学位論文（博士）

The different effects of acacetin and biochanin A on SPC-induced abnormal vascular smooth muscle contraction
(SPC誘発性血管平滑筋の異常収縮に対するアカセチンとビオカニン Aの異なる効果)

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Abstract

Unlike Ca^{2+}-dependent normal vascular contraction, the Rho-kinase-mediated Ca^{2+}-independent abnormal vascular contraction mediates cerebral and coronary vasospasm. As an upstream key molecule of such abnormal Ca^{2+}-independent vasocontraction, we identified sphingosylphosphorylcholine (SPC) and Fyn tyrosine kinase. The ideal therapeutic agent for vasospasm is to specifically inhibit Ca^{2+}-independent contraction without affecting Ca^{2+}-dependent one. We previously found the ideal drug eicosapentaenoic acid (EPA) and indeed EPA clinically suppressed human vasospasm after subarachnoid hemorrhage. However, lipophilic EPA can't be administered intravenously. Therefore, it's urgent to find a new water-soluble compound with the same effects as EPA.

We screened plant-derived compounds and focused on flavonoids, acacetin and its structural isomer biochanin A which is only different in the position of phenyl group in chemical structure. Acacetin slightly inhibited 40 mM K^{+}-induced Ca^{2+}-dependent contraction in porcine coronary vascular smooth muscle (VSM) strips, but inhibited the SPC-induced Ca^{2+}-independent contraction fast and strongly. In contrast, biochanin A inhibited 40 mM K^{+}-induced contraction more strongly than the SPC-induced contraction. Pre-incubation of acacetin and biochanin A showed inhibitory effect on SPC and 40 mM K^{+}-induced contractions indicating that they had a superior preventive effect on vascular contractions. The morphological changes

induced by SPC in human coronary smooth muscle cells were also inhibited by acacetin and biochanin A. Both acacetin and biochanin A strongly inhibited SPC-induced Rho-kinase activation and myosin light chain phosphorylation.

In summary, the difference in the position of the phenyl group on acacetin and biochanin A is involved in the inhibitory effect on SPC-induced abnormal contraction.

Key words: acacetin, biochanin A, SPC, vascular smooth muscle, Rho-kinase

Introduction

The number of deaths due to the fatal vascular diseases such as angina, myocardial infarction and cerebral infarction is almost the second leading cause of death following cancer. The pathophysiological reason of these diseases is not the abnormality of the heart or brain, but the abnormal contraction of blood vessel perfusing these organs. We previously discovered that Rho-kinase plays a pivotal role in the Ca^{2+}-sensitization of smooth muscle contraction, as associated with Ca^{2+}-independent myosin light chain phosphorylation[1)]. While Ca^{2+}-dependent contraction plays a major role in the regulation of physiological blood pressure and circulation, Rho-kinase-mediated Ca^{2+}-independent abnormal contraction causes cerebral vasospasm after subarachnoid hemorrhage and vasospastic angina. We also found sphingosylphosphorylcholine (SPC) is the novel messenger for Rho-kinase-mediated Ca^{2+}-sensitization of arterial smooth muscle[2)]. The ideal

therapeutic agent for vasospasm is to specifically inhibit Ca^{2+}-independent abnormal contraction with little effects on the Ca^{2+}-dependent contraction regulating the normal blood pressure and circulation. We previously found that eicosapentaenoic acid (EPA), an n-3 polyunsaturated fatty acid, specifically inhibited the SPC-induced abnormal contraction without affecting the 40 mM K^{+}-induced contraction in the porcine coronary artery[3)], which satisfied our purpose of treating vasospasm. However, EPA has at least two disadvantages, 1) supply instability and 2) limited administration method. The problem of supply instability is that most of EPA production depends on the purification from fish oil, while overfishing, marine pollution and climate change may influence the quality of fish. The problem with the administration method is that EPA can be administered only orally because of its lipophilicity, therefore it cannot be administered intravenously and thereby it is impossible to save unconscious patients. To solve these problems, it is necessary to find the new water-soluble compound as a therapeutic agent for vasospasm derived from plants which has a stable supply.

