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The inhibitory effect of vitexin on the agonist-induced regulation of vascular contractility

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The present study was undertaken to investigate the influence of vitexin on vascular smooth muscle contractility and to determine the mechanism involved. Intact or denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Vitexin more significantly relaxed phorbol ester-induced vascular contraction than thromboxane A_2 or fluoride-induced contraction suggesting as a possible anti-hypertensive on the agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, vitexin significantly inhibited phorbol ester-induced increases in pERK1/2 levels. On the other hand, it did not significantly inhibit thromboxane A_2-induced increases in pMYPT1 levels suggesting the mechanism involving the primarily inhibition of MEK activity and the subsequent phosphorylation of ERK1/2. This study provides evidence regarding the mechanism underlying the relaxation effect of vitexin on agonist-induced vascular contraction regardless of endothelial function.

1. Introduction

The flavonoid vitexin (Fig. 1) is a flavone C-glycoside (apigenin-8-C-β-D-glucopyranoside) present in several medicinal and other plants (Abbasi et al. 2012; Martino et al. 2008; Zucolotto et al. 2012). Plant extracts containing vitexin are reported to possess antinociceptive, anti-inflammatory, and antioxidant activities (Cao et al. 2011). It has been reported that the c-glycosilated flavone vitexin (5, 7, 4-trihydroxyflavone-8-glucoside) has several pharmacological properties, including antinociceptive, antispasmodic, antimyeloperoxidase and α-glucosidase inhibitory activities (Ragone et al. 2007). We investigated the possible influence and related mechanisms of the anti-inflammatory vitexin on vascular smooth muscle contractility to develop a better antihypertensive agent. Intact or denuded aortic rings from male Sprague-Dawley rats were used and isometric contractions were recorded using a computerized data acquisition system. These data were combined with molecular experiments.

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

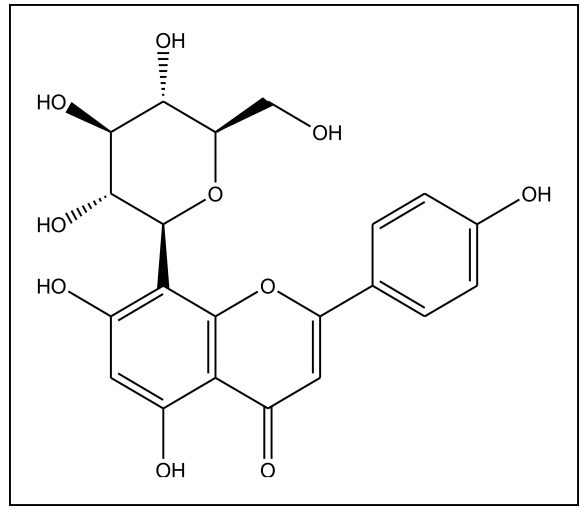

Fig. 1: Chemical structure of vitexin (5, 7, 4-trihydroxyflavone-8-glucoside).

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; Sakurada et al. 2003; Somlyo and Somlyo 1998; Uehata et al. 1997) 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 contractility but is also connected with pathologic hypertrophy, hyperplasia, hypertension and atherosclerosis (Touyz et al. 1999; Xu et al. 1996). 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 vitexin-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator thromboxane A_2 mimetic 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 vitexin-induced relaxation of isolated rat aortas by using RhoA/Rho-kinase activators fluoride or thromboxane A_2 or a MEK activator phorbol ester excluding endothelial nitric oxide synthesis.

2. Investigations and results

2.1. Effect of vitexin 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 vitexin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine (1 μM). Vitexin showed no significant effect on basal tension (data not shown), and did not inhibit the contraction induced by a Rho-kinase activator fluoride at a high concentration regardless of endothelial nitric oxide synthesis (Fig. 2A). This suggests that the relaxation mechanism of vitexin might not involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. Conversely, vitexin at the same concentration significantly inhibited thromboxane AB_{2B} mimetic U46619-induced contraction in denuded or intact muscles (Fig. 2C, 2D) suggesting that thromboxane AB_{2B} mimetic acts differently from fluoride where Rho-kinase activation was the main pathway.

