

## Endothelium Independent Effect of Pelargonidin on Vasoconstriction in Rat Aorta

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### Abstract

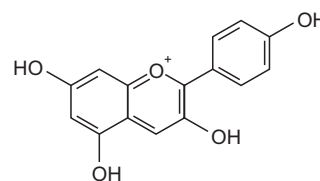
In this study, we investigated the effects of pelargonidin, an anthocyanidin found in many fruits and vegetables, on endothelium-independent vascular contractility to determine the underlying mechanism of relaxation. Isometric contractions of denuded aortic muscles from male rats were recorded, and the data were combined with those obtained in western blot analysis. Pelargonidin significantly inhibited fluoride-, thromboxane A<sub>2</sub>-, and phorbol ester-induced vascular contractions, regardless of the presence or absence of endothelium, suggesting a direct effect of the compound on vascular smooth muscles via a different pathway. Pelargonidin significantly inhibited the fluoride-dependent increase in the level of myosin phosphatase target subunit 1 (MYPT1) phosphorylation at Thr-855 and the phorbol 12,13-dibutyrate-dependent increase in the level of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation at Thr202/Tyr204, suggesting the inhibition of Rho-kinase and mitogen-activated protein kinase (MEK) activities and subsequent phosphorylation of MYPT1 and ERK1/2. These results suggest that the relaxation effect of pelargonidin on agonist-dependent vascular contractions includes inhibition of Rho-kinase and MEK activities, independent of the endothelial function.

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

### INTRODUCTION

Anthocyanins such as cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin (Castaneda-Ovando *et al.*, 2009) possess high free radical scavenging and antioxidant activity and prevents neuronal and cardiovascular diseases, cancer and diabetes (He and Giusti, 2010). Among them, pelargonidin [2-(4-hydroxyphenyl)chromenylium-3,5,7-triol; Fig. 1] is an anthocyanidin responsible for the typical color (blue, purple, or red) and biological effects of many fruits, berries, and vegetables. The compound shows various pharmacological activities such as antioxidant, antidiabetic, anti-inflammatory, anti-atherosclerotic and recently discovered anticarcinogenic activities (Noda *et al.*, 2002; Hamalainen *et al.*, 2007; Roy *et al.*, 2008; Son *et al.*, 2014) including the inhi-

bition of epithelial–mesenchymal transition through the TGFβ<sub>1</sub>/Smad2 signaling pathway (Ouanouki *et al.*, 2017). However, the biological effects of pelargonidin on vascular contractility have not been elucidated thus far. In this study, we investigat-



**Fig. 1.** The chemical structure of pelargonidin [2-(4-hydroxyphenyl)chromenylium-3,5,7-triol].

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ed the mechanisms underlying the endothelium-independent relaxation effect of pelargonidin on vascular smooth muscles with the goal of developing a better antihypertensive using denuded aortic muscle from male Sprague–Dawley rats and recording isometric contractions.

The contraction of vascular smooth muscles influenced by lifestyle modification and pharmacological interventions (Appel *et al.*, 2006) is regulated via both  $\text{Ca}^{2+}$ -dependent and  $\text{Ca}^{2+}$  sensitization mechanisms (Akata, 2007; Kim *et al.*, 2011). The mechanism responsible for  $\text{Ca}^{2+}$  sensitization involves the inhibition of myosin light chain phosphatase (MLCP), leading to the increased phosphorylation of the 20-kDa myosin light chain (MLC20) and subsequent enhanced contraction. The inhibition of MLCP in vascular smooth muscles is mediated by phosphorylation of the myosin phosphatase target subunit of MLCP by Rho-kinase, which leads to sustained phosphorylation of MLC20. Subsequent studies have suggested that inhibition of myosin phosphatase by Rho-kinase (Uehata *et al.*, 1997; Sakurada *et al.*, 2003; Akata, 2007) or thin filament regulation involving the activation of protein kinase C, mitogen-activated protein kinase kinase (MEK), and extracellular signal-regulated kinases (ERKs) 1/2, as well as phosphorylation of the actin-binding protein caldesmon (Wier and Morgan, 2003), may precipitate  $\text{Ca}^{2+}$  sensitization.