Flavonoids are a type of polyphenol and a general term for plant pigments of a group of naturally organic compounds. Many of them have physiological functions for the human and widely distributed in plants. According to the studies in recent years, it has been proved that flavonoids have excellent protective effects against cancer, cardiovascular diseases and osteoporosis[4)]. However, studies of flavonoids on the SPC-induced abnormal vascular contraction has not been clarified yet. In this

study, we focused on the structural isomers acacetin and biochanin A of flavonoids. Acacetin is found in various plants, such as black locust, damiana, birch and safflower species[5)]. Biochanin A is found in red clover, soybeans, peanuts, chickpeas, and other legumes[6)]. We found that acacetin and biochanin A inhibited Ca^{2+}-independent abnormal contraction of VSM caused by SPC and Ca^{2+}-dependent contraction caused by high potassium depolarization in varying degrees and the difference in the position of the phenyl group on acacetin and biochanin A may be involved in the inhibitory effect on the SPC-induced abnormal contraction.

Purpose

In this study, we aimed to find the new plant-derived compounds which were water-soluble and were readily available as a therapeutic agent for vasospasm patients. We tried to explore the effects of acacetin and biochanin A, which are only different in the position of phenyl ring in their chemical structures, on the SPC- and 40 mM K^{+}-induced contractions.

Methods

1. Materials and reagents

Biochanin A (purity ≥ 97%) and acacetin (purity ≥ 97%) were purchased from Sigma-Aldrich (USA). The compounds were dissolved in 100% DMSO to make a 40

mM stock solution to store at -20°C and diluted to final concentration before use. SPC was purchased from Enzo Life Sciences Inc (USA), Bradykinin (BK) was obtained from Peptide Research Institute (Osaka, Japan). All the other reagents were purchased from Katayama and Wako (Japan).

2. Methods

1) Tissue preparation

All the procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University and were conducted in conformity with institutional guidelines. Porcine left anterior descending coronary arteries were obtained from a local public abattoir (Kitakyushu Municipal Meat Inspection and Control Center, Japan). Tissue specimens were placed into ice-cold Krebs solution (123 mM NaCl, 4.7 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 11.5 mM D-glucose) and transported to the laboratory. All solutions were treated with a mixture of 5% CO_2 and 95% O_2 (pH adjusted to 7.4 at 4°C). The fat tissue and adventitia were removed from the arteries with scissors, and the tunica intima was gently scraped off with swabs and the arterial rings were cut into strips (0.7 mm × 4 mm). The complete removal of the endothelium from strips was confirmed by the lack of relaxation response to 1 μM BK.

2) Measurement of the isometric tension in porcine coronary vascular smooth muscle (VSM) strips

Tension measurement was performed in the same way as described in the previous paper[7-10]. Smooth muscle strips were placed perpendicularly into the 8-chamber organ bath LE01086 (Panlab Harvard device, Spain) filled with Krebs solution. The solution was gassed with a mixed gas (5% CO_2 and 95% O_2) and maintained at 37°C during the whole experiment. The force converter TB-612T (Nippon Photoelectric) was used to measure the isometric force. With adjusting the resting tension, 118 mM K^+ (10.9 mM NaCl, 116.8 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO4, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 11.5 mM D-glucose) and Krebs solutions were alternately applied until 118 mM K^+ induced the maximum tension. Effects of the compounds on the force were examined at the steady state of the pre-contraction induced by 30 μM SPC or 40 mM K^+ (88.9 mM NaCl, 38.8 mM KCl, 15.5 mM $NaHCO_3$, 1.2 mM KH_2PO4, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, and 11.5 mM D-glucose). The inhibitory extent of contraction induced by the drugs was described as a percentage of the response compared to the contractions induced by 30 μM SPC or 40 mM K^+. The pretreatment-induced contractions were normalized by the ratio compared with 118 mM K^+ depolarization-induced contraction.

3) Time-lapse recording of human coronary smooth muscle cells (HCASMCs) contraction

HCASMCs (Kurabo, Osaka, Japan) were cultured in HuMedia-SG2 (Kurabo, Osaka, Japan) containing 5% fetal bovine serum (FBS), 0.5 ng/ml human epidermal

growth factor (hEGF), 2 ng/mL human fibroblasts growth factor-B (hFGF-B), 5 μg/mL insulin, 50 μg/mL gentamycin and 50 ng/mL amphotericin B. HCASMCs were used for experiments within 3-9 passages after the initiation of culture. When the cell confluence reached 80-90%, FBS and growth factor-free HuMedia-SB2 (Kurabo, Osaka, Japan) medium was changed to obtain the contractile phenotype of HCASMCs. After treatment with HuMedia-SB2 for 24 h, cells were pretreated with or without 40 μM acacetin and biochanin A for 30 min at 37°C in the incubator. Then, 30 μM SPC was added to the medium and time-lapse recording of HCASMCs contraction was performed with the phase-contrast microscope (KEYENCE BZ9000, Osaka, Japan) in every 32 seconds for 10 minutes and 40 seconds.

