RESEARCH ARTICLE

Effect of N-(*p*-coumaroyl)serotonin and N-feruloylserotonin, major anti-atherogenic polyphenols in safflower seed, on vasodilation, proliferation and migration of vascular smooth muscle cells

Tetsuya Takimoto[1], Katsuya Suzuki[1], Harumi Arisaka[2], Takahisa Murata[3], Hiroshi Ozaki[3] and Naoto Koyama[1]

[1] Institute for Innovation, Ajinomoto Co., Inc., Kawasaki Japan
[2] Exploratory Research Laboratories, Ajinomoto Pharmaceuticals Co. Ltd., Kawasaki Japan
[3] Department Veterinary Pharmacology, Graduate School of Agriculture and Sciences, The University of Tokyo, Tokyo Japan

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Scope: The objective of this study is to investigate a vascular effect of N-(*p*-coumaroyl)serotonin (CS) and N-feruloylserotonin (FS), major antioxidative indolic polyphenols in safflower seeds with anti-atherogenic properties, with emphasis on effects on vascular smooth muscle cells (VSMCs).

Methods and results: Both CS and FS (each 10 to 100 μM) relaxed rat femoral arteries, which were pre-contracted by 10^{-5} M phenylephrine or 50 mM KCl, independently of their endothelium. Both CS and FS also concentration-dependently inhibited the increase of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that was induced by KCl or 5-hydroxytryptamine in cultured rat VSMCs. Next, we examined the effects of CS and FS on platelet-derived growth factor (PDGF)-BB-evoked proliferation and migration of the VSMCs. Both CS and FS inhibited PDGF-BB-evoked proliferation and migration of the VSMCs in a concentration-dependent manner. They also inhibited PDGF-BB-induced phosphorylation of PDGF receptor β and ERK1/2, and Ca^{2+} release from sarcoplasmic reticulum in the VSMCs in a concentration-dependent fashion.

Conclusion: These results indicated a possible vascular effect of CS/FS to inhibit the activation of VSMCs by blocking the increase of $[Ca^{2+}]_i$ and/or blocking PDGF signaling. These may explain a part of anti-atherogenic mechanism that underlies their ability to improve vascular distensibility and to inhibit aortic hyperplasia.

Keywords:
Atherosclerosis / Safflower seed / Serotonin hydroxycinnamic acid amides / Vascular distensibility / Vascular smooth muscle cell

1 Introduction

Development of atherosclerosis is characterized by dysfunction of endothelial cells, oxidative modification of low-density lipoprotein (LDL) followed by foam cell formation, migration of vascular smooth muscle cells (VSMCs) from the arterial media into intima, excessive proliferation of VSMCs in the neointima, and increased extracellular matrix deposition [1–4]. The increment in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays an important role in

Correspondence: Dr. Tetsuya Takimoto, Institute for Innovation, Ajinomoto Co., Inc., 1-1 Suzuki-Cho, Kawasaki-Ku, Kawasaki 210-8681 Japan
E-mail: tetsuya_takimoto@ajinomoto.com
Fax: +81-44-211-7609

Abbreviations: 5-HT, 5-hydroxytryptamine; **ANOVA,** analysis of variance; **CS,** N-(*p*-coumaroyl)serotonin; **$[Ca^{2+}]_I$,** intracellular Ca ion concentration; **EGCG,** epigallocathechin-3-gallate; **ERK,** extracellular signal-regulated kinase; **FBS,** fetal bovine serum; **FS,** *N*-feruloylserotonin; **KHC rabbit,** Kurosawa and Kusanagi-hypercholesterolemic rabbit; **PDGF,** platelet-derived growth factor; **SHAAs,** serotonin hydroxycinnamic acid amides; **SOC,** store operated channel; **SR,** sarcoplasmic reticulum; **VSMCs,** vascular smooth muscle cells

modulating VSMC function (eg; contraction, migration and proliferation), therefore, it is considered to be deeply involved in progression of hypertension and atherosclerosis. Furthermore, abnormal VSMC proliferation has been shown as a crucial factor of development of hypertension [5], arterial stiffness and atherosclerosis [6, 7], and it has been repeatedly pointed out by many researchers that the growth factors such as platelet-derived growth factor (PDGF) play an important role in the process. Therefore, inhibition of frequency of $[Ca^{2+}]_i$ increment in VSMCs and VSMC proliferation has been one of the main targets for prevention of the cardiovascular diseases associated with hypertension and vascular wall thickening, and this is frequently explained as one of the pleiotropic effects of statins or some kind of Ca^{2+} channel blocker [8, 9].

Recently, some dietary factors, which have strong antioxidative effects such as vitamins E and C, polyphenols, carotenoids—mainly lycopene and β-carotene, coenzyme Q10, curcumin, epigallocatechin gallate (EGCG) etc., have been reported to have vascular effects that is vasodilation and/or improvement of VSMC abnormality by attenuating PDGF-receptor signaling [10–13]. N-(*p*-coumaroyl)serotonin (CS) and N-feruloylserotonin (FS) are members of serotonin hydroxycinnamic acid amides (SHAAs; Fig. 1) which are known to be widely distributed in many plants [14–17], especially rich in seed of safflower (*Carthamus tinctorius L.*) [18]. Their protective effects on oxidative modification of LDL [18] and vascular wall distensibility in Kurosawa and Kusanagi-Hypercholesterolemic (KHC) rabbits [19], inhibitory effect on atheromatous plaque formation in apolipoprotein E-deficient mice [18] and the rabbits [19], and reduction of some vascular risk factors in human subjects [20] have been recently reported. Because of recently-reported anti-inflammatory action of CS and FS on cultured aortic endothelial cells [21], we hypothesized that these compounds might have direct vascular effect beneficial for preventing progression of atherosclerosis in addition to their strong antioxidant effect. However, the effect of CS and FS on VSMCs has never been reported.

In this study, we tried to elucidate the anti-atherosclerotic mechanism of CS and FS with emphasis on vascular function. For this purpose, we examined the action of CS and FS on vascular distensibility, PDGF-induced VSMC proliferation and migration, and an involvement of their direct effect on calcium dynamics and PDGF signaling in VSMCs, if any.

