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The Inhibitory Effect of Quercetin on the Agonist-Induced Regulation of Vascular Contractility

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Abstract

The present study was undertaken to investigate the influence of quercetin on vascular smooth muscle contractility and to determine the mechanism involved. Denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Quercetin at a low concentration (0.01-0.03 mM) directly and more significantly relaxed fluoride or thromboxane A_2-induced vascular contraction than phorbol ester-induced contraction suggesting as a possible antihypertensive on the agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, quercetin more significantly inhibited thromboxane A_2-induced increases in pMYPT1 levels than phorbol ester-induced increases. It also more significantly inhibited thromboxane A_2-induced increases in pMYPT1 levels than pERK1/2 levels suggesting the mechanism involving the primarily inhibition of Rho-kinase activity and the subsequent phosphorylation of MYPT1. This study provides evidence regarding the mechanism underlying the relaxation effect of quercetin on agonist-induced vascular contraction regardless of endothelial function.

Key Words: ERK1/2, Fluoride, MYPT1, Phorbol ester, Quercetin, Rho-kinase, Vasodilation

INTRODUCTION

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) is the most abundant of the flavonoids and is commonly used as a food supplement (Boots *et al.*, 2008), but evidence-based data regarding its clinical efficacy are quite scanty (Bischoff, 2008). Flavonoids are a large group of polyphenolic compounds abundantly present in certain vegetables, fruits, seeds and beverages (e.g. tea and wine) (Hollman and Katan, 1999). Dietary intake rich in these compounds has been suggested to decrease the risk of cardiovascular disease and cancer (Ross and Kasum, 2002) and reduce the incidence of cerebrovascular disease in humans (Knekt *et al.*, 2002). The beneficial effects of flavonoids, including quercetin, have been attributed to their anti-oxidant and anti-inflammatory properties (Guardia *et al.*, 2001; Rotelli *et al.*, 2003). Several studies pointed out the beneficial biological activities of quercetin which include antioxidant, antiinflammatory, antiatherosclerotic, and antitumor properties (Naderi *et al.*, 2003; Lotito and Frei, 2006; Mamani-Matsuda *et al.*, 2006). However, little is known about the mechanism responsible for the relaxation of vascular smooth muscle by quercetin, although the influence of endothelial nitric oxide synthesis is well established (Nicholson *et al.*, 2008; Khoo *et al.*, 2010). Furthermore, endothelial dysfunction is known to contribute to the pathophysiologies of conditions like hypertension or diabetes.

It is generally accepted that the initiation of smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in the phosphorylation of a 20 kDa myosin light chain (MLC_{20}) (Somlyo and Somlyo, 1994). However, the degree of MLC_{20} phosphorylation or contraction does not always parallel the intracellular Ca^{2+} concentration. The extent of MLC_{20} phosphorylation or force of contraction induced by agonist stimulation is usually higher than that caused by an increase in the Ca^{2+} concentration referred to as Ca^{2+} sensitization (Somlyo and Somlyo, 1994). Subsequent studies suggested that the inhibition of MLC phosphatase by Rho-kinase (Kitazawa *et al.*, 1991; Uehata *et al.*, 1997; Somlyo and Somlyo, 1998; Sakurada *et al.*, 2003) or thin filament regulation including the activation of protein kinase C (PKC), mitogen-activated protein kinase kinases (MEK) and extracellular signal regulated kinase (ERK) 1/2, and phosphorylation of the actin binding protein caldesmon (Wier and Morgan, 2003) may be major components of the pathway that facilitates in Ca^{2+} sensitization.

In various smooth muscles, fluoride or phorbol ester has been shown to induce contractions, which may be due to en-

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hanced Ca^{2+} sensitivity. In particular, fluoride has been known to induce contractions in blood vessel preparations and to be a potent stimulator of Gs, Gi, Gq and transducin (Gilman, 1984; Kanaho *et al.*, 1985; Blackmore and Exton, 1986; Cockcroft and Taylor, 1987). It is possible that the contractions induced by fluoride involve the RhoA/Rho-kinase pathway (Jeon *et al.*, 2006). However, it has not been reported as to whether this pathway is inhibited during quercetin-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator fluoride or phorbol ester primarily attributing to endothelial nitric oxide synthesis (Nicholson *et al.*, 2008; Khoo *et al.*, 2010). Therefore, the aim of the present study was to investigate the possible roles of Rho-kinase or MEK inhibition on Ca^{2+} desensitization during the quercetin-induced relaxation of isolated rat aortas by using RhoA/Rho-kinase activators such as a full activator thromboxane A_2 or a partial activator phorbol ester excluding endothelial nitric oxide synthesis.