4) Western blot

Porcine coronary arterial pieces were divided into six groups: (1) no pretreated, without SPC stimulation, (2) biochanin A-pretreated (40 μM, 30 min), without SPC stimulation, (3) acacetin-pretreated (40 μM, 30 min), without SPC stimulation, (4) no pretreated, with SPC stimulation (30 μM, 15 min), (5) biochanin A-pretreated (40 μM, 30 min), with SPC stimulation (30 μM, 15 min), (6) acacetin-pretreated (40 μM, 30 min), with SPC stimulation (30 μM, 15 min), and each group was treated according to the experimental group. Those arterial pieces were then quickly immersed in 5% trichloroacetic acid (TCA) for 10 min and washed twice with chilled 10 mM DL-dithiothreitol (DTT)/acetone to remove TCA. Liquid nitrogen was used to freeze

the samples, and SK-Mill Freeze-Crush Apparatus (Diagnocine, USA) was used to smash the sample to powder in tubes in liquid nitrogen. RIPA buffer (Wako, Japan) was added to lysis the tissue samples. The tissue lysates were centrifuged at 12,000 rpm/min at 4°C and the supernatants were collected and subjected to western blot. 30 μg protein was loaded and separated by 10% SDS-PAGE and subjected to immunoblotting with appropriate antibodies against myosin phosphatase target subunit 1 (MYPT1) (Santa Cruz, USA), phospho-Thr853 of MYPT1 (Santa Cruz, USA), myosin light-chain (MLC) (Santa Cruz, USA), phospho-Ser19 MLC (Cell signaling, USA). All the primary antibodies were diluted 1: 1,000 with 5% skim milk in Tris-buffered saline-0.05% Tween-20 (TBS-T). The secondary antibodies were diluted 1:5,000 with TBS-T. The signal was visualized using the Super Signal West Pico (Thermo Fisher, USA) chemical luminescent substrate and evaluated using software named Quantity One with ChemiDoc XRS-J (Bio-Rad, USA).

3. Statistics

The data were analyzed with Excel and expressed as mean ± SEM, and n represented the number of the assays. Student's t-test was used to determine the statistical significance between the two groups. Tukey-Kramer was used to analyze multiple groups. P-values < 0.05 was defined to be statistically significant.

Results

1. Effects of acacetin and biochanin A on the SPC-induced Ca^{2+}-independent abnormal contraction and 40 mM K^{+}-induced Ca^{2+}-dependent contraction

The structural formulas of acacetin and biochanin A are shown in Figure 1A and 1B. Acacetin belongs to flavone (Figure 1C) and biochanin A is the structural isomer of acacetin belonging to isoflavone (Figure 1D). The only difference between the two compounds is the position of the phenyl ring. The effect of this small structural difference on the effect of VSM contraction is significantly different. The traces in Figure 2A - D showed the typical inhibitory effects of 40 μM acacetin and 40 μM biochanin A on SPC or 40 mM K^{+}-induced contractions. 20 - 80 μM acacetin and biochanin A inhibited SPC and 40 mM K^{+}-induced contractions in a dose-dependent manner (Figure 2E, F). 40 - 80 μM acacetin inhibited the SPC-induced contraction significantly stronger than 40 mM K^{+}-induced contraction. But 20 μM biochanin A significantly inhibited 40 mM K^{+}-induced contraction stronger than the SPC-induced contraction, and 40 - 80 μM biochanin A inhibited SPC and 40 mM K^{+}-induced contractions to the same extent (Figure 2E, F). Therefore, acacetin was shown to specifically inhibit the SPC-induced abnormal vascular contraction in post-administration experiments.

2. Effects of pre-incubating acacetin and biochanin A on SPC and 40 mM K^{+}-induced contractions of VSM

Due to the aim of studying on the preventive effect of acacetin and biochanin A

on the VSM contraction, we also investigated the effects of pretreatment of acacetin and biochanin A on SPC and 40 mM K^+-induced contractions in porcine artery VSM strips. Figure 3 showed that pretreatment with acacetin or biochanin A significantly inhibited both the SPC and 40 mM K^+-induced contractions. After pretreating with acacetin, SPC stimulation caused a smaller contraction compared to pretreating with biochanin A, which was consistent with the effects of post-administration. Both pretreatment with acacetin and biochanin A inhibited the 40 mM K^+-induced contraction which was different from the effects of post-administration.

