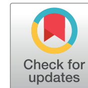

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Effect of Kaempferol on Modulation of Vascular Contractility Mainly through PKC and CPI-17 Inactivation

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Abstract

In this study, we investigated the efficacy of kaempferol (a flavonoid found in plants and plant-derived foods such as kale, beans, tea, spinach and broccoli) on vascular contractibility and aimed to clarify the detailed mechanism underlying the relaxation. Isometric contractions of divested muscles were stored and linked with western blot analysis which was carried out to estimate the phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) and phosphorylation-dependent inhibitory protein for myosin phosphatase (CPI-17) and to estimate the effect of kaempferol on the RhoA/ROCK/CPI-17 pathway. Kaempferol conspicuously impeded phorbol ester-, fluoride- and a thromboxane mimetic-derived contractions regardless of endothelial nitric oxide synthesis, indicating its direct effect on smooth muscles. It also conspicuously impeded the fluoride-derived elevation in phospho-MYPT1 rather than phospho-CPI-17 levels and phorbol 12,13-dibutyrate-derived increase in phospho-CPI-17 and phospho-ERK1/2 levels, suggesting the depression of PKC and MEK activities and subsequent phosphorylation of CPI-17 and ERK1/2. Taken together, these outcomes suggest that kaempferol-derived relaxation incorporates myosin phosphatase retrieval and calcium desensitization, which appear to be modulated by CPI-17 dephosphorylation mainly through PKC inactivation.

Key Words: CPI-17, Fluoride, Kaempferol, MYPT1, Phorbol ester, PKC

INTRODUCTION

Kaempferol (3,4',5,7-tetrahydroxyflavone) (Fig. 1) is an aglycone flavonoid found in various plant parts such as seeds, leaves, fruits, flowers and vegetables (Rajendran *et al*., 2014), and it has various pharmacological activities such as anti-inflammatory, anti-oxidant, anti-microbial, anti-diabetic and anticancer activities (Hung *et al*., 2017; Du *et al*., 2019; Imran *et al*., 2019; Periferakis *et al*., 2022) in many cancers including gastric, breast, lung and renal cancer promoting apoptosis, endoplasmic reticulum stress, autophagy and epigenetic modification. However, the molecular targets responsible for the favorable effects of kaempferol require further investigation.

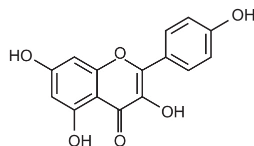

Fig. 1. Chemical structure of kaempferol (3,4',5,7-tetrahydroxyflavone).

Vascular contractility is modulated by 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 have been observed in many cardiovascular diseases. The mechanism responsible for calcium sensitization incorporates the constraint of myosin phosphatase, causing phosphorylation of the myosin light chain 20 kDa (MLC_{20}) and following augmented contractility. The constraint of myosin phosphatase in smooth muscles is modified by the phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) and/

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or phosphorylation-dependent inhibitory protein for myosin phosphatase (CPI-17) by protein kinase C (PKC) or Rho-associated protein kinase (ROCK), which causes decreased dephosphorylation of MLC_{20}. The constraint of myosin phosphatase in smooth muscle is mediated by the phosphorylation of MYPT1 via ROCK, which leads to the reserved phosphorylation of MLC_{20}. PKC is incorporated into contractile filaments that are responsive to calcium. Calcium antagonist-impervious forms of hypertension and coronary vasospasm necessitate treatment patterns that focus on other pathways such as ROCK and PKC. PKC constrains myosin phosphatase activity by activating CPI-17, a myosin phosphatase inhibitor when phosphorylated at Thr38 by PKC or ROCK, resulting in the enhanced phosphorylation of MLC (Kim *et al*., 2012; Yang *et al*., 2018). CPI-17 (Thr38) and MLC phosphorylation proportionately match with vasoconstriction during several physiological processes within vessels and other cells. Extracellular signal regulated kinase (ERK) 1/2 and its activator mitogen-activated protein kinase kinase (MEK) have been shown to be activated via PKC-modulated phosphorylation in various cell types (Ansari *et al*., 2009). Thromboxane mimetics, phorbol esters and fluoride have been shown to induce vascular contractions from developed calcium sensitivity or partially developed calcium concentrations. The activation of ERK1/2 by phenylephrine (Perez-Aso *et al*., 2013), a thromboxane mimetic or phorbol ester triggers ERK1/2-derived cytoskeletal remodeling and blunts the inhibitory action of caldesmon thereby increasing the affinity between myosin and actin and cross-bridge cycling (Gallet *et al*., 2003; Roman *et al*., 2014).