MATERIALS AND METHODS

Tissue preparation

Male Sprague-Dawley rats weighing 300-350 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) as subjected to cervical dislocation, in accord with the procedures approved by the Institutional Animal Care and Use Committee at our institutions. Thoracic aortas were quickly removed and immersed in oxygenated (95% O_2/5% CO_2) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25.0 $NaHCO_3$, 1.2 KH_2PO_4, and 10.0 dextrose (pH 7.4). They were then freed of all adherent connective tissue, and aortic endothelia were removed by gentle abrasion using a cell scraper.

Contraction measurements

Care was taken to avoid rubbing the endothelial surface of the vessels with intact endothelium. Two stainless-steel triangles were inserted through each vessel ring and each aortic ring was then suspended in a water-jacketed organ bath (10 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 applying an optimal resting tension of 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 60 min before contractile responses to 50 mM KCl were measured. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, AD Instruments, Castle Hill, NSW, Australia).

The direct effect of quercetin was determined by addition of it after KCl (50 mM), thromboxane A_2 (0.1 μM), phorbol ester (1 μM) or fluoride (8 mM) induced contractions had plateaued in normal Krebs' solution.

Western blot analysis

Muscle strips 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 in a dry ice/acetone/ TCA/DTT mixture and then homogenized in a buffer containing 20 mM MOPS, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 μM leupeptin, 5.5 μM pepstatin, 20 kIU aprotinin, 2 mM Na_3VO_4, 1 mM NaF, 100 μM $ZnCl_2$, 20 μM 4-(2-aminoethyl) benzenesulphonyl fluoride (AEBSF) and 5 mM EGTA. Protein-matched samples (modified Lowry protein assay, DC Protein Assay Kit, Bio-Rad) were electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE (Protogel, National Diagnostics), transferred to polyvinylidene fluoride PVDF membranes, and subjected to immunostaining and densitometry using appropriate antibodies. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and by densitometry of the actin band. Lane loading variations were corrected by normalization versus β-actin. Sets of samples produced during individual experiments were run in the same gel and densitometry was performed on the same film.

Chemicals and antibodies

Drugs and chemicals were obtained from the following sources. Sodium fluoride, KCl, acetylcholine, quercetin,

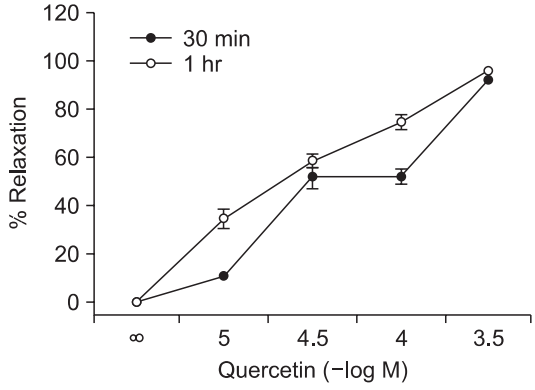

Fig. 1. Effect of quercetin on fluoride-induced vascular contraction. Each ring was equilibrated in the organ bath solution for 30 or 60 min before relaxation responses to quercetin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs.

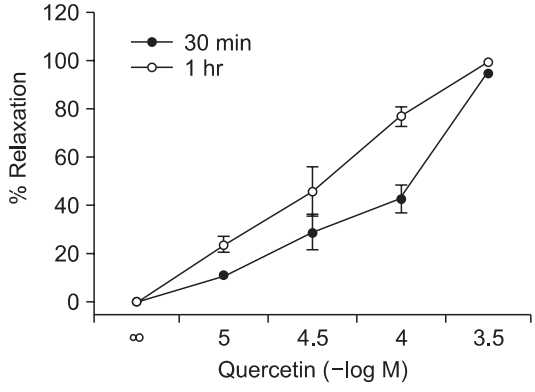

Fig. 2. Effect of quercetin on thromboxane A_2-induced vascular contraction. Each ring was equilibrated in the organ bath solution for 30 or 60 min before relaxation responses to quercetin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs.