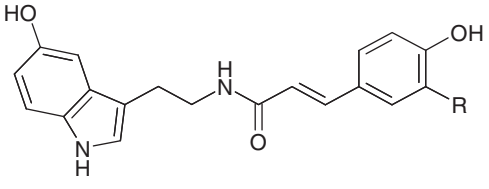

Figure 1. structure of serotonin hydroxycinnamic acid amides. CS; R = H, FS; R = OCH_3.

2 Materials and Methods

2.1 Safflower seed SHAAs

CS and FS were synthesized from 5-HT HCl, *p*-coumaric acid and trans-ferulic acid [18]. The purity of CS and FS were determined to be 97.1% and 99.5%, respectively, with the use of a charged aerosol detector (Corona CAD, Dionex Corp., USA).

2.2 Tissue preparations and contraction-relaxation studies in isolated rat femoral arterial rings

Femoral arteries were isolated from male Sprague Dawley rats (aged 8–12 weeks), and cleaned to remove fat and connective tissue. To prepare arterial rings, femoral arteries were sectioned into approximately 1-mm segments. The endothelium was removed by gently rubbing the intimal space with a pipette tip. The intact or endothelium-denuded artery rings were placed in organ chambers containing Tyrode buffer (10 mM $NaHCO_3$, 5 mM Glucose, 158 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 4.2 μM NaH_2PO_4) aerated with 95% O_2/5% CO_2.

Femoral arterial rings were equilibrated for at least 1 hour at a resting tension of 0.3 g and 2 g. During this time, the bath solution was replaced with prewarmed and oxygenated Tyrode buffer every 15 min. Thereafter, 50 mM KCl was added to induce repeated, reproducible contractions. After contractions, arterial rings were rinsed several times with prewarmed and oxygenated Tyrode buffer until muscle tension returned to the basal level.

2.3 Phenylephrine- or KCl- induced contraction

After 10^{-5} M phenylephrine (Sigma, St. Lois, MO, USA) or 50 mM KCl induced sustained contractions, CS or FS (each 10 to 100 μM) was added to induce relaxation in the rings.

2.4 Cell cultures

We used the A7r5 cell line, which is a well-established vascular smooth muscle cell line obtained from embryonic rat aorta (American Type Culture Collection; CRL1446; Rockville, MD, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Corp., Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C with 10% CO_2 atmosphere.

2.5 $[Ca^{2+}]_i$ imaging experiments

For the calcium imaging study, A7r5 cells (10 000 cells/300 μl/well) seeded in Lab-Tek™ II chambered coverglass (Nunc, Naperville, IL, USA) pre-treated with Attachment Factor™ (Cell Systems, Kirkland, WA, USA) were incubated for 90 min at 37°C in Krebs-HEPES buffer (140 mM NaCl, 3 mM KCl 2 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM Glucose, and 10 mM HEPES (pH 7.4)) containing 4 μM fluo-4 acetoxymethyl ester (fluo-4 AM; Dojindo, Osaka, Japan). Cells were gently washed twice, and allowed to rest for at least 10 min on a heated plate at 37°C. Cells were visualized under an inverted microscope. For experiments in extracellular Ca^{2+}-free medium, $CaCl_2$ was replaced by 0.5 mM EGTA.

Fluorescence images were obtained at 1 second intervals (excitation, 470 nm; emission, 525 nm). Digital images were stored and analyzed using Leica FW4000 software (Leica Microsystems, Wetzlar, Germany).

Each CS, FS or DMSO as a vehicle control was added in volumes of 200 μl to obtain a final incubation volume of 300 μl bathing solution. To evaluate the effect of CS and FS on intracellular calcium ion concentration ($[Ca^{2+}]_i$) increased by KCl, 5-hydroxytriptamine (5-HT; Tokyo Chemical Industry, Co., Ltd. Tokyo, Japan) or PDGF-BB (R&D Systems, Inc. Minneapolis, MN, U.S.A) stimulation, CS or FS (each 10 or 66 μM) was added 15 seconds before addition of 50 mM KCl, 5 μM 5-HT or 50 ng/ml PDGF-BB. To evaluate the effect of CS and FS on Ca^{2+} influx through the store-operated channels (SOCs), 5 μM thapsigargin was added 4 min before addition of 2 mM $CaCl_2$ and CS or FS (66 μM each). All experiments were performed at 37°C. For each cell, fluorescence intensity increased by $[Ca^{2+}]_i$ was determined by the proportion of pixels that were altered from the 0 time point. The value for each time point was expressed as relative to that at the 0 time point.

2.6 Rabbit mesenteric artery culture

Male Japanese White rabbits (2 kg) were euthanized by stunning and exsanguination. Animal experiments and care were approved by local ethical review and were performed in strict compliance with the guidelines outlined within the Guide to Animal Use and Care from the University of Tokyo which conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The organ culture procedure was performed as described previously [22]. In brief, main branches of the superior mesenteric arteries were isolated aseptically. After removing fat and adventitia in sterile HBSS, each artery was cut into rings approximately 1.5-mm in length. The endothelium was removed by gently rubbing the internal surface with tweezers.

Arterial rings were placed in one of the following settings: serum-free DMEM (Serum-free), DMEM containing 3% FBS (3% FBS), DMEM containing 3% FBS + 10 μM CS (+10 μM CS) or DMEM containing 3% FBS + 10 μM FS (+10 μM FS), maintained at 37°C in an atmosphere of 95% air and 5% CO_2 for 7 days. DMEM were supplemented with 1% penicillin-streptomycin. The medium was changed every other day until the end of the experiment.

2.7 Vascular smooth muscle cells proliferation assay

A7r5 cells (2,000 cells/well) were seeded in 96 well microplate and pre-incubated for 24 hours in serum-free medium consisting of DMEM. Then CS, FS (each 1, 10 or 100 μM) or DMSO as a vehicle control was added to medium and A7r5 cells were stimulated with 10% FBS or 10 ng/ml PDGF-BB for 24 hours. Cell proliferation activity was measured using the Cell Counting Kit-8 (Dojindo), which measure formazan dye produced by living cell reducing enzyme, according to the manufacturer's guidelines.

