The Effect of Galangin on the Regulation of Vascular Contractility via the Holoenzyme Reactivation Suppressing ROCK/CPI-17 rather than PKC/CPI-17

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Abstract
In this study, we investigated the influence of galangin on vascular contractility and to determine the mechanism underlying the relaxation. Isometric contractions of denuded aortic muscles were recorded and combined with western blot analysis which was performed to measure the phosphorylation of phosphorylation-dependent inhibitory protein of myosin phosphatase (CPI-17) and myosin phosphatase targeting subunit 1 (MYPT1) and to evaluate the effect of galangin on the RhoA/ROCK/CPI-17 pathway. Galangin significantly inhibited phorbol ester-, fluoride- and thromboxane mimetic-induced vasoconstrictions regardless of endothelial nitric oxide synthesis, suggesting its direct effect on vascular smooth muscle. Galangin significantly inhibited the fluoride-dependent increase in pMYPT1 and pCPI-17 levels and phorbol 12,13-dibutyrate-dependent increase in pERK1/2 level, suggesting repression of ROCK and MEK activity and subsequent phosphorylation of MYPT1, CPI-17 and ERK1/2. Taken together, these results suggest that galangin-induced relaxation involves myosin phosphatase reactivation and calcium desensitization, which appears to be mediated by CPI-17 dephosphorylation via not PKC but ROCK inactivation.

Key Words: CPI-17, Fluoride, Galangin, MYPT1, Phorbol ester, ROCK

INTRODUCTION

Galangin (3,5,7-trihydroxyflavone, Fig. 1) is a bioflavonoid derived primarily from propolis, a natural compound produced by honeybee and rhizome of Alpinia officinarum, and it has various pharmacological activities such as anti-oxidant, anti-inflammatory, antidiabetic and anti-carcinogenic activities (Kim et al., 2013; Zha et al., 2013; Devadoss et al., 2018) including the inhibition of proliferation, induced apoptosis and promoted autophagy in hepatocellular and esophageal carcinoma cells (Zhang et al., 2012; Ren et al., 2016) and cell invasion by suppressing the epithelial-mesenchymal transition (Cao et al., 2016). The scientific rationale on experimental design was to investigate the influence of galangin nonspecifically most potent on vascular contractility and determine the mechanism involved using denuded muscles from male rats and recording isometric contractions combined with molecular experiments. The vascular contractility is is regulated via both Ca²⁺-dependent and Ca²⁺ sensitization mechanisms (Kuriyama et al., 2012; Sasahara et al., 2015; Liu and Khalil, 2018) and dysregulated contractility and Ca²⁺ sensitization in blood vessels is observed in many cardiovascular diseases. The mechanism responsible for Ca²⁺ sensitization involves repression of myosin phosphatase, leading to the phosphorylation of 20-kDa myosin light chain (MLC20) and subsequent enhanced contractility. The inhibition of myosin phosphatase in vascular smooth muscle is mediated by phosphorylation of either the phosphorylation-dependent inhibitory protein of myosin phosphatase (CPI-17) or the myosin phosphatase targeting subunit 1 (MYPT1) via either Rho-kinase (ROCK) or protein kinase C (PKC), which leads to attenuated dephosphorylation of MLC20. Inhibition of myosin phosphatase in smooth muscle
is mediated by phosphorylation of myosin phosphatase target subunit via ROCK, which leads to sustained phosphorylation of MLC$_{20}$. PKC is an important kinase involved in increasing the contractile filament sensitivity to calcium, Ca$^{2+}$ antagonists-insensitive forms of hypertension and coronary vasospasm require other treatment modalities that target other pathways such as ROCK and PKC. PKC inhibits myosin phosphatase activity by activating CPI-17, a myosin phosphatase inhibitor when it is phosphorylated at Thr38 by PKC or ROCK, resulting in increased levels of MLC phosphorylation (Kim et al., 2012; Yang et al., 2018). CPI-17 (Thr38) and MLC phosphorylation levels coordinately correspond with smooth muscle contraction during many physiological processes within smooth muscles and other cell types. Extracellular signal regulated kinase (ERK) 1/2 and its activator mitogen-activated protein kinase kinase (MEK) have been shown to be activated via PKC-mediated phosphorylation in various cell types (Ansari et al., 2009). Thromboxane A$_2$ mimetics, fluoride, and phorbol esters have been shown to induce contractions of vascular muscles, which may be due to enhanced Ca$^{2+}$ sensitivity or partially due to an increased Ca$^{2+}$ concentration. Activation of ERK1/2 induced by a thromboxane A$_2$ mimetic (Gallet et al., 2003) or phorbol ester triggers ERK1/2-dependent cytoskeletal remodeling and relieves the inhibitory action of caldesmon increasing the affinity between myosin and actin and cross-bridge cycling (Hedges et al., 2000).