However, the specific protein kinases and attendant cellular pathways responsible for calcium desensitization in response to kaempferol remain unknown. Therefore, the objective of this study was to identify the specific protein kinases and associated signaling pathways responsible for kaempferol-induced myosin phosphatase restoration and calcium desensitization.

MATERIALS AND METHODS

Smooth muscle preparation

Male Sprague-Dawley rats (200-230 g) were anaesthetized with 0.3 mg/kg etomidate and euthanatized by exsanguination and thoracotomy in compliance with the experimental procedures recognized by the Institutional Committee at Chung-Ang University and Daegu Catholic University (IACUC-2018-006) and the National Institutes of Health guide for the care and use of laboratory animals. After euthanasia, the thoracic aorta was discreetly and swiftly extracted and placed in an oxygenated saline solution incorporating 115.0 mM sodium chloride, 25.0 mM sodium bicarbonate, 10.0 mM dextrose, 4.7 mM potassium chloride, 2.5 mM calcium chloride, 1.2 mM magnesium chloride and 1.2 mM potassium phosphate monobasic. The adjacent connective tissue was removed from the muscle and the endothelia were divested by moderate scraping using a pipette tip and/or N^G-monomethyl-L-arginine (L-NMMA) if needed.

Estimation of muscle contraction

To estimate the functional alterations in the vessel in response to a vasoconstrictor, each vessel was shortened with an agonist in a water-jacketed organ bath aerated with oxygen. The vessels were extended before a resting tension of 2.0 g was attained, and fluctuations in their tension were estimated using a force-displacement transducer (FT03C, Grass Instruments, Quincy, MA, USA) associated with a PowerLab recording system (AD Instruments, Castle Hill, NSW, Australia). After stabilization (for 60 min), the arterial integrity was estimated by shortening the vessels with 1 μM phenylephrine or 50 mM KCl, succeeded by relaxation with acetylcholine (1 μM).

The relaxation effect of kaempferol was identified by its injection after KCl (50 mM), phenylephrine (1 μM), a thromboxane mimetic (0.1 μM), fluoride (6 mM), or phorbol ester (1 μM)-stimulated shortening plateaued in a normal Krebs solution.

Western blot analysis

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

Chemicals and antibodies

Potassium chloride, sodium chloride, sodium bicarbonate, acetylcholine, kaempferol, phenylephrine, phorbol 12,13-dibutyrate (PDBu), sodium fluoride and U-46619 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetone, TCA and DTT were acquired from Fisher Scientific (Pittsburgh, PA, USA) and enhanced chemiluminescence kits were acquired from Pierce Biotechnology (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, ERK or phosphoERK at Thr202/Tyr204 (Cell Signaling Technology, Danvers, MA, USA or Upstate Biotechnology, Lake Placid, NY, USA) were utilized to estimate the extent of RhoA/ROCK activity (Kitazawa *et al*., 2000; 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 utilized as secondary antibodies (1:2,000; Upstate Biotechnology). Antibodies against MLC_{20} (1:1,500; Sigma-Aldrich) and anti-mouse IgG (goat) conjugated with horseradish peroxidase (1:2,000; Upstate Biotechnology) were utilized to confirm the extent of LC20 phosphorylation. Kaempferol was dissolved in dimethyl sulfoxide as a 0.1 M stock solution and stored at –20°C for later dilution.

Statistics

Values concerning western blot analyses and isometric tension measurements are exhibited as mean ± standard error of the mean (SEM) of at least three separate experiments. Statistical comparisons between groups were performed using Student's t-test or one-way analysis of variance followed by Bonferroni's post hoc comparisons. Statistical analyses were carried out using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Differences between groups were considered significant at $p<0.05$.

