学位論文（博士）

Doctoral dissertation

Inhibitory effects of tangeretin on the sphingosylphosphorylcholine (SPC)-induced vascular smooth muscle contraction through suppressing the Fyn/Rho-kinase signaling pathway

(Fyn / Rho-キナーゼシグナル伝達経路の抑制を介したスフィンゴシルホスホリルコリン（SPC）誘発血管平滑筋収縮に対するタンゲレチンの阻害効果)

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Major (専攻) Medicine (医学専攻)

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Contents

Abstract

We previously discovered that the SPC/Fyn/Rho-kinase (ROK) pathway mediates the Ca^{2+}-sensitization of coronary arterial smooth muscle (CASM) contraction leading to vasospasm, a major cause of sudden death. Lately, we have been trying to find and develop more natural edible compounds which can treat and/or prevent the SPC-induced abnormal CASM contraction, and finally the first to discover that tangeretin (5,6,7,8,4'-pentamethoxyflavone), a natural compound extracted from citrus plants, can inhibit the SPC-induced CASM contraction both in the pretreatment and posttreatment. In porcine CASM tissues, tangeretin showed remarkable inhibitory effects on the SPC-induced contraction with modest inhibitory effects on the high K^+-depolarization-induced Ca^{2+}-dependent contraction, both in pretreatment and posttreatment at the optimal concentrations; Regarding the mechanisms, tangeretin markedly abolished the SPC-induced cell contraction through inhibiting the SPC-induced activation and translocation of Fyn and ROK from the cytoplasm to the cell membrane in cultured CASM cells, resulting in the reduction of phosphorylation of myosin light chain. Taken together, these findings indicate that tangeretin, upon pre- or post-treatment, inhibits the SPC-induced CASM contraction through suppressing the Fyn/ROK signaling pathway, thereby suggesting that tangeretin can be a potential candidate for the treatment and/or prevention of vasospasm.

Background

Cerebral and coronary vasospasm have high mortality rates and threaten human health which need to be solved urgently [1, 2]. Rho-kinase (ROK) mediated Ca^{2+}-sensitization plays a pivotal role in the pathology of vasospasm [3, 4]. We previously found that sphingosylphosphorylcholine (SPC), a naturally occurring bioactive sphingolipid, induced the ROK-mediated Ca^{2+}-sensitization in bovine cerebral arteries [5] and porcine coronary arteries [6, 7]. Furthermore, we revealed that SPC concentration was elevated in the cerebrospinal fluid of subarachnoid hemorrhage (SAH) patients, indicating the possible role of SPC as a spasmogen [5, 8]. Moreover, our previous study indicates that the SPC-induced CASM contraction is mediated by the sequential activation of Fyn and ROK. The digitized immunocytochemical technology analysis in CASM cells elucidated that SPC can induce the translocation of Fyn and ROK, from the cytosol to cell membrane [5, 6]. The translocation of ROK from the cytoplasm to the cell membrane, is supposed to be essential for the activation of the enzyme in the SPC-induced Ca^{2+}-sensitization of cerebral and coronary artery vascular smooth muscle (VSM) contractions [6]. Activated ROK can phosphorylate myosin phosphatase targeting subunit 1 (MYPT1) to inhibit myosin light chain phosphatase (MLCP) or phosphorylate MLC directly [9], leading to continuous phosphorylation of MLC. The ROK-mediated inhibition of MLCP and MLC phosphorylation promotes actomyosin-based contractility which contributes to Ca^{2+}-sensitization of the VSM contraction [9, 10]. Therefore, the pathogenic mechanism of the SPC-induced Ca^{2+}-sensitization of CASM contraction by mediating the Fyn/ROK signaling pathway has been investigated extensively. Representative ROK inhibitors such as fasudil, which can

effectively treat cerebral vasospasm by inhibiting ROK activity, have been widely used in clinical practice [11, 12]. Based on the trend of returning to nature, using natural medicines, the development and clinical practice of natural medicines are still the focus of global attention. Therefore, we are committed to finding and developing more natural and easily available edible compounds that can effectively inhibit the Fyn/ROK pathway, leading to the treatment and/or prevention of the SPC-induced abnormal contraction of CASM.

Epidemiological studies have shown that higher intake of flavonoids is negatively correlated with higher risk of coronary heart disease [13-15]. Tangeretin (5,6,7,8,4'-pentamethoxyflavone), concentrated in the peel of citrus fruits, has shown relatively few studies on the treatment of vascular diseases. Tangeretin suppresses platelet-derived growth factor (PDGF)-induced proliferation and migration of rat aortic smooth muscle cells through PI3K/AKT signaling pathway, expecting to prevent atherosclerosis [16]. In addition, tangeretin ameliorates oxygen‐glucose deprivation‐induced human brain microvascular endothelial cell injury through the JNK signaling pathway, therefore it might be used as a therapeutic strategy for ischemia‐reperfusion brain injury [17]. Accordingly, it is worthwhile to investigate whether tangeretin can effectively treat and/or prevent the SPC-induced CASM contraction. Herein, we hypothesized that tangeretin has an inhibitory effect on the SPC-induced Ca^{2+}-sensitization of CASM contraction. We demonstrated that tangeretin inhibits the SPC-induced CASM contraction with little or no inhibitory effects on the Ca^{2+}-dependent contraction through suppressing the Fyn/ROK signaling pathway, suggesting that tangeretin can be a potential candidate for the treatment and/or prevention of vasospasm.

Aim

Our study aimed to explore whether tangeretin has an inhibitory effect on the SPC-induced Ca^{2+}-sensitization of VSM contraction. Futhermore, whether tangeretin can inhibit the SPC-induced VSM contraction with little or no inhibitory effects on the Ca^{2+}-dependent contraction through suppressing the Fyn/ROK signaling pathway. We would like to contribute some new ideas that tangeretin could be a potential candidate for the treatment and/or prevention of vasospasm.

Methods

Subjects

The protocols were followed Institutional Ethics Committee of Yamaguchi University. The porcine (age: 5-6 months; weight: 120 ± 20 kg). Porcine coronary arteries (20 to 30 mm from the origin of the proximal portion of left anterior descending arteries) were obtained from Kitakyushu Municipal Meat Center. Human coronary artery smooth muscle cells (CASMCs) were purchased from Kurbao Industries, Ltd. (Osaka, Japan).

Methods

Reagents and antibodies

Tangeretin (purity ≧ 98%) was purchased from Wako, Osaka, Japan, and was dissolved in dimethyl sulfoxide to make a 10 mM stock solution, stored at −80°C. Sphingosylphosphorylcholine (SPC) and bradykinin (BK) were purchased from Biomol (Plymouth Meeting, PA, USA) and Peptide institute, Inc. (Osaka, Japan) respectively.

The following antibodies were used: anti-phospho-MYPT1 (Thr853) (#36-003, Merck Millipore, CA, USA), anti-MYPT1 (#sc-514261, Santa Cruz, Dallas, USA), anti-GAPDH monoclonal antibody (#016-25523, Wako, Osaka, Japan), anti-phospho-myosin light chain 2 (Ser19) monoclonal antibody and anti-Phospho-Src Family (Tyr416) antibody (#3671 and #2101, Cell Signaling, MA, USA), anti-myosin light chain monoclonal antibody (#M4401, Sigma Aldrich, St. Louis, MO), anti-ROCKII antibody and anti-Fyn antibody (#610623 and #610613, BD transduction laboratories, Null, USA), anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 546 (#A32723 and #A-11010, Invitrogen, Waltham,

USA). Secondary HRP-conjugated antibodies (anti-mouse and anti-rabbit) were purchased from Promega (#W4021 and #W4011, Madison, WI).