2.2. Effect of vitexin on the contractions of denuded aortas induced by a MEK activator phorbol ester

Phorbol esters used have been proved to be MEK activators and partial RhoA/Rho-kinase activators (data not shown). Interestingly, phorbol 12,13-dibutyrate-induced contraction was significantly inhibited by vitexin at a low concentration regardless of endothelial nitric oxide synthesis (Fig. 2B), which suggested that thin or actin filament regulation including MEK/ERK were significantly inhibited.

2.3. Effect of vitexin on levels of ERK1/2 phosphorylation at Thr-202/Tyr-204

To confirm the role of vitexin 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 vitexin 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 ERK 1/2 phosphorylation at Thr202/Tyr204 was led by vitexin in these vitexin (0.1 mM)-treated rat aortas in the absence of endothelium (Fig. 3) showing full vasorelaxation (Fig. 2B) 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 vitexin at a low concentration (0.03 mM). Thus, the intensity of relaxation seems to directly proportional to the level of inhibition of ERK1/2 phosphorylation by vitexin.

2.4. Effect of vitexin on the level of MYPT1 phosphorylation at Thr-855

To confirm the role of vitexin 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 vitexin for the equilibration. Each relaxing ring was precontracted with 0.1 μM U-46619. This work was done using quick frozen vitexin (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, no significant decrease in thromboxane AB_2-induced MYPT1 phosphorylation at Thr855 was found to be led by vitexin (Fig. 4). Thus, thick or myosin filament regulation including myosin phosphatase activation via RhoA/Rho-kinase inactivation might not be involved in the reduced contractility of vitexin-treated rat aorta.

3. Discussion

The present study demonstrates that vitexin 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 MEK activity and the partial inhibition of Rho-kinase. Vitexin 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 vitexin-induced vascular relaxation in rat aortas denuded and precontracted by a RhoA/Rho-kinase activator fluoride or thromboxane A_2 or by a MEK activator phorbol ester.

The mechanism by which phorbol ester activates MEK/ERK has been established (Gu et al. 2007; Kordowska et al. 2006). On the other hand, previous studies that examined the mechanisms underlying arterial contractions induced by fluoride or thromboxane AB_{2B} have reported variable findings with regard to the contraction triggered by Rho-kinase activation (Tsai and Jiang 2006; Wilson et al. 2005). These findings are consistent with the notion that vitexin can decrease phorbol ester or thromboxane AB_2 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.

