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# Endothelium-Independent Effect of Fisetin on the Agonist-Induced Regulation of Vascular Contractility

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## Abstract

Fisetin, a natural flavonoid found in a variety of vegetables and fruits, has been shown to possess many biological functions. The present study was undertaken to investigate the influence of fisetin on vascular smooth muscle contractility and to determine the mechanism involved. Denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Fisetin significantly relaxed fluoride-, thromboxane $A_2$- or phorbol ester-induced vascular contraction suggesting as a possible anti-hypertensive on the agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, fisetin significantly inhibited fluoride-induced increases in pMYPT1 levels and phorbol ester-induced increases in pERK1/2 levels suggesting the mechanism involving the inhibition of Rho-kinase activity and the subsequent phosphorylation of MYPT1 and MEK activity and the subsequent phosphorylation of ERK1/2. This study provides evidence regarding the mechanism underlying the relaxation effect of fisetin on agonist-induced vascular contraction regardless of endothelial function.

**Key Words:** ERK1/2, Fisetin, Fluoride, MYPT1, Phorbol ester, Rho-kinase

## INTRODUCTION

Fisetin (3,3′,4′,7-tetrahydroxyflavone, Fig. 1), a tetrahydroxyflavone, is a flavonoid rich in strawberries and other edible fruits or vegetables (Ross and Kasum, 2002). Fisetin has a wide variety of pharmacological activities such as anti-allergic, cancer chemo-preventive and neuroprotective activities (Cheong *et al*., 1998; Ravichandran *et al*., 2011; Patel *et al*., 2012). Studies have shown that fisetin possesses the anti-cancer effect through its anti-proliferative, antioxidant and ROS generating activities (Lee *et al*., 2002; Jang *et al*., 2012) and recently through increased generation of NO and elevated $Ca^{2+}$ entry activating the caspase dependent apoptotic pathways (Ash *et al*., 2015). We investigated the possible influence and related mechanisms of the fisetin on vascular smooth muscle contractility to develop a better antihypertensive. Intact or denuded aortic rings from male Sprague-Dawley rats were used and isometric contractions were recorded using a computerized data acquisition system. These data were combined with molecular experiments.

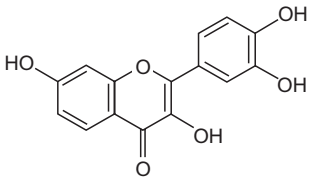


**Fig. 1.** The chemical structure of fisetin (3,3′,4′,7-tetrahydroxyflavone).

Hypertension is the most prevalent modifiable risk factor for cardiovascular morbidity and mortality. More importantly, stroke and ischemic heart disease are directly attributable to hypertension that is a multifactorial disorder and involves many mechanisms including endothelial dysfunction and leading to risk factors for cardiovascular diseases, thus primarily responsible for one quarter of deaths recorded globally. Besides endothelial dysfunction, it is generally accepted that vascular smooth muscle contractility is predominantly controlled by $Ca^{2+}$ signaling involving $Ca^{2+}$ influx, release or sensitization and regulating a $Ca^{2+}$-dependent increase in the phosphorylation of a 20 kDa myosin light chain ($MLC_{20}$) (Somlyo and

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Somlyo, 1994). The extent of $MLC_{20}$ phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the cytosolic $Ca^{2+}$ concentration referred to as $Ca^{2+}$ sensitization (Somlyo and Somlyo, 1994). Subsequent studies suggested that the inhibition of MLC phosphatase by Rho-kinase (Kitazawa *et al*., 1991; Uehata *et al*., 1997; Somlyo and Somlyo, 1998; Sakurada *et al*., 2003) or thin filament regulation including the activation of protein kinase C (PKC), mitogen-activated protein kinase kinases (MEK) and extracellular signal regulated kinase (ERK) 1/2, and phosphorylation of the actin binding protein caldesmon (Wier and Morgan, 2003) may be major components of the pathway that facilitates in $Ca^{2+}$ sensitization.