Cell culture

Human coronary artery smooth muscle cells (CASMCs) were grown in HuMedia SG2 (Kurabo, Osaka, Japan) which contains 5% fetal bovine serum (FBS), 0.5 ng/ml human epidermal growth factor (hEGF), 2 ng/ml human fibroblast growth factor-B (hFGF-B), 5 μg/ml insulin, 50 μg/ml gentamycin, and 50 ng/ml amphotericin B. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Between the fourth and ninth passages of human CASMCs were used in the present experiments.

Force recording of CASM

All procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University and were conducted in conformity with institutional guidelines. Porcine coronary arteries (20 to 30 mm from the origin of the proximal portion of left anterior descending arteries) were obtained from Kitakyushu Municipal Meat Center. Tissues were stored in ice-cold Krebs solution with the following composition: 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 $CaCl_2$, and 11.5 mM D-glucose, transported to our laboratory. Fat, adventitia, and endothelium were removed and then the arteries were cut into strips (1 × 4 mm). These CASM strips were mounted vertically at the organ bath filled with Krebs, gassed with mixture of 5% CO_2 and 95% O_2 (37°C). The force-transducer TB-612T (Nihon Koden, Tokyo, Japan) was used to measure isometric force. The resting tension was adjusted so that the CASM strips can develop the maximal 118 mM K^+-depolarization-induced contraction. The complete denudation of the endothelium was confirmed by the

lack of relaxation induced by 1 μM BK. Effects of tangeretin were examined at the maximum force and steady state of the precontraction induced by 30 μM SPC or 40 mM K^+. The inhibition ratio of contraction induced by tangeretin was described as a percentage of the response to the contraction induced by 30 μM SPC or 40 mM K^+.

Time-lapse recording of CASM cell contraction

Human CASMCs were starved with HuMedia SB2 (Kurabo, Osaka, Japan) for 24 h to obtain the hypercontractile type of CASMCs. Then cells were pretreated with tangeretin (1 μM, 5 μM and 10 μM) for 30 min at 37°C, subsequently, 30 μM SPC was added to the medium for 10 min. Morphological changes of human CASMCs were recorded by time-lapse recording and relative mean cell area was analyzed by Keyence BZ-II analysis application (KEYENCE, Osaka, Japan).

Immunofluorescence staining

Human CASMCs were starved with HuMedia SB2 for 24 h and tangeretin group and tangeretin + SPC group were pretreated with tangeretin (10 μM) for 30 min. Then SPC group and tangeretin + SPC group were stimulated with SPC (30 μM) for 10 min. Subsequently, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, washed with PBS (-), blocked with NanoBio Blocker (NanoBioTech, Yokohama, Japan) and then incubated with the primary antibodies such as anti-Fyn antibody or anti-ROCKII antibody, washed with PBS (-) and followed by the secondary antibody staining using AF488-conjugated anti-mouse IgG antibody and AF546-conjugated anti-rabbit IgG antibody. The nuclei were stained with DAPI (Thermo Fisher Scientific, Waltham, USA). Stained cells

were observed using fluorescent microscope (KEYENCE, Osaka, Japan). Fluorescence intensity profile analysis was performed by Keyence BZ-II analysis application.

Immunoprecipitation

Cells were lysed in immunoprecipitation (IP) buffer [RIPA Buffer (Wako, Osaka, Japan) supplemented with 100 mM Na_3VO_4, 10 mM NaF, 1/1000 Aprotinin, 1/1000 Leupeptin and 1/100 protease inhibitor cocktail]. The cell extracts (100 μg) were first pre-cleared with 1 μg control IgG, 20 μl resuspended protein A/G agarose (Santa Cruz, Dallas, USA) in IP buffer (4°C, 30 min for rotation), to precipitate nonspecific proteins. The supernatant was incubated with 1 μg of anti-Phospho-Src Family (Tyr416) antibody and protein A/G agarose at 4°C overnight for rotation, to precipitate target protein. Then, protein A/G agarose-antigen-antibody complexes were collected by centrifugation at 1,000 × g at 4°C for 5 min. Subsequently, the sediments were washed four times with IP buffer, dissolved in 1 × sodium dodecyl sulfate (SDS) loading buffer and boiled at 95°C for 5 min. Immunoprecipitated protein samples were separated by SDS-PAGE and immunoblotted with anti-Fyn antibody and p-Src (Tyr416) antibody for analysis.

Western blot analysis

The CASM tissue strips were pretreated with or without tangeretin (10 μM) in Krebs (37°C, 30 min), SPC group and SPC + tangeretin group were stimulated with SPC (30 μM, 15 min, 37°C). Then, tissue strips were pretreated with 5% TCA (Wako, Osaka, Japan) on the ice (10 min) to stop the reaction; The TCA-treated CASM tissue strips were immersed in 10 mM Dithiothreitol (Thermo Fisher Scientific, Waltham, USA) in acetone, to remove the TCA and prevent the formation of intermolecular disulfide

bonds in the sample protein. Subsequently, CASM tissue strips were crushed in the steel cylindrical device (SK mill, Tokken, Kashiwa, Japan) which is frozen by liquid nitrogen. Finally, the crushed samples were dissolved in 1 × SDS loading buffer and boiled at 95°C for 5 min for western blot analysis.

Cell samples of each group (control, tangeretin, SPC and SPC + tangeretin) were treated with the corresponding drugs and then lysed and boiled in 1 × SDS loading buffer. Tissue or cell samples were separated by SDS-PAGE (10%) and subjected to immunoblotting with proper antibodies against phospho-MYPT1 (Thr853), MYPT1, p-Tyr416 of SrcPTKs, Fyn, phospho-MLC (Ser 19), MLC and GAPDH. The bands were incubated with proper secondary antibodies and visualized by the Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo scientific, Waltham, MA) and analyzed by software named Quantity One with ChemiDoc XRS-J (Bio-Rad, California, USA).

Analysis

Statistical analyses were performed using Graph Pad Prism 8.0. The data are expressed as mean ± SD or mean ± SEM from three independent experiments. Multigroup comparisons of the means were carried out by one-way ANOVA test with post hoc contrasts by Bonferroni's test or Tukey's test. Values of $P < 0.05$ were considered statistically significant.

Results

Tangeretin inhibits the SPC-induced CASM contraction without affecting 40 mM K^+-induced Ca^{2+}-dependent contraction.

Firstly, to examine the effects of tangeretin on the SPC-induced CASM contraction in the porcine tissue strips for posttreatment, we chose four different concentrations (2.5 μM, 5 μM, 10 μM and 20 μM). As shown in Fig. 1A-C, at the optimal concentration of 2.5 μM, tangeretin exhibited an inhibition rate on the SPC-induced CASM contraction, up to 85.40 ± 8.23 %, meanwhile with the slightly inhibitory effect on the 40 mM K^+-induced Ca^{2+}-dependent contraction, only 1.53 ± 2.57 %.