U-46619 and phorbol 12,13-dibutyrate were purchased from Sigma (St. Louis, MO, USA). DTT, TCA and acetone were obtained from Fisher Scientific (Hampton, NH, USA). Enhanced chemiluminescence (ECL) kits were from Pierce (Rockford, IL, USA). Antibodies against phospho-myosin phosphatase targeting subunit 1 (phospho-MYPT1) at Thr855 (1:5,000), myosin phosphatase targeting subunit 1 (MYPT1), ERK or phosphoERK at Thr202/Tyr204 were purchased from Upstate Biotechnology (Lake Placid, NY, USA), BD Biosciences (San Jose, CA, USA) or Cell Signaling Technology (Danvers, MA, USA) to determine levels of RhoA/Rho-kinase activity (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 used as secondary antibodies (1:2,000 and 1:2,000, respectively, Upstate, Lake Placid, NY). Quercetin was prepared in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and frozen at −20°C for later use. DMSO alone had no observable effect at concentrations used (data not shown).

Statistical analysis

Data are expressed as means ± standard errors of the means (SEMs). Student's unpaired t test was used to determine the statistical significance of the difference between two groups, and one-way analysis of variance (ANOVA) followed by post-hoc test was carried out for comparisons among three groups. Statistical analyses were done using SPSS 12.0 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was accepted for p-values of <0.05.

RESULTS

Effect of quercetin on contractions of endothelium-denuded aortas induced by a full RhoA/Rho-kinase activator fluoride or thromboxane A_2

Endothelium was removed by gentle abrasion with a cell scraper to identify the direct effect of quercetin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine (1 μM). Quercetin showed no significant effect on basal tension (data not shown), but significantly inhibited the contraction induced by a full activator fluoride at a low concentration (0.01-0.03 mM) regardless of endothelial nitric oxide synthesis (Fig. 1). This suggests that the relaxation mechanism of quercetin might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. Coincidentally, quercetin at the same concentration significantly inhibited thromboxane A_2 mimetic U46619-induced contraction (Fig. 2) suggesting that thromboxane A_2 mimetic acts as almost a full activator where Rho-kinase activation was the main pathway.

Effect of quercetin on the contractions of denuded aortas induced by a partial RhoA/Rho-kinase activator phorbol ester

The vasoconstrictors used have been proved to be partial RhoA/Rho-kinase activators (data not shown). Interestingly, phorbol 12,13-dibutyrate-induced contraction was not significantly inhibited by quercetin at a low concentration (0.01-0.03 mM) regardless of endothelial nitric oxide synthesis (Fig. 3), which suggested that other pathways including thin or actin filament regulation were not inhibited.

Effect of quercetin on the level of MYPT1 phosphorylation at Thr-855

To confirm the role of quercetin on the thick filament regulation of smooth muscle contractility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in muscles quick frozen after 60 min exposure to quercetin for the equilibration. Each relaxing ring was precontracted with 0.1 μM thromboxane A_2 or 1 μM phorbol ester (phorbol 12,13-dibutyrate). This work was done using quick

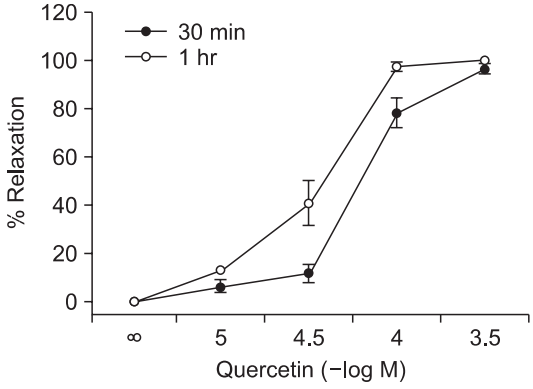

Fig. 3. Effect of quercetin on phorbol ester-induced vascular contraction. Each ring was equilibrated in the organ bath solution for 30 or 60 min before relaxation responses to quercetin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs.