Activation of ERK1/2, in addition to regulating vascular contractility, is also connected to the pathologic hypertrophy, hyperplasia, hypertension, and atherosclerosis (Ruppert *et al.*, 2013; Brietz *et al.*, 2016). ERK1/2 are activated by threonine and tyrosine phosphorylation by RAF-activated MEK. Sodium fluoride, phorbol 12,13-dibutyrate, and U-46619 have been shown to induce the contractions of smooth muscles, which may be primarily due to enhanced  $\text{Ca}^{2+}$  sensitivity or partially due to an increased  $\text{Ca}^{2+}$  concentration. ERK1/2 activation is induced by a thromboxane A2 mimetic (Gallet *et al.*, 2003) or phorbol ester. Activation of ERK1/2 triggers the ERK1/2-dependent cytoskeletal remodeling and podosome formation (Gu *et al.*, 2007). It is possible that the contractions caused by fluoride or thromboxane A2 mimetics involve the RhoA/Rho-kinase pathway (Jeon *et al.*, 2006). However, it is unknown whether this pathway is inhibited during the pelargonidin-dependent vascular smooth muscle relaxation in aortic muscles precontracted with vasoconstrictors. Our aim was to investigate the roles of inhibition of MYPT1 phosphorylation at Thr-855 and ERK 1/2 phosphorylation at Thr202/Tyr204 in  $\text{Ca}^{2+}$  desensitization during pelargonidin-induced relaxation of isolated, denuded rat aortas using Rho-kinase (fluoride) and MEK (phorbol ester) activators.

## MATERIALS AND METHODS

### Tissue preparation

Male Sprague–Dawley rats weighing 200–250 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and then subjected to cervical dislocation, in accordance with procedures approved by the Institutional Animal Care and Use Committee of Chung-Ang University and Catholic University of Daegu (IACUC-2016-045). Thoracic aortas were quickly separated and placed in an oxygenated physiological saline solution (pH 7.4) containing (mM): NaCl, 115.0; KCl, 4.7;  $\text{CaCl}_2$ , 2.5;  $\text{MgCl}_2$ , 1.2;  $\text{NaHCO}_3$ , 25.0;  $\text{KH}_2\text{PO}_4$ , 1.2; and dextrose, 10.0. The aortas were then freed of all adherent connective

tissue, and aortic endothelia were cleaned by gentle abrasion using a cell scraper, if necessary.

### Contraction measurements

Two stainless steel triangles were inserted through each vessel ring. Each aortic ring was suspended in a water-jacketed organ bath (10 mL) maintained at 37°C and was aerated with a mixture of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . One triangle was anchored to a stationary support, and the other was connected to an isometric force transducer (FT03C; Grass Instrument Company, Quincy, MA, USA). Muscles were passively stretched by applying an optimal resting tension of 2.0 g, which was maintained throughout the experiment. Each muscle was equilibrated in the organ bath solution for 60 min before contractile responses to 50 mM KCl or 1  $\mu\text{M}$  phenylephrine were measured. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP; AD Instruments, Castle Hill, NSW, Australia).

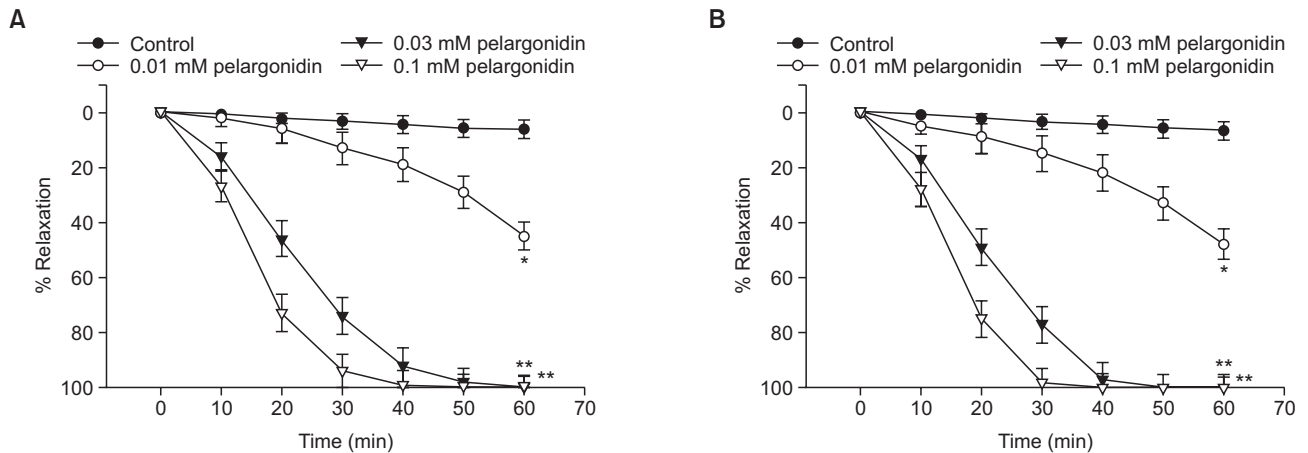
The relaxation effect of pelargonidin was determined by applying the compound after thromboxane A2 (0.1  $\mu\text{M}$ )-, phorbol ester (1  $\mu\text{M}$ )-, or fluoride (6 mM)-induced contractions plateaued in a normal Krebs' solution.