Next, we examined the effects of tangeretin pretreatment on the SPC-induced contraction in the CASM tissue strips with the same four concentrations, and the statistical evaluation (Fig. 1F) showed that the inhibition rates of the SPC and 40 mM K^+-induced contraction were both dependent on the concentration of tangeretin. At 10 μM, tangeretin showed a marked inhibitory effect on the SPC-induced abnormal CASM contraction, for 72.51 ± 10.04 %, whereas the inhibition rate for the 40 mM K^+-induced normal Ca^{2+}-dependent contraction was 31.89 ± 9.74 %, showing the significant difference (Fig. 1D, E). Comprehensively, tangeretin showed remarkable inhibitory effects on the SPC-induced CASM contraction with modest inhibitory effects on the high K^+-depolarization-induced Ca^{2+}-dependent contraction, whether it was pretreatment or posttreatment at the optimal concentrations. The IC50 values for tangeretin inhibition of SPC-induced contraction were 1.13 μM and 4.20 μM, for post- and pre-treatment respectively.

Fig.1

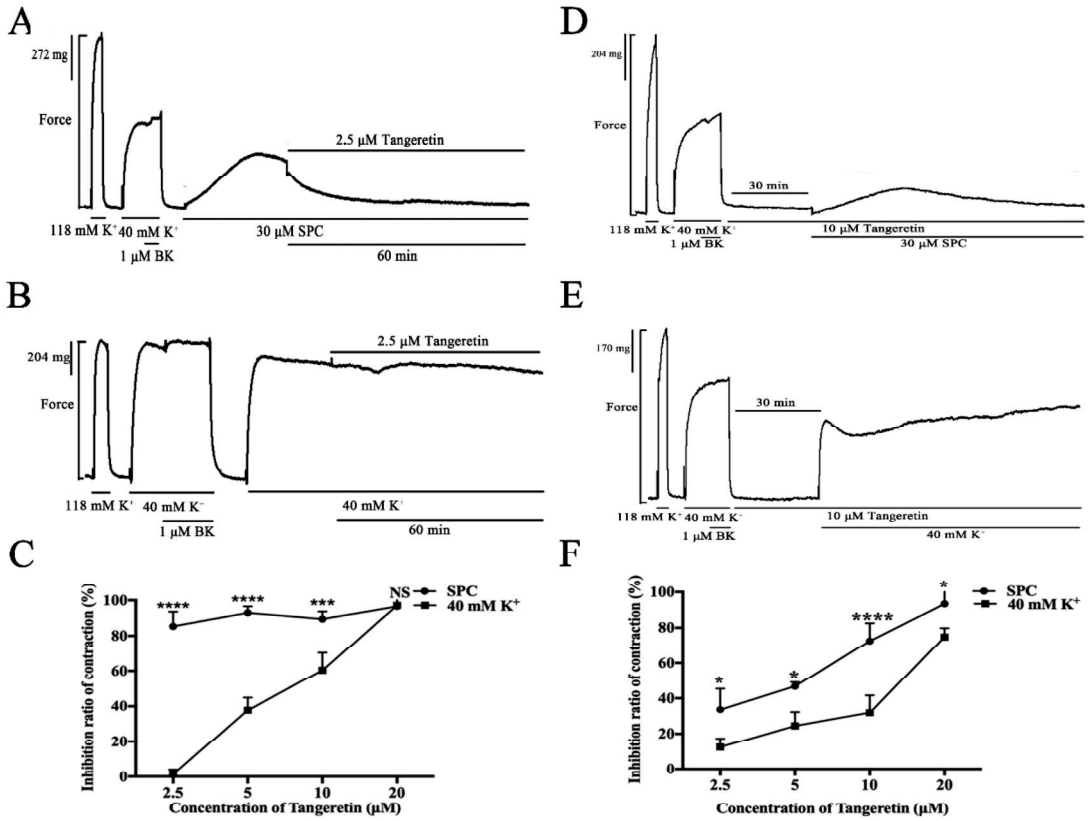


Fig. 1. Effects of tangeretin posttreatment and pretreatment on the SPC-induced CASM contraction and the 40 mM K^+-induced Ca^{2+}-dependent CASM contraction. (A-C) The inhibitory effects of tangeretin (2.5 μM) posttreatment on the SPC-induced CASM contraction (A) and the 40 mM K^+-induced Ca^{2+}-dependent contraction (B) of CASM, respectively. (C) Statistical evaluation between tangeretin concentrations and inhibition ratios of the SPC-induced and the 40 mM K^+-induced Ca^{2+}-dependent contraction of CASM for the posttreatment. (D-F) The inhibitory effects of tangeretin (10 μM) pretreatment on the SPC-induced contraction (D) and the 40 mM K^+-induced Ca^{2+}-dependent contraction (E) of CASM, respectively. (F) Statistical evaluation of dose-dependency of tangeretin concentrations and inhibition ratios of the SPC-induced Ca^{2+}-independent and the 40 mM K^+-induced Ca^{2+}-dependent contraction of CASM for the pretreatment ($n = 3 \sim 5$, $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P <$

0.0001, compared with the inhibition ratio in each 40 mM K^+ group at the same concentration).

Tangeretin abolishes the SPC-induced contraction in human CASMCs.

Next, we observed the effect of tangeretin on the SPC-induced contraction in human CASMCs by time-lapse recording images (Fig. 2A). In the SPC group, about half of the CASMCs were contracted and morphologically changed at 20 min compared with 0 min; Until 30 min, most of the CASMCs were contracted and deformed and even fell off the cell culture dish (see Supplementary Video 1). However, at 30 min, in the CASMCs which were pretreated with different concentrations of tangeretin (1 μM, 5 μM and 10 μM), the cell morphology showed less remarkable change compared with SPC group. Additionally, there was no cell detached from the cell culture dishes (see Supplementary Video 2-4). Furthermore, we defined the relative mean cell area as a reference to confirm the effect of tangeretin against SPC-induced cell contraction more accurately. As shown in Fig. 2B, from 0 min to 30 min, the mean cell area in the SPC group decreased rapidly from 1196.20 ± 92.01- to 175.97 ± 28.14 pixels; The mean cell area of the other three groups which were pretreated with tangeretin also showed decreasing trend, but at the same time point (10 min, 20 min, 30 min) the mean cell area of the three groups were higher than that of the SPC group with statistical difference distinctly. The cells pretreated with 10 μM tangeretin had the relative best effect against the SPC-induced contraction and remained 944.83 ± 69.74 pixels at 30 min. Taken together, those data suggest that tangeretin also has a significant inhibitory effect on the SPC-induced contraction in human CASMCs.