2.8 Vascular smooth muscle cells migration assay

Cell migration was studied using a modified micro Boyden chamber across an 8 μm pore polycarbonate filter (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). 10 ng/ml PDGF-BB was added to the lower chambers and CS, FS (each 1, 10 or 66 μM) or DMSO as a vehicle control was added to the upper chamber. A7r5 cells (100,000 cells), which were pre-incubated for 24 hours in 0.2% FBS contained medium consisting of DMEM, were cultured in the upper chamber and incubated at 37°C for 4 hours. The cells that had migrated to the lower surface of the filter were fixed and stained with Diff Quick (Baxter, Thetford, Norfolk UK). The excess stain was rinsed with PBS, and the residual cell-bound stain was eluted in 100 μl of 10% acetic acid. Ninety microliters of this solution were transferred into 96-well plates, and absorbance was read at 600 nm.

2.9 Effect of CS and FS on PDGF-BB signaling in VSMCs

A7r5 cells (150 000 cells/dish) were seeded in 60 mm dishes and pre-incubated over night in serum-free medium consisting DMEM. Then CS, FS (each 10 or 66 μM) or DMSO as a vehicle control was added to the medium and A7r5 cells were stimulated with 10 ng/ml PDGF-BB for different time periods. After removing the medium, cells were twice washed with PBS 500 μl and lysed with 200 μl sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 6% β-mercaptoethanol, 10% glycerol) containing 10 μl/ml proteinase inhibitor (Sigma) and phosphatase inhibitor (Sigma). Cell lysates were sonicated by ultrasonic cell homogenizer (VP-5S, TAITEC, Koshigaya, Japan) on ice. Protein determination of the supernatant was performed

using the BCA protein assay reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA). Samples were incubated at 95°C for 5 min. Proteins in the supernatant were separated in a 7.5% SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol at 26 mA. After 1 hour incubation in blocking solution (5% BSA in PBS-T (PBS, 0.05% Tween 20)), the membrane was incubated for over night at 4°C with the primary polyclonal rabbit anti-phospho-ERK (1:1000, Cell Signaling Technology, Inc., Danvers, MA, USA), anti-ERK (1:1000, Cell Signaling Technology), anti-phospho-PDGF receptor β (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or anti-PDGF receptor β (1:200, Santa Cruz Biotechnology, Inc.). After washing three times with PBS-T, bound rabbit IgG was detected by anti-rabbit IgG conjugated to horseradish peroxidase (1:10,000 dilution in 3% BSA PBS-T, GE Healthcare UK Ltd., Little Chalfont, UK) to incubate for 1 hour at room temperature. Immunoblots were visualized by LAS-3000 (Fujifilm Corp., Tokyo, Japan) with ECL Plus Western Blotting Detection System (GE Healthcare). Degree of protein phosphorylation was quantitated by calculating the ratio of the densities of phosphoproteins to total proteins for each blot.

2.10 Statistical analysis

Data in figures are represented as means ± SEM. Significant differences were determined by one-way analysis of variance