3. Effects of acacetin and biochanin A on the SPC-stimulated morphological changes of HCASMCs

The inhibitory effects of acacetin and biochanin A on the SPC-induced abnormal contraction were clarified in porcine coronary arterial strips (tissues), and the effects could also be confirmed in HCASMCs. In the SPC group, the morphology of HCASMCs changed from spindle shape to round in a time-dependent manner, while the cell-free areas expanded at the same time. After the pre-incubation of acacetin and biochanin A, HCASMCs had a delayed rate of morphological changes to SPC stimulation compared with the SPC group. Therefore, the inhibitory effects of acacetin and biochanin A on the SPC-induced contraction were observed not only in the porcine coronary artery tissue (Figure 2, 3), but also in human cells (Figure 4). These findings suggest that the inhibitory effects of acacetin and biochanin A on the

SPC-induced contraction are preserved between species.

4. Effects of acacetin and biochanin A on the SPC-induced Rho-kinase activation and MLC phosphorylation

Previous studies showed that SPC activated Rho-kinase, then Rho-kinase phosphorylated MYPT1 which is a regulatory subunit 1 of myosin-light chain phosphatase, and enhanced Ca^{2+} sensitization to induce the contraction of VSM[2, 11-13)]. In this study, we performed western blot experiments on the phosphorylation of MYPT1 (phospho-thr-853) to read out the Rho-kinase activity. It was evaluated whether the SPC-induced Rho-kinase activation was inhibited by acacetin and biochanin A or not[14, 15)]. 30 μM SPC significantly increased the phosphorylation level on Thr-853 of MYPT1. But the phosphorylation of MYPT1 on Thr-853 was significantly inhibited by both acacetin and biochanin A pretreatment (30 minutes), and the inhibitory degree was more obvious in acacetin group. The phosphorylation of myosin light chain (MLC) on Ser-19 was measured at the same time (Figure 5). As expected, both acacetin and biochanin A inhibited the SPC-induced phosphorylation at the site Ser-19 of MLC in the same way as they inhibited phosphorylation of MYPT1 (Figure 5). These results were also consistent with the results of post- and pre-administration experiments (Figure 2 and 3).

Discussion

In the treatment of vasospasm, the vasodilator Ca^{2+} channel antagonist is contraindicated because it may cause hypotension and exacerbate the condition of the patients. Therefore, acacetin, which selectively inhibited the SPC-induced contraction, is recommended as a candidate for the treatment of vasospasm rather than biochanin A, which inhibited 40 mM K^+-induced contraction more effectively.

Hypertension has been shown as a risk factor for subarachnoid cerebral aneurysm rupture, and the Stroke Treatment Guidelines 2015 clearly stated that the improvement of hypertension was strongly recommended for the patients. The most recommended drug for hypertension is Ca^{2+} channel antagonists, which have the effect of inhibiting the 40 mM K^+-induced Ca^{2+}-dependent contraction. In pretreatment experiments, 40 μM acacetin and 40 μM biochanin A were pretreated with the VSM strips for 30 min. Both of them significantly inhibited 40 mM K^+ and the SPC-induced contractions compared to the control group (Figure 3). These findings suggest both acacetin and biochanin A have the capability to inhibit the hypertension in the case of prophylactic administration for vasospasm after subarachnoid hemorrhage. In hypertensive patients, it may be possible to prevent subarachnoid hemorrhage itself. Similar to the results of post-administration in Figure 2, acacetin had a higher inhibitory ratio on the SPC-induced contraction than 40 mM K^+-induced contraction, and biochanin A had the opposite effect.

Acacetin is known to have anti-inflammatory, cardioprotective, and antitumor

effects[5]. AMPK activation is known to be one of the mechanisms of acacetin[16, 17]. In recent years, plant-derived natural compound madagascine has been reported to suppress the SPC/Rho-kinase pathway through AMPK activation[18, 19]. Therefore, acacetin may also inhibit the SPC/Rho-kinase pathway through AMPK activation. Biochanin A is also known to have anti-inflammatory, antitumor, and estrogen effects, but as far as we know, biochanin A has not been reported to activate AMPK or Rho-kinase.