Fig. 2

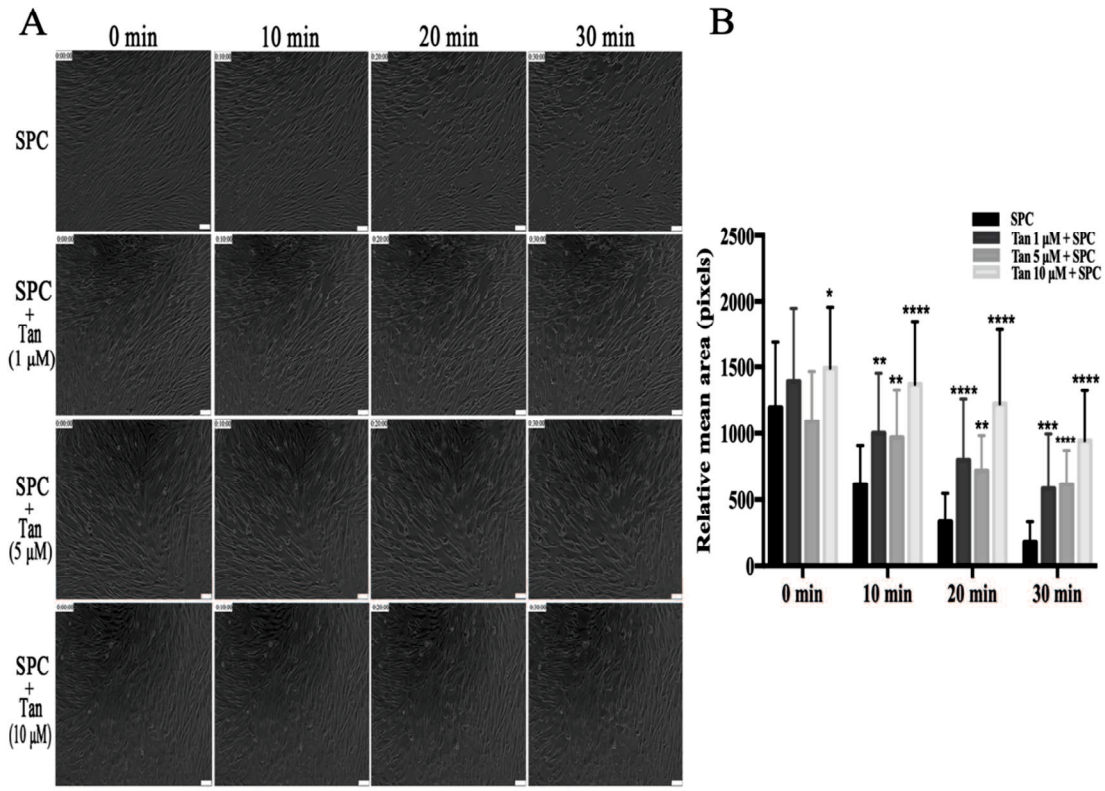


Fig. 2. Effects of tangeretin pretreatment on the SPC-induced contraction in human CASMCs. (A) Records of CASMCs cell morphology changes at different time points, vehicle control and cells pretreated with different concentrations of tangeretin (1 μM, 5 μM, 10 μM, for 30 min) were stimulated by 30 mM SPC. Scale bar = 100 μm. (B) Statistical analysis of the relative mean cell area in the SPC group, Tan (1 μM, 5 μM, 10 μM) + SPC groups at 0 min, 10 min, 20 min and 30 min. $n \geq 29$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, compared with the relative mean cell area in each SPC group at the same time point, expressed as mean ± SEM. (Tan: tangeretin; n: the number of cells analyzed).

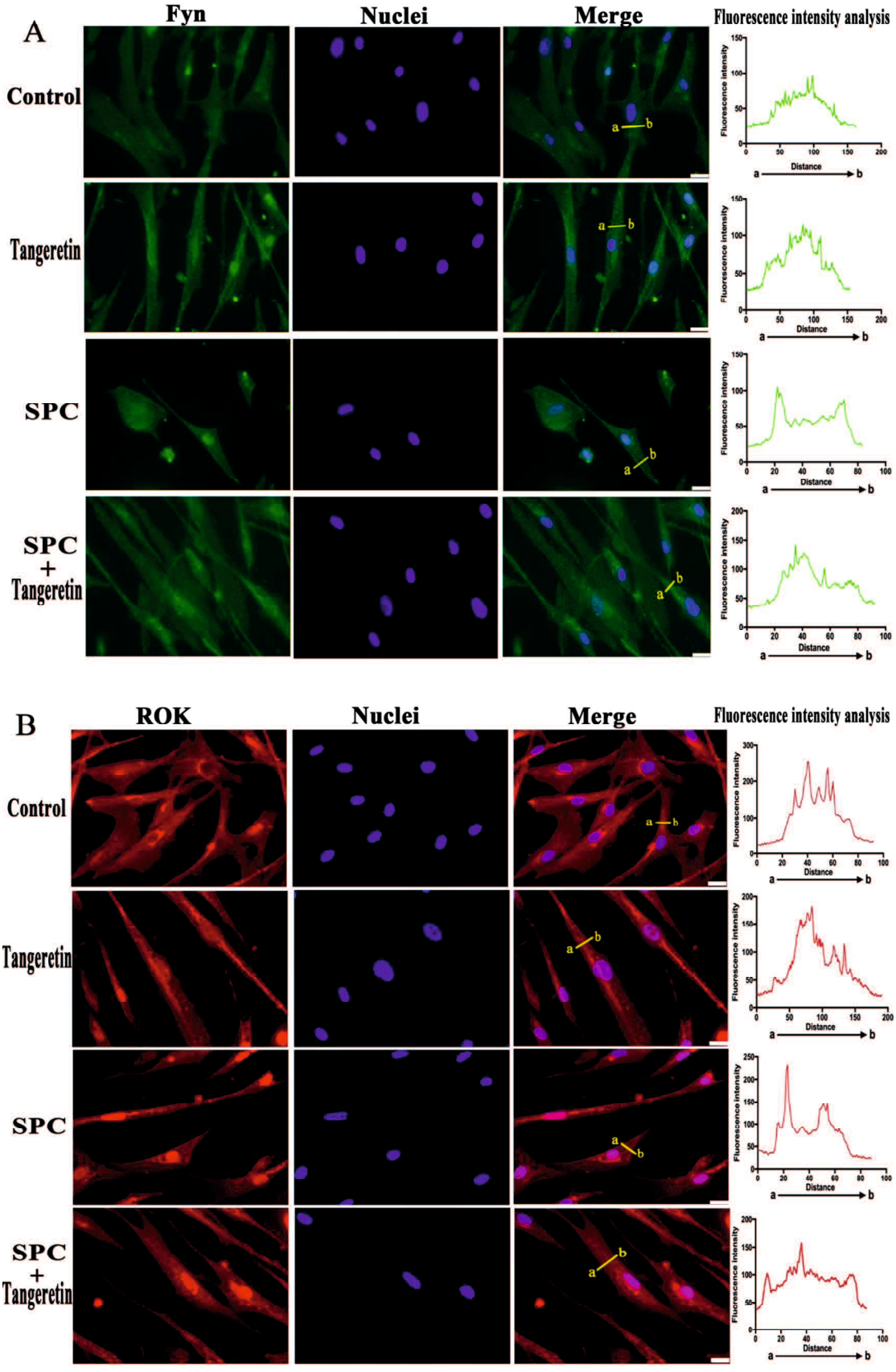
Tangeretin inhibits the SPC-induced Fyn and ROK translocation in CASMCs.