However, the specific protein kinases and associated cellular pathways primarily responsible for increased calcium desensitization in response to galangin remain unknown. Therefore, the purpose of this study was to investigate the specific protein kinase and associated cellular signaling pathways responsible for myosin phosphatase reactivation and calcium desensitization induced by galangin.

**MATERIALS AND METHODS**

**Preparation of aorta**

Male Sprague-Dawley rats (210-240 g) were anesthetized with etomidate (0.3 mg/kg i.v.) and euthanized by thoracotomy and exsanguination according to the guidance approved by the Institutional Committee at Chung-Ang University and Daegu Catholic University (IACUC-2016-040). After euthanasia performed in accordance with the National Institutes of Health guide for the care and use of Laboratory animals, the thoracic aorta was carefully and rapidly isolated and placed in oxygenated physiological saline solution consisting (mM) of 115.0 NaCl, 4.7 KCl, 25.0 NaHCO$_3$, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 1.2 KH$_2$PO$_4$, and 10.0 glucose. The aorta was separated from the surrounding connective tissue and the endothelia were cleaned by gentle abrasion using a pipette tip and N$^\circ$-monomethyl-L-arginine (L-NMMA) if necessary.

**Evaluation of vascular contraction**

To examine functional changes of the muscle in response of a vasoconstrictor, each muscle was incubated with the vasoconstrictor ex vivo in a water-jacketed organ bath aerated with gas mixture. Muscles were stretched until an optimal resting tension of 2.0 g was enforced, and changes in their tension were analyzed using a force-displacement transducer (FT03C, Grass, Quincy, MA, USA) linked to a PowerLab recording system (AD Instruments, Castle Hill, NSW, Australia). After equilibration (for 60 min), arterial integrity was examined by contracting the rings with 50 mM KCl or 1 $\mu$M phenylephrine, followed by relaxation with acetylcholine (1 $\mu$M).

The relaxation effect of galangin was determined by its application after KCl- (50 mM), phenylephrine- (1 $\mu$M), thromboxane A$_2$- (0.1 $\mu$M), phorbol ester- (1 $\mu$M) or fluoride- (6 mM) evoked contractions that had plateaued in normal Krebs’ solution.

**Western blot analysis**

Protein expression was quantified using immunoblotting, as reported previously (Jeon et al., 2006; Je and Sohn, 2009). Aortic tissues were quick-frozen in a dry ice/acetone slurry including 10 mM dithiothreitol (DTT) and 10% trichloroacetic acid (TCA), washed several times in room temperature with the washing buffer including aceton and DTT, and homogenized with the homogenization buffer including antioxidants. Protein-matched samples were subjected to sodium dodecyl sulfate-polyacrylamide denaturing gel electrophoresis (Ponceau gel, National Diagnostics, Atlanta, GA, USA), transferred to polyvinylidene difluoride or nitrocellulose membranes, and subjected to immunostaining incubating with primary and secondary antibodies. Lane loading variations were corrected by normalization with beta-actin. Sets of samples produced during individual experiments were conducted on the same gel and the densitometry was performed on the same image.