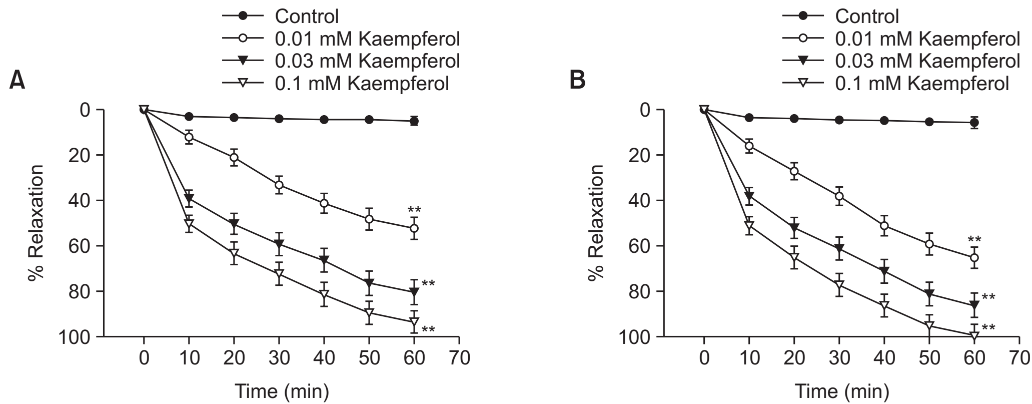

Fig. 2. Effect of kaempferol on fluoride-derived vasoconstriction in divested (A) or intact (B) vessels. Each vessel was equilibrated in the organ bath for 40-50 min until relaxation responses to kaempferol were estimated. Data are exhibited as the mean of three to five discrete experiments with a vertical line displaying SEM. ***p*<0.01, absence versus presence of kaempferol.

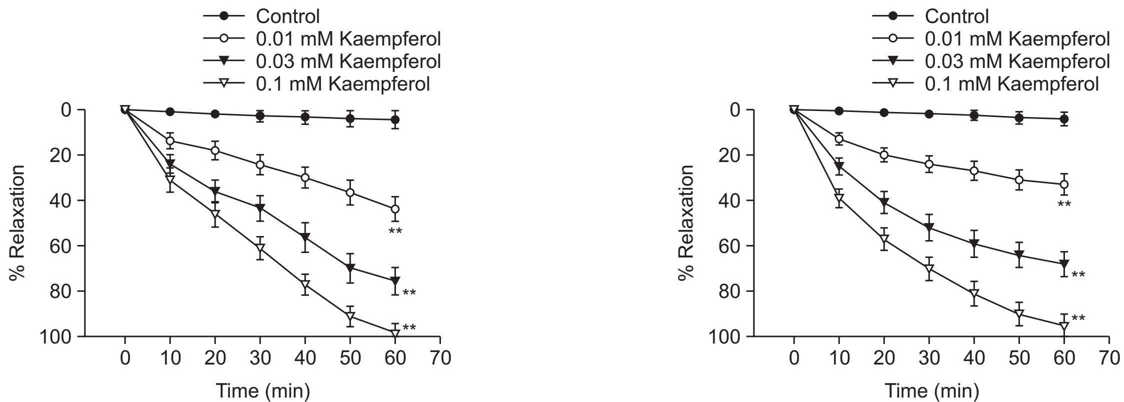

Fig. 3. Effect of kaempferol on a thromboxane mimetic-derived vasoconstriction in divested vessels. Each vessel was stabilized in the bath for 40-50 min until relaxation responses to kaempferol were estimated. Data are exhibited as the mean of three to five discrete experiments with a vertical line displaying SEM. ***p*<0.01, absence versus presence of kaempferol.

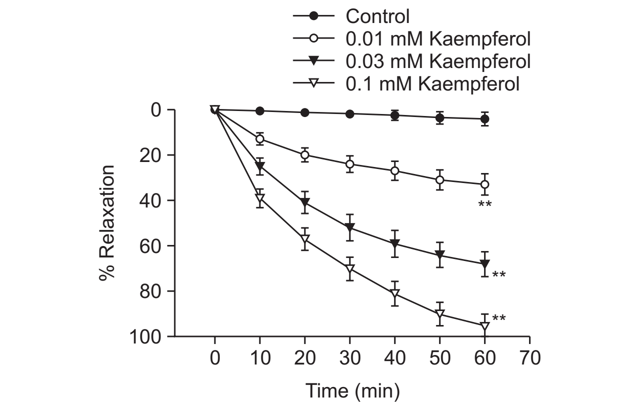

Fig. 4. Effect of kaempferol on phorbol ester-derived vasoconstriction in divested vessels. Each vessel was stabilized in the bath for 40-50 min until relaxation responses to kaempferol were estimated. Data are exhibited as the mean of three to five experiments with a vertical line displaying SEM. ***p*<0.01, absence versus presence of kaempferol.

