# The Effect of Luteolin on the Modulation of Vascular Contractility via ROCK and CPI-17 Inactivation

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## Abstract

In this investigation, we made a study of the efficacy of luteolin (a flavonoid found in plants such as vegetables, herbs and fruits) on vascular contractibility and to elucidate the mechanism underlying the relaxation. Isometric contractions of denuded muscles were stored and combined with western blot analysis which was conducted to assess the phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) and phosphorylation-dependent inhibitory protein for myosin phosphatase (CPI-17) and to examine the effect of luteolin on the RhoA/ROCK/CPI-17 pathway. Luteolin significantly alleviated phorbol ester-, fluoride- and thromboxane mimetic-elicited contractions regardless of endothelial nitric oxide synthesis, implying its direct effect on smooth muscle. It also significantly alleviated the fluoride-elicited elevation in pCPI-17 and pMYPT1 levels and phorbol 12,13-dibutyrate-elicited increase in pERK1/2 level, suggesting depression of ROCK and PKC/MEK activity and ensuing phosphorylation of MYPT1, CPI-17 and ERK1/2. Taken together, these results suggest that luteolin-elicited relaxation includes myosin phosphatase reactivation and calcium desensitization, which seems to be arbitrated by CPI-17 dephosphorylation via ROCK/PKC inhibition.

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

## INTRODUCTION

Luteolin (3,4,5,7-tetrahydroxy flavone, Fig. 1) is a flavonoid derived primarily from fruits and vegetables such as celery, chrysanthemum flowers, sweet bell peppers, carrots, onion leaves, broccoli and parsley (Burton *et al*., 2016), and it has various pharmacological activities such as anti-oxidant, anti-inflammatory, anti-allergic and anti-carcinogenic activities (Lin



**Fig. 1.** The chemical structure of luteolin (3,4,5,7-tetrahydroxy flavone).

*et al*., 2008; Wei *et al*., 2018; Iida *et al*., 2020) in many cancers including colon, pancreatic, prostate and lung cancer promoting signaling through peroxiredoxin II, upregulating p21 and inhibiting mTOR signaling. However, the molecular target for the beneficial effect of luteolin requires further investigation.

The vascular contractility is modulated via both calcium-dependent and calcium sensitization mechanisms (Kuriyama *et al*., 2012; Sasahara *et al*., 2015; Liu and Khalil, 2018) and dysregulated contractility and calcium sensitization in blood vessels is observed in many cardiovascular diseases. The mechanism responsible for calcium sensitization involves restriction of myosin phosphatase, resulting in the phosphorylation of myosin light chain 20 kDa (MLC) and ensuing augmented contractility. The repression of myosin phosphatase in smooth muscle is adjusted by phosphorylation of either the myosin phosphatase targeting subunit 1 (MYPT1) or the phosphorylation-dependent inhibitory protein for myosin phosphatase (CPI-17) via Rho-kinase (ROCK) or protein kinase C (PKC), which results in regressed dephosphorylation of

$MLC_{20}$. Repression of myosin phosphatase in smooth muscle is arbitrated by phosphorylation of MYPT1 via ROCK, which results in retained phosphorylation of $MLC_{20}$. PKC is a kinase involved in contractile filament sensitivity to calcium. Calcium antagonist-insensitive forms of hypertension and coronary vasospasm need other treatment modalities that focus other pathways such as ROCK and PKC. PKC/ROCK restricts myosin phosphatase activity by activating CPI-17, a myosin phosphatase inhibitor when it is phosphorylated at Thr38 by PKC or ROCK, resulting in enhanced levels of MLC phosphorylation (Kim *et al*., 2012; Yang *et al*., 2018; Sun *et al*., 2019; Eto *et al*., 2022). CPI-17 (Thr38) and MLC phosphorylation scales commensurately correspond with vasoconstriction during numerous physiological processes within vessels 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-arbitrated phosphorylation in various cell types (Ansari *et al*., 2009). Thromboxane mimetic, phorbol esters and fluoride have been shown to elicit vascular contractions, which may be due to enhanced calcium sensitivity or partially due to an enhanced calcium concentration. Activation of ERK1/2 elicited by a phenylephrine (Perez-Aso *et al*., 2013), thromboxane mimetic or phorbol ester triggers ERK1/2-elicited cytoskeletal remodeling and relieves the inhibitory action of caldesmon increasing the affinity between myosin and actin and cross-bridge cycling (Hedges *et al*., 2000; Gallet *et al*., 2003).