We previously proved that SPC induces the activation of Fyn, facilitating the translocation of Fyn from the cytoplasm to the membrane [6]. Herein, we verified whether tangeretin could inhibit the SPC-induced translocation of Fyn by immunofluorescence staining. The fluorescence intensity analysis graphs showed the intensity of Fyn fluorescence on the cell membrane in the SPC group was significantly

higher compared with that in the control group (Fig. 3A, C), which confirmed the correctness of our previous results demonstrating that SPC can induce Fyn translocation from the cytoplasm to the cell membrane. However, as shown in the SPC + tangeretin group, tangeretin reversed this effect; Fyn did not transfer to the cell membrane even in the presence of SPC and the CASMC cells did not show contracted state. These results indicate that tangeretin can inhibit the SPC-induced translocation of Fyn.

In addition, as the downstream of Fyn, the translocation of ROK from the cytoplasm to the cell membrane is the key factor for the SPC-induced abnormal CASM contraction. We therefore conducted immunofluorescence staining to confirm whether tangeretin could inhibit the SPC-induced translocation of ROK from the cytoplasm to the membrane. As shown in Fig. 3B and D, the fluorescence intensity of ROK on the cell membrane was significantly lower in the SPC + tangeretin group compared with that in the SPC group, the M/C ratio decreased markedly; the vast majority of ROK remain in the cytoplasm, indicating that tangeretin could effectively inhibit the SPC-induced translocation of ROK from the cytoplasm to the membrane.

Fig. 3


A
Fyn
Nuclei
Merge
Fluorescence intensity analysis
Control
Tangeretin
SPC
SPC + Tangeretin
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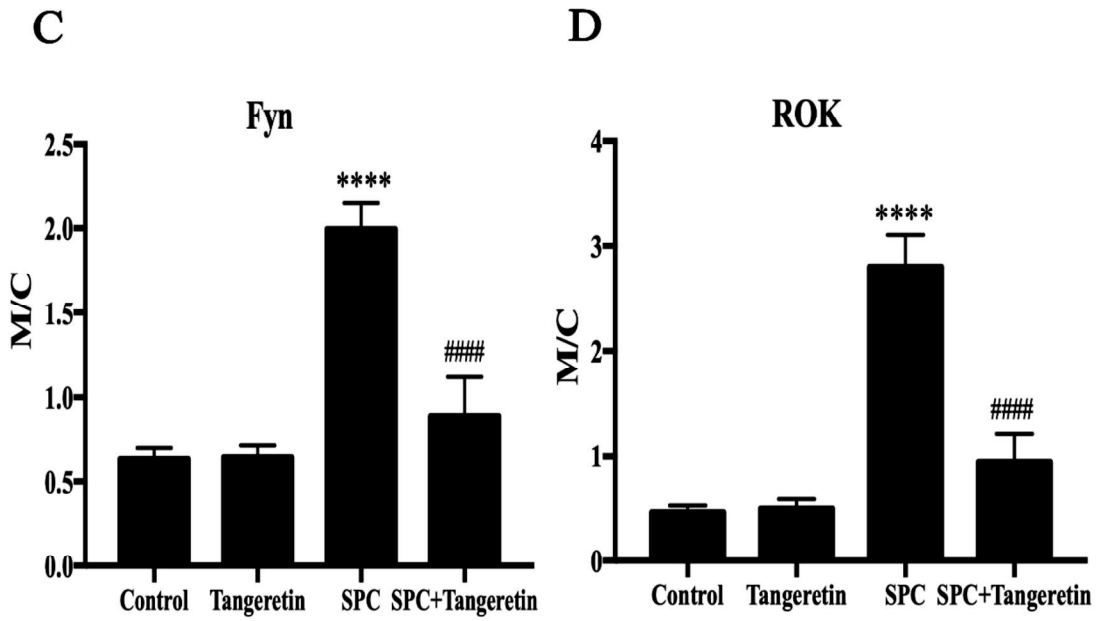


Fig. 3. The inhibitory effects of tangeretin on the SPC-induced translocation of Fyn and ROK. (A) Fluorescent images showing the effects of tangeretin (10 μM, 30 min) and SPC (30 μM, 15 min) on the translocation of Fyn in CASMCs. Fyn were labeled with Alexa 488 in green, the nuclei were labeled with Hoechst nuclear stain in blue. Fyn fluorescence intensities were analyzed along the arrows (a → b). Scale bar = 20 μm (B) CASMCs were also treated in the same way as described above, ROK were labeled with Alexa 546 in red, the nuclei were labeled with Hoechst nuclear stain in blue. ROK fluorescence intensities were analyzed along the arrows (a → b). Scale bar = 20 μm. (C, D) Statistical analysis of the ratios of Fyn (C) and ROK (D) fluorescence intensities on membrane (M) to that on cytosol (C), expressed as M/C. $^{****}P < 0.0001$ compared with control group; $^{####}P < 0.0001$ compared with SPC group (n = 10, n: the number of cells analyzed).

Tangeretin inhibits the SPC-induced the activation of Fyn in CASMCs.

Since Fyn is a member of Src family tyrosine kinase which requires autophosphorylation of Y416 (in c-Src) at the activation loop of the kinase domain for full activation [18, 19], we conducted immunoprecipitation to precipitate Fyn and observed the degree of phosphorylation of the SrcY416 site in each group to verify whether tangeretin could inhibit the SPC-induced activation of Fyn in CASMCs. As shown in Fig. 4, SPC increased the phosphorylation of the SrcY416 site 1.89 ± 0.12- folds in the SPC group compared with the control, whereas pretreatment of tangeretin significantly inhibited the SPC-induced phosphorylation of the SrcY416 site 1.27 ± 0.07- folds in the SPC + tangeretin group, indicating that tangeretin could effectively inhibit the SPC-induced activation of Fyn in CASMCs.

Fig. 4

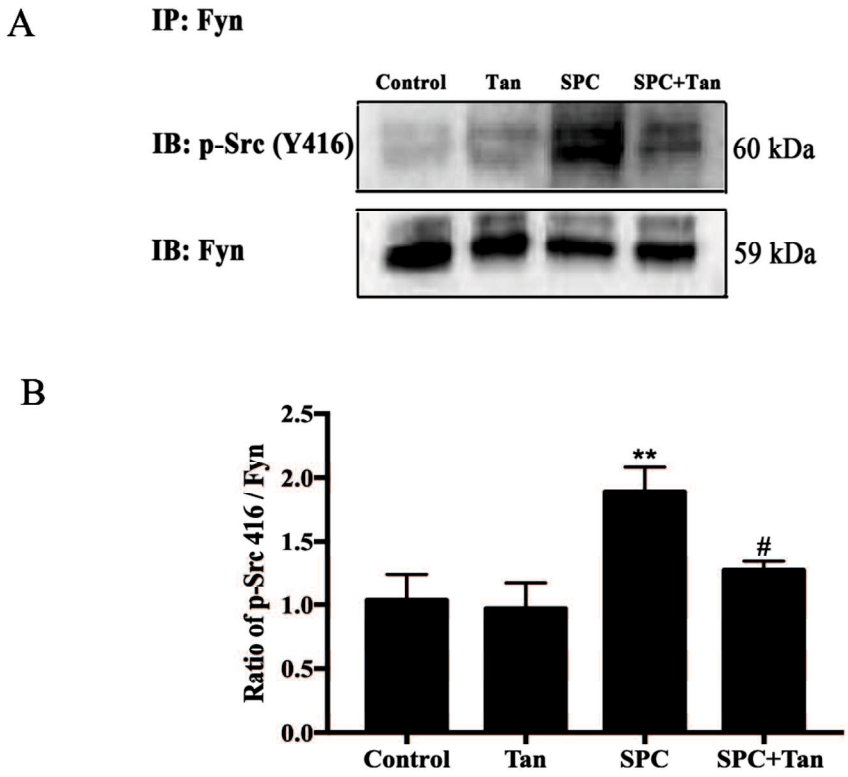


Fig. 4. The inhibitory effects of tangeretin on the SPC-induced activation of Fyn in human CASMCs. (A) The activation and expression levels of Fyn were evaluated by immunoblotting using

anti-Fyn or anti-p-SrcY416 antibodies after immunoprecipitation using anti-Fyn antibody. (B) Statistical evaluation of the effect of tangeretin on the activation of Fyn was quantified by the ratio of p-SrcY416 to Fyn. $^{**}P < 0.01$ compared with control group; $^{\#}P < 0.05$ compared with SPC group (n = 3). (Tan: tangeretin).