### Western blot analysis

Muscles were quick-frozen by immersion in a dry ice/acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT). Muscles were stored at -80°C until use. Tissues were brought up to room temperature from the freezer in the mixture including dry ice, acetone, TCA and DTT and then homogenized in a buffer containing 20 mM MOPS, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM  $\beta$ -glycerophosphate, 5.5  $\mu\text{M}$  leupeptin, 5.5  $\mu\text{M}$  pepstatin, 20 kIU aprotinin, 2 mM  $\text{Na}_3\text{VO}_4$ , 1 mM NaF, 100  $\mu\text{M}$   $\text{ZnCl}_2$ , 20  $\mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 mM EGTA. Protein-matched samples (modified Lowry protein assay, DC protein assay kit, Bio-Rad) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (Protogel, Atlanta, GA, USA), then transferred to polyvinylidene difluoride membranes, and subjected to immunostaining and densitometry using primary and secondary antibodies. Lane loading variations were corrected by normalization versus  $\beta$ -actin. Sets of samples produced during individual experiments were run in the same gel, and densitometry was performed on the same image.

### Chemicals and antibodies

Sodium chloride, sodium fluoride, potassium chloride, acetylcholine, pelargonidin, U46619, and phorbol 12,13-dibutyrate (PDBu) were purchased from Sigma (St. Louis, MO, USA). DTT, TCA, and acetone were obtained from Fisher Scientific (Hampton, NH, USA). The enhanced chemiluminescence kit was from Pierce (Rockford, IL, USA). Antibodies against myosin phosphatase target subunit 1 phosphorylated at Thr-855 (phospho-MYPT1; 1:5,000), MYPT1, ERK, and phospho-ERK (Thr-202/Tyr-204) (Cell Signaling Technology, Danvers, MA, USA or Upstate Biotechnology, Lake Placid, NY, USA) were used to determine the levels of RhoA/Rho-kinase activity (Woodbridge *et al.*, 2004; Wilson *et al.*, 2005) or MEK activity. Anti-mouse IgM (goat) and anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1:2,000 dilutions for both; Upstate Biotechnology). Pelargonidin was prepared in dimethyl sulfoxide (DMSO) as



**Fig. 2.** Effects of pelargonidin on fluoride-dependent vascular contractions in denuded (A) and intact (B) muscles. Each muscle was equilibrated in the organ bath solution for 30-60 min before relaxation responses to pelargonidin were measured. Data are expressed as the mean of 3-5 experiments with the error bar representing SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , presence versus absence of pelargonidin.

a 100 mM stock solution and frozen at  $-20^{\circ}\text{C}$  for later use. DMSO alone had no observable effect at the concentrations used (data not shown).

**Statistics**

Data were expressed as the mean  $\pm$  standard error of the mean (SEM). The Student's unpaired *t*-test or ANOVA was used to determine statistical significance of the differences between two groups using SPSS 12.0 (SPSS, Inc., Chicago, IL, USA). *p*-values of  $< 0.05$  were regarded as statistically significant.