Activation of ERK1/2 cannot only regulate vascular contractility but also is connected with pathologic hypertrophy, hyperplasia, hypertension and atherosclerosis (Xu *et al*., 1996; Touyz *et al*., 1999). ERK1/2 is activated by threonine and tyrosine phosphorylation by the specific kinase MEK activated by Raf. In various smooth muscles, fluoride, phorbol ester or thromboxane $A_2$ mimetic has been shown to induce contractions, which may be due to primarily enhanced $Ca^{2+}$ sensitivity or partially increased $Ca^{2+}$ concentration only in thromboxane $A_2$ mimetic. ERK1/2 activation was induced by the phorbol ester, phorbol 12,13-dibutyrate (PDBu). The stimulus PDBu triggers ERK1/2 dependent cytoskeletal remodeling and formation of podosomes inducing ERK1/2 activation (Gu *et al*., 2007). On the other hand, it is possible that the contractions induced by fluoride or thromboxane $A_2$ mimetic involve the RhoA/Rho-kinase pathway (Jeon *et al*., 2006). However, it has not been reported as to whether this pathway is inhibited during fisetin-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator fluoride or MEK activator phorbol ester. Therefore, the aim of the present study was to investigate the possible roles of Rho-kinase or MEK inhibition on $Ca^{2+}$ desensitization during the fisetin-induced relaxation of isolated rat aortas by using RhoA/Rho-kinase activators fluoride or thromboxane $A_2$ or a MEK activator phorbol ester excluding endothelial nitric oxide synthesis.

## MATERIALS AND METHODS

### Tissue preparation

Male Sprague-Dawley rats weighing 250-300 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) as subjected to cervical dislocation, in accord with the procedures approved by the Institutional Animal Care and Use Committee at our institutions. Thoracic aortas were quickly removed and immersed in oxygenated (95% $O_2$/5% $CO_2$) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.0 $NaHCO_3$, 1.2 $KH_2PO_4$, and 10.0 dextrose (pH 7.4). They were then freed of all adherent connective tissue, and aortic endothelia were removed by gentle abrasion using a cell scraper if necessary.

### Contraction measurements

Two stainless-steel triangles were inserted through each vessel ring and each aortic ring was then suspended in a water-jacketed organ bath (10 ml) maintained at 37°C and aerated with a mixture of 95% $O_2$ and 5% $CO_2$. One triangle was anchored to a stationary support, and the other was connected to an isometric force transducer (Grass FT03C, Quincy, Mass., USA). The rings were stretched passively by applying an optimal resting tension of 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 60 min before contractile responses to 50 mM KCl or 1 μM phenylephrine were measured. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, AD Instruments, Castle Hill, NSW, Australia).

The direct effect of fisetin was determined by addition of it after KCl (50 mM), thromboxane $A_2$ (0.1 μM), phorbol ester (1 μM) or fluoride (6 mM) induced contractions had plateaued in normal Krebs' solution.

### Western blot analysis

Muscle strips were quick-frozen by immersion in a dry ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Muscles were stored at -80°C until use. Tissues were brought up to room temperature in a dry ice/acetone/TCA/DTT mixture and then homogenized in a buffer containing 20 mM MOPS, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 μM leupeptin, 5.5 μM pepstatin, 20 kIU aprotinin, 2 mM $Na_3VO_4$, 1 mM NaF, 100 μM $ZnCl_2$, 20 μM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) and 5 mM EGTA. Protein-matched samples (modified Lowry protein assay, DC Protein Assay Kit, Bio-Rad) were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (Protogel, National Diagnostics), transferred to polyvinylidene fluoride PVDF membranes, and subjected to immunostaining and densitometry using primary and secondary antibodies. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and by densitometry of the actin band. Lane loading variations were corrected by normalization versus β-actin. Sets of samples produced during individual experiments were run in the same gel and densitometry was performed on the same image.