However, the specific protein kinases and related cellular pathways mainly responsible for enhanced calcium desensitization in response to luteolin remain unknown. Therefore, the purpose of this study was to examine the specific protein kinase and associated signaling pathways responsible for myosin phosphatase activation and calcium desensitization elicited by luteolin.

## MATERIALS AND METHODS

### Preparation of muscle

Male Sprague-Dawley rats (200-230 g) were anesthetized with 0.3 mg/kg etomidate and euthanized by exsanguination and thoracotomy in accordance with the guidance validated by the Institutional Committee at Chung-Ang University (Seoul, Korea) and Daegu Catholic University (Gyeongsan, Korea) (IACUC-2017-045) and the National Institutes of Health guide for the care and use of Laboratory animals. After euthanasia performed, the thoracic aorta was carefully and rapidly extracted and placed in oxygenated saline solution consisting (mM) of 25.0 $NaHCO_3$, 4.7 KCl, 115.0 NaCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 $KH_2PO_4$ and 10.0 glucose. The surrounding connective tissue was separated from the muscle and the endothelia were avulsed by smooth rubbing using a pipette tip and $N^G$-monomethyl-L-arginine (L-NMMA) in case of necessity.

### Assessment of muscle contraction

To evaluate functional changes of the vessel in response of a vasoconstrictor, each vessel was stimulated with the vasoconstrictor in a water-jacketed organ bath aerated with oxygen mixture. Vessels were stretched until an ideal resting tension of 2.0 g was applied, and alterations in their tension were calculated using a force-displacement transducer (FT03C, Grass, Quincy, MA, USA) connected to a PowerLab recording system (AD Instruments, Castle Hill, NSW, Australia). After equilibration (for 60 min), arterial integrity was assessed by contracting the vessels with 50 mM KCl or 1 $\mu$M phenylephrine, ensued by relaxation with acetylcholine (1 $\mu$M).

The relaxation effect of luteolin was identified by its treatment after KCl- (50 mM), phenylephrine- (1 $\mu$M), thromboxane mimetic- (0.1 $\mu$M), phorbol ester- (1 $\mu$M) or fluoride- (6 mM) stimulated contractions had plateaued in normal Krebs' solution.
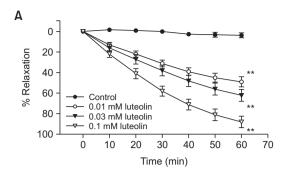
### Western blot analysis

Protein expression was quantified using immunoblotting, as reported previously (Jeon *et al*., 2006; Je and Sohn, 2009). The vessels were rapidly frozen in a dry ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Protein-consistent samples were subjected to sodium dodecyl sulfate-polyacrylamide denaturing gel electrophoresis (Protogel, National Diagnostics, Atlanta, GA, USA), transferred to nitrocellulose membranes or polyvinylidene difluoride, and subjected to immunostaining incubating with primary and secondary antibodies. Lane loading variations were rectified by normalization with beta-actin. Sets of samples generated during individual experiments were performed on the same gel and the densitometry was conducted on the same image.

### Chemicals and antibodies

Potassium chloride, sodium chloride, sodium fluoride, acetylcholine, luteolin, phenylephrine, phorbol 12,13-dibutyrate and U-46619 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetone, TCA and DTT were purchased from Fisher Scientific (Hampton, NH, USA) and enhanced chemiluminescence (ECL) kits were purchased from Pierce (Rockford, IL, USA). Antibodies against phospho-CPI-17 at Thr38 (1:1,000), CPI-17, phospho-MYPT1 at Thr855 (1:5,000), MYPT1, adducin or phospho-adducin at Ser662, $\beta$-actin, ERK or phospho-ERK 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, Upstate Biotechnology). A specific antibody against $MLC_{20}$ (1:1,500, Sigma-Aldrich) and anti-mouse IgG (Goat) conjugated with horseradish peroxidase (1:2,000, Upstate Biotechnology) were used to verify the level of $LC_{20}$ phosphorylation. Luteolin 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 t-test or ANOVA. These statistical analyses were executed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant when $p<0.05$.
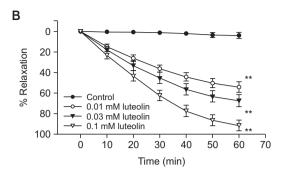
A



B



**Fig. 2.** Effect of luteolin on fluoride-elicited vasoconstriction in denuded (A) or intact (B) vessels. Each vessel was stabilized in the organ bath for 40-50 min before relaxation responses to luteolin were assessed. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. **$p<0.01$, absence versus presence of luteolin.
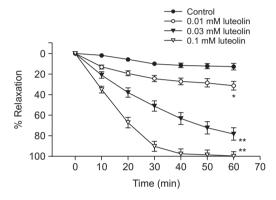


**Fig. 3.** Effect of luteolin on thromboxane mimetic-elicited vasoconstriction in denuded vessels. Each vessel was stabilized in the organ bath for 40-50 min before relaxation responses to luteolin were assessed. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. *$p<0.05$, **$p<0.01$, absence versus presence of luteolin.