Tangeretin inhibits the SPC-induced ROK activation in CASM tissues and cells.

Because the activation of ROK plays a pivotal role in its translocation, whether tangeretin can effectively inhibit the activation of ROK or not is also crucial [20]. As the phosphorylation of MYPT1 at Thr853 site is an important indicator of ROK activation [21, 22], so we conducted western blot to detect ROK activation using anti-phospho-MYPT1 (Thr853) antibody. As shown in Fig. 5, SPC increased the phosphorylation of MYPT1 at Thr853 in CASM tissues (Fig. 5 A, B) and human CASMCs (Fig. 5 C, D) 1.67 ± 0.25- and 1.62 ± 0.19- folds compared to the controls, respectively. However, tangeretin pretreatment significantly decreased the SPC-induced ROK activation 0.97 ± 0.15- and 1.13 ± 0.15- folds respectively, indicating that tangeretin can obviously inhibit the SPC-induced ROK activation.

Fig. 5

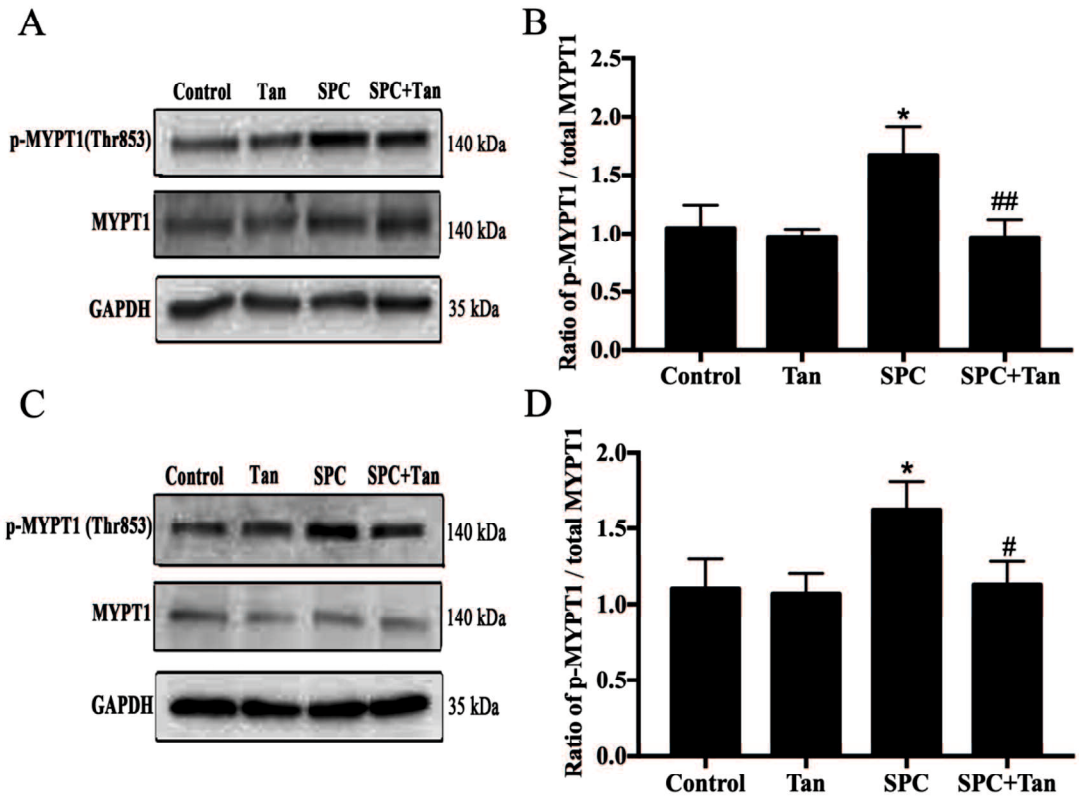


Fig. 5. The inhibitory effects of tangeretin on the SPC-induced activation of ROK. (A, C) Strips of CASM and CASMCs were pretreated with 10 μM tangeretin for 30 min, and then SPC (30 μM) were applied for 15 min. CASM tissue (A) and CASMC (C) samples were analyzed by western blot using anti-p-MYPT1 (Thr853), anti-MYPT1 and anti-GAPDH antibodies. (B, D) Statistical analysis of the inhibitory effects of tangeretin on the SPC-induced ROK activation. The relative expression levels of p-MYPT1 (Thr853) were normalized to total MYPT1 levels. $^{*}P < 0.05$, compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with SPC group (n = 3). (Tan: tangeretin).

Tangeretin inhibits the SPC-induced phosphorylation of MLC in CASM tissues and cells.

MLC phosphorylation induced by ROK activation also plays an important role in the SPC-induced abnormal contraction [23-25]. Therefore, we tested whether tangeretin could inhibit the phosphorylation of MLC at Ser19 site. As shown in Fig. 6, SPC increased the MLC phosphorylation 2.25 ± 0.47- and 2.29

± 0.28- folds compared to the controls in CASM tissues (Fig. 6 A, B) and human CASMCs (Fig. 6 C, D), respectively. However, tangeretin pretreatment significantly reduced the MLC phosphorylation 0.80 ± 0.36- and 1.17 ± 0.25- folds, respectively, indicating that tangeretin can effectively inhibit the phosphorylation of MLC, leading to inhibit the SPC-induced abnormal CASM contraction.