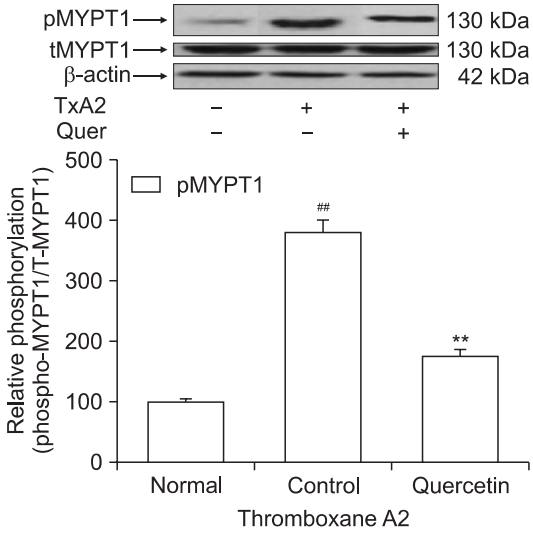

Fig. 4. Effect of quercetin on thromboxane A_2-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen querctin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. $^{**}p<0.01$, $^{\#\#}p<0.01$, versus control or normal group respectively. Quer: 0.1 mM quercetin, TxA_2: 0.1 μM U-46619.

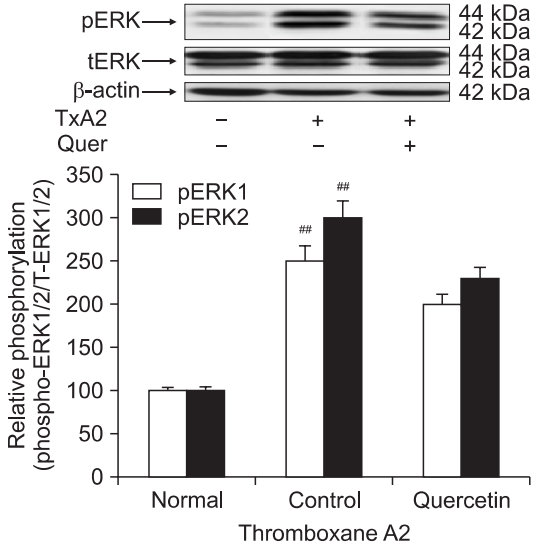

Fig. 5. Effect of quercetin on thromboxane A_2-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were sustained in quick frozen quercetin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with fluoride. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-ERK1/2. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. $^{\#\#}p<0.01$, versus normal group. Quer: 0.1 mM quercetin, TxA_2: 0.1 μM U-46619.

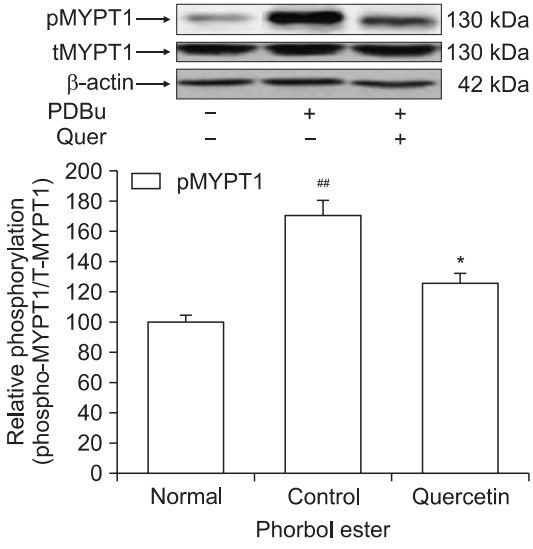

Fig. 6. Effect of quercetin on phorbol ester-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were partially decreased in quick-frozen quercetin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-MYPT1. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. $^{*}p<0.05$, $^{\#\#}p<0.01$, versus control or normal group respectively. Quer: 0.1 mM quercetin, PDBu: 1 μM phorbol 12,13-dibutyrate.

frozen quercetin (0.1 mM)-treated rat aortas in the absence of endothelium and the levels were compared to those of vehicle-treated rat aortas (Fig. 4). Interestingly, a significant decrease in thromboxane A_2-induced MYPT1 phosphorylation at Thr855 was found to be led by quercetin (Fig. 4). Thus, thick or myosin filament regulation including myosin phosphatase activation via RhoA/Rho-kinase inactivation might be involved in the reduced contractility of quercetin-treated rat aorta. On the other hand, a slight reduction in phorbol ester-induced MYPT1 phosphorylation at Thr855 was found to be led by quercetin (Fig. 6). Thus, the intensity of relaxation seems to directly proportional to the level of inhibition of MYPT1 phosphorylation by quercetin.