Nakao et al. of our lab suggested that the SPC-induced Ca^{2+}-sensitization in porcine coronary artery was mediated specifically by Src family tyrosine kinase (Src-TK) and showed that SPC induced the translocation of Fyn, a member of Src-TK, from cytosol to cell membrane. But the translocation of Rho-kinase and Fyn from the cytosol to the cell membrane induced by SPC were inhibited by EPA and PP1 (an SrcPTKs inhibitor)[3]. As acacetin had the similar effect with EPA which selectively inhibited the SPC-induced Ca^{2+}-sensitization of VSM contraction with little effect on Ca^{2+}-dependent contraction, we doubted that acacetin might also inhibit the translocation of Rho-kinase and Fyn from the cytosol to the cell membrane induced by SPC, which in turn affected the activity of Rho-kinase. But the further experiments should be done to prove it.

Conclusion

Acacetin selectively inhibited the SPC-induced Rho-kinase-mediated Ca^{2+}-independent vascular abnormal contraction compared to biochanin A. The position of the phenyl group in the structural isomers acacetin and biochanin A might explain the different effects of acacetin and biochanin A on the SPC-induced Ca^{2+}-independent abnormal VSM contraction and the inhibition of Rho-kinase activation.

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References

1. Kureishi Y, Kobayashi S, Amano M, et al. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *The Journal of Biological Chemistry.* 1997;272(19):12257-12260.

2. Shirao S, Kashiwagi S, Sato M, et al. Sphingosylphosphorylcholine is a novel messenger for

Rho-kinase-mediated Ca^{2+} sensitization in the bovine cerebral artery: unimportant role for protein kinase C. *Circulation Research.* 2002;91(2):112-119.

3. Nakao F, Kobayashi S, Mogami K, et al. Involvement of Src family protein tyrosine kinases in Ca^{2+} sensitization of coronary artery contraction mediated by a sphingosylphosphorylcholine-Rho-kinase pathway. *Circulation Research.* 2002;91(10):953-960.

4. Perez-Vizcaino F, Fraga CG. Research trends in flavonoids and health. *Archives of Biochemistry and Biophysics.* 2018;646:107-112.

5. Singh S, Gupta P, Meena A, et al. Acacetin, a flavone with diverse therapeutic potential in cancer, inflammation, infections and other metabolic disorders. *Food and Chemical Toxicology.* 2020;145:111708.

6. Liu X, Wang T, Liu X, et al. Biochanin A protects lipopolysaccharide/D-galactosamine-induced acute liver injury in mice by activating the Nrf2 pathway and inhibiting NLRP3 inflammasome activation. *International Immunopharmacology.* 2016;38:324-331.

7. Todoroki-Ikeda N, Mizukami Y, Mogami K, et al. Sphingosylphosphorylcholine induces Ca^{2+}-sensitization of vascular smooth muscle contraction: possible involvement of rho-kinase. *FEBS Letters.* 2000;482(1-2):85-90.

8. Mogami K, Mizukami Y, Todoroki-Ikeda N, et al. Sphingosylphosphorylcholine induces cytosolic Ca^{2+} elevation in endothelial cells in situ and causes endothelium-dependent relaxation through nitric oxide production in bovine coronary artery. *FEBS Letters.* 1999;457(3):375-380.

9. Omura M, Kobayashi S, Mizukami Y, et al. Eicosapentaenoic acid (EPA) induces

Ca^{2+}-independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation. *FEBS Letters.* 2001;487(3):361-366.

10. Mogami K, Kishi H, Kobayashi S. Sphingomyelinase causes endothelium-dependent vasorelaxation through endothelial nitric oxide production without cytosolic Ca^{2+} elevation. *FEBS Letters.* 2005;579(2):393-397.

11. Choi SK, Ahn DS, Lee YH. Comparison of contractile mechanisms of sphingosylphosphorylcholine and sphingosine-1-phosphate in rabbit coronary artery. *Cardiovascular Research.* 2009;82(2):324-332.

12. Zhang Y, Zhang M, Lyu B, et al. Omega-3 and omega-6 DPA equally inhibit the sphingosylphosphorylcholine-induced Ca^{2+}-sensitization of vascular smooth muscle contraction via inhibiting Rho-kinase activation and translocation. *Scientific Reports.* 2017;7:36368.

13. Xu D, Kishi H, Kawamichi H, et al. Involvement of Fyn tyrosine kinase in actin stress fiber formation in fibroblasts. *FEBS Letters.* 2007;581(27):5227-5233.

