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Flavone Attenuates Vascular Contractions by Inhibiting RhoA/Rho Kinase Pathway

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Our previous study demonstrated that flavone inhibits vascular contractions by decreasing the phosphorylation levelof the myosin phosphatase target subunit (MYPT1). In the present study, we hypothesized that flavone attenuates vascular contractions through the inhibition of the RhoA/Rho kinase pathway. Rat aortic rings were denuded of endothelium, mounted in organ baths, and contracted with either 30 nM U46619 (a thromboxane A2 analogue) or 8.0 mM NaF 30 min after pretreatment with either flavone (100 or 300 μM) or vehicle. We determined the phosphorylation level of the myosin light chain (MLC_{20}), the myosin phophatase targeting subunit 1 (MYPT1) and the protein kinase C-potentiated inhibitory protein for heterotrimeric myosin light chain phophatase of 17-kDa (CPI17) by means of Western blot analysis. Flavone inhibited, not only vascular contractions induced by these contractors, but also the levels of MLC_{20} phosphorylation. Furthermore, flavone inhibited the activation of RhoA which had been induced by either U46619 or NaF. Incubation with flavone attenuated U46619- or NaF-induced phosphorylation of $MYPT1^{Thr855}$ and $CPI17^{Thr38}$, the downstream effectors of Rho-kinase. In regards to the Ca^{2+}-free solution, flavone inhibited the phosphorylation of $MYPT1^{Thr855}$ and $CPI17^{Thr38}$, as well as vascular contractions induced by U46619. These results indicate that flavone attenuates vascular contractions, at least in part, through the inhibition of the RhoA/Rho-kinase pathway.

Key Words: Flavone, RhoA, Rho kinase, CPI-17, MYPT1, Vasorelaxation

INTRODUCTION

Flavonoids have been known to have cardioprotective effects (Machha and Mustafa, 2005; Zern and Fernandez, 2005), which are chiefly ascribed to their antioxidant and vasodilatory actions (Zenebe et al., 2001). Since the DASH (Dietary Approaches to Stop Hypertension) diet has high compositions of phytochemicals, its health benefits are also attributable to phytochemicals, such as flavonoids (Most, 2004). While most flavonoids usually induce endothelium-dependent vasorelaxation (Fitzpatrick et al., 2000; Ajay et al., 2007), some also exert endothelium-independent vasorelaxation (Ajay et al., 2003; Ajay et al., 2007). Flavone has the highest efficacy in regards to endothelium-independent vasorelaxation among flavonoids (Ajay et al., 2003). Several mechanisms have been proposed to explain the vasorelaxing action of flavonoids including the inhibition of enzymes such as protein kinase C or cAMP or cGMP phosphodiesterase, as well as the inhibition of the Ca^{2+} release from intracellular stores or the Ca^{2+} influx from extracellular fluids (Duarte et al., 1993; Herrera et al., 1996).

The small guanosine triphosphatase RhoA plays an important role as a molecular switch in regards to the enhancement of Ca^{2+} sensitivity during smooth muscle contractions (Hirata et al., 1992). Activated RhoA increases the Ca^{2+} sensitivity of both MLC phosphorylation and smooth muscle contractions (Fukata et al., 2001). Rho-kinase is a downstream effector molecule of RhoA and a major cellular regulator for Ca^{2+}-sensitization in regards to smooth muscle contractions. Rho-kinase inhibits the activity of the myosin light chain phosphatase (MLCP) by means of phosphorylation of either MYPT1 at Thr855 of MLCP or protein kinase C (PKC)-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (CPI17) which inhibits the catalytic domain of MLCP (Eto et al., 1995). CPI17 can be phosphorylated by PKC as well as the Rho-kinase.

In regards to our previous study, we showed that flavone inhibited vascular contractions by decreasing the phosphorylation level of both MLC_{20} and MYPT1 (Jeon et al., 2007). In the present study, we hypothesized that flavone attenuates vascular contractions by inhibiting the RhoA/Rho-kinase signaling pathway.

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ABBREVIATIONS: MLC20, myosin light chain; MYPT1, myosin phosphatase-targeting subunit 1; MLCP, myosin light chain phosphatase; CPI17, PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa; TCA, trichloroacetic acid; DTT, dithiothreitol; ANOVA, analysis of variance; U46619, 9,11-dideoxy-11,9-epoxymethanoprostaglandin $F_{2\alpha}$; Y27632, trans-4-[(1R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride.

METHODS

Chemicals

U46619, NaF, EGTA and flavone were obtained from the Sigma Chemical Co (St. Louis, MO, USA). Stock solutions of U46619 and flavone were prepared in DMSO. All other reagents were analytical grade.

Tissue preparation and tension measurement

The investigation is in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (National Institutes of Health, 1996). Male Sprague-Dawley rats, weighing 320~350 g, were used. With animals under anesthesia (sodium pentobarbital 50 mg kg^{-1} i.p.), the thoracic aorta was immediately excised and immersed in an ice-cold, modified Krebs solution composed of (in mM) NaCl, 115.0; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and dextrose, 10.0. To make a Ca^{2+}-free solution, $CaCl_2$ was omitted and 2 mM EGTA was added. The aorta was cleaned of all adherent connective tissue on wet filter paper, soaked in the Krebs-bicarbonate solution and cut into four ring segments (4 mm in length) as described by Kim et al. (2007). All rings were denuded of endothelium by gently rubbing the internal surface with the edge of forceps. Two stainless-steel triangles were inserted through each vessel ring. Each aortic ring was suspended in a water-jacketed organ bath (20 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 imposing the optimal resting tension, 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 90 min before the experiment involving the contractile response to 50 mM of KCl. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia).