### Chemicals and antibodies

Drugs and chemicals were obtained from the following sources. Sodium fluoride, KCl, acetylcholine, fisetin, U-46619 and phorbol 12,13-dibutyrate were purchased from Sigma (St. Louis, MO, USA). DTT, TCA and acetone were obtained from Fisher Scientific (Hampton, NH, USA). Enhanced chemiluminescence (ECL) kits were from Pierce (Rockford, IL, USA). Antibodies against phospho-myosin phosphatase targeting subunit protein 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, ERK or phosphoERK at Thr202/Tyr204 were purchased from Cell Signaling Technology (Danvers, MA, USA) or Upstate Biotechnology (Lake Placid, NY, USA) to determine levels of RhoA/Rho-kinase activity (Wooldridge *et al*., 2004; Wilson *et al*, 2005) or MEK activity. Anti-mouse IgM (goat) and anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1:2,000 and 1:2,000, respectively, Upstate, Lake Placid, NY). Fisetin solution was prepared in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and frozen at -20°C for later use. DMSO alone had no observable effect at concentrations used (data not shown).

### Statistics

The data were expressed as mean ± standard error of the mean (SEM). The student's unpaired *t* test or ANOVA was used to determine the statistical significance of the means

between two groups using SPSS 12.0 (SPSS Inc., Chicago, Illinois, U.S.A.). *p*-values<0.05 were regarded as statistically significant.

## RESULTS

### Effect of fisetin on contractions of endothelium-denuded aortas induced by a full RhoA/Rho-kinase activator fluoride

Endothelium was removed by gentle abrasion with a cell scraper to identify the direct effect of fisetin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine (1 μM). Fisetin showed no significant effect on basal tension (data not shown), and significantly inhibited the contraction induced by a Rho-kinase activator fluoride at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 2). This suggests that the relaxation mechanism of fisetin might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase.

### Effect of pretreated fisetin on contraction of denuded aortas induced by a full RhoA/Rho-kinase activator thromboxane $A_2$

The addition of the thromboxane $A_2$ mimetic U-46619 (0.001-1 μM) produced concentration-dependent contractions in denuded (Fig. 3A) or intact (Fig. 3B) muscles. Interestingly, this response was significantly inhibited by fisetin with endothelium denuded (Fig. 3A) or intact endothelium (Fig. 3B); and this was true regardless of endothelial function in either pretreatment or direct relaxation suggesting that thromboxane $A_2$ mimetic acts similarly from fluoride where Rho-kinase activation was the main pathway.

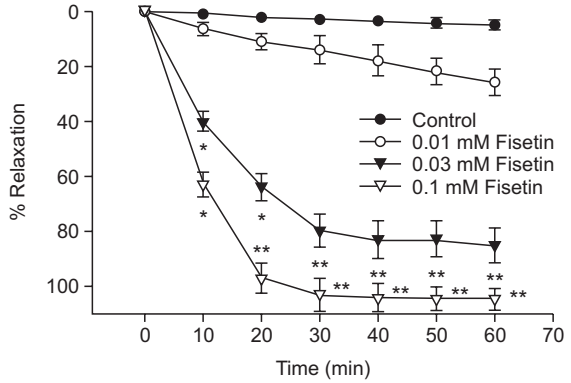


**Fig. 2.** Effect of fisetin on fluoride-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to fisetin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ***p*<0.01, presence versus absence of fisetin.

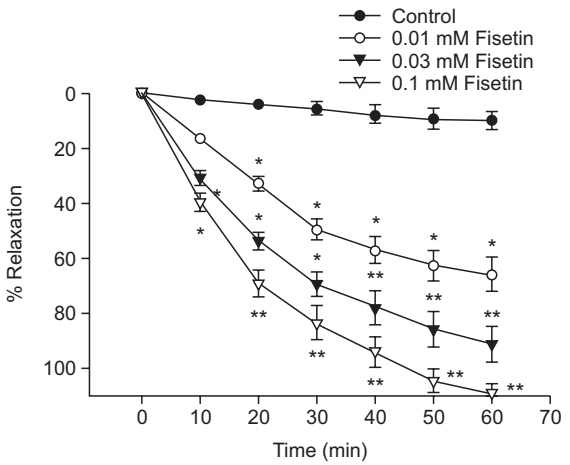


**Fig. 4.** Effect of fisetin on phorbol ester-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to fisetin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ***p*<0.01, presence versus absence of fisetin.