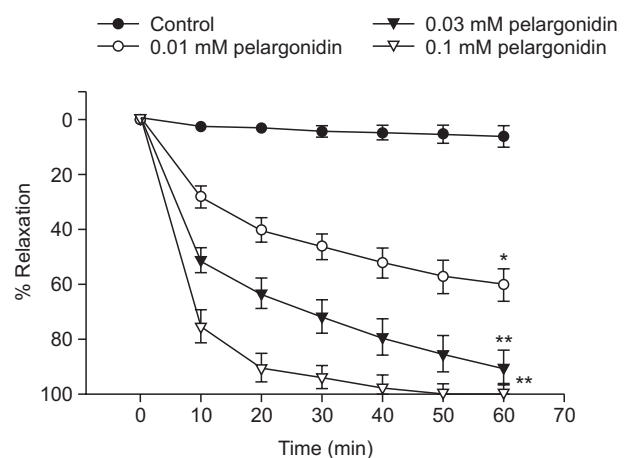
**RESULTS**

**Effect of pelargonidin on contractions of endothelium-denuded aortas, induced by a full RhoA/Rho-kinase activator, fluoride**

The endothelium was removed by gentle abrasion with a cell scraper to detect the relaxation effect of pelargonidin on vascular smooth muscles. The absence of the endothelium was confirmed by the lack of relaxation after treating precontracted ring segments with acetylcholine ( $1 \mu\text{M}$ ). Pelargonidin showed no significant effect on basal tension (data not shown) but significantly inhibited the contraction evoked by the Rho-kinase activator fluoride, regardless of the absence or presence of endothelial nitric oxide synthesis in denuded (Fig. 2A) or intact (Fig. 2B) muscles, respectively. This suggests that the relaxation mechanism of pelargonidin may include inhibition of Rho-kinase activity and is independent of the endothelial function.

**Effect of pelargonidin on contractions of denuded aortas, induced by the dual Rho-kinase and MEK activator thromboxane A2**

Pelargonidin significantly inhibited the thromboxane A2 mimetic U46619-induced contraction in denuded muscles (Fig. 3), suggesting that the dual activator (thromboxane A2 mimetic) acts similarly to full activators targeting Rho-kinase.



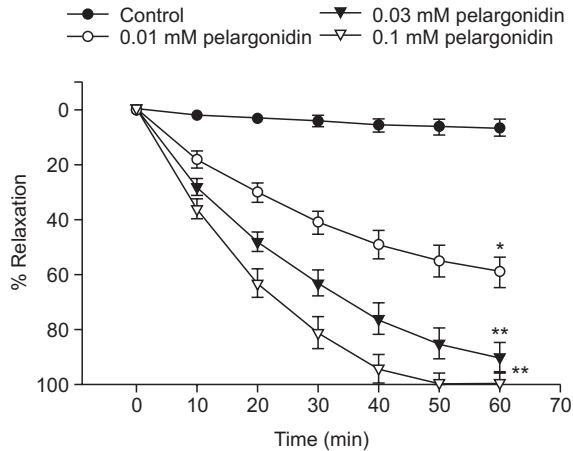
**Fig. 3.** Effects of pelargonidin on thromboxane A2-dependent vascular contractions in denuded muscles. The contractility in each muscle was equilibrated in the organ bath solution for 30-60 min before relaxation responses to pelargonidin were measured. Data are expressed as the mean of 3-5 experiments with the error bar representing SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , presence versus absence of pelargonidin.

**Effect of pelargonidin on contractions of denuded aortas, induced by an MEK activator, phorbol 12,13-dibutyrate**

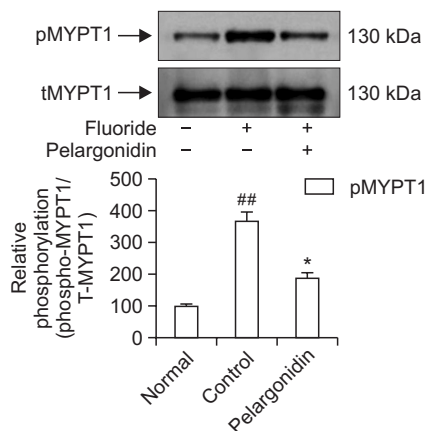
Phorbol esters are primarily MEK activators and partial Rho-kinase activators (Goyal *et al.*, 2009; Je and Sohn, 2009). Interestingly, PDBu-induced contractions were inhibited by pelargonidin at a low concentration, despite the absence of the endothelial nitric oxide synthesis in denuded muscles (Fig. 4), which suggested that the thin or actin filament regulation, including MEK/ERK, was inhibited.