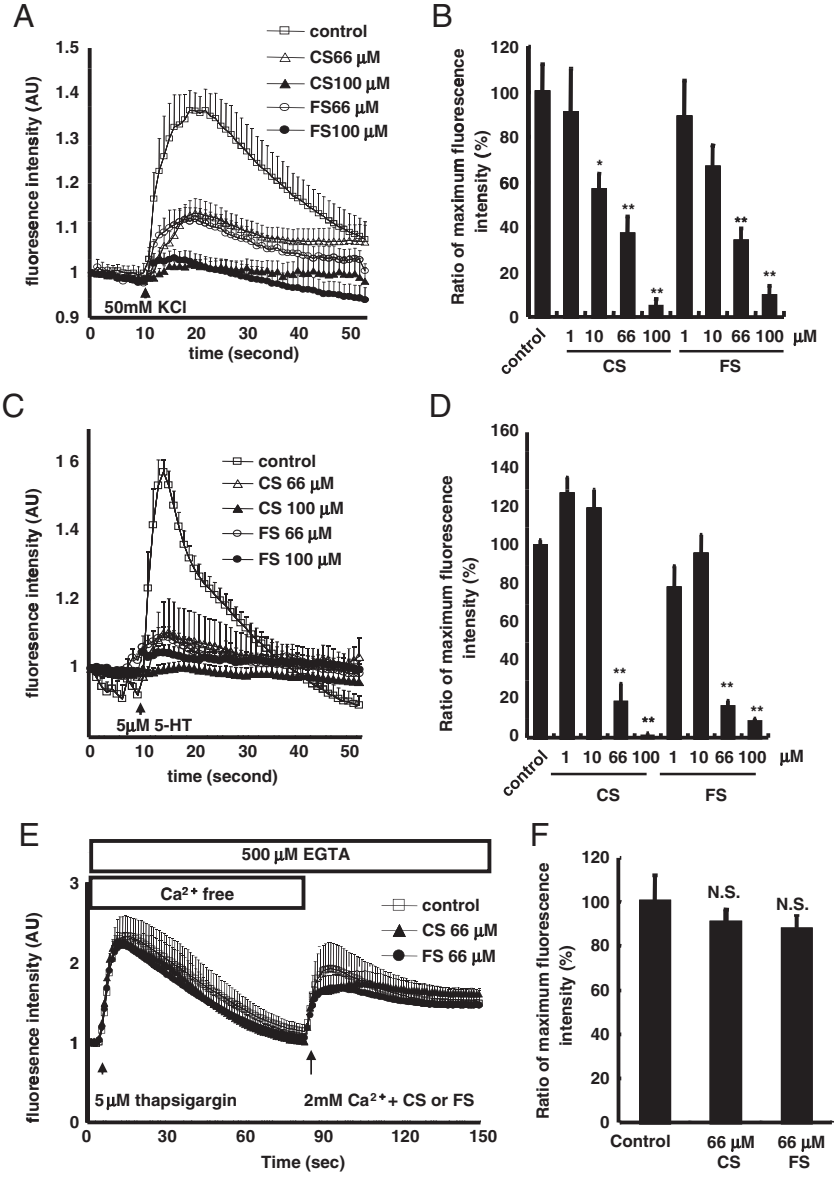

Figure 2. Effects of serotonin hydroxycinnamic acid amides (CS and FS) on Ca ion influx through voltage-dependent Ca ion channels (A)(B), receptor-operated Ca ion channels (C)(D) and store-operated channels (E)(F) in A7r5 cells; kinetics of intracellular fluorescence intensity derived from fluo-4 induced by 50 mM KCl (A), 5 μM 5-HT (C) or intra-SR Ca ion deprivation by 5 μM thapsigargin (E), concentration-dependent effect of serotonin hydroxycinnamic acid amides on maximum Ca ion influx in A7r5 cells induced by KCl (B; $n = 4$–9) or 5-HT (D; $n = 5$–13), and effect of serotonin hydroxycinnamic acid amides on maximum Ca ion influx through store-operated channels (F). Maximum fluorescence intensity of control = 100%. Values are represented as means ± SEM; * and ** indicate significant differences compared with control at $p<0.05$ and $p<0.01$, respectively. N.S; no significant difference compared with control.

(ANOVA) followed by multiple Dunnett's test or Fisher's protected least significant difference (PLSD).

3 Results

3.1 Effect of CS and FS on pre-contracted rat femoral artery rings

Both CS and FS relaxed rat femoral artery rings that were pre-contracted by 10 μM phenylephrine. Rings were relaxed in a concentration-dependent manner, and relaxation occurred whether or not the endothelium was present. Both CS and FS resulted in slow relaxation of the vessels, and relaxation reached a plateau 30 min after each compound was added. The minimum concentration of both CS and FS that was required to achieve the plateaued relaxation was 25 μM. The EC_{50} values (the concentration required to cause 50% relaxation) of the dilative effect of CS and FS for the intact artery and the endothelium-denuded one were as follows: 16.7 ± 4.2 μM (CS, intact); 18.6 ± 1.0 μM (FS, intact); 29.0 ± 0.7 μM (CS, endothelium-denuded); and 22.1 ± 1.2 μM (FS, endothelium-denuded). The EC_{50} value of the relaxant effect of CS for the endothelium-denuded artery was significantly ($P < 0.05$) higher than that for the intact artery.

In addition, CS and FS also relaxed the rat femoral arterial ring pre-contracted by 50mM KCl in a concentration-dependent manner. This vasodilative effect was observed over time after the addition of each 25 μM CS or FS.

3.2 Effect of CS and FS on the KCl-, 5-HT- or sarcoplasmic reticulum Ca^{2+} deprivation-induced increase of $[Ca^{2+}]_i$ in A7r5

To clarify the effect of CS and FS on Ca^{2+} dynamics in vascular smooth muscle cells, KCl and 5-HT were used to stimulate release of Ca^{2+} from the sarcoplasmic reticulum (SR) and to stimulate Ca^{2+} influx from extracellular spaces. The addition of 50mM KCl or 5 μM 5-HT to the medium increased maximum fluorescence intensity in fluo-4 AM-loaded A7r5 cells by 1.4 times or 1.8 times, respectively (Fig. 2(A) and Fig. 2(C)). This increase in fluorescence intensity indicates that the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) increased in the A7r5 cells. Both CS and FS reduced the $[Ca^{2+}]_i$ increase induced by KCl or 5-HT in a concentration-dependent manner (Fig. 2(A)(B)). CS at concentrations ≥ 10 μM and FS at ≥ 66 μM significantly reduced the increase of $[Ca^{2+}]_i$ in cells exposed to KCl. (Fig. 2(B)). Similarly, CS and FS at ≥ 66 μM reduced the increase of $[Ca^{2+}]_i$ in cells exposed to 5 μM 5-HT (Fig. 2(C)(D)). When cells were pretreated with CS and FS for 18 hours then washed to remove CS and FS, $[Ca^{2+}]_i$ was not affected by treatment in response to 5-HT (data not shown).

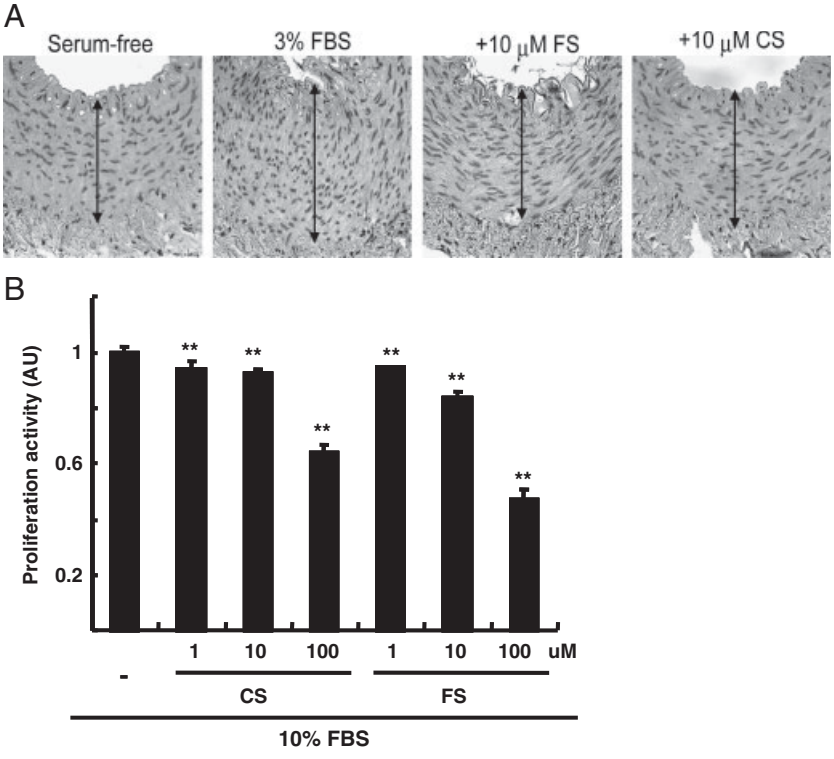

Figure 3. Effect of serotonin hydroxycinnamic acid amides (CS or FS (each 10 μM)) on rabbit arterial wall thickening (A) and A7r5 proliferation (B) evoked by FBS. In panel (A), the arrow indicates width of the medial layer in the vascular wall. Values in panel (B) are represented as means ±SEM of 3 well determinations. **: $p < 0.01$ shows statistically significant differences when compared with '10% FBS'.