For the contractile response, aortic rings were pretreated with 100 and 300 μM flavone for 30 min, and subjected to treatment U46619 or NaF.

Western blot

After functional study, muscle strips were quickly immersed in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) precooled to −80°C. The aortic rings were washed in acetone containing 5 mM DTT to remove TCA, air-dried and stored at −80°C until use. Previously stored samples were homogenized in a buffer containing 320 mM sucrose, 50 mM TRIS, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors leupeptin (10 μg/ml), trypsin inhibitor (10 μg/ml), aprotinin (2 μg/ml), phenylmethylsulphonyl fluoride (PMSF; 100 μg/ml) and phosphatase inhibitor β-glycerophosphate (50 mM). Protein-matched samples (Bradford assay) were electrophoresed (SDS-PAGE), transferred to nitrocellulose membranes, and subjected to an immunoblot with a pMYPT1 antibody (1 : 4,000, Upstate Biotechnology, Lake Placid, NY, USA) and a pCPI17 antibody (1 : 250, Santa Cruz Biotechnology, Inc) that detects phosphorylated MYPT1 and CPI17. Anti-rabbit IgG and anti-goat IgG, conjugated with horseradish peroxidase, were used as secondary antibody (1 : 4,000, Sigma, St. Louis, MO, USA and 1 : 1,000, Santa Cruz Biotechnology, Inc). The bands containing pMYPT1 and pCPI17 were detected with enhanced chemiluminescence (ECL) visualized on films. The nitrocellulose membranes were stripped of the pMYPT1 and pCPI17 antibody and reblotted with total form of MYPT1 antibody (1 : 4,000, BD Biosciences Pharmingen, San Diego, CA, USA) and CPI17 antibody (1 : 500, Upstate Biotechnology, Lake Placid, NY, USA). Anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were used as secondary antibody (1 : 4,000, Sigma, St. Louis, MO, USA and 1 : 1,000, Upstate Biotechnology, Lake Placid, NY, USA).

MLC_{20} phosphorylation

Muscle strips were quick frozen by immersion in acetone containing 10% TCA and 10 mM DTT precooled to −80°C. Muscles were washed four times with acetone containing 5 M DTT for 15 min each to remove TCA and were soaked 1 hr with frequent vortex in 100 μl of urea sample buffer containing 20 mM Tris base/23 mM glycine (pH 8.6), 8.0 M urea, 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue. 12.5% polyacrylamide gels containing 40% glycerol were pre-electrophoresed for 30 min at 200 V. The running buffer consists of 20 mM Tris base/23 mM glycine (pH 8.6), 2.3 M thioglycolate, and 2.3 mM DTT. The urea-extracted samples (12 μl) were electrophoresed at 300 V for 6 hours, transferred to nitrocellulose membranes, and subjected to immunoblotting with a specific myosin light chain 20 antibody (1 : 2,000, Sigma, St. Louis, MO, USA). Anti-mouse IgM (goat), conjugated with horseradish peroxidase, was used as a secondary antibody (1 : 4,000, Assay design, Ann Arbor, MI, USA). The bands containing myosin light chains were visualized with enhanced chemiluminescence (ECL) on films, and then analyzed by NIH image as described (Kim et al., 2004).

Assay for RhoA activation

Muscle strips were quick frozen by liquid nitrogen, and stored at −80°C. For RhoA activation assays, the procedure followed the manufacture's instruction regarding RhoA G-LISA™ Activation Assay (Cytoskeleton Inc, Denver, CO, USA). Briefly, previously stored samples were homogenized in a lysis buffer, and were centrifuged at 13,200 rpm for 15 min at 4°C. The supernatant, containing 37 μg of proteins, was transferred into a plate and equal volumes of ice-cold binding buffer mixtures were added into each well. The plate was shaken on a cold orbital microplate shaker (300 rpm) for 30 min at 4°C, flicked out of the solution from wells, and incubated with diluted anti-RhoA primary antibodies followed by secondary antibodies on a microplate shaker (300 rpm) at room temperature for 45 min each. The plate was incubated with an HRP detection reagent for 15 min at 37°C and, after addition of an HRP stop buffer, the absorbance was immediately recorded at 490 nm.

Statistical analysis

Data are expressed as mean±S.E.M and were analyzed by repeated measures ANOVA and one-way ANOVA followed by the post-hoc Tukey's test for dose-response curves and protein phosphorylation, respectively, in order to determine statistical significance. p values of less than 0.05 were regarded as significant.