**Effect of pelargonidin on the level of MYPT1 phosphorylation at Thr-855**

To confirm the role of pelargonidin in the thick filament regulation of smooth muscle contractility, we measured the levels of MYPT1 and phospho-MYPT1 in quick-frozen aortas after the relaxation by pelargonidin for equilibration. Each relax-

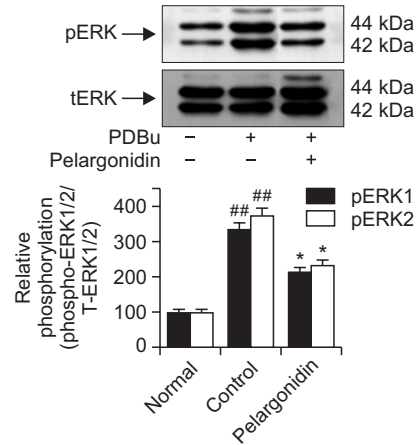


**Fig. 4.** Effects of pelargonidin on phorbol ester-dependent vascular contractions in denuded muscles. Phorbol ester-induced contractility was equilibrated in the organ bath solution for 30-60 min before relaxation responses to pelargonidin were measured. Data are expressed as the mean of 3-5 experiments with the error bar representing SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , presence versus absence of pelargonidin.



**Fig. 5.** Effects of pelargonidin on fluoride-dependent increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels decreased in rapidly frozen pelargonidin-treated rat aortas in the absence of the endothelium as compared to the levels in vehicle-treated rat aortas precontracted with fluoride. The upper panel shows a typical blot, and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the mean of 3-5 experiments with the error bar representing SEM. \* $p < 0.05$ , \*\* $p < 0.01$  versus control or normal group, respectively. Pelargonidin, 0.1 mM pelargonidin; Fluoride, 6 mM sodium fluoride.

ing muscle was precontracted with 6 mM fluoride. This experiment was conducted using quick-frozen pelargonidin (0.1 mM)-treated rat aortas in the absence of the endothelium, and the levels were compared to those in vehicle-treated rat aortas (Fig. 5). Interestingly, a significant decrease in fluoride-induced MYPT1 phosphorylation at Thr-855 was observed in response to pelargonidin treatment (Fig. 5). Thus, thick or myosin filament regulation, including myosin phosphatase activation via RhoA/Rho-kinase inactivation, might be involved in the reduced contractility of pelargonidin-treated rat aortas.



**Fig. 6.** Effects of pelargonidin on phorbol ester-dependent increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels decreased in rapidly frozen pelargonidin-treated rat aortas in the absence of the endothelium as compared to the levels in vehicle-treated rat aortas precontracted with a phorbol ester. The upper panel shows a typical blot, and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as the mean of 3-5 experiments with the error bar representing SEM. \* $p < 0.05$ , \*\* $p < 0.01$  versus control or normal group, respectively. Pelargonidin, 0.1 mM pelargonidin; PDBu, 1  $\mu$ M phorbol 12,13-dibutyrate.

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

To confirm the role of pelargonidin in the thin filament regulation of smooth muscle contractility, we measured the levels of ERK1/2 and phospho-ERK1/2 in quick-frozen aortas after the relaxation by pelargonidin for equilibration. Each relaxing muscle was precontracted with 1  $\mu$ M PDBu. Compared with that in the vehicle-treated rat aortas, a significant decrease in ERK1/2 phosphorylation at Thr-202/Tyr-204 was observed in the pelargonidin (0.1 mM)-treated rat aortas in the absence of the endothelium (Fig. 6); full vasorelaxation (Fig. 4) and thin filament regulation were observed. These findings indicate that thin or actin filament regulation, including ERK1/2 phosphorylation via MEK activation, plays an important role in pelargonidin-induced relaxation.