Fig. 6

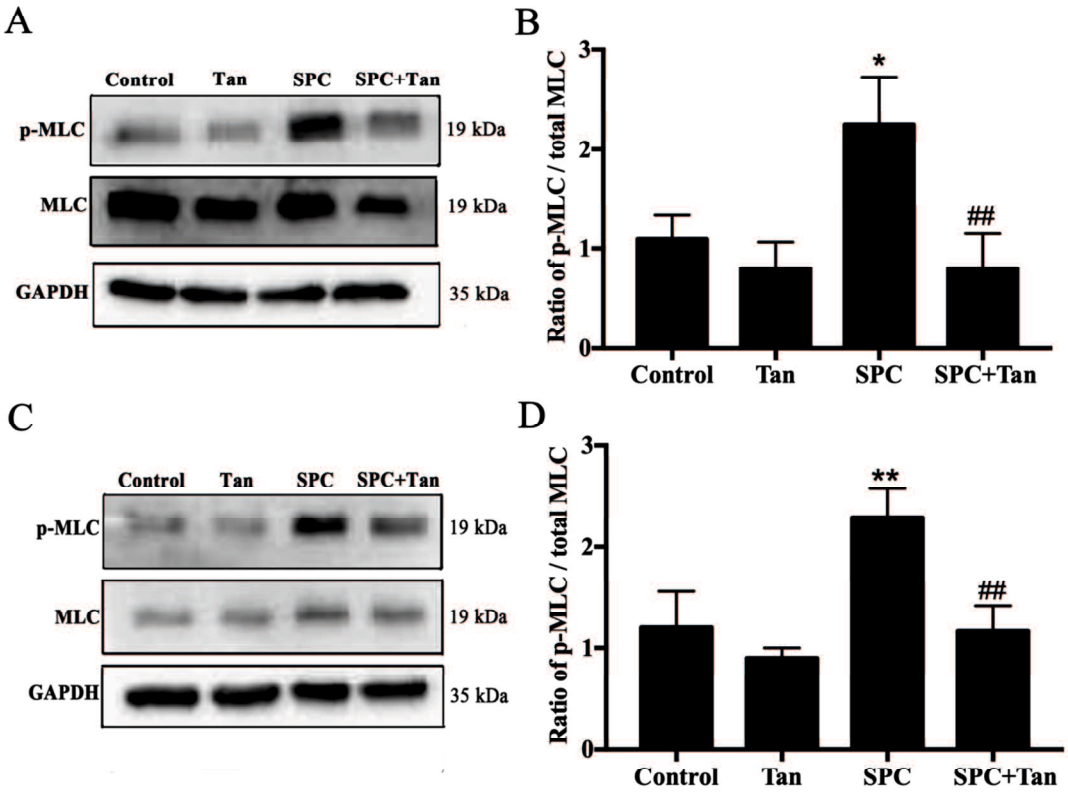
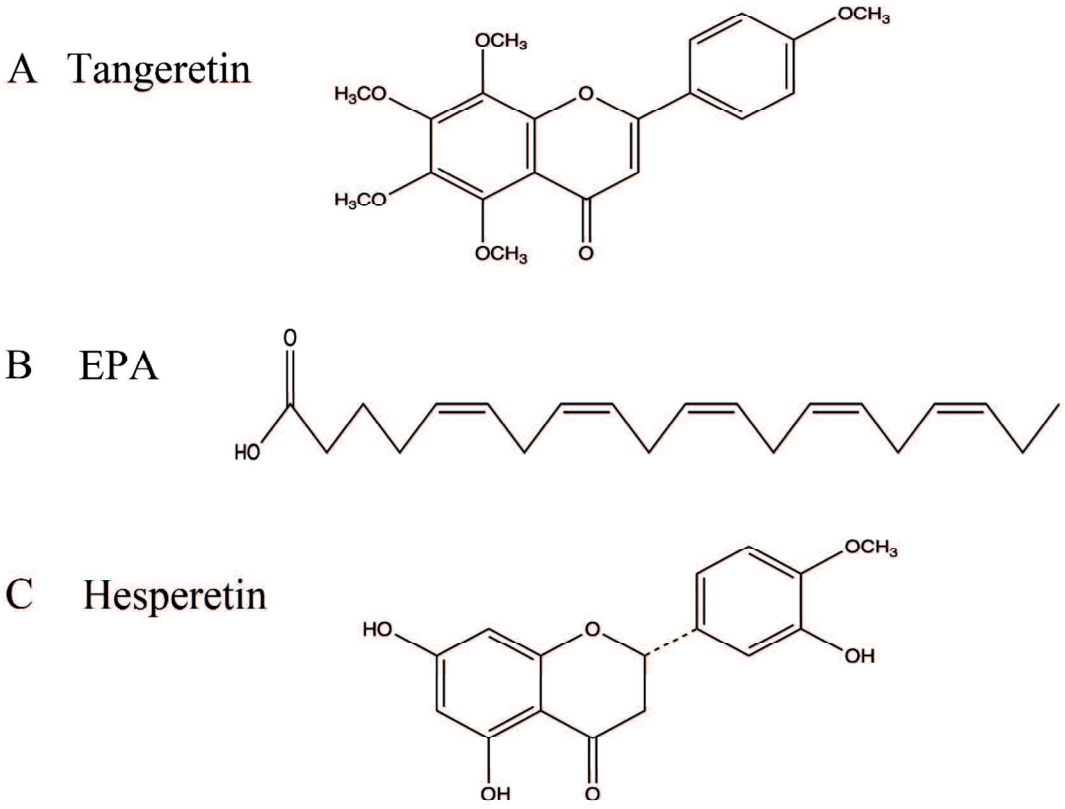


Fig. 6. The inhibitory effects of tangeretin on the SPC-induced MLC phosphorylation. (A, C) Strips of CASM and CASMCs were pretreated with 10 μM tangeretin for 30 min, and then SPC (30 μM) were applied for 15 min. CASM tissue (A) and CASMC (C) samples were analyzed by western blot using anti-p-MLC (Ser19), anti-MLC and anti-GAPDH antibodies. (B, D) Statistical analysis of the inhibitory effects of tangeretin on the SPC-induced MLC phosphorylation. The relative expression levels of p-MLC

(Ser19) were normalized to total MLC levels. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control group; $^{\#\#}P < 0.01$ compared with SPC group (n = 3). (Tan: tangeretin).

Supplementary figure 1. The chemical structures of tangeretin, EPA and hesperetin.



Discussion

In this study, we demonstrated that tangeretin has an inhibitory effect on the SPC-induced CASM contraction by inhibiting Fyn/ROK pathway, both in pre- and post-treatment. We previously found that eicosapentaenoic acid (EPA) selectively inhibited the SPC-induced Ca^{2+}-sensitization of VSM contraction without affecting high potassium depolarization-induced Ca^{2+}-dependent VSM contraction both in vitro and in vivo [6, 26, 27]. However, to overcome the disadvantages of EPA source limitation and low yield, we are trying to find and develop more natural compounds that are rich in edible plants and have no toxic side effects. Epidemiological studies have reported that regular intake of citrus fruits has a certain relationship with reducing the risk of cardiovascular disease [28-30]. Tangeretin belongs to flavonoid, which is extracted from the peel of citrus fruits, is reported to inhibit PGDF-BB-induced proliferation and migration of aortic smooth muscle cells by blocking AKT activation [16] and to protects human brain microvascular endothelial cells against oxygen-glucose deprivation-induced injury [17]. However, relatively few studies reported the effects of tangeretin on the CASM contraction. To our best knowledge, our study is the first to show that tangeretin has a marked inhibitory effect on the SPC-induced CASM contraction both for pretreatment and posttreatment.