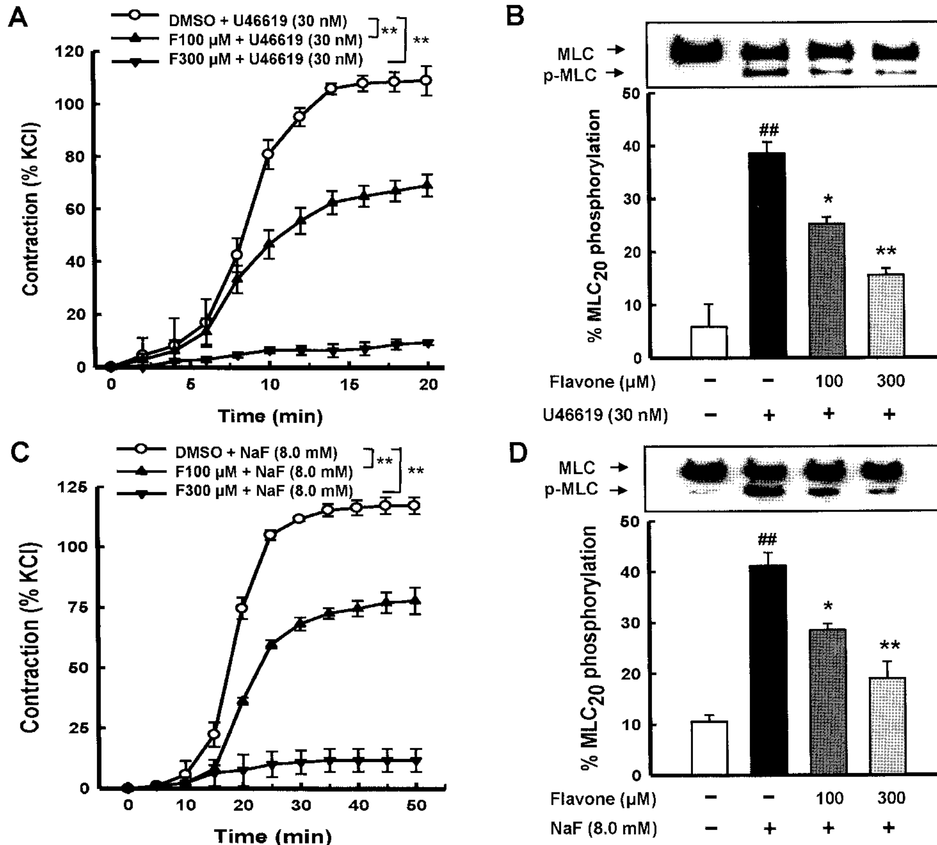

Fig. 1. Inhibitory effect of flavone on contractions and the phosphorylation level of MLC_{20} induced by U46619 or NaF in rat aorta. U46619 (30 nM) (A) or NaF (8.0 mM) (C) was each added to elicit tension 30 min after pretreatment with flavone (100 or 300 μM) or vehicle to denuded aortic rings. Developed tension is expressed as a percentage of the maximum contractions to 50 mM KCl (4 aortic rings). When the tension reached plateaus, the phosphorylation levelof MLC_{20} in response to U46619 (B) or NaF (D) was measured and was expressed as a percentage of the total MLC_{20} (4 aortic rings). Data are expressed as means with vertical bars showing S.E.M. $^{\#\#}p<0.01$ vs. basal. $^{*}p<0.05$, $^{**}p<0.01$ vs. U46619 or NaF alone. (A) and (C) were analyzed by repeated measures ANOVA whereas (B) and (D) by one-way ANOVA followed by the post-hoc Tukey's test.

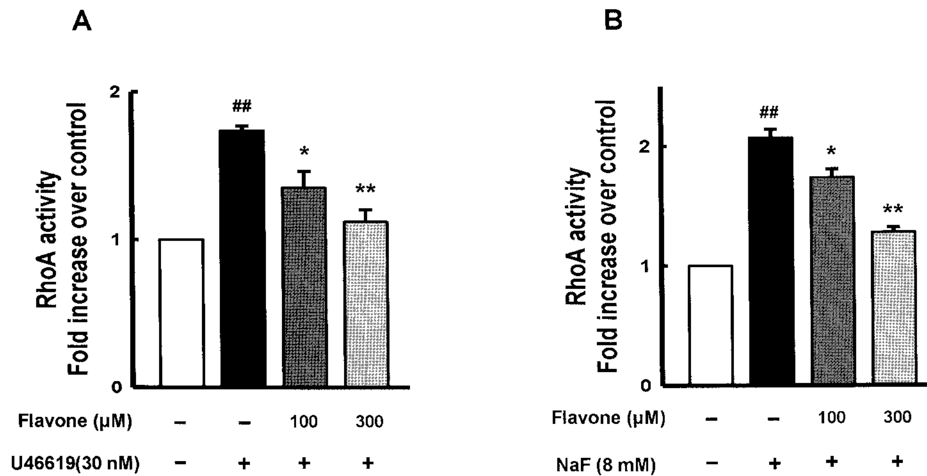

Fig. 2. Inhibitory effect of flavone on RhoA activation induced by U46619 or NaF in rat aorta. RhoA activation was assessed by G-LISA™ Activation Assay (4 aortic rings). Thirty nM U46619 (A) or 8 mM NaF (B) was each added to elicit tension 30 min after pretreatment with flavone (100 or 300 μM) or vehicle to denuded aortic rings. Absorbance at the control (OD of about 0.4) was expressed as 1 arbitrary unit. Data were expressed as means with vertical bars showing S.E.M. $^{*}p<0.05$, $^{**}p<0.01$ vs. U46619 or NaF alone. $^{\#\#}p<0.01$ vs. basal (one-way ANOVA followed by post-hoc Tukey's test).

RESULTS

Inhibitory effects of flavone on contractions as well as MLC_{20} phosphorylation induced by U46619 or NaF

The concentration-response relationships to either U46619 or NaF were obtained 30 min after pretreatment with flavone (100 or 300 μM) or vehicle in regards to endothelium-denuded aortic rings (Fig. 1A, C). The tension is expressed as the percentage of the initial contractile response to 50 mM of KCl. Pretreatment with flavone inhibited the concentration-response curves to U46619 or NaF in regards to concentration-dependent manners. In Fig. 1C, NaF slowly increased the vascular tension, which reached its plateaus in 45 min.

In order to investigate whether flavone affects U46619- or NaF-induced MLC_{20} phosphorylation, aortic rings were pretreated with flavone or vehicle for 30 min before the addition of U46619 or NaF. Flavone (100 or 300 μM) significantly decreased the U46619- or NaF-induced MLC_{20} phosphorylation (Fig. 1B, D).