RESULTS

Effect of kaempferol on contractions of endothelium-divested muscles derived by a full RhoA/ROCK activator fluoride

Deprivation of endothelium, the modulator of vascular homeostasis, was accomplished by smooth scrub with a pipette tip and/or L-NMMA to estimate the relaxation effect of kaempferol on vascular smooth muscle. Endothelial deprivation was identified by the sparsity of relaxation after treating contracted segments with acetylcholine (1 μM). Kaempferol was devoid of any effect when tested on basal tension (data not shown), but it conspicuously impeded contraction induced by the ROCK activator fluoride in divested muscles (Fig. 2A) free from endothelial nitric oxide synthesis or intact muscles (Fig. 2B). This implies that the relaxation mechanism of kaempferol might involve the restriction of ROCK activity and myosin phosphatase retrieval excluding endothelial nitric oxide synthesis and following activation of guanylyl cyclase.

Effect of kaempferol on contractions of divested aortas derived by the binary ROCK and MEK activator thromboxane mimetic

Kaempferol conspicuously weakened thromboxane mimetic-derived contractions in divested muscles (Fig. 3), suggesting that the mechanism incorporates restriction of ROCK activity and myosin phosphatase invigoration and a dual activator (a thromboxane mimetic) acts similarly to a potent activator focusing on ROCK.

Effect of kaempferol on contractions of divested muscles derived by a MEK activator PDBu

Phorbol esters are primarily MEK activators and partial ROCK activators (Goyal *et al*, 2009; Je and Sohn, 2009). Interestingly, PDBu-derived contractions were conspicuously weakened by kaempferol, regardless of endothelial nitric oxide synthesis in the divested vessels (Fig. 4), which insinuated that thin filament adjustment including MEK/ERK was regressed.

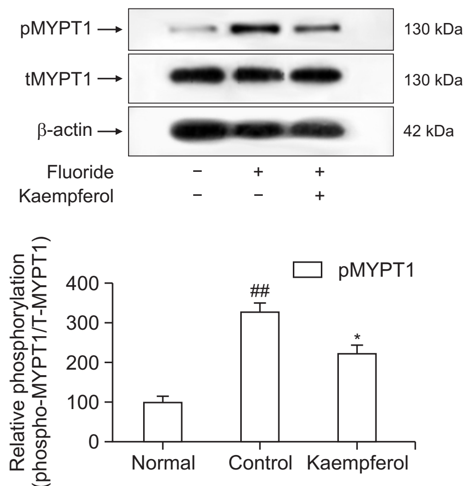

Fig. 5. Effect of kaempferol on fluoride-derived invigoration in phospho-MYPT1 protein levels. Phospho-MYPT1 levels were restricted in rapidly frozen kaempferol-treated vessels free from endothelium compared to vehicle-processed vessels contracted with fluoride. Upper panel shows a typical blot, and lower panel shows average densitometric outcomes for relative levels of phospho-MYPT1. Data are exhibited as the mean of three to five discrete experiments with a vertical line displaying SEM. $^{##}p<0.01$, $^{*}p<0.05$, versus normal or control group respectively. Kaempferol: 0.1 mM kaempferol; Fluoride: 6 mM sodium fluoride.