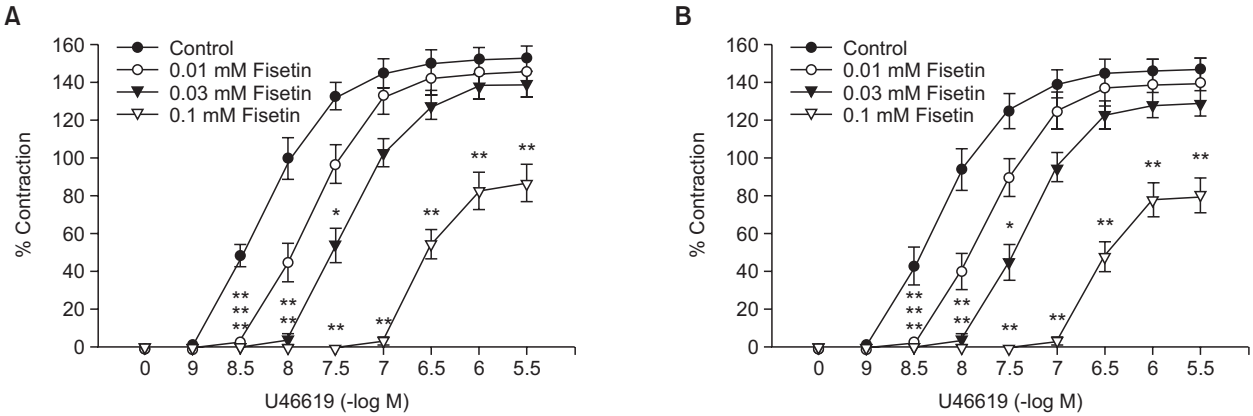


**Fig. 3.** Effect of pretreated fisetin on thromboxane $A_2$-induced vascular contraction in denuded (A) or intact (B) muscles. U46619 was added to elicit tension in the presence or absence of fisetin for 30 min in aortic rings with endothelium denuded (A) or intact endothelium (B). Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as the means of 3-5 experiments with vertical lines representing SEM. **p*<0.05, ***p*<0.01, presence versus absence of fisetin.

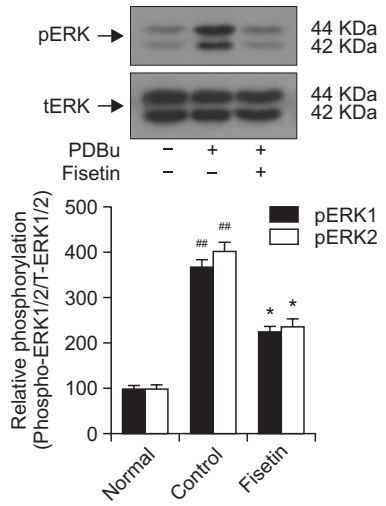


**Fig. 5.** Effect of fisetin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were decreased in quick frozen fisetin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. *$p$<0.05, $^{\#\#}p$<0.01, versus control or normal group respectively. Fisetin: 0.1 mM fisetin; PDBu: 1 μM phorbol 12,13-dibutyrate.

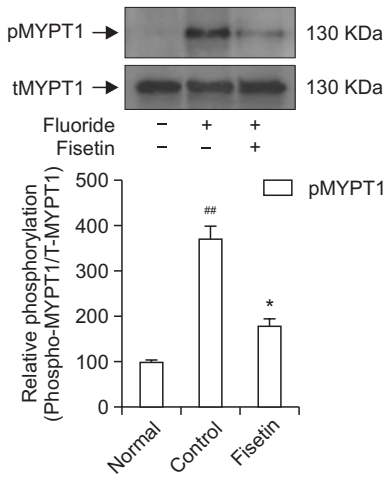


**Fig. 6.** Effect of fisetin on fluoride-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen fisetin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. *$p$<0.05, $^{\#\#}p$<0.01, versus control or normal group respectively. Fisetin: 0.1 mM fisetin; Fluoride: 6 mM sodium fluoride.