Effect of quercetin on levels of ERK1/2 phosphorylation at Thr-202/Tyr-204

To confirm the role of quercetin on thin filament regulation of smooth muscle contractility, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick frozen after 60 min of exposure to quercetin for the equilibration. Each relaxing ring was precontracted with 0.1 μM thromboxane A_2 or 1 μM phorbol 12,13-dibutyrate. As compared with vehicle-treated rat aortas, no significant decrease in ERK 1/2 phosphorylation at Thr202/Tyr204 was led by quercetin in these quercetin (0.1 mM)-treated rat aortas in the absence of endothelium (Fig. 5) showing full vasorelaxation (Fig. 2) and weak thin filament regulation. On the other hand, a significant decrease in phorbol ester-induced ERK 1/2 phosphorylation was led by quercetin in quick frozen quercetin at a high concentration (0.1 mM)-treated aortas in the absence of endothelium (Fig. 7). These findings show that thin or actin filament regulation including ERK1/2 phosphorylation via MEK activation might be of lesser importance in the decreased contractility induced by quercetin at a low concentration (0.03 mM).

DISCUSSION

The present study demonstrates that quercetin can modulate the vascular contractility in an agonist-dependent manner. Interestingly, the mechanism involved seems to be not only endothelium-dependent but also to involve the inhibition of Rho-kinase and the partial inhibition of MEK activity. It has been reported that the health benefits of quercetin include relief of menopausal symptoms, lower plasma cholesterol levels, a reduction in the risks of certain hormone-related cancers, enhanced endothelium-dependent vasorelaxation (Nicholson *et al.*, 2008; Khoo *et al.*, 2010) and a reduced risk of cardiovascular disease due to its anti-atherosclerotic properties (Zang *et al.*, 2006). Although these effects of quercetin suggest that it could protect against vascular disease, the mechanism involved seems to be primarily endothelium-dependent and it is known to be deficient in several adult or metabolic diseases such as hypertension or diabetes. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to quercetin-induced vascular relaxation in rat aortas denuded and precontracted by a full RhoA/Rho-kinase activator thromboxane A_2 or by a partial activator phorbol ester.

The mechanism by which fluoride activates G-proteins has been established (Kanaho *et al.*, 1985; Blackmore and Exton, 1986; Cockcroft and Taylor, 1987). It has been reported that the effect of fluoride on heterotrimeric G protein is due to the formation of AlF_4^- from fluoride and contamination of glassware (Zeng *et al.*, 1989; Chabre, 1990), which mimics

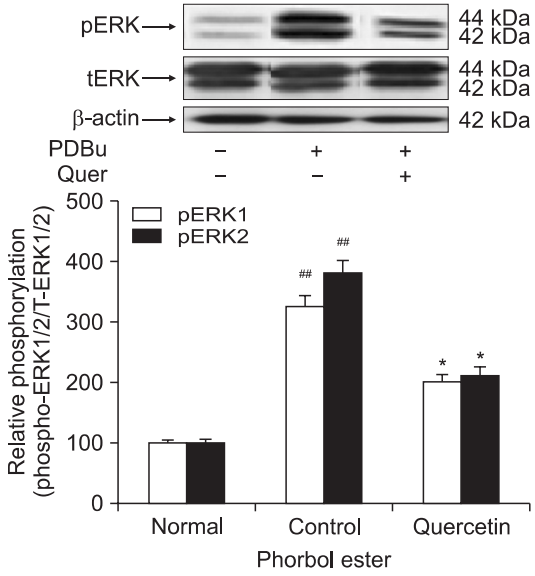

Fig. 7. Effect of quercetin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were partially decreased in quick frozen quercetin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester (phorbol 12,13-dibutyrate). 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 means of 3-5 experiments with vertical lines representing SEMs. *p<0.05, $^{\#\#}p$<0.01, versus control or normal group respectively. Quer: 0.1 mM quercetin, PDBu: 1 μM phorbol 12,13-dibutyrate.