Next, we investigated effects of CS and FS (each 66 μM) on Ca^{2+} influx through the SOCs. We used the Ca^{2+}-ATPase inhibitor, thapsigargin, to deplete Ca^{2+} stored in the SR. Upon addition of $CaCl_2$, the fluorescence intensity was approximately doubled (Fig. 2E). Addition of CS (66 μM) and FS (66 μM) did not affect the increase in fluorescence intensity (Fig. 2E, 2F)).

3.3 Effect on FBS- induced rabbit arterial wall thickening and rat VSMCs proliferation

In the serum-free condition, the arterial wall maintained its intact morphology during the 7-day organ culture; the smooth muscle cells in the media were well arranged and had typical spindle-shaped nuclei as reported previously (Fig. 3A, [22]). However, in the medial layer of the arteries treated with 3% FBS-treated for 7 days, the smooth muscle cells got disarranged and degenerative in appearance, and round nuclei predominated. These morphological changes were significantly inhibited by addition of each 10 μM FS or CS in the cultured medium containing 3% FBS (we confirmed that both SHAAs alone did not affect the vascular morphology up to 7 days at this concentration under serum-free condition). We next quantified the hyperplastic changes in the vascular wall by measuring the width of the media layer (indicated by arrow). 3% FBS treatment significantly ($p<0.05$) increased the width of the media (serum-free; 87.5 ± 12.9 μm, 3% FBS; 183.6 ± 20.2 μm, $n=5$ each). Each 10 μM FS and CS significantly ($p<0.05$) restored the hyperplastic responses to 3% FBS (3% FBS+10 μM FS; 133.3 ± 15.1 μm, 3% FBS+10 μM CS; 148.1 ± 14.6 μm, $n=5$ each).

Next, to confirm the wall thickening inhibition effect was due to the direct inhibition of VSMCs proliferation, effect of CS and FS (each 1 to 100 μM) on A7r5 proliferation was examined. Both compounds inhibited FBS (10%)-stimulated cell proliferation in a concentration-dependent manner, in which 40 or 60% inhibition was observed at each 100 μM CS or FS, respectively (Fig. 3B).

3.4 Effects on PDGF-BB-induced VSMCs proliferation and migration

CS and FS (each 10 to 100 μM) inhibited PDGF-BB (10 ng/ml)-stimulated cell proliferation in a concentration-dependent manner (Fig. 4A). The lack of cytotoxicity of CS and FS at the concentrations used in these experiments was ascertained by the trypan blue exclusion assay (data not shown).

Boyden chamber assay was performed to clarify the effect of SHAAs on VSMCs chemotaxis induced by PDGF-BB. As shown in Fig. 4(B), PDGF-BB up-regulated the VSMCs transmigration across the membrane, and the treatment with CS and FS (each 1 to 66 μM) significantly attenuated the migration.

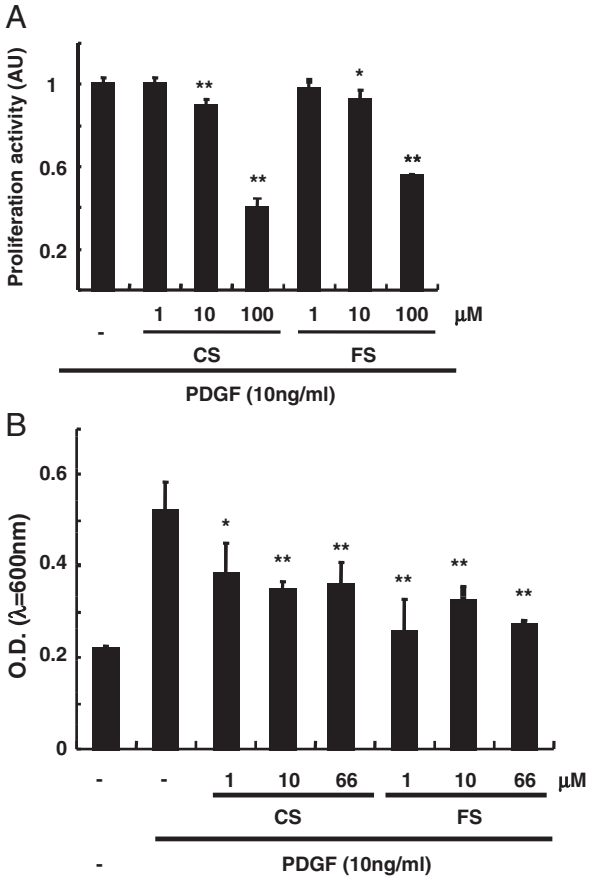

Figure 4. Effect of serotonin hydroxycinnamic acid amides (CS and FS) on A7r5 proliferation (A) and migration (B) induced by 10 ng/ml PDGF-BB. Values are represented as means ± SEM. of 3 well determinations. *: $p<0.05$, **: $p<0.01$ shows statistically significant differences when compared with value in PDGF-BB stimulated without CS and FS.

3.5 Effect on PDGF-BB-induced tyrosine phosphorylation of PDGF receptor β and ERK1/2 phosphorylation

As shown in Fig. 5, PDGF-BB (10 ng/ml) dramatically increased phosphotyrosine levels of PDGF receptor β, whereas CS and FS attenuated this effect in a concentration-dependent manner at 5 min after PDGF-BB stimulation but not at 10 min after stimulation (Fig. 5A, B). FS (10 to 66 μM) significantly inhibited ERK1/2 phosphorylation, one of PDGF-receptor's downstream effects, from 5 to 10 min after the stimulation (Fig. 5C, D).

