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

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## Abstract

Shikonin, a natural flavonoid found in the roots of *Lithospermum erythrorhizon*, has been shown to possess many biological functions. The present study was undertaken to investigate the influence of shikonin 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. Shikonin significantly relaxed fluoride-, thromboxane $A_2$- or phorbol ester-induced vascular contraction suggesting as a possible anti-hypertensive on the agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, shikonin significantly inhibited fluoride-induced increases in pMYPT1 levels and phorbol ester-induced increases in pERK1/2 levels suggesting the mechanism involving the inhibition of Rho-kinase activity and the subsequent phosphorylation of MYPT1 and the inhibition of MEK activity and the subsequent phosphorylation of ERK1/2. This study provides evidence regarding the mechanism underlying the relaxation effect of shikonin on agonist-induced vascular contraction regardless of endothelial function.

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

## INTRODUCTION

Shikonin (5,8-Dihydroxy-2-[(1*S*)-1-hydroxy-4-methylpent-3-en-1-yl]naphthalene-1,4-dione), a naphthoquinone, is an effective constituent of *Lithospermum erythrorhizon*, a Chinese medicinal herb (Andujar *et al*., 2013). Shikonin has antioxidant, anti-inflammatory (Chen *et al*., 2002; Lu *et al*., 2011), antithrombotic, antiviral, antimicrobial, as well as anticancer (Wang *et al*., 2014) potency and fosters wound healing. Studies have shown that shikonin possesses the anti-cancer effect inhibiting tumor cell proliferation, induce tumor cell apoptosis, and change cell cycle (Han *et al*., 2007; Chang *et al*., 2010). We investigated the possible influence and related mechanisms of the anti-inflammatory shikonin on vascular smooth muscle contractility to develop a better antihypertensive.

Alterations in the arterial tone are frequently associated with cardiovascular diseases constituting an important cause of morbidity and mortality in humans, one of which is hypertension that is a multifactorial disorder that involves many mechanisms including endothelial dysfunction and leading to risk factors for cardiovascular diseases. Besides endothelial dysfunction, it is generally accepted that vascular smooth muscle contractility is predominantly controlled by $Ca^{2+}$ signaling involving $Ca^{2+}$ influx, release or sensitization and regulating a $Ca^{2+}$-dependent increase in the phosphorylation of a 20 kDa myosin light chain ($MLC_{20}$) (Somlyo and Somlyo, 1994). 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 cytosolic $Ca^{2+}$ 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.

Activation of ERK1/2 cannot only regulate vascular con-

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tractility but also is connected with pathologic hypertrophy, hyperplasia, hypertension and atherosclerosis (Xu *et al*., 1996; Touyz *et al*., 1999). ERK1/2 is activated by threonine and tyrosine phosphorylation by the specific kinase MEK activated by Raf. In various smooth muscles, fluoride, phorbol ester or thromboxane $A_2$ mimetic has been shown to induce contractions, which may be due to primarily enhanced $Ca^{2+}$ sensitivity or partially increased $Ca^{2+}$ concentration only in thromboxane $A_2$ mimetic. ERK1/2 activation was induced by the phorbol ester, phorbol 12,13-dibutyrate (PDBu). The stimulus PDBu triggers ERK1/2 dependent cytoskeletal remodeling and formation of podosomes inducing ERK1/2 activation (Gu *et al*., 2007). On the other hand, it is possible that the contractions induced by fluoride or thromboxane $A_2$ mimetic involve the RhoA/Rho-kinase pathway (Jeon *et al*., 2006). However, it has not been reported as to whether this pathway is inhibited during shikonin-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator fluoride or MEK activator phorbol ester. 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 shikonin-induced relaxation of isolated rat aortas by using RhoA/Rho-kinase activators fluoride or thromboxane $A_2$ or an MEK and preceding PKC activator phorbol ester excluding endothelial nitric oxide synthesis.

## MATERIALS AND METHODS

### Tissue preparation

Male Sprague-Dawley rats weighing 250-300 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 if necessary.

### Contraction measurements

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 1 μM phenylephrine or 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 shikonin was determined by addition of it after KCl (50 mM), phenylephrine (1 μM), thromboxane $A_2$ (0.1 μM), phorbol ester (1 μM) or fluoride (6 mM) induced contractions had plateaued in normal Krebs' solution.