**Fig. 4.** Effect of luteolin on phorbol ester-elicited vasoconstriction in denuded vessels. Each vessel was stabilized in the organ bath for 40-50 min before relaxation responses to luteolin were assessed. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. *$p<0.05$, **$p<0.01$, absence versus presence of luteolin.

## RESULTS

### Effect of luteolin on contractions of endothelium-denuded muscles elicited by a full RhoA/ROCK activator fluoride

Impairment of endothelium, the regulator of vascular homeostasis, was attained by smooth abrasion with a pipette tip and $N^G$-mono-methyl-L-arginine (L-NMMA) to verify the relaxation effect of luteolin on vascular smooth muscle. The absence of endothelium was identified by a dearth of relaxation after treating contracted vessel segments with acetylcholine (1 μM). Luteolin had little effect on basal tension (data not shown), but it remarkably alleviated the contraction elicited by a ROCK activator fluoride in denuded (Fig. 2A) freed from endothelial nitric oxide synthesis or intact (Fig. 2B) muscles. This implies that the relaxation mechanism of luteolin might include the inhibition of ROCK activity and myosin phosphatase reactivation excluding endothelial nitric oxide synthesis and ensuing activation of guanylyl cyclase.

### Effect of luteolin on contractions of denuded aortas elicited by the dual ROCK and MEK activator thromboxane mimetic

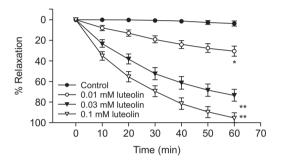Luteolin alleviated thromboxane mimetic-elicited contrac-

tion in denuded muscles (Fig. 3), suggesting that the mechanism includes inhibition of ROCK activity and myosin phosphatase activation and a dual activator (thromboxane mimetic) takes action similar to a potent activator focusing ROCK.

### Effect of luteolin on contractions of denuded muscles elicited 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)-elicited contraction was alleviated by luteolin, regardless of endothelial nitric oxide synthesis in denuded vessels (Fig. 4), which implied that thin filament regulation including MEK/ERK was regressed.

### Effect of luteolin on the levels of MYPT1 phosphorylation at Thr-855

To verify the role of luteolin on thick filament regulation of vascular contractibility, we assessed levels of MYPT1 and phospho-MYPT1 in aortas rapidly frozen after a 60-min exposure to luteolin for equilibration. 6 mM fluoride augmented the force of contraction in each relaxing vessel. This work was executed using quick frozen luteolin (0.1 mM)-treated vessels freed from endothelium, and the levels were compared to those of vehicle-treated vessels (Fig. 5). A significant reduction in fluoride-elicited MYPT1 phosphorylation at Thr855 in response to luteolin treatment was observed (Fig. 5). Further-
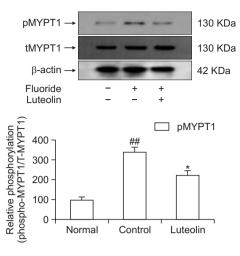
**Fig. 5.** Effect of luteolin on fluoride-elicited enhancement in phospho-MYPT1 protein levels. Phospho-MYPT1 levels were reduced in rapidly frozen luteolin-treated vessels freed from endothelium compared to vehicle-treated vessels contracted with fluoride. Upper panel shows a typical blot, and lower panel shows average densitometric results for relative levels of phospho-MYPT1. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. $*p<0.05$, $\#\#p<0.01$, versus control or normal group respectively. Luteolin: 0.1 mM luteolin; Fluoride: 6 mM sodium fluoride.



**Fig. 6.** Effect of luteolin on fluoride-induced enhancement in phospho-$MLC_{20}$ level. Phospho-$MLC_{20}$ levels expressed as a percentage of total $MLC_{20}$ were attenuated in rapidly frozen luteolin-treated vessels freed from endothelium compared to vehicle-treated vessels contracted with fluoride (6 mM). Data are exhibited as the means of 3-5 experiments with a vertical line implying SEM. $*p<0.05$, $\#\#p<0.01$, versus control or normal group respectively. Luteolin: 0.1 mM luteolin; Fluoride: 6 mM sodium fluoride.

more, a reduction in fluoride-stimulated $LC_{20}$ phosphorylation was found in response to luteolin treatment (Fig. 6). Therefore, thick filament regulation, containing myosin phosphatase activation via RhoA/ROCK inactivation might be implicated in the repressed contractility of luteolin-treated rat aortas.