3.6 Effect on PDGF-BB-induced Ca^{2+} release from sarcoplasmic reticulum

To clarify whether CS and FS affect PDGF-induced Ca^{2+} release from SR via PDGF-receptor phosphorylation

and trigger cell activation, the calcium imaging study was performed. $[Ca^{2+}]_i$ was increased from 20 sec after PDGF-BB (50 ng/ml) treatment in VSMCs without extracellular Ca^{2+} (Fig. 6A). CS and FS (each 10 to 66 μM) inhibited $[Ca^{2+}]_i$ increase in a concentration-dependent manner. The area under the curve of $[Ca^{2+}]_i$ (Fig. 6B) and maximum $[Ca^{2+}]_i$ (Fig. 6C) was also significantly attenuated by the addition of each 66 μM CS or FS.

4 Discussion

We have previously reported the anti-atherogenic effect of CS and FS in apoE-deficient mice [18] and discussed that the effect was partly due to the suppression of oxidative modification of LDL by their antioxidative action. This notion was supported by a pilot human study in which a parallel reduction of serum oxidative LDL and autoantibody titers to malone dialdehyde-LDL was observed at the end of the 4-week intervention of safflower seed extract [20]. Recently, Piga et al. demonstrated a protective effect of these compounds against the short-term high glucose-induced pro-inflammatory insult in cultured aortic endothelial cells [21]. Katsuda et al. demonstrated that the supplementation of CS and FS ameliorated vascular distensibility and stiffness index in heritable hypercholesterolemic spontaneous atherosclerotic rabbits (KHC rabbit) [19]. Moreover, recent progress in plant phenolic research suggested an involvement of direct vascular effects in their cardioprotective

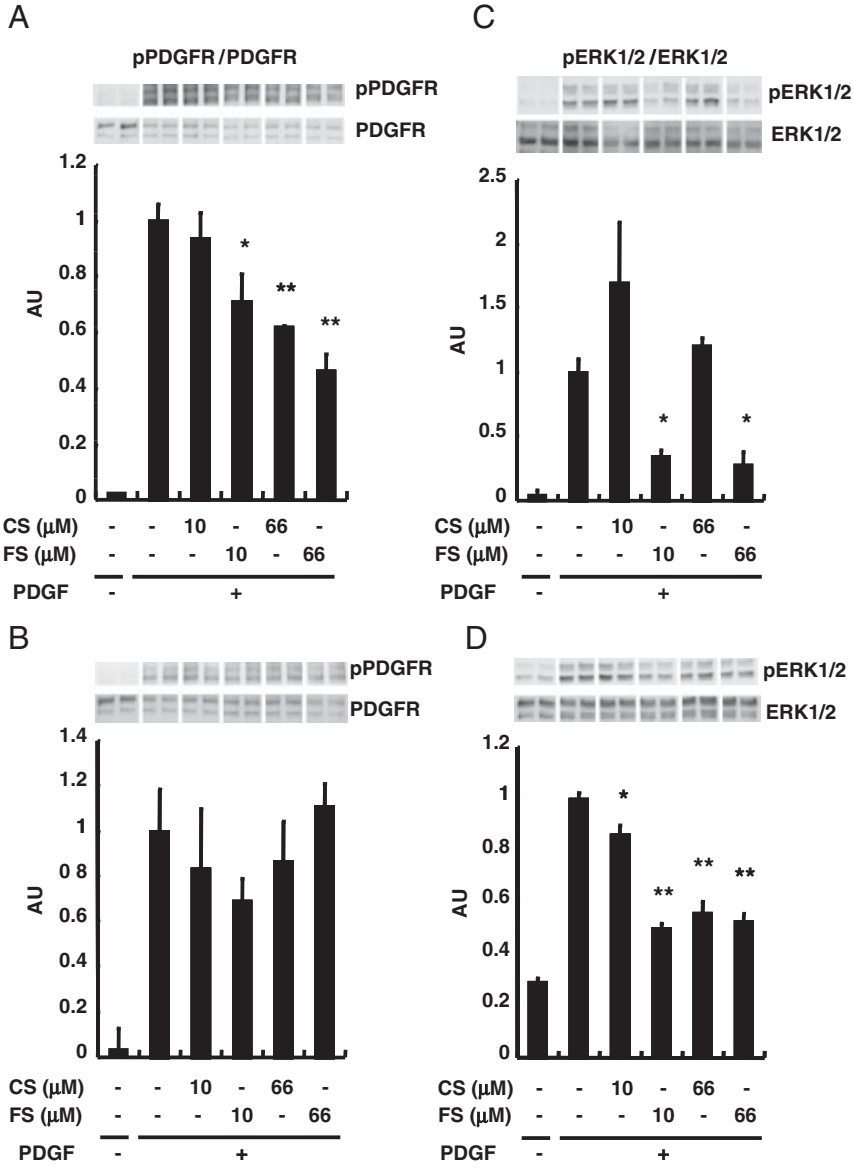

Figure 5. Effect of serotonin hydroxycinnamic acid amides (CS and FS) on PDGF receptor β phosphorylation (A) (B) and ERK1/2 phosphorylation (C) (D) induced by PDGF-BB. A7r5 cells were exposed to 10 ng/ml PDGF-BB with CS or FS (each 10 or 66 μM) for 5 (A) (C) or 10 min (B) (D). Arbitrary unit (AU) represents the proportion of phosphorylation protein to non phosphorylation protein. Value of PDGF-BB stimulated without CS and FS was represented as 1. Values are represented as means ±S.E.M. of 3 determinations. *: $p<0.05$, **: $p<0.01$ shows statistically significant differences when compared with value in PDGF-BB stimulated without CS and FS.