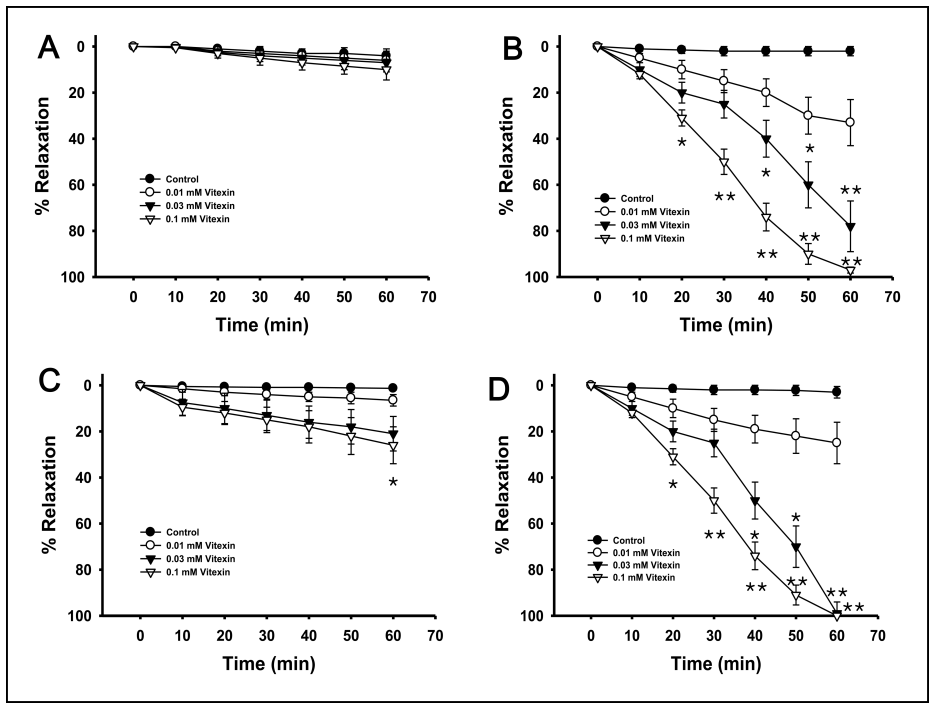

Fig. 2: Effect of vitexin on fluoride-induced (A), phorbol ester-induced (B) vascular contraction and thromboxane AB_2-induced vascular contraction in denuded (C) or intact muscles (D). Each ring was equilibrated in the organ bath solution for 30 - 60 min before relaxation responses to vitexin were measured. Data are expressed as the means of 3–5 experiments with vertical lines representing SEMs. *$P < 0.05$, presence versus absence of vitexin. **$P < 0.01$, presence versus absence of vitexin.

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 vitexin ameliorates the maximal or submaximal contraction induced by vasoconstrictor thromboxane AB_{2B} or phorbol ester endothelium-independently or dependently (Fig. 2B, 2C, 2D), and that this ameliorative mechanism primarily involves the MEK/ERK 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, vitexin at a low concentration significantly inhibited phorbol ester- or thromboxane AB_{2B}-induced contraction regardless of endothelial function (Fig. 2B, 2C, 2D), but not fluoride-induced contraction (Fig. 2A). Therefore, we postulated that pathways other than the RhoA/Rho-kinase pathway might be involved in CaP^{2+P} sensitization induced by the phorbol ester. Thus, vitexin at a low concentration might not inhibit CaP^{2+P} mobilization (Davis et al. 2001; Low 1996) or the phosphorylation of myosin phos-

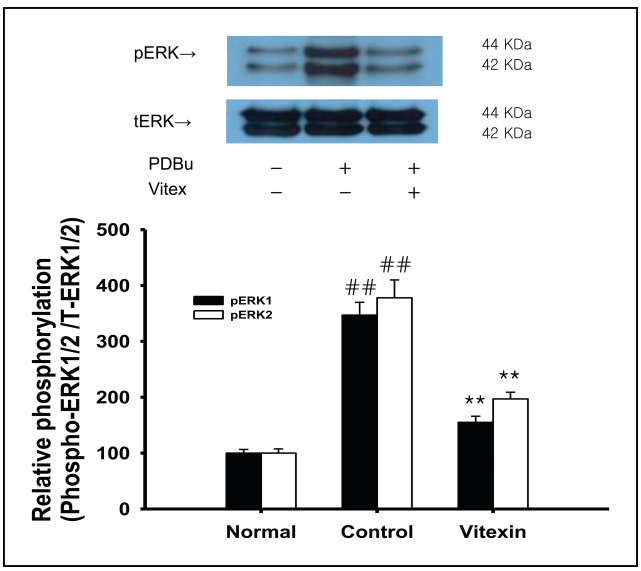

Fig. 3: Effect of vitexin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were not decreased in quick frozen vitexin-treated rat aortas in the absence of endothelium compared to vehicle-treated rat aortas precontracted with phorbol ester. The upper panel shows 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. **$P < 0.01$, ##$P < 0.01$, versus control or normal group respectively. Vitex: 0.1 mM vitexin; PDBu: 1 μM phorbol 12,13-dibutyrate.