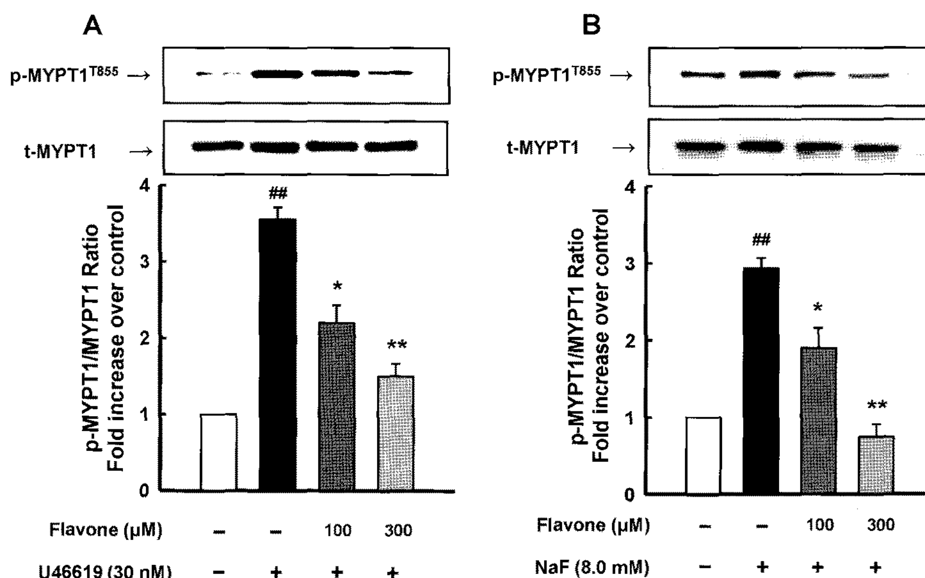

Fig. 3. Inhibitory effect of flavone on the phosphorylation of MYPT1 induced by U46619 or NaF in rat aorta. U46619 (A) or NaF (B) was added to elicit tension 30 min after pretreatment with flavone (100 or 300 μM) or vehicle to denuded aortic rings. MYPT1 phosphorylation at Thr855 was assessed by Western blot analysis after treatment with 30 nM U46619 or 8 mM NaF. Upper and lower bands in representative Western blots were probed with anti-pMYPT1 and anti-MYPT1 antibodies, respectively. Densitometry shows that the ratio of density of phosphorylated MYPT1 (upper) to that of total MYPT1 (lower) for the control was expressed as 1 arbitrary unit (5 aortic rings). Data are expressed as means with vertical bars showing S.E.M. *$p<0.05$, **$p<0.01$ vs. U46619 or NaF alone. ##$p<0.01$ vs. basal (one-way ANOVA followed by post-hoc Tukey's test).

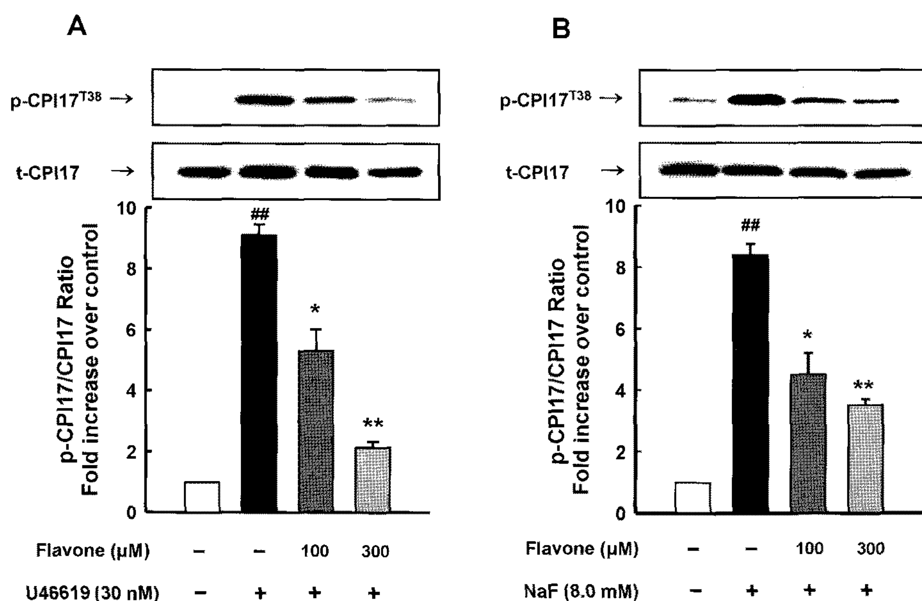

Fig. 4. Inhibitory effect of flavone on the phosphorylation of CPI17 induced by U46619 or NaF in rat aorta. U46619 (A) or NaF (B) was added to elicit tension 30 min after pretreatment with flavone (100 or 300 μM) or vehicle to denuded aortic rings. The phosphorylation of CPI17 at Thr38 was assessed by Western blots after treatment with 30 nM U46619 or 8 mM NaF. Upper and lower bands in representative Western blots were probed with anti-pCPI17 and anti-CPI17 antibodies, respectively. Densitometry shows that the ratio of density of phosphorylated CPI17 (upper) to that of total CPI17 (lower) for the control was expressed as 1 arbitrary unit (5 aortic rings). Data are expressed as means with vertical bars showing S.E.M. *$p<0.05$, **$p<0.01$ vs. U46619 or NaF alone. ##$p<0.01$ vs. basal (one-way ANOVA followed by post-hoc Tukey's test).