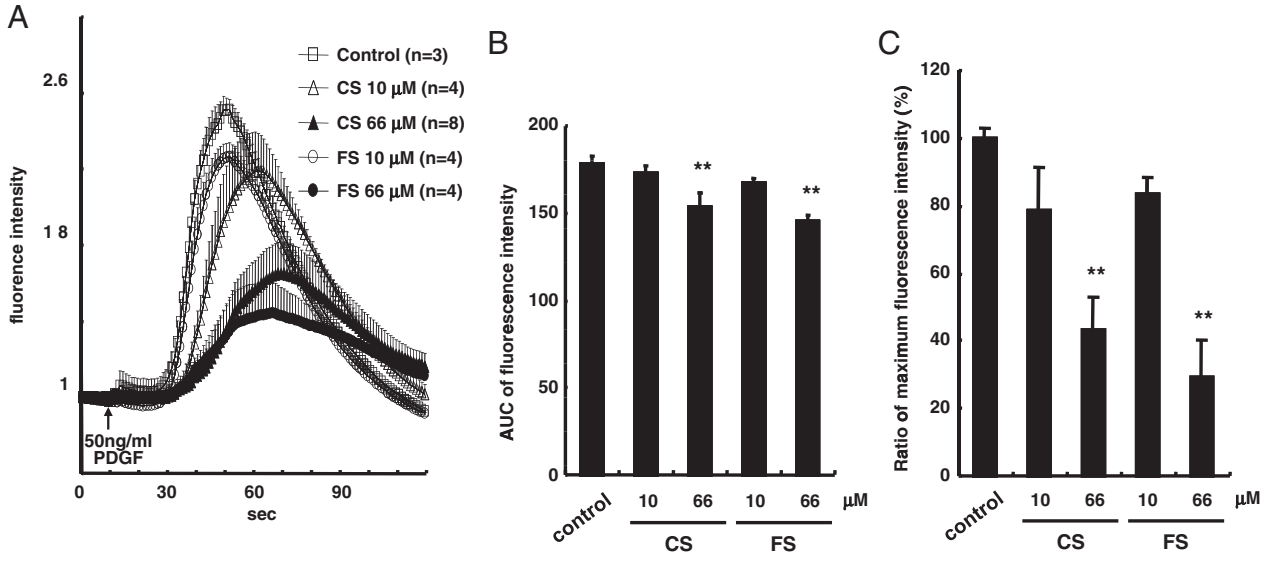

Figure 6. Effect of serotonin hydroxycinnamic acid amides (CS and FS) on increase of intracellular Ca ion concentration induced by 50 ng/ml PDGF-BB. Time course of intracellular Ca ion concentration in A7r5 cells for 120 sec, area under the curve (AUC) of changes of intracellular Ca ion concentration over time in the cells, and the maximum intracellular Ca ion concentration in the cells are indicated in panel (A), (B) and (C), respectively. Value of fluorescence intensity at start point was represented as 1. Values are represented as means ±SEM of 3~8 determinations. **: $p<0.01$ shows statistically significant differences when compared with value of control.

effects, such as vasorelaxation [23] and attenuation of VSMC abnormal activation [24]. Such information prompted us to examine the direct vascular effect of CS and FS to gain a mechanistic insight into the anti-atherogenic effects of them.

Both CS and FS inhibited the KCl-induced increase in $[Ca^{2+}]_i$. This result suggests that CS and FS may inhibit Ca^{2+} influx into VSMCs. As well, CS and FS inhibited the 5-HT-induced increase in $[Ca^{2+}]_i$. The release of Ca^{2+} from SR stimulated by 5-HT is mediated by 5-HT_2 receptors, which are coupled to the production of inositol triphosphate (IP_3) in A7r5 [25]. We speculate that CS and FS interfere with the signal transduction that leads to the release of Ca^{2+} from the SR, which eventually results in inhibition of the $[Ca^{2+}]_i$ increase. This is possibly a result of antagonistic binding to the 5-HT_2 receptors, since CS and FS both possess a 5-HT moiety. Because this effect disappeared when these compounds were washed out, these SHAAs may act as competitive antagonists which displace 5-HT from its binding site at sufficient concentration, not as non-competitive antagonists which bind to the receptor irreversibly nor affecting binding constant of 5-HT. To confirm this hypothesis, a precise characterization of interaction between these SHAAs and 5-HT_2 receptor should be done by competitive binding analysis.

Neither CS nor FS had any effect on the increase of $[Ca^{2+}]_i$ induced by SOCs activation which was caused by the deprivation of Ca^{2+} in SR by thapsigargin treatment. Therefore, it is unlikely that CS or FS interact with SOCs to affect the Ca^{2+} dynamics.

Many natural compounds derived from plants show vasodilation effects. The mechanisms of some natural compounds have already been discussed [10, 23], such as apigenin, which inhibits Ca^{2+} uptake [26]. The vasodilation effect of some flavonoids including apigenin is thought to result from inhibition of the increase in $[Ca^{2+}]_i$ [27]. This is the first time to report that SHAAs, which do not have flavonoid-like structures, were able to inhibit the increase in $[Ca^{2+}]_i$. To understand the structure-activity relationship of these polyphenols by comparing vasodilative activity among structurally-related phenolic compounds (eg; flavonoids, SHAAs, curcuminoids, chemically-modified phenolic compounds derivatives, etc) may gain more insight into the regulation of cellular Ca^{2+} dynamics.

EC_{50} values for vasodilative effect of CS in the endothelium-denuded artery were significantly higher than that in the intact one. Piga et al. recently reported that both CS and FS directly interact with endothelial cells and attenuate proinflammatory reaction elicited by high glucose [21]. They demonstrated the localization of these compounds in the mitochondria in the cells where the suppression of high glucose-induced reactive oxygen species was also observed by the treatment. Thus, we do not exclude the possibility that CS and FS has modulated smooth muscle tone via regulating endothelial function. Whether these compounds indirectly regulate smooth muscle tone via interaction between endothelial cells and which signaling pathway is involved remained to be elucidated.