### Effect of fisetin on the contractions of denuded aortas induced by a MEK activator phorbol ester

Phorbol esters used have been proved to be MEK activators and partial RhoA/Rho-kinase activators (Goyal *et al*., 2009; Je and Sohn, 2009). Interestingly, phorbol 12,13-dibutyrate-induced contraction was significantly inhibited by fisetin at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 4), which suggested that thin or actin filament regulation including MEK/ERK were significantly inhibited.

### Effect of fisetin on levels of ERK1/2 phosphorylation at Thr-202/Tyr-204

To confirm the role of fisetin on thin filament regulation of smooth muscle contractility, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick frozen after 60 minutes of exposure to fisetin for the equilibration. Each relaxing ring was precontracted with 1 μM phorbol ester (phorbol 12,13-dibutyrate). As compared with vehicle-treated rat aortas, a significant decrease in ERK 1/2 phosphorylation at Thr202/Tyr204 was led by fisetin in these fisetin (0.1 mM)-treated rat aortas in the absence of endothelium (Fig. 5) showing full vasorelaxation (Fig. 4) and thin filament regulation. These findings show that thin or actin filament regulation including ERK1/2 phosphorylation via MEK activation might be of importance in the decreased contractility induced by fisetin.

### Effect of fisetin on the level of MYPT1 phosphorylation at Thr-855

To confirm the role of fisetin on the thick filament regulation of smooth muscle contractility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in muscles quick frozen after 60 min exposure to fisetin for the equilibration. Each relaxing ring was precontracted with 6 mM fluoride. This work was done using quick frozen fisetin (0.1 mM)-treated rat aortas in the absence of endothelium and the levels were compared to those of vehicle-treated rat aortas (Fig. 6). Interestingly, significant decrease in fluoride-induced MYPT1 phosphorylation at Thr855 was found to be led by fisetin (Fig. 6). Thus, thick or myosin filament regulation including myosin phosphatase activation via RhoA/Rho-kinase inactivation might be involved in the reduced contractility of fisetin-treated rat aorta.

## DISCUSSION

The present study demonstrates that fisetin can modulate the vascular contractility in an agonist-dependent manner. Interestingly, the mechanism involved seems to be not only endothelium-dependent but also to involve the equal inhibition of MEK and Rho-kinase activity. Fisetin has been previously recognized for its anti-inflammatory or antioxidant activity. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to fisetin-induced vascular relaxation in rat aortas denuded and precontracted by a RhoA/Rho-kinase activator fluoride or by a MEK activator phorbol ester.

The mechanism by which phorbol ester activates MEK/ERK has been established (Kordowska *et al*., 2006; Gu *et al*., 2007). On the other hand, previous studies that examined the mechanisms underlying arterial contractions induced by fluoride or thromboxane $A_2$ have reported variable findings with regard to the contraction triggered by Rho-kinase activation (Wilson *et al*., 2005; Tsai and Jiang, 2006). These findings are consistent with the notion that fisetin can decrease phorbol ester or fluoride-induced contraction by inhibiting MEK or Rho-kinase activity.

The mechanisms by which MEK activation causes vascular contraction is an area of intense study, and several possibilities exist. The phosphorylation of caldesmon by MEK/ERK appears to regulate smooth muscle contractility (Kordowska *et al*., 2006). In this process MEK/ERK is activated by PKC which in turn can be stimulated by phorbol esters or GPCR receptor agonists.