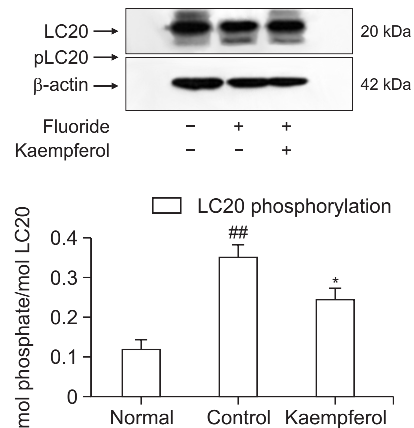

Fig. 6. Effect of kaempferol on fluoride-derived invigoration in phospho-MLC_{20} level. Phospho-MLC_{20} levels displayed as a percentage of total MLC_{20} were restricted in rapidly frozen kaempferol-treated vessels free from endothelium compared to vehicle-processed vessels contracted with fluoride (6 mM). Data are exhibited as the means of three to five discrete experiments with a vertical line displaying SEM. $^{##}p<0.01$, $^{*}p<0.05$, versus normal or control group respectively. Kaempferol: 0.1 mM kaempferol; Fluoride: 6 mM sodium fluoride.

Effect of kaempferol on the extent of MYPT1 phosphorylation at Thr-855

To estimate the role of kaempferol in the thick filament adjustment of vascular contractibility, we estimated the extent of MYPT1 and phospho-MYPT1 in vessels that were swiftly frozen after a 60-min exposure to kaempferol for equilibration. Fluoride (6 mM) invigorated the contraction force in each vessel. This study was conducted using quick-frozen kaempferol (0.1 mM)-treated vessels free from endothelial function, and the extents were compared with those of vehicle-processed vessels (Fig. 5). A conspicuous constraint of fluoride-derived MYPT1 phosphorylation at Thr855 in response to kaempferol treatment was observed (Fig. 5). Furthermore, a constraint of fluoride-invigorated LC20 phosphorylation was observed in response to kaempferol treatment (Fig. 6). Therefore, thick filament control, incorporating myosin phosphatase activation by way of RhoA/ROCK inactivation may be incorporated into the restricted contractility of kaempferol-treated rat aortas.

Effect of kaempferol on the extent of CPI-17 phosphorylation at Thr-38

The myosin phosphatase inhibitor CPI-17 is phosphorylated by PKC or ROCK. CPI-17 phosphorylation is usually invigorated during the contraction as it is one mechanism that heightens myofilament calcium sensitivity. PDBu or fluoride was utilized as a control for CPI-17 phosphorylation as it directly invigorates PKC or ROCK generating a significant increment in CPI-17 phosphorylation. To confirm the role of kaempferol in the thin or thick filament adjustment of smooth muscle contractility, we estimated the extent of phospho-CPI-17 and CPI-17 in vessels that were swiftly frozen after a 60-min exposure to kaempferol for equilibration. Fluoride (6 mM) or phorbol ester (1 μM) invigorated the contraction force of each vessel. This work was conducted using swiftly frozen flavonol (0.1 mM)-treated vessels free from endothelial function, and the extent was compared to that of vehicle-processed vessels (Fig. 7). Interestingly, a significant reduction in phorbol ester-invigorated CPI-17 phosphorylation at Thr-38 in response to kaempferol treatment was observed (Fig. 7B). The reduction in CPI-17 phosphorylation with kaempferol during phorbol ester application suggests that PKC is inactivated in the kaempferol-derived constraints on contractile force, MLC phosphorylation and myosin phosphatase restriction.

Effect of kaempferol on the extent of adducin phosphorylation at Ser662 and ERK1/2 phosphorylation at Thr-202/Tyr-204

To substantiate the role of kaempferol in thin filament disinhibition of vasoconstriction, we estimated the extent of phospho-adducin and adducin and phospho-ERK1/2 and ERK1/2 in vessels that were swiftly frozen after 60 min of exposure to kaempferol for equilibration. PDBu (1 μM) invigorated the contractile force in each vessel. Compared to vehicle-processed vessels, a constraint in adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 was discerned in kaempferol (0.1 mM)-treated vessels with endothelial deficiency (Fig. 8); conspicuous relaxation (Fig. 4) and thin filament adjustments were discerned. These findings showed that thin filament adjustment, incorporating adducin and ERK1/2 phosphorylation by way of PKC and MEK invigoration, plays a role in kaempferol-derived relaxation.