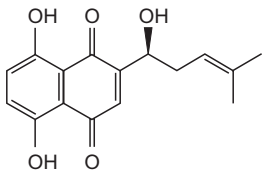


**Fig. 1.** The chemical structure of shikonin (5,8-Dihydroxy-2-[(1S)-1-hydroxy-4-methylpent-3-en-1-yl]naphthalene-1,4-dione).

### 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 30%, National Diagnostics), transferred to polyvinylidene fluoride PVDF membranes, and subjected to immunostaining and densitometry using primary and secondary 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 image.

### Chemicals and antibodies

Drugs and chemicals were obtained from the following sources. Sodium fluoride, KCl, acetylcholine, shikonin (S7576, ≥98% (HPLC)), U-46619 (D8174, solution, 10 mg/mL in methyl acetate) and phorbol 12,13-dibutyrate (P1269, ≥98% (TLC), powder) 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 phosphomyosin phosphatase targeting subunit protein 1 (phospho-MYPT1) at Thr855 (1:5,000), MYPT1, ERK or phosphoERK at Thr202/Tyr204 were purchased from Cell Signaling Technology (Danvers, MA, USA) or Upstate Biotechnology (Lake Placid, NY, 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, USA). Shikonin solution 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).

### Statistics

The data were expressed as mean ± standard error of the mean (SEM). The student's unpaired *t* test or ANOVA was used to determine the statistical significance of the means

between two groups using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). *p*-values < 0.05 were regarded as statistically significant.

## RESULTS

### Effect of shikonin 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 shikonin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine (1 μM). Shikonin showed no significant effect on basal tension (data not shown), and significantly inhibited the contraction induced by a Rho-kinase activator fluoride at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 2). This suggests that the relaxation mechanism of shikonin might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. On the other hand, shikonin at the same concentration significantly inhibited thromboxane $A_2$ mimetic U46619-induced contraction in denuded muscles (Fig. 3) suggesting that thromboxane $A_2$ mimetic acts similarly from fluoride where Rho-kinase activation was the main pathway.

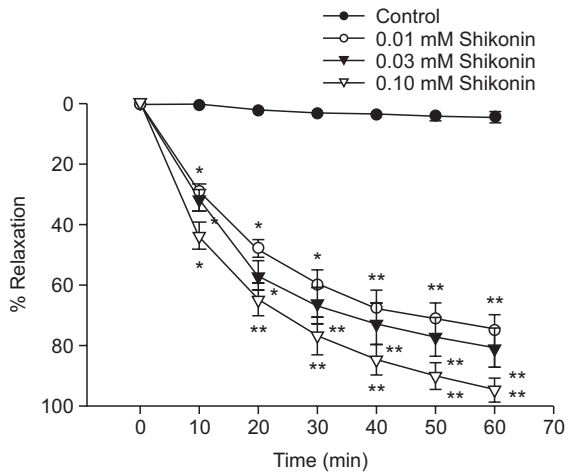


**Fig. 2.** Effect of shikonin on fluoride-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to shikonin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ***p*<0.01, presence versus absence of shikonin.

### Effect of shikonin on the contractions of denuded aortas induced by an MEK and preceding PKC activator phorbol ester

Phorbol esters used have been proved to be MEK and preceding PKC activators and partial RhoA/Rho-kinase activators (data not shown). Interestingly, phorbol 12,13-dibutyrate-induced contraction was significantly inhibited by shikonin at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 4), which suggested that thin or actin filament regulation including MEK/ERK were significantly inhibited.