Inhibitory effect of flavone on RhoA activation induced by U46619 or NaF

The addition of U46619 (30 nM) or NaF (8 mM) induced vascular contractions, which reached plateaus in 20 min or 45 min, respectively. We froze the clamped aortic rings contracted with U46619 or NaF 30 minutes after pretreatment with flavone (100 or 300 μM) or vehicle, and determined the amounts of GTP-RhoA by using the RhoA G-LISA™ Activation Assay kit. Both U46619 and NaF increased GTP-RhoA as compared with the basal level (1.74±0.05 fold or 2.07±0.07 fold, respectively) in regards to endothelium-denuded aortic rings, which were almost completely suppressed by means of 300 μM flavone (Fig. 2).

Inhibitory effect of flavone on $MYPT1^{Thr855}$ phosphorylation induced by U46619 or NaF

Our preceding report showed that U46619 (30 nM) increased the phosphorylation level of MYPT1 at Thr855 but not at Thr697 (Jeon et al., 2007). When flavone (300 μM) was pretreated for 30 min, it almost completely decreased the phosphorylation level of $MYPT1^{Thr855}$ induced by U46619 or NaF (Fig. 3).

Inhibitory effect of flavone on $CPI17^{Thr38}$ phosphorylation induced by U46619 or NaF

We investigated whether flavone affects the level of phos-

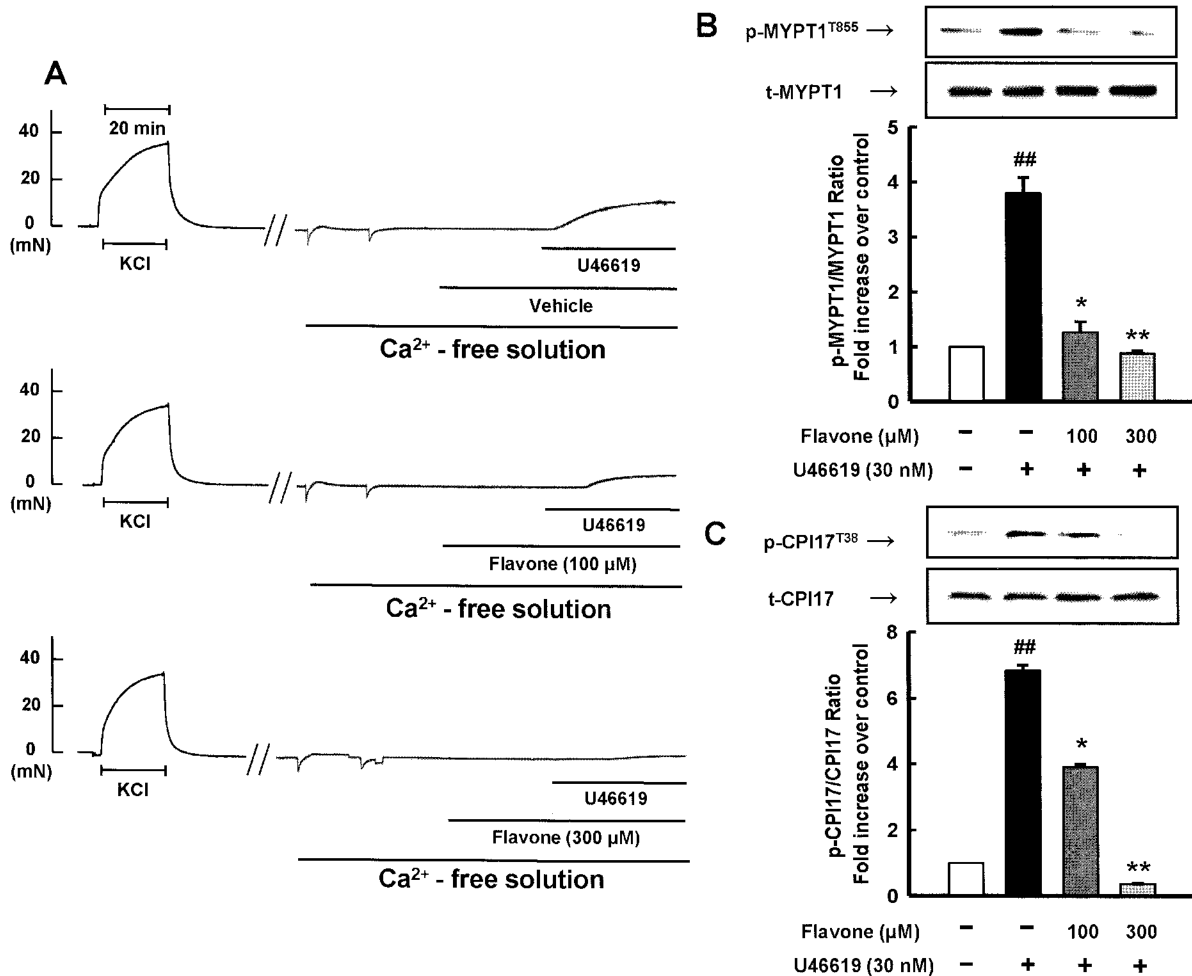

Fig. 5. Inhibitory effect of flavone on contractions and phosphorylation of MYPT1^{Thr855} and CPI17^{Thr38} induced by U46619 in a Ca^{2+}-free solution. (A) Representative tracings of the development of isometric tension showed that, after initial reference contractions to KCl (50 mM) were obtained, the aortic rings were incubated in a Ca^{2+}-free solution for 40 min, pretreated with either 100 or 300 μM flavone or vehicle for 30 min and then challenged with the addition of U46619 in a Ca^{2+}-free solution (3 aortic rings). Phosphorylation of MYPT1 at Thr855 (B) and CPI17 at Thr38 (C) was assessed by western blots 15 min after treatment with U46619 (30 nM) or vehicle. Data are expressed as means with vertical bars showing S.E.M. *$p<0.05$, **$p<0.01$ vs. U46619 or NaF alone. ##$p<0.01$ vs. basal (one-way ANOVA followed by post-hoc Tukey's test).

phorylated CPI17, another Rho-kinase downstream effector, which is induced by either U46619 (30 nM) or NaF (8 mM). Both U46619 and NaF increased the phosphorylation level of CPI17^{Thr38}, which was significantly decreased by 100 or 300 μM of flavone (Fig. 4).