DISCUSSION

This is the study to indicate that kaempferol constrains tonic tension and restricts calcium sensitization through the blockade of primarily PKC-mediated CPI-17 phosphorylation rather than ROCK-mediated CPI-17 phosphorylation. Pharmaco-

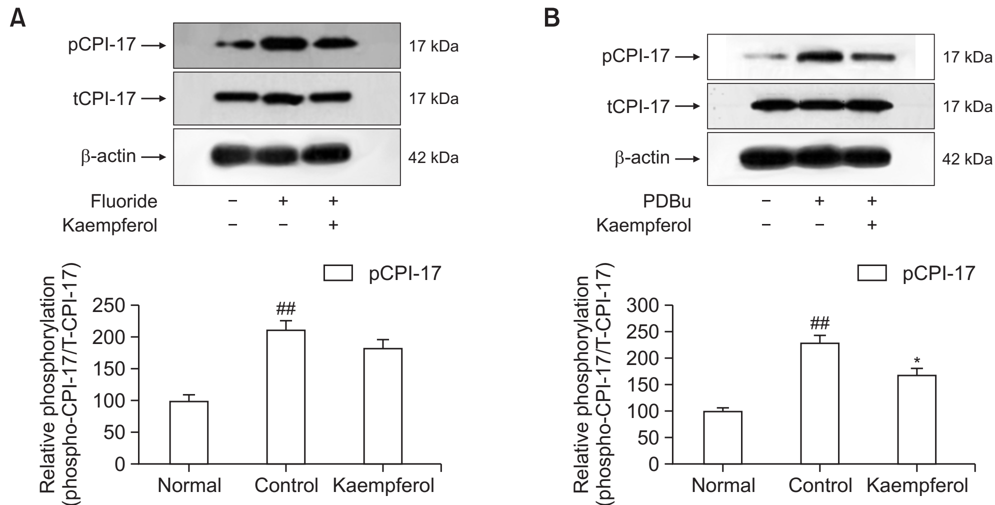

Fig. 7. Effect of kaempferol on fluoride (A) or phorbol ester (B)-derived invigoration in phospho-CPI-17 protein extents. Phospho-CPI-17 extents were restricted in rapidly frozen flavonol-treated vessels free from endothelial function compared to vehicle-processed vessels contracted with fluoride or phorbol ester. Upper panel indicates a typical blot, and lower panel shows average densitometric outcomes for relative levels of phospho-CPI-17. Data are exhibited as the mean of three to five discrete experiments with a vertical line displaying SEM. $^{\#\#}p$<0.01, $^{*}p$<0.05, versus normal or control group respectively. Kaempferol: 0.1 mM kaempferol; Fluoride: 6 mM sodium fluoride; PDBu: 1 μM phorbol 12,13-dibutyrate.