We examined the effects of CS and FS on PDGF-evoked proliferation and migration of cultured rat aortic smooth muscle cell line (A7r5) and demonstrated that both CS and FS inhibited them in a concentration-dependent manner. Serum-stimulated hyperplastic change in the rabbit

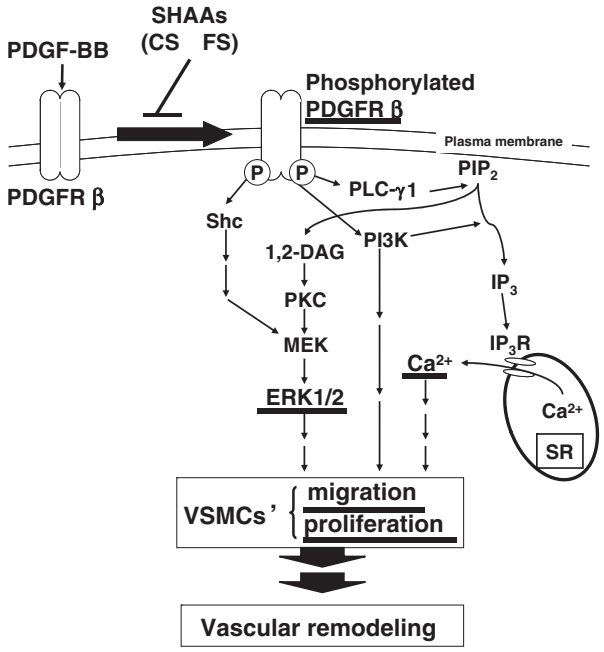

Figure 7. Schematic diagram of PDGF signaling and the site of action of serotonin hydroxycinnamic acid amides (SHAAs; CS and FS). The effects of SHAAs on the underlined factors are evaluated in this study. CS, N-(*p*-coumaroyl)serotonin; DAG, diacylglycerol; ERK, extracellular signal-regulated kinase; FS, N-feruloylserotonin; IP_3, Inositol triphosphate; IP_3R, IP_3 receptor; MEK, mitogen-activated protein kinase or ERK kinase PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PI3K, phosphatidylinositol 3-kinase; PIP_2, Phosphatidylinositol 3,4-bisphosphate; PKC, phosphokinase C; PLC, phospholipase C; SHAAs, serotonin hydroxycinnamic acid amides; Shc, SH2-containing collagen-related proteins; SR, sarcoplasmic reticulum; VSMCs, vascular smooth muscle cells.

mesenteric arterial ring was also suppressed when cultured with these serotonin conjugates. To the best of our knowledge, this is the first report which demonstrates the direct interaction between VSMCs and the SHAAs. Proliferation and migration of VSMCs in arterial wall and resulting thickened intima-media complex is generally recognized as one of the crucial events in atherogenesis [28]. Moreover, enhanced PDGF action and abnormal proliferation and migration of VSMCs in apoE-deficient mice has been reported [29–31], therefore, the effects on VSMCs may be responsible at least in part for the anti-atherogenic effect of CS and FS besides their effect on LDL oxidation. Reported significant reduction of aortic wall thickness in KHC rabbits fed on CS and FS-containing atherogenic diet [19] may be relevant to the result obtained from rabbit arterial ring organ culture study.

Then we questioned whether the PDGF downstream signaling was affected by these compounds and found that CS and FS attenuated PDGF action on PDGFR tyrosine phosphorylation, downstream ERK1/2 phosphorylation and Ca^{2+} release from SR. PDGF-BB propagates mitogenic signals through autophosphorylation of its cognate receptor and causes subsequent phosphorylation of other proteins, including phospholipase C-γ(PLC-γ), Shc, and phosphatidylinositol 3-kinase (PI3K) [32]. PLC-γ and PI3K stimulate production of IP_3 which subsequently interacts with IP_3-receptor on SR to release Ca^{2+} from SR, and elevated intracellular Ca^{2+} in turn leads to proliferation and migration of VSMCs. It has also been reported that Shc could activate Ras, and consequently activated MEK-ERK stimulated cell growth [32, 33]. Thus, it seems plausible that the inhibition of intracellular Ca^{2+} rise evoked by PDGF is one of the consequences of the inhibitory effect of SHAAs on PDGFR phosphorylation. (see Fig. 7)

To date, a body of research has demonstrated that several natural compounds, such as luteolin [34], cudraflavanone [35], cudratricusxanthone [36], fangchinoline [37], astrapterocarpan [38], catechins [12], curcumin [11] and lycopene [13], attenuate VSMC proliferation by down-regulating PDGF signaling. A relationship between the function and their structure has been discussed; however, it is still controversial. CS and FS belong to a class of phenylpropanoid amides with an indole ring, which is considered as a structurally different class of polyphenols from those described above. It is intriguing that many compounds exert similar effects on PDGF signaling without structural kinship. Although there is no obvious structural kinship, these compounds share a functional similarity in varying degree, such as antioxidant action. It has been postulated that oxidation of protein tyrosine phosphatases (PTPs) is a candidate mechanism for ROS-meditated effects on VSMC proliferation and migration, and Kappert et al. demonstrated that some antioxidants attenuated PDGFR tyrosine phosphorylation and prevented thickening of vessel wall by protecting PTPs from oxidative insult [39]. Antioxidant action of CS and FS on VSMC to negatively regulate PDGF intracellular signaling may have significant impact on their anti-atherogenic effect as well as the action on LDL to protect them from oxidation.

Since some previously-reported experiments showed that some kind of calcium blockers had preventive effect against atherosclerosis [8, 40], Ca^{2+} influx inhibitory effect of CS and FS may not be only involved in regulation of vascular contraction, but also in inhibition of atherosclerosis progression by regulating VSMCs' migration and proliferation like the calcium blockers. If it is true, the anti-atherosclerotic action of CS and FS shown in rabbits [19] and mice [18] may be a consequence of pleiotropic effects of these compounds such as attenuation of PDGF signaling and inhibition of $[Ca^{2+}]_i$ increase in addition to inhibition of LDL oxidation caused by their anti-oxidative activity.

Because arterial stiffness is affected in nature by many factors such as vascular tone and wall thickness, the reported reduction of arterial stiffness in KHC rabbits [19] and human volunteers [41] may also be a consequence of vasorelaxation and/or the reduction of VSMC proliferation and migration by CS and FS.

Because a part of orally- administered CS and/or FS were found without being metabolized in plasma [18] and in rat aorta [42], it is possible to link the vascular effects found in this study to various in vivo effects of these SHAAs previously reported [18–20, 41].

In conclusion, our results suggest that CS and FS may contribute to vascular health and prevention of cardiovascular disease.

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