**Chemicals and antibodies**

Sodium chloride, potassium chloride, sodium fluoride, acetylcholine, galangin, U46619 and phorbol 12,13-dibutyrate were obtained from Sigma (St. Louis, MO, USA). DTT, TCA and aceton were purchased from Fisher Scientific (Hampston, NH, USA). Enhanced chemiluminescence (ECL) kits were purchased from Pierce (Rockford, IL, USA). Antibodies against phospho-myo 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, phosphophosphorylation-dependent inhibitory protein of myosin phosphatase (phospho-CPI-17) at Thr38 (1:1,000), CPI-17, adducin or phospho-adducin at Ser662, ERK or phosphoERK at Thr202/Tyr204 (Upstate Biotechnology, Lake Placid, NY, USA) or Cell Signaling Technology, Danvers, MA, USA) were used to determine levels of RhoA/ROCK activity (Kitazawa et al., 2000; Wooldridge et al., 2004; Wilson et al., 2005) or MEK activity. Anti-rabbit IgG (goat) and anti-mouse IgM (goat) conjugated with horseradish peroxidase were used as secondary antibodies (1:2,000 dilutions for both, Upstate B.). A specific MLC$_{20}$ antibody (1:1,500, Sigma) and anti-mouse IgG (goat) conjugated with horseradish peroxidase (1:2,000, Upstate B.) were used to determine the level of myosin light chain (LC$_{20}$).
phosphorylation. Galangin was prepared in dimethyl sulfoxide (DMSO) as a 0.1 M stock solution and frozen at –20°C for later use.

**Statistics**

The data are presented as mean ± standard error of the mean (SEM). Statistical evaluations between two groups were performed using student’s unpaired t-test or ANOVA. These statistical analyses were made using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when p<0.05.

**RESULTS**

**Effect of galangin on contractions of endothelium-denuded muscles induced by a full RhoA/ROCK activator fluoride**

Removal of endothelium, the regulator of vascular homeostasis, was achieved by gently rubbing with a pipette tip and Nω-mono-methyl-L-arginine (L-NMMA) to identify the relaxation effect of galangin on vascular smooth muscle. The absence of endothelium was identified by a lack of relaxation after treated contract muscle segments with acetylcholine (1 μM). Galangin had no observable effect on basal tension (data not shown), but it significantly inhibited the contraction evoked by a ROCK activator fluoride, regardless of the absence of endothelial nitric oxide synthesis in denuded (Fig. 2A) or intact (Fig. 2B) muscles. This suggests that the relaxation mechanism of galangin might include the repression of ROCK activity and myosin phosphatase reactivation besides endothelial nitric oxide synthesis and the activation of guanylyl cyclase.

**Effect of galangin on contractions of denuded aortas induced by the dual ROCK and MEK activator thromboxane A₂**

Galangin inhibited thromboxane A₂ mimetic U46619-induced contraction in denuded muscles (Fig. 3), suggesting that the mechanism includes repression of ROCK activity and myosin phosphatase reactivation and a dual activator (thromboxane mimetic) acts similar to a full activator targeting ROCK.

**Effect of galangin on contractions of denuded muscles induced by a MEK activator phorbol 12, 13-dibutyrate**

Phorbol esters are primarily MEK activators and partial ROCK activators (Goyal et al., 2009; Je and Sohn, 2009). Interestingly, phorbol 12,13-dibutyrate (PDBu)-induced contraction was inhibited by galangin, regardless of endothelial nitric oxide synthesis in denuded muscles (Fig. 4), which suggested that thin filament regulation including MEK/ERK was inhibited.
was observed (Fig. 5). Furthermore, a decrease in fluoride-induced LC20 phosphorylation was found in response to galangin treatment (Fig. 6). Therefore, thick filament regulation, including myosin phosphatase reactivation via RhoA/ROCK inactivation might be involved in the decreased contractility of galangin-treated rat aortas.

### Effect of galangin on the level of CPI-17 phosphorylation at Thr-855

To identify the role of galangin on thick filament regulation of vascular contractility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in aortas quick-frozen after a 60-min exposure to galangin for equilibration. Each relaxing muscle was contracted with 6 mM fluoride. This work was conducted using quick frozen galangin (0.1 mM)-treated muscles in the absence of endothelium, and levels were compared to those of vehicle-treated muscles (Fig. 5). A significant decrease in fluoride-induced MYPT1 phosphorylation at Thr-855 in response to galangin treatment

**Fig. 5.** Effect of galangin on fluoride-dependent increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were decreased in rapidly-frozen galangin-treated muscles in the absence of endothelium compared to vehicle-treated muscles precontracted with fluoride. Upper panel indicates a typical blot, and lower panel indicates average densitometric results for relative levels of phospho-MYPT1. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. **p**<0.01, *p*<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride.