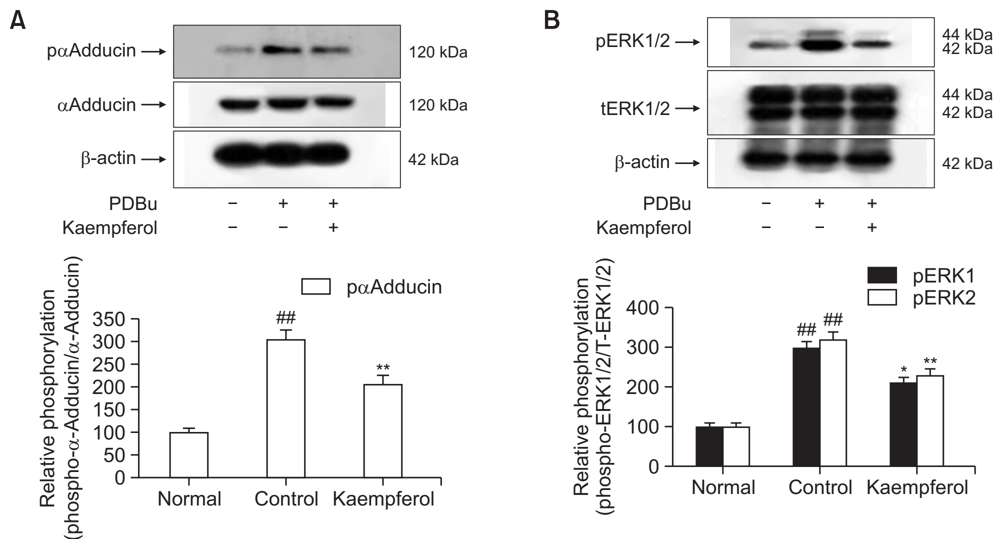

Fig. 8. Effect of kaempferol on phorbol ester-derived invigorations in phospho-alpha-adducin (A) and phospho-ERK1/2 protein extents (B). Phospho-α-adducin and phospho-ERK1/2 levels were attenuated in swiftly frozen kaempferol-treated vessels free from endothelium compared to vehicle-processed vessels contracted with phorbol ester. Lower panel implies average densitometric outcomes for relative extents of phospho-ERK1/2. Data are displayed as the mean of three to five discrete experiments with a vertical line displaying SEM. $^{\#\#}p$<0.01, $^{**}p$<0.01, $^{*}p$<0.05, versus normal or control group respectively. Kaempferol: 0.1 mM kaempferol; PDBu: 1 μM phorbol 12,13-dibutyrate.

logical activators of ROCK (fluoride), MEK (phorbol ester) or both (a thromboxane mimetic) were utilized to determine their incorporation in the flavonol-derived suppression. The CPI-17-modulated and calcium-sensitized contraction, derived by various agonists, was potentiated consistently. Kaempferol constrains tonic tension and restricts calcium sensitization through the blockade of PKC or ROCK-modulated myosin phosphatase constraint. Importantly, kaempferol selectively affected not fluoride but PDBu-mediated phosphorylation of CPI-17 and fluoride-mediated phosphorylation of MYPT1, so forstering myosin phosphatase activities, which resulted in a restricted extent of MLC phosphorylation. With this discrete mode of action, kaempferol restricted fluoride, phorbol 12,13-dibutyrate and a thromboxane mimetic-derived vasoconstriction; thus exhibiting a target for the development of novel antihypertensives.

Invigoration of PKC or ROCK, phosphorylation of CPI-17 or MYPT1, and following constraint of myosin phosphatase

are part of the calcium sensitization route that invigorates enhanced MLC phosphorylation without requiring an increment in calcium influx or release. ROCK/CPI-17 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/CPI-17 was reported to be incorporated in vascular contractions induced by fluoride, phorbol ester or a thromboxane mimetic (Wilson *et al*., 2005; Jeon *et al*., 2006; Tsai and Jiang, 2006).

The present study demonstrates that kaempferol attenuates contractions derived from contractile agonists (phorbol ester or fluoride) in an endothelium-unrelated route (Fig. 2-4), and that the mechanisms incorporate the PKC/MEK/ERK and RhoA/ROCK routes. Kaempferol constrained not fluoride but phorbol ester-derived phosphorylation of CPI-17 at Thr38, implicating that CPI-17 incorporated in phorbol ester-derived contraction would be a downstream effector invigorated by PKC. Furthermore, kaempferol conspicuously restricted the contractility and the phosphorylation of CPI-17 at Thr-38 (Fig. 7B) and alpha-adducin and ERK 1/2 phosphorylation at Ser662 and Thr202/Tyr204 derived from a phorbol ester (Fig. 8A, 8B) with the adequate relaxation (Fig. 4), suggesting that constraint of PKC/MEK activity is a central mechanism regarding the effects of kaempferol 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) partially impeded by kaempferol (Fig. 6). Therefore, thick or myosin filament control involving pCPI-17 restriction or myosin phosphatase invigoration through RhoA/ROCK and PKC/CPI-17 restriction might be partially incorporated in kaempferol-derived inhibition of vascular contractility.

In summary, kaempferol used safely in humans (Akiyama *et al*., 2023) impedes the RhoA/ROCK activator fluoride-derived contractions decreasing MYPT1 phosphorylation and restricts phorbol ester-derived contraction due to PKC/MEK activation and CPI-17 phosphorylation. Thus, the mechanism underlying the flavonol-derived relaxation of phorbol ester- or fluoride-derived contractions incorporates constraint of PKC/MEK and ROCK activity. Repression of PKC activity and following CPI-17/ERK12 phosphorylation derived by kaempferol during agonists-induced contraction suggests that primarily PKC/CPI-17 restriction is required for myosin phosphatase retrieval and vessel relaxation.

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