Inhibitory effects of flavone on contractions and phosphorylation of MYPT1^{Thr855} and CPI17^{Thr38} induced by U46619 in a Ca^{2+}-free solution

In the absence of extracellular Ca^{2+}, U46619 (30 nM) still induced contractions with an amplitude of 11±0.2 mN. Pretreatment of the aortic rings with 100 and 300 μM flavone for 30 min significantly decreased the contractions to U46619 by 57% and 89% respectively (Fig. 5A). In a Ca^{2+}-free solution, U46619 increased the phosphorylation level of MYPT1 and CPI-17, which were significantly inhibited by means of pretreatment with flavone (Fig. 5B, C).

DISCUSSION

The present study demonstrates that flavone attenuates vascular contractions through the inhibition of the RhoA/Rho-kinase signaling pathway. Flavone inhibited the activation of RhoA and the subsequent phosphorylation of MYPT1 and CPI17 by means of Rho-kinase. In addition, flavone inhibited the phosphorylation level of both MYPT1^{Thr855} and CPI17^{Thr38} as well as vascular contractions induced by U46619 in a Ca^{2+}-free solution.

U46619, an analogue of thromboxane A_2, as well as NaF activated RhoA, increased the phosphorylation of MLC_{20}, and also induced contractions which were all inhibited by pretreatment with flavone (Fig. 1, 2). The activation of RhoA/Rho-kinase plays an important role in the tonic component of U46619- or NaF-induced contraction and MLC_{20} phosphorylation. Flavone also inhibited the phosphorylation level of both MYPT1^{Thr855} and CPI17^{Thr38}, down-

stream effectors of Rho-kinase, induced by either U46619 or NaF (Fig. 3, 4). These results indicate that flavone attenuates the RhoA/Rho-kinase signaling pathway activated by U46619 or NaF.

GDP dissociation inhibitor (RhoGDI), guanine nucleotide exchange factors (RhoGEFs), Rho GTPase activating proteins (RhoGAPs), and GTP RhoA are all on upstream of the Rho-kinase signaling pathway. In the resting state, GDP RhoA resides in cytosol with its prenylated, hydrophobic tail buried within its partner, RhoGDI. The activation of receptors coupled to trimeric G proteins ($G_{\alpha 12,13}$), through the activity of GEFs, leads to the exchange of GTP for GDP on RhoA. The GTP RhoA translocates to the membrane in which it recruits and activates Rho-kinase. When RhoGAPs catalyze the hydrolysis of GTP bound to RhoA, the GDP RhoA reassociates with RhoGDI (Somlyo and Somlyo, 2003). In addition, phosphoinositide 3-kinase (PI3K-C2α) is essential for the Ca^{2+}-dependent activation of RhoA in regards to the vascular smooth muscle (Wang et al., 2006; Azam et al., 2007). Flavone suppresses the activation of GTP RhoA induced by either U46619 or NaF likely through the interaction with RhoGEFs, RhoGAPs, RhoGDI, PI3K or other proteins. The molecular mechanism is being studied in this laboratory.

There are two major effectors of Rho-kinase: One effector is MYPT1, a myosin targeting subunit 1 of MLCP, in which phosphorylation at Thr-855 by the Rho-kinase decreases the activity of the MLCP holoenzyme. Another effector is CPI-17, a specific inhibitor of the catalytic subunit of MLCP, which requires phosphorylation at Thr-38 for purpose of strong inhibitory activity. Both PKC and Rho-kinase are able to phosphorylate CPI-17 (Koyama et al., 2000). We examined the phosphorylation level of $MYPT1^{Thr855}$, which is an inhibitory site upon phosphorylation. The activation of Rho-kinase by U46619 phosphorylates MYPT1 at Thr855, but not MYPT1 at Thr697 (Jeon et al., 2007). We observed that flavone inhibited $MYPT1^{Thr855}$ phosphorylation induced by either U46619 or NaF (Fig. 3).

CPI17 is another potential mediator of Ca^{2+} sensitization which is independent of MYPT1 phosphorylation. CPI17 is a 17-kDa peptide first isolated from porcine aorta (Eto et al., 1995), in which its phosphorylation enhances its potency for inhibiting the catalytic subunit of the myosin phosphatase (Pang et al., 2005). These results showed that U46619 or NaF increased the phosphorylation levelof CPI17, which were inhibited by flavone (Fig. 4).

MLC_{20} is known to be phosphorylated by both MLCK and Rho-kinase (Somlyo and Somlyo, 2003). The activation of Rho-kinase by U46619 or NaF inhibits the activity of myosin light chain phosphatase through the phosphorylation of $MYPT1^{Thr855}$, leading to an increased MLC_{20} phosphorylation as well as contractions (Sakurada et al., 2003; Wilson et al., 2005). Both U46619 and NaF phosphorylated MLC_{20}, which was inhibited by flavone (Fig. 1B, D).

U46619, via binding to the thromboxane A_2 receptor which couples to $G_{12/13}$, can activate RhoGEF (Somyo and Somyo, 2003). Activated GEF dissociates GDI (guanine nucleotide dissociation inhibitor) from RhoA, allows the GDP/GTP exchange, and leads to RhoA activation (Kimura et al., 1996). The fluoride increased contractions in the rat aorta and mesenteric artery, which were decreased by Y-27632, a Rho-kinase inhibitor (Ghisdal et al., 2003). NaF has been found to induce vascular contractions through the activation of the RhoA/Rho kinase pathway (Jeon et al., 2006).