### Effect of luteolin on the level of CPI-17 phosphorylation at Thr-38

The myosin phosphatase inhibitor CPI-17 is phosphorylated by PKC or ROCK. CPI-17 phosphorylation is usually augmented during contraction as it is one mechanism that intensifies myofilament calcium sensitivity. Phorbol 12,13-dibutyrate or fluoride was used as a control for CPI-17 phosphorylation as it directly activates PKC or ROCK generating a significant increase in CPI-17 phosphorylation. To confirm the role of luteolin in thin or thick filament disinhibition of smooth muscle contractility, we assessed levels of phospho-CPI-17 and CPI-17 in aortas rapidly frozen after a 60-min exposure to luteolin for equilibration. 6 mM fluoride or 1 μM phorbol ester augmented the force of contraction in each relaxing vessel. This work was executed using quick frozen flavone (0.1 mM)-treated vessels freed from endothelium, and the levels were compared to those of vehicle-treated vessels (Fig. 7). Interestingly, a significant reduction in fluoride- or phorbol ester-stimulated CPI-17 phosphorylation at Thr-38 in reaction to luteolin treatment was observed (Fig. 7). The reduction in CPI-17 phosphorylation with luteolin during agonists application suggests that ROCK is inactivated in the luteolin-elicited restriction in the force of contraction, MLC phosphorylation and myosin phosphatase inactivation.

### Effect of luteolin on the level of adducin phosphorylation at Ser662 and ERK1/2 phosphorylation at Thr-202/Tyr-204

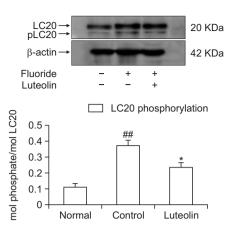To verify the role of luteolin on thin filament disinhibition of

vascular contractibility, we assessed levels of phospho-adducin and adducin and phospho-ERK1/2 and ERK1/2 in aortas rapidly frozen after 60 minutes of exposure to luteolin for equilibration. 1 μM phorbol 12,13-dibutyrate augmented the force of contraction in each vessel. As compared with vehicle-treated vessels, a reduction in adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 was observed in luteolin (0.1 mM)-treated vessels in the absence of endothelium (Fig. 8); significant relaxation (Fig. 4) and thin filament modulation were observed. These findings represent that thin filament modulation, containing adducin and ERK1/2 phosphorylation via PKC and MEK activation, plays a role in luteolin-elicited relaxation.

## DISCUSSION

This is the study to suggest that luteolin restricts tonic tension and represses calcium sensitization through the blockade of not only PKC-mediated CPI-17 phosphorylation but also ROCK-mediated CPI-17 phosphorylation. Pharmacological activators of ROCK (fluoride), MEK (phorbol 12,13-dibutyrate) or both (thromboxane mimetic) were used to determine their involvement in suppressed contraction. The CPI-17-arbitrated and calcium-sensitized contraction, elicited by various agonists, was potentiated consistently. Luteolin restricts tonic tension and represses calcium sensitization through the blockade of ROCK-arbitrated myosin phosphatase restriction. Importantly, luteolin nonselectively affected PDBu-stimulated phosphorylation of CPI-17 and fluoride-stimulated phosphorylation of CPI-17 and MYPT1, so promoting myosin phosphatase activities, which resulted in a reduced level of LC phosphorylation. With this distinct mode of action, luteolin attenuated fluoride, phorbol 12,13-dibutyrate and thromboxane mimetic-elicited vasoconstriction; thus displaying a therapeutic target for the development of novel antihypertensives.