The present study demonstrates that fisetin ameliorates the maximal or submaximal contraction induced by vasoconstrictor fluoride or phorbol ester endothelium-independently (Fig. 2, 4), and that this ameliorative mechanism involves the MEK/ERK and RhoA/Rho-kinase pathway. Previously, most vasodilation was believed to be caused by endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase (Taubert *et al*., 2002; Ajay *et al*., 2003). In the present study, fisetin at a low concentration significantly inhibited phorbol ester- or fluoride-induced contraction regardless of endothelial function (Fig. 2, 4). Furthermore, fisetin decreased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by phorbol ester (Fig. 5) and significantly decreased the phosphorylation of MYPT1 at Thr855 induced by fluoride (Fig. 6) with full relaxation (Fig. 2) suggesting the inhibition of Rho-kinase or MEK activity as a major mechanism.

In summary, fisetin at a low concentration significantly attenuates the contractions induced by a MEK activator phorbol ester regardless of endothelial function. Furthermore, a Rho-kinase activator fluoride-induced contraction was significantly inhibited by fisetin at this low concentration. Thus, the mechanism underlying the relaxation induced by fisetin at a low concentration in phorbol ester or fluoride-induced contractions involves the inhibition of MEK activity and Rho-kinase activity which work independently or synergistically (Lim *et al*., 2014). Interestingly, during fluoride-induced contraction, the inhibition of Rho-kinase activity and subsequent MYPT1 phosphorylation induced by fisetin suggest that Rho-kinase inactivation is required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis in intact muscle which makes synergism, both MEK and Rho-kinase inhibition make a major contribution to the mechanism responsible for fisetin-induced vasorelaxation in the denuded muscle.

## REFERENCES


Ajay, M., Gilani, A. U. and Mustafa, M. R. (2003) Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci.* **74**, 603-612.

Ash, D., Subramanian, M., Surolia, A. and Shaha, C. (2015) Nitric oxide is the key mediator of death induced by fisetin in human acute monocytic leukemia cells. *Am. J. Cancer Res.* **5**, 481-497.

Cheong, H. Ryu, S. Y., Oak, M. H., Cheon, S. H., Yoo, G. S. and Kim, K. M. (1998) Studies of structure activity relationship of flavonoids for the anti-allergic actions. *Arch. Pharm. Res.* **21**, 478-480.

Goyal, R., Mittal, A., Chu, N., Shi, L., Zhang, L. and Longo L. D. (2009) Maturation and the role of PKC-mediated contractility in ovine cerebral arteries. *Am. J. Physiol. Heart Circ. Physiol.* **297**, H2242-2252.

Gu, Z., Kordowska, J., Williams, G. L., Wang, C. L. and Hai, C. M. (2007) Erk1/2 MAPK and caldesmon differentially regulate podosome dynamics in A7r5 vascular smooth muscle cells. *Exp. Cell Res.* **313**, 849-866.

Jang, K. Y., Jeong, S. J., Kim, S. H., Jung, J. H., Kim, J. H., Koh, W., Chen, C. Y. and Kim, S. H. (2012) Activation of reactive oxygen species/AMP activated protein kinase signaling mediates fisetin-induced apoptosis in multiple myeloma U266 cells. *Cancer Lett.* **319**, 197-202.

Je, H. D. and Sohn, U. D. (2009) Inhibitory effect of genistein on agonist-induced modulation of vascular contractility. *Mol. Cells* **27**, 191-198.

Jeon, S. B., Jin, F., Kim, J. I., Kim, S. H., Suk, K., Chae, S. C., Jun, J. E., Park, W. H. and Kim, I. K. (2006) A role for Rho kinase in vascular contraction evoked by sodium fluoride. *Biochem. Biophys. Res. Commun.* **343**, 27-33.

Kitazawa, T., Masuo, M. and Somlyo, A. P. (1991) G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9307-9310.

Kordowska, J., Huang, R. and Wang, C. L. (2006) Phosphorylation of caldesmon during smooth muscle contraction and cell migration or proliferation. *J. Biomed. Sci.* **13**, 159-172.