The facts that flavone inhibited vascular contractions induced by two activators of different mechanisms suggest that flavone inhibits their common signaling pathway (Fig. 1A, C, Fig. 2). A potential common pathway is the RhoA/Rho kinase pathway, which is generally activated through the $G_{12/13}$ stimulation of Rho GEFs (Hart et al., 1998; Gohla et al., 2000; Fukata et al., 2001). The activation of RhoA leads to the subsequent activation of an effector, the Rho kinase (Matsui et al., 1996). The physiological significance of the RhoA-ROCK pathway in regards to the regulation of vascular smooth muscle contractions has been exhibited in numerous studies (Kureishi et al., 1997; Fukata et al., 2001; Tsai and Jiang, 2006).

In many studies, the relaxant actions of the flavonoids involve other mechanisms, including the inhibition of Ca^{2+} release from intracellular stores (Ko et al., 1991; Chan et al., 2000). 17β-Estradiol also inhibits vascular contractions in a Ca^{2+}-dependent manner (Lee et al., 2005) whereas flavone inhibits vascular contractions, even in the absence of Ca^{2+} (Ajay et al., 2003). Even in a Ca^{2+}-free solution, flavone completely inhibited the contractions induced by U46619, although the contraction was smaller than that obtained in a calcium-containing normal Krebs' solution (Fig. 5). In addition, flavone also significantly inhibited the increased phosphorylation levelof $MYPT1^{Thr855}$ and $CPI17^{Thr38}$ induced by U46619 in a Ca^{2+}-free solution. These results show that the relaxant responses of flavone involve the inhibition of RhoA/Rho-kinase signaling, even in the absence of Ca^{2+}.

Flavonoids are chemicals used for the operation of defense mechanisms against stress from various origins in plants, and comprise the most common group of polyphenolic compounds. Flavone, a prototypic member of the class flavones of the flavonoids, is present in high concentrations in yellow plants. In regards to adult human beings, diets rich in flavonoids lead to several estrogen-mimetic effects, such as lowering the levels of serum cholesterol, low-density lipoprotein, and triglycerides (Ricketts et al., 2005), as well as the low incidence of cardiovascular diseases (Cassidy et al., 2000). The estrogen-mimetic effects of compounds are currently assumed to prevent symptoms associated with estrogen deficiency in women during menopause (Fitzpatrick, 2003; Duffy et al., 2007). Our results show that flavone may suppress cardiovascular diseases through the inhibition of RhoA/Rho-kinase among the estrogen-mimetic effects. It has recently been proven that the activation of the RhoA/Rho-kinase signal transduction pathway is one of the principal mechanisms of vasocontraction in regards to arterial hypertension, and that this pathway is a novel therapeutic target for regulating arterial hypertension (Johns et al., 2000, Wirth et al., 2008).

In conclusion, flavone attenuates vascular contractions through the inhibition of the RhoA/Rho-kinase pathway and the subsequent phosphorylation of both $MYPT1^{Thr855}$ and $CPI17^{Thr38}$. These results suggest that flavone inhibited vascular contractions induced by U46619 or NaF through the disinhibition of MLCP.

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REFERENCES

Ajay M, Achike FI, Mustafa MR. Modulation of vascular reactivity in normal, hypertensive and diabetic rat aortae by a non-antioxidant flavonoid. *Pharmacol Res* 55: 385–391, 2007.

Ajay M, Gilani AU, Mustafa MR. Effects of flavonoids on vascular smooth muscle of the isolated rat thoracic aorta. *Life Sci* 74: 603-612, 2003.

Azam MA, Yoshioka K, Ohkura S, Takuwa N, Sugimoto N, Sato K, Takuwa Y. Ca^{2+}-independent, inhibitory effects of cyclic adenosine 5'-monophosphate on Ca^{2+} regulation of phosphoinositide 3-kinase C2alpha, Rho, and myosin phosphatase in vascular smooth muscle. *J Pharmacol Exp Ther* 320: 907–916, 2007.

Cassidy A, Hanley B, Lamuela-Raventos RM. Isoflavones, lignans and stilbenes - origins, metabolism and potential importance to human health. *J Sci Food Agricult* 80: 1044–1062, 2000.

Chan EC, Pannangpetch P, Woodman OL. Relaxation to flavones and flavonols in rat isolated thoracic aorta: mechanism of action and structure-activity relationships. *J Cardiovasc Pharmacol* 35: 326–333, 2000.

Duarte J, Perez Vizcaino F, Utrilla P, Jiménez J, Tamargo J, Zarzuelo A. Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. *Gen Pharmacol* 24: 857–862, 1993.

Duffy C, Perez K, Partridge A. Implications of phytoestrogen intake for breast cancer. *CA Cancer J Clin* 57: 260–277, 2007.

Eto M, Ohmori T, Suzuki M, Furuya K, Morita F. A novel protein phosphatase-1 inhibitory protein potentiated by protein kinase C. Isolation from porcine aorta media and characterization. *J Biochem (Tokyo)* 118: 1104–1107, 1995.

Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol* 265: H774-778, 2000.

Fitzpatrick LA. Alternatives to estrogen. *Med Clin North Am* 87: 1091–1113, 2003.

Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci* 22: 32–39, 2001.

Ghisdal P, Vandenberg G, Morel N. Rho-dependent kinase is involved in agonist-activated calcium entry in rat arteries. *J Physiol* 551: 855–867, 2003.