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

To confirm the role of shikonin on thin filament regulation of smooth muscle contractility, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick frozen after 60 minutes of exposure to shikonin for the equilibration. Each relaxing ring was precontracted with 1 μM phorbol ester (phorbol 12,13-dibutyrate). As compared with vehicle-treated rat aortas, a significant decrease in ERK1/2 phosphorylation at Thr202/Tyr204 was led by shikonin in these shikonin (0.03 mM)-treat-

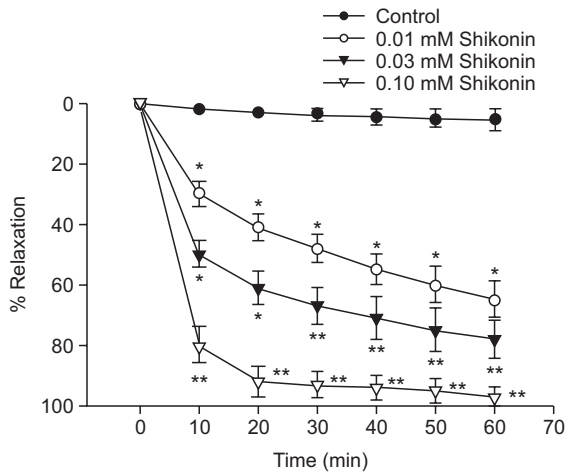


**Fig. 3.** Effect of shikonin on thromboxane $A_2$-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to shikonin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ***p*<0.01, presence versus absence of shikonin.

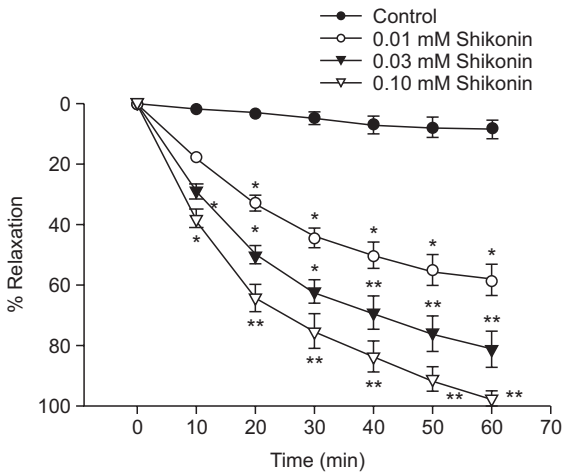


**Fig. 4.** Effect of shikonin on phorbol ester-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to shikonin were measured. Data are expressed as the means of 3-5 experiments with vertical lines representing SEMs. **p*<0.05, ***p*<0.01, presence versus absence of shikonin.

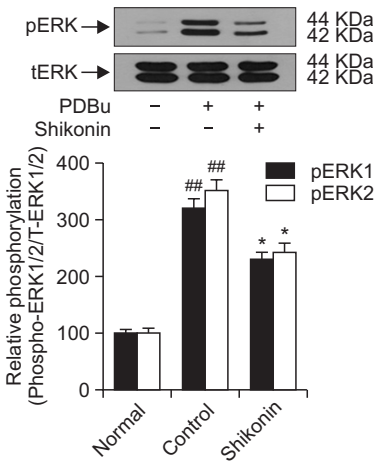


**Fig. 5.** Effect of shikonin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were not decreased in quick frozen shikonin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester. The upper panel shows a typical blot and the lower panel shows average densitometry results for relative levels of phospho-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. Shikonin: 0.03 mM shikonin; PDBu: 1 μM phorbol 12,13-dibutyrate.

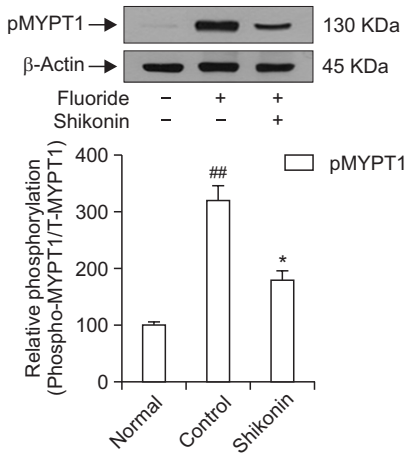


**Fig. 6.** Effect of shikonin on fluoride-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen shikonin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. The upper panel shows 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. *$p$<0.05, $^{\#\#}p$<0.01, versus control or normal group respectively. Shikonin: 0.03 mM shikonin; Fluoride: 6 mM sodium fluoride.

ed rat aortas in the absence of endothelium (Fig. 5) showing full vasorelaxation (Fig. 4) and thin filament regulation. These findings show that thin or actin filament regulation including ERK1/2 phosphorylation via MEK activation might be of importance in the decreased contractility induced by shikonin.