We found that tangeretin showed strong inhibitory effects on the SPC-induced CASM contraction both for pretreatment and posttreatment at the optimal concentrations. As shown in Figure 1C and F, the inhibition ratios of tangeretin on the SPC-induced contraction was concentration-dependent in the pretreatment whereas it was concentration-independent in the posttreatment. We speculate that 30-min incubation with tangeretin may play a key role. During this time, tangeretin is likely to permeate cell

membrane and disperse inside the cells so that intracellular concentration of tangeretin get uniform. In contrast, in the posttreatment, most of tangeretin may accumulate on cell membrane because of a shorter contact time with the tissues, exhibiting more potent inhibitory effect on the translocation of Fyn to cell membrane compared with pretreatment. Since Fyn requires S-palmitoylation at Cys3 and Cys6 for its membrane localization, which is required for the activation of Src family tyrosine kinase [31], we speculate that tangeretin in the cell membrane may inhibit the S-palmitoylation of Fyn and thereby inhibit the activation of Fyn and the downstream signaling pathway. Although further studies will be required to focus on the dose-response relationship in vitro and in vivo, the present study suggests that tangeretin may have a potential benefit for the prevention and treatment of cardiovascular diseases such as coronary and cerebral artery vasospasm. Furthermore, there is a statistical difference between the "SPC at 0 min" and "Tan10 uM + SPC at 0 min" groups in Fig. 2B, which might suggest that tangeretin itself may have an effect on cell area. Since tangeretin is reported to increase cell surface area in MO_4 cells [32], we speculate that tangeretin may influence cellular adhesion and thereby affect cell area in our study.

In addition, tangeretin is also effective in abolishing the SPC-induced abnormal contraction in CASMCs. Therefore, whether the inhibitory mechanism of tangeretin acts on the SPC/Fyn/ROK signal pathway is worth to be explored. In our study, the results of immunoprecipitation and immunofluorescence staining confirmed that tangeretin can specifically inhibit the SPC-induced Fyn activation and translocation from the cytoplasm to the cell membrane. Thus, the inhibitory effects of tangeretin on the SPC-induced Fyn activation and translocation can play a key role in alleviating abnormal CASM contraction. Our previous study suggested that EPA may inhibit the translocation of Fyn from rafts mainly through specific inhibition of fat acylation, rather than non-specific effects on raft structure, which in turn inhibits the SPC-induced abnormal contraction [6]. The chemical structure of

tangeretin, as a member of the flavonoid family, is completely different from that of EPA (see Supplementary Fig1. A and B). How tangeretin inhibits the translocation of Fyn is still under investigation, and it is more intriguing to compare the difference of its inhibitory mechanism with that of EPA.

The pivotal role of ROK activation in cerebral vasospasm has been extensively studied by using ROK inhibitors such as Y27632 and fasudil [33-35]. ROK can be activated when the SPC concentration exceeds 5 μmol/L and blocking the ROK activation and translocation can efficiently alleviate the SPC-induced Ca^{2+}-sensitization of VSM contraction [5, 36, 37]. All the evidence indicate that ROK is an important new therapeutic target in vasospasm [37]. In the present study, tangeretin showed marked inhibitory effects on repressing the SPC-induced ROK activation both in CASM tissues and cells. Furthermore, immunofluorescence staining result showed that tangeretin can inhibit the SPC-induced translocation of ROK from the cytoplasm to the cell membrane in CASM cells. Subsequently, tangeretin decreased the level of MLC phosphorylation both in CASM tissues and cells to abolish the SPC-induced CASM contraction. Our study fully demonstrates that tangeretin can precisely suppress the crucial enzyme, ROK, to alleviate the SPC-induced abnormal CASM contraction. Additionally, according to the chemical structure, tangeretin belongs to flavones and only has methoxy substitution in the side chains; The vasodilatory function of flavonoids is closely related to the types and numbers of substitutions carried by flavonoids, and the existence of hydroxyl group is particularly important [39-41]. Moreover, flavone can attenuate vascular contraction by inhibiting of the RhoA/ROK signaling pathway [42, 43]; however, the question how can tangeretin inhibit the SPC-induced ROK activation and translocation when only methoxy (one methoxy group in the B-benzene ring, and four methoxy groups in the A-benzene ring) and no hydroxyl groups are present remains to be elucidated. We endeavor to conduct further studies to

enhance knowledge of the relationship between the structure and the inhibitory effects of tangeretin against both the SPC-induced abnormal CASM contraction and ROK activation.

Recently we discovered that hesperetin can also inhibit the SPC-induced CASM contraction by regulating the Fyn/Rho-kinase pathway [44]. The IC50 of hesperetin is about 14 μM to the SPC-induced contraction on posttreatment, whereas that of tangeretin is 1.13 mM in the present study, suggesting that tangeretin has more potent inhibitory effect on the SPC-induced abnormal CASM contraction. Lin et al [45] confirmed that, compared with hesperetin, which has C-5 and C-7 position hydroxylation on A-ring, tangeretin, which has a fully methoxylated A-ring, had a more potent inhibitory effect on apoB secretion (see Supplementary Fig1. A and C); in addition, they also verified that tangeretin inhibited the cholesterol synthesis modestly while hesperetin exhibited weaker effects on the cholesterol synthesis in HepG2 cells. Chen at el. indicated that the high degree of methoxylation on the tangeretin's A ring, which are more easily permeable to cell membranes and demonstrate more reducing lipid accumulation to regulate adipogenesis, compared with hesperetin which has only one methoxylation in its B ring [46]. Therefore, we speculate that (i). tangeretin is likely to rely on its own structure-activity, the A ring is fully methylated, and it has an inhibitory effect on the SPC-induced CASM contraction; (ii). Since cholesterol primes VSM contraction by the SPC/Fyn/ROK pathway [47], tangeretin might have the possibility to inhibit the SPC-induced CASM contraction through reducing the part of cholesterol synthesis. The above two assumptions are likely to be the advantages of tangeretin compared with hesperetin in the treatment of the SPC-induced CASM contraction, and also the directions of our future research.

Conclusion

Taken together, tangeretin is shown for the first to effectively repress the SPC-induced abnormal CASM contraction. This effect is achieved by inhibiting the SPC-induced activation of Fyn and ROK and their translocation from the cytoplasm to the cell membrane, and consequently inhibiting phosphorylation of MLC to ultimately alleviate abnormal CASM contraction. Our study provides a new perspective on tangeretin for the treatment or prevention of vasospasm and an effective basis for the development of tangeretin's clinical use.

Acknowledgment

My story of studying abroad began in the midsummer August of 2018 and ended on the autumnal equinox in September 2022, in a small quiet coastal city in Japan; Four years have flown by, and I have experienced the bitterness and sweetness of life before I present this doctoral dissertation to you.

In August 2018, I came to Yamaguchi University alone with my curiosity and dream to study for a medicine doctor. If there is one word to describe the life of studying abroad, there is no doubt that it is loneliness. Empty, almost deserted streets; No dazzling entertainment facilities; Differences in language and culture with foreigners; Mechanical repetition of experiments, and the often-bottlenecked scientific research life, all of which have converged into unprecedented loneliness. Until the beginning of 2020, the COVID-19 epidemic started sweeping the world, resulting in countries shut down, homecoming becomes an extravagant demand; During this period, my grandfather who accompanied me throughout my childhood passed away, and I was overwhelmed with powerlessness and sadness. Fortunately, I realized at such an early age that sorrow and joy, self-transfer, no one is who is redeemed. All the hardships I have endured only made my inner world stronger and stronger, creating a new self; With the firm belief in my mind: one day I will reach the realm of succeed. Learn to be friends with loneliness and enjoy it, for it's the best time to improve yourself.

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Life goes on, still walking, no end for learning. There are too many beauty and romance in the world worthy of our efforts, dedication, and pursuit. Youth in our heart, and light in our eyes, forever and always.

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