Gohla A, Schultz G, Offermanns S. Role for G(12)/G(13) in agonist-induced vascular smooth muscle cell contraction. *Circ Res* 87: 221–227, 2000.

Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G. Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. *Science* 280: 2112–2114, 1998.

Herrera MD, Zarzuelo A, Jimenez J, Marhuenda E, Duarte J. Effects of flavonoids on rat aortic smooth muscle contractility: Structure-activity relationships. *Gen Pharmacol* 27: 273–277, 1996.

Hirata K, Kikuchi A, Sasaki T, Kuroda S, Kaibuchi K, Matsuura Y, Seki H, Saida K, Takai Y. Involvement of rho p21 in the GTP-enhanced calcium ion sensitivity of smooth muscle contraction. *J Biol Chem* 267: 8719–8722, 1992.

Jeon SB, Jin F, Kim JI, Kim SH, Suk K, Chae SC, Jun JE, Park WH, Kim IK. A role for Rho kinase in vascular contraction evoked by sodium fluoride. *Biochem Biophys Res Commun* 343: 27–33, 2006.

Jeon SB, Kim G, Kim JI, Seok YM, Kim SH, Suk K, Shin HM, Lee YH, Kim IK. Flavone inhibits vascular contraction by decreasing phosphorylation of the myosin phosphatase target subunit. *Clin Exp Pharmacol Physiol* 34: 1116–1120, 2007.

Johns DG, Dorrance AM, Leite R, Weber DS, Webb RC. Novel signaling pathways contributing to vascular changes in hypertension. *J Biomed Sci* 7: 431–443, 2000.

Kim IK, Park TG, Kim YH, Cho JW, Kang BS, Kim CY. Heat-shock response is associated with enhanced contractility of vascular smooth muscle in isolated rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 369: 402–407, 2004.

Kim JI, Jeon SB, Baek I, Seok YM, Shin HM, Kim IK. Heat shock augments myosin phosphatase target-subunit phosphorylation. *Biochem Biophys Res Commun* 356: 718–722, 2007.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* 273: 245–248, 1996.

Ko F, Huang T, Teng C. Vasodilatory action mechanisms of apigenin isolated from *Apium graveolens* in rat thoracic aorta. *Biochim Biophys Acta* 1115: 69–74, 1991.

Koyama M, Ito M, Feng J, Seko T, Shiraki K, Takase K, Hartshorne DJ, Nakano T. Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. *FEBS Lett* 475: 197–200, 2000.

Kureishi Y, Kobayashi S, Amano M, Kimura K, Kanaide H, Nakano T, Kaibuchi K, Ito M. Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J Biol Chem* 272: 12257–12260, 1997.

Lee HA, Seong Y, Lee WJ, Kim I. 17β-Estradiol inhibits calcium-dependent, but not calcium-independent, contraction in isolated rat aorta. *Naunyn Schmiedebergs Arch Pharmacol* 371: 152–157, 2005.

Machha A, Mustafa MR. Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta. *J Cardiovasc Pharmacol* 46: 36–40, 2005.

Most MM. Estimated phytochemical content of the dietary approaches to stop hypertension (DASH) diet is higher than in the Control Study Diet. *J Am Diet Assoc* 104: 1725–1727, 2004.

National Institutes of Health. Guide for the care and use of laboratory animals. NIH publication No. 85–23, revised edn. NIH, Bethesda, MD, 1996.

Pang H, Guo Z, Su W, Xie Z, Eto M, Gong MC. RhoA-Rho kinase pathway mediates thrombin- and U-46619-induced phosphorylation of a myosin phosphatase inhibitor, CPI-17, in vascular smooth muscle cells. *Am J Physiol Cell Physiol* 289: 352–360, 2005.

Ricketts ML, Moore DD, Banz WJ, Mezei O, Shay NF. Molecular mechanisms of action of the soy isoflavones includes activation of promiscuous nuclear receptors. *A review J Nutr Biochem* 16: 321–330, 2005.

Sakurada S, Takuwa N, Sugimoto N, Takuwa Y. Rho activation in excitatory agonist-stimulated vascular smooth muscle. *Am J Physiol Cell Physiol* 281: C571–578, 2001.

Somyo AP, Somyo AV. Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: Modulated by G proteins, kinases, and myosin phosphatase. *Physiol Rev* 83: 1325–1358, 2003.

Tsai MH, Jiang MJ. Rho-kinase-mediated regulation of receptor-agonist-stimulated smooth muscle contraction. *Pflugers Arch* 435: 223–232, 2006.

Wang Y, Yoshioka K, Azam MA, Takuwa N, Sakurada S, Kayaba Y, Sugimoto N, Inoki I, Kimura T, Kuwaki T, Takuwa Y. Class II phosphoinositide 3-kinase alpha-isoform regulates Rho, myosin phosphatase and contraction in vascular smooth muscle. *Biochem J* 394: 581–592, 2006.

Wilson DP, Susnjar M, Kiss E, Sutherland C, Walsh MP. 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, 2005.

Wirth A, Benyó Z, Lukasova M, Leutgeb B, Wettschureck N, Gorbey S, Orsy P, Horváth B, Maser-Gluth C, Greiner E, Lemmer B, Schütz G, Gutkind JS, Offermanns S. G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat Med* 14: 64–68, 2008.

Zenebe W, Pechanova O, Bernatova I. Protective effects of red wine polyphenolic compounds on the cardiovascular system. *Exp Clin Cardiol* 6: 153–158, 2001.

Zern TL, Fernandez ML. Cardioprotective effects of dietary polyphenols. *J Nutr* 135: 2291–2294, 2005.