the control group.

Effects of CAPE on the expression of AKT, MEK, ERK

To study the signaling molecules involved in CAPE-mediated changes in the HCSMCs, we analyzed the expression of phosphorylated AKT1. MEK1/2, and ERK1/2 in cells. The decrease of phosphorylated AKT1, MEK1/2, and ERK1/2 in cells after the treatment of CAPE indicated that the inhibitory effects of CAPE on the growth and the migration of HCSMVs may be associated with a down-regulation of AKT and MEK signaling pathways. (Fig. 5A). The average quantitative results on the level of

specific protein and its phosphorylation were presented in figure 5B.

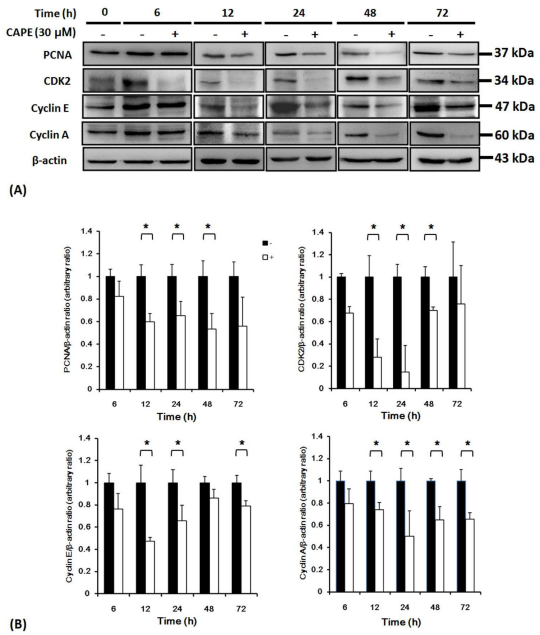

Figure 4. The expression of cell cycle genes in the presence of CAPE at 6, 12, 24, 48, and 72 h. (A) The levels of Cyclin E, CDK2 and Cyclin A decreased in cells after treatment with 30 μM CAPE. (B) The quantitative results on the level of specific protein were presented. *p<0.05, compared to the control group.

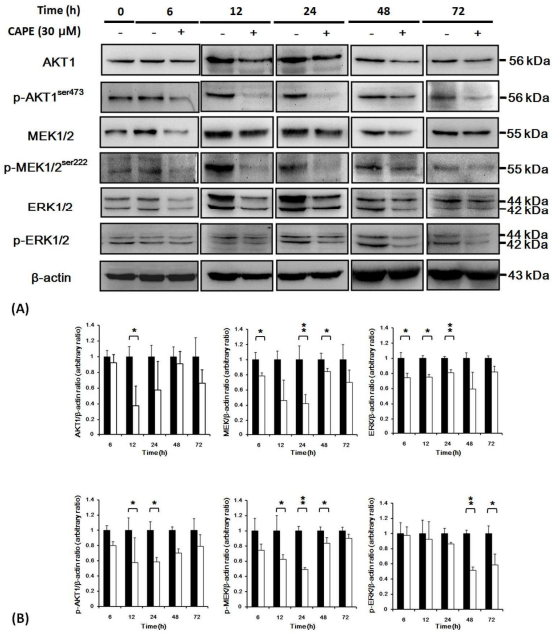

Figure 5. CAPE inhibited the expression of phosphorylated AKT1, MEK1/2, and ERK1/2. (A) The phosphorylated-AKT1, -MEK1/2, and -ERK1/2, as well as total AKT1, MEK1/2, and ERK1/2 were detected by Western blotting using specific antibodies. The expressions of AKT1, MEK1/2, ERK1/2, phosphorylated-AKT1, -MEK1/2, and -ERK1/2 decreased in cells after treatment with 30 μM CAPE. (B) The quantitative results on the level of specific protein were presented. *p<0.05, **p<0.01, compared to the control group.

Animal study

In hypercholesterolemic rabbits not subjected to balloon injury, intimal thickening was observed (Fig. 6A). After balloon injury, intimal thickening became more prominent in the vessel wall (Fig. 6 B) and more severe followed by IABC local saline infusion (Fig. 6C). However, after CAPE infusion, intimal thickening decreased significantly (Fig. 6D). The percentages of intimal thickening in the aforementioned groups was 48.2 ± 2.89, 70.0 ± 1.29, 98.3 ± 0.41 and 55.0 ± 0.12, respectively (Fig. 6E)