14. Szasz T, Webb RC. Rho-Mancing to Sensitize Calcium Signaling for Contraction in the Vasculature: Role of Rho Kinase. *Advances in Pharmacology.* 2017;78:303-322.

15. MacDonald JA, Walsh MP. Regulation of Smooth Muscle Myosin Light Chain Phosphatase by Multisite Phosphorylation of the Myosin Targeting Subunit, MYPT1. *Cardiovascular & Hematological Disorders-Drug Targets.* 2018;18(1):4-13.

16. Wu WY, Li YD, Cui YK, et al. The Natural Flavone Acacetin Confers Cardiomyocyte Protection Against Hypoxia/Reoxygenation Injury via AMPK-Mediated Activation of Nrf2 Signaling Pathway.

Frontiers in Pharmacology. 2018;9:497.

17. Wu WY, Cui YK, Hong YX, et al. Doxorubicin cardiomyopathy is ameliorated by acacetin via Sirt1-mediated activation of AMPK/Nrf2 signal molecules. *J Cell Mol Med.* 2020;24(20):12141-12153.

18. Chen D, Lv B, Kobayashi S, et al. Madagascine Induces Vasodilatation via Activation of AMPK. *Frontiers in Pharmacology.* 2016;7:435.

19. Yu C, Zhang P, Lou L, et al. Perspectives Regarding the Role of Biochanin A in Humans. *Frontiers in Pharmacology*. 2019;10:793.

Figure 1

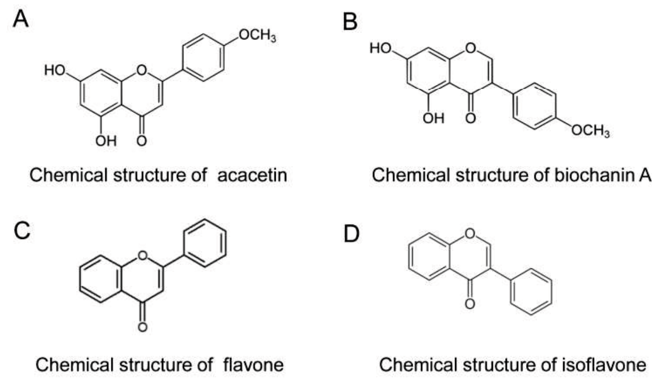

Figure 1 The chemical structures of acacetin, biochanin A, flavone, and isoflavone. (A) The chemical structure of acacetin. (B) The chemical structure of biochanin A. (C) The chemical structure of flavone. (D) The chemical structure of isoflavone.