## DISCUSSION

The present study demonstrates that pelargonidin can modulate the vascular contractility in an endothelium-independent manner. Interestingly, the mechanism involved seems to be not only endothelium-dependent but to also involve the inhibition of MEK and Rho-kinase activities. Pelargonidin has been previously recognized for its antioxidant and antiinflammatory activities. In this study, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributed to the pelargonidin-induced vascular relaxation in denuded rat aortas precontracted with a Rho-kinase activator (fluoride), MEK activator (PDBu), or dual activator (thromboxane A2).

The mechanism by which phorbol esters or thromboxane A2 mimetics activate MEK/ERK has been established (Gu et al., 2007). In contrast, the previous studies that examined the mechanisms underlying arterial contractions induced by

fluoride or thromboxane A2 have reported conflicting findings with regard to contractions triggered by Rho-kinase activation (Wilson *et al.*, 2005; Tsai and Jiang, 2006). These findings are consistent with the notion that pelargonidin can decrease fluoride-, thromboxane A2 mimetic-, and phorbol ester-dependent contractions by decreasing the MEK or Rho-kinase activity.

The mechanisms by which MEK or Rho-kinase activation causes vascular contractions have been extensively studied, and several possibilities exist. Rho-kinase phosphorylates MLCP, which inhibits the phosphatase activity and leads to accumulation of phosphorylated myosin light chains (Pfizer 2001; Akata, 2007). Rho-kinase has also been proven to phosphorylate myosin light chains directly and independently of myosin light chain kinase (MLCK) and MLCP activity (Amano *et al.*, 1996). Recently, Rho-kinase has been reported to be involved in vascular contractions induced by fluoride, phorbol ester, or thromboxane A2 (Wilson *et al.*, 2005; Jeon *et al.*, 2006; Tsai and Jiang, 2006).

The present study demonstrates that pelargonidin ameliorates the maximal or submaximal contractions induced by vasoconstrictors (fluoride or phorbol ester) in an endothelium-independent manner (Fig. 2-4) and that the mechanism involves the MEK/ERK and RhoA/Rho-kinase pathways. Previously, vasodilation has been thought to be mostly caused by endothelial nitric oxide synthesis and subsequent activation of guanylyl cyclase (Taubert *et al.*, 2002; Ajay *et al.*, 2003; Choi *et al.*, 2015; Zou *et al.*, 2015; Choi *et al.*, 2016; Nong *et al.*, 2016). In the present study, pelargonidin at a low concentration significantly inhibited the fluoride- or phorbol ester-induced contraction, independent of the endothelial function (Fig. 2, 4). Furthermore, pelargonidin significantly decreased the phosphorylation of MYPT1 at Thr-855, evoked by fluoride (Fig. 5), with full relaxation (Fig. 2), and ERK1/2 phosphorylation at Thr-202/Tyr-204, induced by a phorbol ester (Fig. 6), suggesting that inhibition of MEK or Rho-kinase activity is the major mechanism underlying the effects of pelargonidin on smooth muscle contractility. MLC20 is thought to be phosphorylated by both MLCK and Rho-kinase (Somlyo and Somlyo, 2003). Activation of Rho-kinase by fluoride decreases the activity of MLCP through phosphorylation of MYPT1, resulting in an increase in MLC20 phosphorylation and in contractions (Sakurada *et al.*, 2003; Wilson *et al.*, 2005).

In summary, pelargonidin at a low concentration significantly attenuated smooth muscle contractions evoked by the RhoA/Rho-kinase activator fluoride, independent of the endothelial function. Furthermore, a low concentration of pelargonidin significantly inhibited the phorbol ester-induced contractions involving MEK activation. Thus, the mechanism underlying the relaxation of fluoride- or phorbol ester-induced contractions by a low concentration of pelargonidin involves the inhibition of MEK and Rho-kinase activities and subsequent MYPT1 phosphorylation. In conclusion, in addition to the synthesis of endothelial nitric oxide as a mediator of vasodilation in intact muscles, pelargonidin-induced relaxation of denuded muscles indicates the existence of a different mechanism, involving inactivation of MEK and Rho-kinase.

## CONFLICT OF INTEREST

The authors disclose no conflicts of interest.

## ACKNOWLEDGMENTS

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