**Fig. 6.** Effect of galangin on fluoride-induced increase in phospho-MLC20 level. Phospho-MLC20 levels expressed as a percentage of total MLC20, were decreased in rapidly-frozen galangin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with fluoride (6 mM). Data are presented as the means of 3-5 experiments with a vertical line indicating SEM. **p**<0.01, *p*<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride.

**Fig. 7.** Effect of galangin on fluoride (A) or phorbol ester (B)-dependent increases in phospho-CPI-17 levels. Phospho-CPI-17 protein levels were decreased in rapidly-frozen flavonol-treated muscles in the absence of endothelium compared to vehicle-treated muscles precontracted with fluoride. Upper panel indicates a typical blot, and lower panel indicates average densitometric results for relative levels of phospho-CPI-17. Data are presented as the mean of 3-5 experiments with a vertical line indicating SEM. **p**<0.01, *p*<0.05, versus normal or control group respectively. Galangin: 0.1 mM galangin; Fluoride: 6 mM sodium fluoride; PDBu: 1 µM phorbol 12,13-dibutyrate.
Inhibited fluoride, phorbol 12,13-dibutyrate and thromboxane mimetic-induced vasoconstriction; thus revealing a novel therapeutic target for the development of novel antihypertensive agents.

Activation of ROCK or PKC, phosphorylation of CPI-17 or MYPT1, and subsequent inhibition of myosin phosphatase are part of the Ca\textsuperscript{2+} sensitization pathway that promotes increased MLC phosphorylation without requiring an increase in Ca\textsuperscript{2+} influx or release. ROCK phosphorylates myosin phosphatase, which inhibits phosphatase activity and leads to an accumulation of phosphorylated MLCs (Johnson et al., 2009; Qi et al., 2009; Qiao et al., 2014) and phosphorylates MLCs directly and independently of myosin light chain kinase and phosphatase activity (Amano et al., 1998). ROCK was reported to be involved in vascular contractions induced by fluoride, phorbol ester or thromboxane A\textsubscript{2} (Wilson et al., 2005; Jeon et al., 2006; Tsai and Jiang, 2006).

The present study demonstrates that galangin ameliorates contractions induced by vasoconstrictors (phorbol ester or fluoride) in an endothelium-independent manner (Fig. 2-4), and that the mechanism included the PKC/MEK/ERK and RhoA/ROCK pathways. Galangin attenuated the fluoride-evoked phosphorylation of CPI-17 at Thr38, suggesting that CPI-17 included in fluoride-induced contraction is a downstream effector activated by ROCK. Furthermore, galangin significantly decreased the contraction and the phosphorylation of MYPT1 at Thr855 and CPI-17 at Thr38 evoked by fluoride (Fig. 5, 7A) with the full relaxation (Fig. 2) and \(\alpha\)-adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 induced by a phorbol ester (Fig. 8), suggesting that inhibition of PKC/MEK or ROCK activity is a major mechanism underlying the effects of galangin on smooth muscle contractility. Activation of ROCK by fluoride decreases the activity of myosin phosphatase through phosphorylation of MYPT1 and CPI-17, resulting in an increase in MLC\textsubscript{20} phosphorylation and contractions (Sakurada et al. et al., 2003; Somlyo and Somlyo, 2003; Wilson et al., 2005) inhibited by galangin (Fig. 6). Therefore, thick or myosin filament regulation including pCPI-17 inactivation or myosin phosphatase activation through RhoA/ROCK inactivation rather than PKC/CPI-17 might be involved in galangin-induced inhibition of vascular contractility.

In summary, galangin without newly reported adverse effects (Aloud et al., 2018) significantly attenuates the RhoA/ROCK activator fluoride-induced contractions decreasing CPI-17 phosphorylation and inhibits phorbol ester-induced contraction due to PKC/MEK activation. Thus, the mechanism underlying the flavonol-evoked relaxation of phorbol ester- or fluoride-induced contractions involves inhibition of PKC/MEK and ROCK activity. Inhibition of ROCK activity and subsequent CPI-17/MYPT1 phosphorylation evoked by galangin during fluoride-induced contraction suggests that ROCK/CPI-17 rather than PKC/CPI-17 inactivation is required for myosin phosphatase reactivation and relaxation.

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