Figure 2

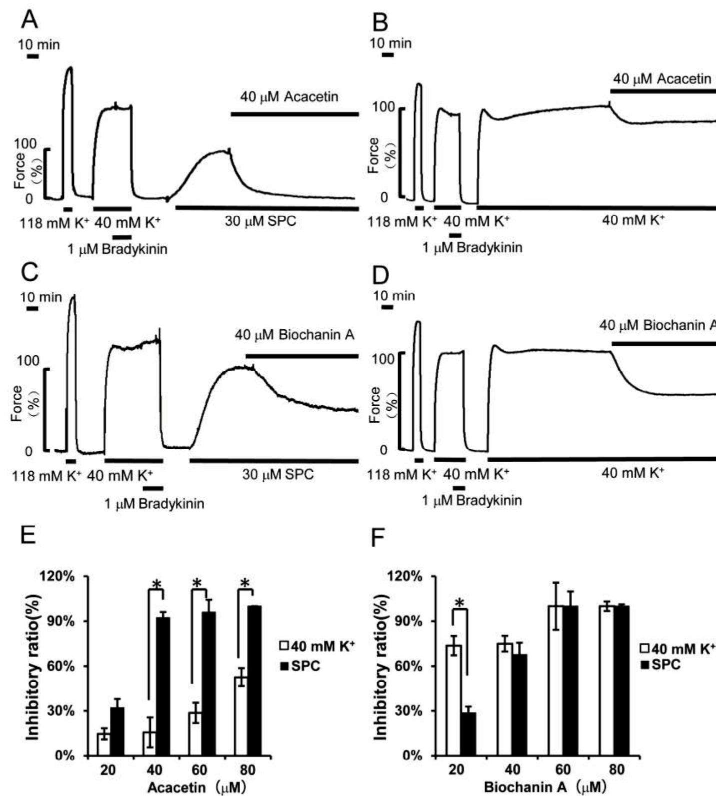

Figure 2 Inhibitory effects of acacetin and biochanin A on the SPC-induced abnormal contraction and 40 mM K^+-induced Ca^{2+}-dependent contraction in porcine coronary vascular smooth muscle strips (A-D) The effects of acacetin and biochanin A (40 μM) on the SPC-induced (30 μM) abnormal contraction or 40 mM K^+-induced contraction in vascular smooth muscle strips. (E, F) Acacetin and biochanin A inhibited the SPC-induced Ca^{2+}-independent abnormal contraction and 40 mM K^+-induced Ca^{2+}-dependent contraction in dose-response manner in vascular smooth muscle strips. Data were expressed as mean ± SEM (n = 3). The inhibitory ratio was calculated as comparing the tension before adding the compounds with the tension 1 hour after adding the compounds. * $P < 0.05$, SPC: sphingosylphosphorylcholine, BK: bradykinin, 40 mM K^+: 40 mM K^+ solution.

Figure 3

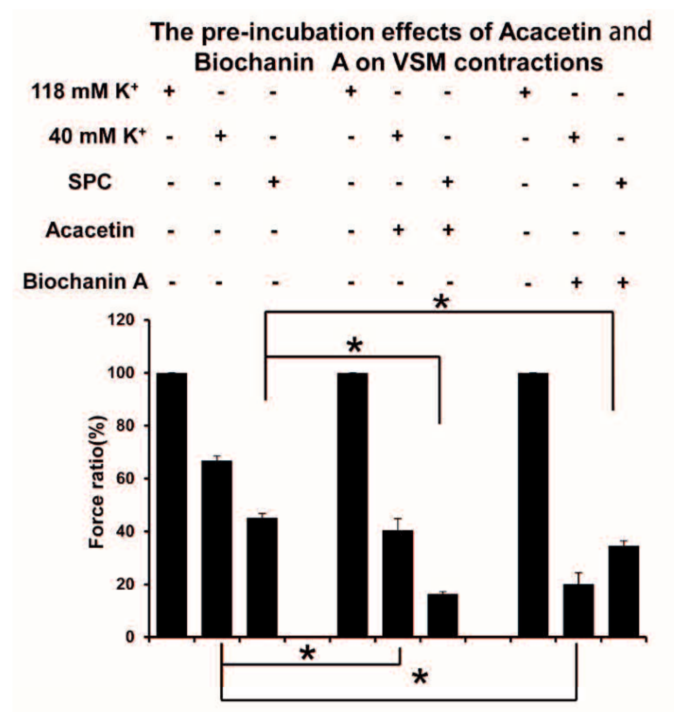

Figure 3 The pre-incubation effects of acacetin and biochanin A on SPC and 40 mM K^+-induced

contractions in porcine coronary arterial strips. The VSM strips were pretreated with acacetin or biochanin A (40 μM) for 30 min at 37°C in Krebs solution, SPC or 40 mM K^+ solutions with compounds were added to stimulate the contractions. The pre-incubation-induced contractions were normalized by the ratio compared with 118 mM K^+ depolarization-induced contraction. The statistical evaluation showed pre-incubating acacetin and biochanin A with VSM tissue inhibited the SPC and 40 mM K^+-induced contractions significantly. Data were expressed as mean ± SEM. (n = 3, *$P < 0.05$).