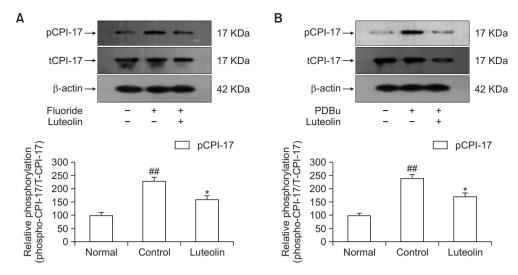Activation of PKC or ROCK, phosphorylation of CPI-17 or

**Fig. 7.** Effect of luteolin on fluoride (A) or phorbol ester (B)-dependent enhancement in phospho-CPI-17 protein levels. Phospho-CPI-17 levels were attenuated in rapidly frozen flavone-treated vessels freed from endothelium compared to vehicle-treated vessels contracted with fluoride or phorbol ester. Upper panel shows indicates a typical blot, and lower panel shows average densitometric results for relative levels of phospho-CPI-17. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. *$p<0.05$, ##$p<0.01$, versus control or normal group respectively. Luteolin: 0.1 mM luteolin; Fluoride: 6 mM sodium fluoride; PDBu: 1 $\mu$M phorbol 12,13-dibutyrate.
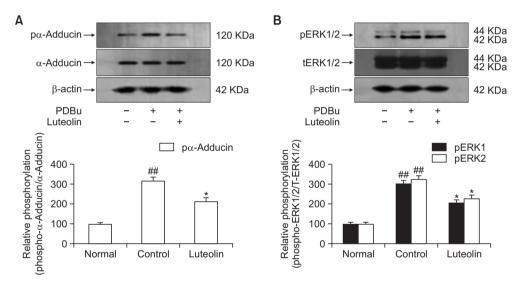


**Fig. 8.** Effect of luteolin on phorbol ester-dependent augmentations in phospho-$\alpha$-adducin (A) and phospho-ERK1/2 protein levels (B). Phospho-$\alpha$-adducin and ERK1/2 levels were attenuated in rapidly frozen luteolin-treated vessels freed from endothelium compared to vehicle-treated vessels contracted with phorbol ester. Upper panel shows indicates a typical blot, and lower panel implies average densitometric results for relative levels of phospho-adducin or ERK1/2. Data are exhibited as the mean of 3-5 experiments with a vertical line implying SEM. *$p<0.05$, ##$p<0.01$, versus control or normal group respectively. Luteolin: 0.1 mM luteolin; PDBu: 1 $\mu$M phorbol 12,13-dibutyrate.

MYPT1, and ensuing restriction of myosin phosphatase are part of the calcium sensitization pathway that potentiates enhanced MLC phosphorylation without requiring an increase in calcium 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.*, 1996). ROCK was reported to be involved in vascular contractions induced by fluoride, phorbol ester or thromboxane mimetic (Wilson *et al.*,

2005; Jeon *et al.*, 2006; Tsai and Jiang, 2006).

The present study demonstrates that luteolin attenuates contractions elicited by vasoconstrictors (phorbol ester or fluoride) in an endothelium-independent cascade (Fig. 2-4), and that the mechanisms include the PKC/MEK/ERK and RhoA/ROCK pathways. Luteolin restricted the fluoride-elicited phosphorylation of CPI-17 at Thr38, implying that CPI-17 included in fluoride-elicited contraction would be a downstream effector activated by ROCK. Furthermore, luteolin significantly attenuated the vasoconstriction and the phosphorylation of MYPT1 at Thr855 and CPI-17 at Thr-38 elicited by fluoride (Fig. 5, 7A)

with the sufficient relaxation (Fig. 2) and $\alpha$-adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 elicited by a phorbol ester (Fig. 8), suggesting that reduction of PKC/MEK or ROCK activity is a main mechanism concerning the effects of luteolin on smooth muscle contractility. Activation of ROCK by fluoride attenuates the activity of myosin phosphatase through phosphorylation of MYPT1 and CPI-17, resulting in an increase in $MLC_{20}$ phosphorylation and contractions (Sakurada *et al.*, 2003; Somlyo and Somlyo, 2003; Wilson *et al.*, 2005; Akata, 2007) alleviated by luteolin (Fig. 6). Therefore, thick or myosin filament regulation involving pCPI-17 inactivation or myosin phosphatase activation through RhoA/ROCK and PKC/CPI-17 inactivation might be involved in luteolin-elicited inhibition of vascular contractility.

In summary, luteolin used safely (Taheri *et al.*, 2021) significantly alleviates the RhoA/ROCK activator fluoride-elicited contractions decreasing CPI-17 phosphorylation and attenuates phorbol ester-elicited contraction due to PKC/MEK activation. Thus, the mechanism underlying the flavone-elicited relaxation of phorbol ester- or fluoride-evoked contractions includes inhibition of PKC/MEK and ROCK activity. Repression of ROCK/PKC activity and ensuing CPI-17/MYPT1 phosphorylation elicited by luteolin during agonists-induced contraction suggests that ROCK/CPI-17 and PKC/CPI-17 inactivation is needed for myosin phosphatase reactivation and vessel relaxation.

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