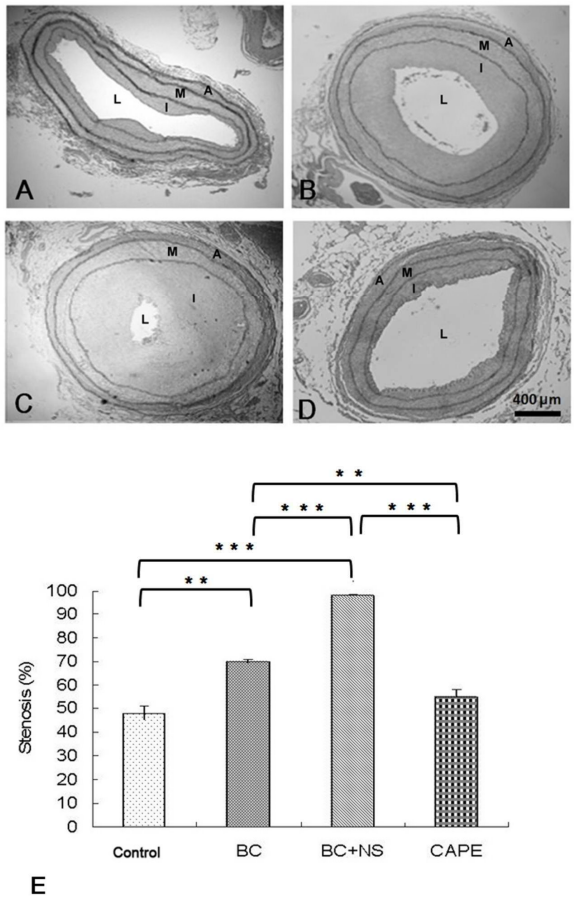

Figure 6. Cross sections of iliac arteries from non-operated rabbits (A), rabbits subjected to balloon injury only (B), rabbits after balloon injury followed by IABC local normal saline infusion (C), and rabbits after balloon injury followed by IABC local CAPE infusion (D). In rabbits fed with high-fat diet, a partial intimal hyperplasia was observed (A); after balloon injury, a more severe hyperplasia was observed (B). After balloon injury followed by IABC local normal saline infusion, severe hyperplasia was noticed (C). However, after IABC local CAPE infusion, intima thickening was decreased significantly (D). Quantitative analysis indicated that the percentage of area with intima thickening in four groups were 48.0 ± 2.89, 70.0 ± 1.29, 98.3 ± 0.41 and 55.0 ± 0.12, respectively (E). **p<0.01, ***p<0.001. L: lumen, I: intima, M: media, A: adventatia, Bar: 400 μm.

DISCUSSION

CAPE has been shown to have various biological activities in cancer cell lines. Its effect on the primary culture, however, is not fully understood. In the present study, we clearly demonstrated that CAPE treatment above 3 μM potently inhibited proliferation of HCSMCs. The anti-proliferative effect of CAPE was not due to apoptosis (data not shown). To further investigate the pattern of the antiproliferative and antimigration effects of CAPE on HCSMCs, flow cytometry analysis was performed. The results showed that the anti-proliferative effect was associated with an increase in cell population in the S phase of cell cycle. Although CAPE treatment (30 μM) for 24 h had no effect, it has been reported that cell cycle arrest occurred in the G0/G1 phase after CAPE treatment at a concentration of 25 μM and 50 μM for 24 h in the C6 glioma cells (11). The present study clearly suggests that cell cycle arrest by CAPE is involved in the growth inhibition of HCSMCs. The inhibition of cell growth occurs as early as at 6-hr CAPE treatment, but the flow cytometry study indicates that the cell cycle was arrested 48-hr CAPE treatment. The reason for this discreptancy is because it takes time for the cells to accumulate at the various phases of the cell cycle after CAPE treatment, Several other studies also demonstrated that the changes in the distribution of cell cycle occurred until 24 to 48 hr after treatment.

Western blot showed that CAPE inhibited not only the expression of Cyclin A, Cyclin E, CDK2 and PCNA but also the downregulation of phosphorylated AKT1, MEK1/2, and ERK1/2 signaling molecules. It has been demonstrated that ERK1/2 plays an essential role in the transition of vascular SMCs from a contractile to a synthetic phenotype (19). MEK and ERK are important signaling molecules mediating the activities of cell proliferation and cell migration. Furthermore, AKT, which is down stream of PI3K, is a major mediator for growth factors in cell survival. Increased SMC proliferation and migration are major events associated with the development of neointimal formation. The present study indicated that CAPE significantly down-regulated AKT 1, MEK1/2 and ERK1/2 phosphorylation, thereby potentially inhibiting the proliferation and migration of HCSMCs. From the western blot data, the decrease in the amount of phosphorylated protein after CAPE treatment is much more prominent than those in the total protein. We speculate that the decreasing phosphorylated signaling protein does not completely result from their decreasing total protein. This issue, however, needs to be further investigated.

It has been reported that downregulated SMC apoptosis is a key contributor to intimal lesions (1, 22), however, the present study showed that CAPE did not induce apoptosis in HCSMCs up to 90 μM in vitro (data not shown). It has been reported that CAPE is an apoptosis-inducing agent in human leukemic cell line HL-60 cells and in the colorectal tumor cell line SW480 (23). Therefore, it appears that the mechanisms of CAPE in the inhibition of cell growth in culture may vary with cell type.

In the animal experiments, using IABC for local CAPE delivery to the rabbit iliac artery, we demonstrated that CAPE exhibited its potential to inhibit intimal thickening 3 weeks after intervention. No significant differences of intimal thickening were observed between CAPE-infused animals and control animals. Neither inflammatory nor systematic side effects occurred at any time point during the study. Apparently, IABC-based CAPE delivery has prominent advantages over the drug eluting stent which puts patients at risk for sub-thrombus due to delayed or limited re-endothelization (4, 20). The results showed that 100 μM CAPE could reduce the stenosis in the animal study. Whether the reduction in stenosis by CAPE in the vessel wall was a result of apoptosis or other mechanisms involved is an interesting issue and needs to be further investigated.

In summary, CAPE has potent inhibitory effects on HCSMC proliferation and migration in vitro, as a result of cell cycle arrest. Furthermore, CAPE treatment markedly decreased the expression of cell cycle genes and decreased phosphorylated AKT, MEK, and ERK associated signaling pathways which modulate the proliferation and migration of HCSMCs. Preliminary animal studies indicate that intimal thickening induced by the combination of high-fat diet and balloon injury could be regressed by IABC infusion with CAPE. This novel finding may enable us to innovate a therapeutic approach to reduce atherosclerotic progression and the occurrence of restenosis.

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