Figure 4

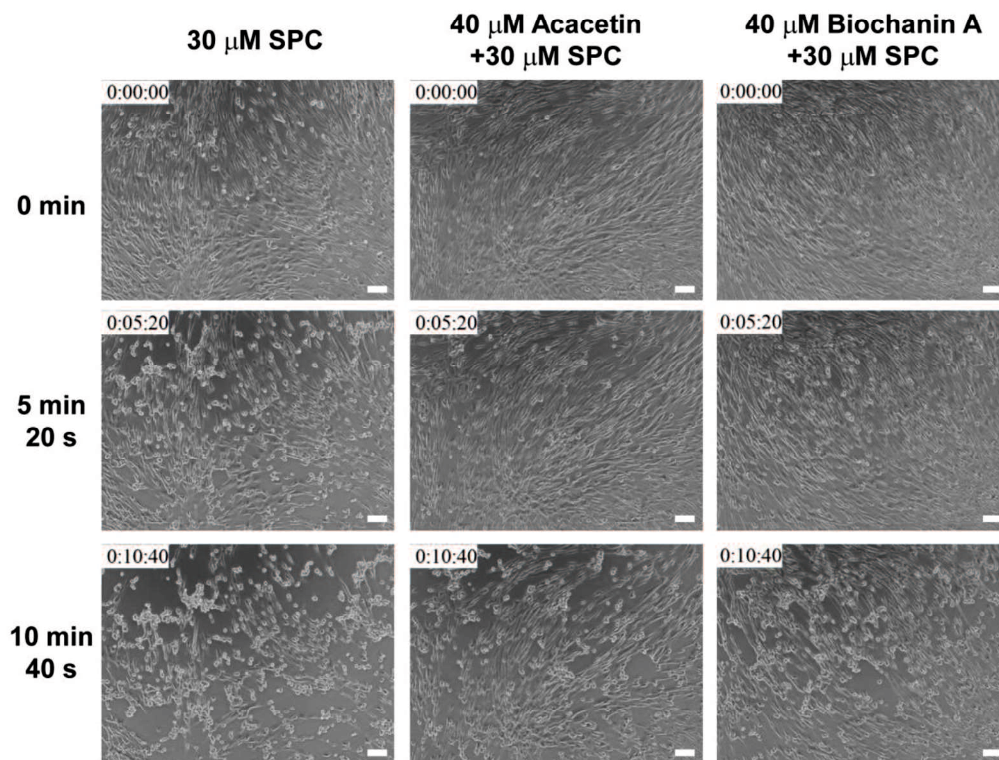

Figure 4 Acacetin and biochanin A inhibited the SPC-induced abnormal contraction in human coronary smooth muscle cells (HCASMC). HCASMCs were serum starved for 24 hours before the experiment. Pre-incubating the cells with or without acacetin and biochanin A for 30 min, and then

stimulated with SPC (30 μM). The time-lapse observation with phase-contrast microscope showed the morphological changes in cells of the control group, acacetin group, and biochanin A group. Scale bar: 100 μm.

Figure 5

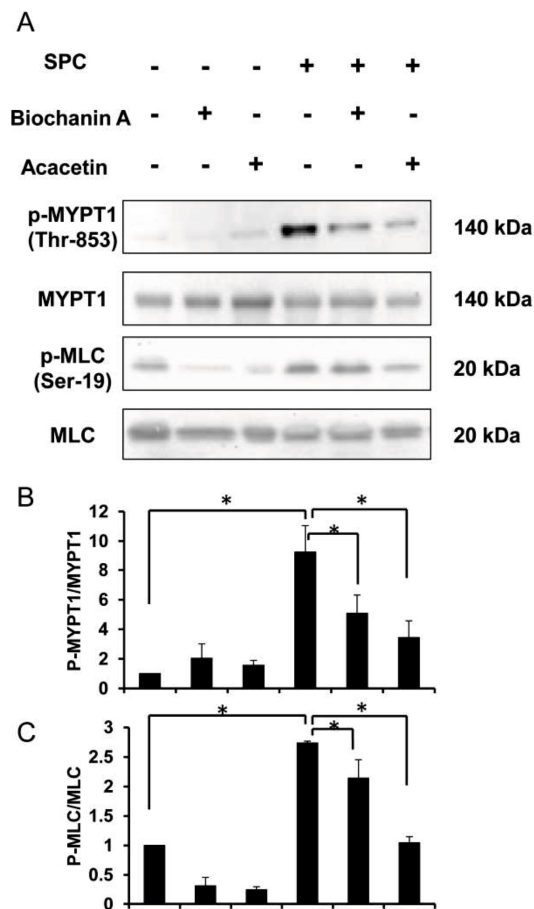

Figure 5 Acacetin and biochanin A inhibited the SPC-induced Rho-kinase activation and MLC phosphorylation in vascular smooth muscle strips. (A). After pre-incubating with acacetin or biochanin A (40 μM, 30 min) in vascular smooth muscle strips in the presence or absence of SPC (30 μM, 15 min), western blot using anti-p-MYPT1 (Thr853), anti-MYPT1,

anti-p-MLC (Ser19), anti-MLC antibodies were showed. (B,C) Statistical analysis of Rho-kinase activation (proportion of MYPT1 phosphorylation on Thr853 to total MYPT1) and MLC phosphorylation (proportion of MLC phosphorylation on Ser19 to total MLC). Data are expressed as mean ± SEM (n = 3). * P <0.05. p-MLC: Phosphorylation of myosin light chain, p-MYPT1: Phosphorylation of myosin phosphatase regulatory subunit 1.