### Effect of shikonin on the level of MYPT1 phosphorylation at Thr-855

To confirm the role of shikonin 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 shikonin for the equilibration. Each relaxing ring was precontracted with 6 mM fluoride. This work was done using quick frozen shikonin (0.03 mM)-treated rat aortas in the absence of endothelium and the levels were compared to those of vehicle-treated rat aortas (Fig. 6). Interestingly, significant decrease in fluoride-induced MYPT1 phosphorylation at Thr855 was found to be led by shikonin (Fig. 6). Thus, thick or myosin filament regulation including myosin phosphatase activation via RhoA/Rho-kinase inactivation might be involved in the reduced contractility of shikonin-treated rat aorta.

## DISCUSSION

The present study demonstrates that shikonin 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 equal inhibition of MEK and Rho-kinase activity. Shikonin has been previously recognized for its anti-inflammatory or antioxidant activity. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to shikonin-induced vascular relaxation in rat aortas denuded and precontracted by a RhoA/Rho-kinase activator fluoride or by an MEK and preceding PKC activator phorbol ester.

The mechanism by which phorbol ester activates MEK/ERK has been established (Kordowska *et al*., 2006; Gu *et al*., 2007). On the other hand, previous studies that examined the mechanisms underlying arterial contractions induced by fluoride 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 shikonin can decrease phorbol ester or fluoride-induced contraction by inhibiting MEK or Rho-kinase activity.

The mechanisms by which MEK activation causes vascular contraction is an area of intense study, and several possibilities exist. The phosphorylation of caldesmon by MEK/ERK appears to regulate smooth muscle contractility (Kordowska *et al*., 2006). In this process MEK/ERK is activated by PKC which in turn can be stimulated by phorbol esters or GPCR receptor agonists.

The present study demonstrates that shikonin ameliorates the maximal or submaximal contraction induced by vasoconstrictor fluoride or phorbol ester endothelium-independently (Fig. 2, 4), and that this ameliorative mechanism involves the MEK/ERK and RhoA/Rho-kinase pathway. Previously, the vasodilation was believed to be caused by endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase (Hu *et al*., 2004). In the present study, shikonin at a low concentration significantly inhibited phorbol ester- or fluorideB-induced contraction regardless of endothelial function (Fig. 2, 4). Furthermore, shikonin at a low concentration significantly decreased the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by phorbol ester (Fig. 5) and the phosphorylation of MYPT1 at Thr855 induced by fluoride (Fig.

6) with full relaxation (Fig. 2, 4) suggesting the inhibition of Rho-kinase or MEK activity as a major mechanism.

Meanwhile, endothelium can be damaged with aging and adult diseases such as diabetes, atherosclerosis or hypertension. Because most flavonoids have some effect on endothelial NO synthesis and shikonin was reported to show the endothelium-dependent relaxation (Hu *et al*., 2004), the effect of shikonin on the smooth muscle itself without endothelium should be elucidated for use in elderly or diabetic patients in the future. In addition, $MLC_{20}$ is known to be phosphorylated by both MLCK and Rho-kinase. The activation of Rho-kinase by U46619 or fluoride inhibits the activity of myosin light chain phosphatase through the phosphorylation of MYPT1, leading to an increased $MLC_{20}$ phosphorylation as well as contractions (Sakurada *et al*., 2003; Wilson *et al*., 2005), which was thought to be inhibited by shikonin.

In summary, shikonin at a low concentration significantly attenuates the contractions induced by an MEK and preceding PKC activator phorbol ester regardless of endothelial function. Furthermore, a Rho-kinase activator fluoride-induced contraction was significantly inhibited by shikonin at this low concentration. Thus, the mechanism underlying the relaxation induced by shikonin at a low concentration in phorbol ester or fluoride-induced contractions involves the inhibition of MEK activity and Rho-kinase activity. Interestingly, during fluoride-induced contraction, the inhibition of Rho-kinase activity and subsequent MYPT1 phosphorylation induced by shikonin suggest that Rho-kinase inactivation is required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis, both MEK and Rho-kinase inhibition make a major contribution to the mechanism responsible for shikonin-induced vasorelaxation in the denuded muscle.

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