the effect of GTP (Bigay *et al.*, 1985). Fluoride is also a classical Ser/Thr phosphatase inhibitor (Shenolikar and Nairn, 1991) and is routinely included in extraction buffers to prevent the dephosphorylations of proteins at Ser and Thr residues by endogenous phosphatases. On the other hand, previous studies that examined the mechanisms underlying arterial contractions induced by the phorbol ester or thromboxane A_2 have reported variable findings with regard to the contraction triggered by Rho-kinase activation (Wilson *et al.*, 2005; Tsai and Jiang, 2006). These findings are consistent with the notion that quercetin can decrease fluoride, phorbol ester or thromboxane A_2 induced contraction by inhibiting Rho-kinase activity.

The mechanisms by which Rho-kinase activation causes vascular contraction is an area of intense study, and several possibilities exist. For example, Rho-kinase phosphorylates myosin light chain phosphatase, which decreases phosphatase activity and causes a buildup of phosphorylated myosin light chains (Somlyo and Somlyo, 2000; Pfitzer, 2001). Rho-kinase has also been demonstrated to phosphorylate myosin light chains directly and independently of myosin light chain kinase and phosphatase activity (Amano *et al.*, 1996). Recently, Rho-kinase was found to be involved in vascular contractions evoked by fluoride, phorbol ester or thromboxane A_2 (Wilson *et al.*, 2005; Jeon *et al.*, 2006; Tsai and Jiang, 2006).

The present study demonstrates that quercetin ameliorates the maximal or submaximal contraction induced by vasoconstrictor fluoride, thromboxane A_2 or phorbol ester endothelium-independently (Fig. 1-3), and that this ameliorative mechanism primarily involves the RhoA/Rho-kinase pathway. Previously, most vasodilation was believed to be caused by endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. In the present study, quercetin at a low concentration (0.01-0.03 mM) significantly inhibited fluoride or thromboxane A_2-induced contraction regardless of endothelial function (Fig. 1, 2), but not phorbol ester-induced contraction (Fig. 3). This suggests that the vascular contractions elicited by RhoA/Rho-kinase activators such as a full activator fluoride or thromboxane A_2 and a partial activator phorbol ester are achieved via different mechanisms (Fig. 4-7). Therefore, we postulated that pathways other than the RhoA/Rho-kinase pathway might be involved in Ca^{2+} sensitization induced by the phorbol ester. Thus, quercetin at a low concentration might not inhibit Ca^{2+} mobilization (Low, 1996; Davis *et al.*, 2001) or the phosphorylation of ERK (Shimizu and Weinstein, 2005), protein kinase C-potentiated inhibitory protein for protein phosphatase type 1 (CPI-17) or integrin-linked kinase (ILK) (Deng *et al.*, 2001; Muranyi *et al.*, 2002). Furthermore, quercetin decreased phosphorylation of MYPT1 at Thr855 induced by thromboxane A_2 or phorbol ester (Fig. 4, 6), suggesting the inhibition of Rho-kinase activity as the major mechanism. However, quercetin at the high concentration (0.1 mM) significantly decreased the phosphorylation of ERK1/2 induced by phorbol ester (Fig. 7) with full relaxation (Fig. 3) suggesting the inhibition of MEK activity as a minor mechanism.

In summary, quercetin at a low concentration significantly attenuates the contractions induced by a full activator thromboxane A_2 regardless of endothelial function. In contrast, a partial activator phorbol ester-induced contraction was not significantly inhibited by quercetin at this low concentration suggesting additional Ca^{2+} mobilization or the phosphorylations of ERK, CPI-17, ILK or ZIPK required for the partial activator-induced contractions. Thus, the mechanism underlying the relaxation induced by quercetin at a low concentration in thromboxane A_2-induced contractions involves the inhibition of Rho-kinase activity and not the inhibition of MEK activity. Interestingly, during phorbol ester-induced contraction, the inhibition of MEK activity and subsequent ERK1/2 phosphorylation or Ca^{2+} mobilization induced by quercetin at a high concentration suggest that MEK activity or Ca^{2+} mobilization is not importantly required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis, Rho-kinase inhibition makes a major contribution to the mechanism responsible for quercetin-induced vasorelaxation in denuded muscle.

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