Lee, W. R., Shen, S. C., Lin, H. Y., Hou, W. C., Yang, L. L. and Chen, Y. C. (2002) Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase 3 and Ca(2+)-dependent endonuclease. *Biochem. Pharmacol.* **63**, 225-236.

Lim, K. M., Kwon, J. H., Kim, K., Noh, J. Y., Kang, S., Park, J. M., Lee, M. Y., Bae, O. N. and Chung, J. H. (2014) Emodin inhibits tonic tension through suppressing PKCδ-mediated inhibition of myosin phosphatase in rat isolated thoracic aorta. *Br. J. Pharmacol.* **171**, 4300-4310.

Patel, M. Y., Panchal, H. V., Ghribi, O. and Benzeroual, K. E. (2012) The neuroprotective effect of fisetin in the MPTP model of Parkinson's disease. *J. Parkinsons Dis.* **2**, 287-302.

Ravichandran, N., Suresh, G., Ramesh, B. and Siva, G. V. (2011) Fisetin, a novel flavonol attenuates benzo(a)pyrene-induced lung carcinogenesis in Swiss albino mice. *Food Chem. Toxicol*. **49**, 1141-1147.

Ross, J. A. and Kasum, C. M. (2002) Dietary flavonoids: bioavailability, metabolic effects, and safety. *Annu. Rev. Nutr.* **22**, 19-34.

Sakurada, S., Takuwa, N., Sugimoto, N., Wang, Y., Seto, M., Sasaki, Y. and Takuwa, Y. (2003) $Ca^{2+}$-dependent activation of Rho and Rho-kinase in membrane depolarization-induced and receptor stimulation-induced vascular smooth muscle contraction. *Circ. Res.* **93**, 548-556.

Somlyo, A. P. and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature* **372**, 231-236.

Somlyo, A. P. and Somlyo, A. V. (1998) From pharmacomechanical coupling to G-proteins and myosin phosphatase. *Acta Physiol. Scand.* **164**, 437-448.

Taubert, D., Berkels, R., Klaus, W. and Roesen, R. (2002) Nitric oxide formation and corresponding relaxation of porcine coronary arteries induced by plant phenols: essential structural features. *J. Cardiovasc. Pharmacol.* **40**, 701-13.

Touyz, R. M., Deng, L. Y., He, G., Wu, X. H. and Schiffrin, E. L. (1999) Angiotensin II stimulates DNA and protein synthesis in vascular smooth muscle cells from human arteries: role of extracellular signal-regulated kinases. *J. Hypertens.* **17**, 907-916.

Tsai, M. H. and Jiang, M. J. (2006) Rho-kinase-mediated regulation of receptor-agonist-stimulated smooth muscle contraction. *Pflugers Arch.* **453**, 223-232.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. and Narumiya, S. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990-994.

Wier, W. G. and Morgan, K. G. (2003) $\alpha$1-Adrenergic signaling mechanisms in contraction of resistance arteries. *Rev. Physiol. Biochem. Pharmacol.* **150**, 91-139.

Wilson, D. P., Susnjar, M., Kiss, E., Sutherland, C. and Walsh, M. P. (2005) Thromboxane A2-induced contraction of rat caudal arterial smooth muscle involves activation of $Ca^{2+}$ entry and $Ca^{2+}$ sensitization: Rho-associated kinase-mediated phosphorylation of MYPT1 at Thr-855, but not Thr-697. *Biochem. J.* **389**, 763-774.

Wooldridge, A. A., MacDonald, J. A., Erdodi, F., Ma, C., Borman, M. A., Hartshorne, D. J. and Haystead, T. A. (2004) Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of Serine 695 in response to cyclic nucleotides. *J. Biol. Chem.* **279**, 34496-34504.

Xu, Q., Liu, Y., Gorospe, M., Udelsman, R. and Holbrook, N. J. (1996) Acute hypertension activates mitogen-activated protein kinases in arterial wall. *J. Clin. Invest.* **97**, 508-514.