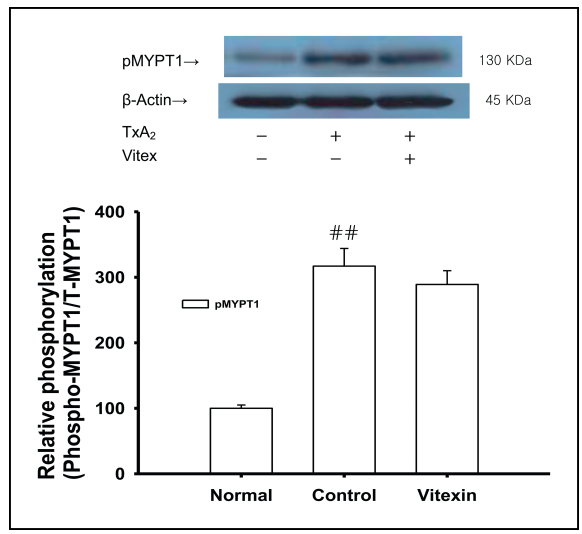

Fig. 4: Effect of vitexin on thromboxane AB_2-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen vitexin-treated rat aorta in the absence of endothelium compared to vehicle-treated rat aorta precontracted with fluoride. The upper panel 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. ##$P < 0.01$, versus normal group. Vitex: 0.1 mM vitexin; TxA_2: 0.1 μM U-46619.

phatase targeting subunit protein 1 (MYPT1), 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, vitexin decreased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 induced by phorbol ester (Fig. 3), suggesting the inhibition of MEK activity as the major mechanism. However, vitexin at the high concentration did not significantly decrease the phosphorylation of MYPT1 at Thr855 induced by thromboxane A_2 (Fig. 4) with slight relaxation (Fig. 2C) suggesting the inhibition of Rho-kinase activity as a minor mechanism.

In summary, vitexin at a low concentration significantly attenuates the contractions induced by a MEK activator phorbol ester regardless of endothelial function. In contrast, a Rho-kinase activator thromboxane A_2-induced contraction was not significantly inhibited by vitexin at this low concentration suggesting additional CaP^{2+P} mobilization or the phosphorylations of MYPT1, CPI-17, ILK or ZIPK required for the Rho-kinase activator-induced contractions. Thus, the mechanism underlying the relaxation induced by vitexin at a low concentration in phorbol ester-induced contractions involves the inhibition of MEK activity and not the inhibition of Rho-kinase activity. Interestingly, during thromboxane A_2-induced contraction, no inhibition of Rho-kinase activity and subsequent MYPT1 phosphorylation induced by vitexin at a high concentration suggest that Rho-kinase activity is not importantly required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis (Fig. 2D), MEK inhibition makes a major contribution to the mechanism responsible for vitexin-induced vasorelaxation in denuded muscle.

4. Experimental

4.1. 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 % OB_{2B}/5% COB_{2B}) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 $CaClB_{2B}$, 1.2 $MgClB_{2B}$, 25.0 $NaHCOB_{3B}$, 1.2 $KHB_{2B}POB_{4B}$, 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.

4.2. 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% OB_{2B} and 5% COB_{2B}. 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 vitexin was determined by addition of it after KCl (50 mM), thromboxane A_2 (0.1 μM), phorbol ester (1 μM) or fluoride (6 mM) induced contractions had plateaued in normal Krebs' solution.

4.3. 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 $NaB_{3B}VOB_{4B}$, 1 mM NaF, 100 μM $ZnClB_{2B}$, 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.

4.4. Chemicals and antibodies

Drugs and chemicals were obtained from the following sources. Sodium fluoride, KCl, acetylcholine, vitexin, 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 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 (Wilson et al. 2005; Wooldridge et al. 2004) 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). Vitexin 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).

4.5. Statistics

The data were expressed as mean ± standard error of the mean (SEM). The student's unpaired *t* test was used to determine the statistical significance of the means between two groups using SPSS 12.0 (SPSS Inc., Chicago, Illinois, U.S.A.). P values < 0.05 were regarded as statistically significant.

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