INTRODUCTION

Artemisia asiatica Nakai (family Asteraceae) is an herbal medicine used as a hepatoprotective, antioxidative, anti-inflammatory, and antibacterial agent [1,2]. Eupatilin, a flavone or a type of flavonoid, is isolated from Artemisia asiatica Nakai and used in the treatment of acid-related disorders. We investigated the possible influence and related mechanisms of the anti-inflammatory eupatilin on vascular smooth muscle contractility to determine the mechanism involved. Denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Eupatilin more significantly relaxed fluoride-induced vascular contraction than thromboxane A_{2} or phorbol ester-induced contraction suggesting as a possible anti-hypertensive on the agonist-induced vascular contraction regardless of endothelial nitric oxide synthesis. Furthermore, eupatilin significantly inhibited fluoride-induced increases in pMYPT1 levels. On the other hand, it didn’t significantly inhibit phorbol ester-induced increases in pERK1/2 levels suggesting the mechanism involving the primary inhibition of Rho-kinase activity and the subsequent phosphorylation of MYPT1. This study provides evidence regarding the mechanism underlying the relaxation effect of eupatilin on agonist-induced vascular contraction regardless of endothelial function.

Key Words: ERK1/2, Eupatilin, MYPT1, Rho-kinase, Vasodilation
METHODS

Tissue preparation

Male Sprague-Dawley rats weighing 300−350 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) as subject 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% O2/5% CO2) physiological saline solution composed of (mM): 115.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgCl2, 25.0 NaHCO3, 1.2 KH2PO4, and 10 mM dithiothreitol (DTT). They were then freed of all adherent connective tissue, and aortic endothelia were removed by gentle abrasion using a cell scraper.

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% O2 and 5% CO2. 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 eupatilin was determined by addition of it after KCl (50 mM), thromboxane A2 (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/TCACDTT 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 Na3VO4, 1 mM NaF, 100 μM ZnCl2, 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 (Proteogel, 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, eupatilin, 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 [13,14] 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). Eupatilin 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 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.

RESULTS

Effect of eupatilin on contractions of endothelium-denuded aortas induced by a full RhoA/Rho-kinase activator fluoride or thromboxane A2

Endothelium was removed by gentle abrasion with a cell scraper to identify the direct effect of eupatilin on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating precontracted ring segments with acetylcholine (1 μM). Eupatilin 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 regardless of endothelial nitric oxide synthesis (Fig. 1, 2). This suggests that the relaxation mechanism of eupatilin might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase. Coincidentally, eupatilin at the same concentration slightly inhibited thromboxane A2 mimetic U46619-induced contraction (Fig. 1, 3) suggesting that thromboxane A2 mimetic acts differently from a full activator where
Mechanisms Involved in the Effect of Eupatilin

Rho-kinase activation was the main pathway.

**Effect of eupatilin 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 slightly inhibited by eupatilin at a high concentration regardless of endothelial nitric oxide synthesis (Fig. 1, 4), which suggested that other pathways including thin or actin filament regulation were slightly inhibited.

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

To confirm the role of eupatilin on the thick filament regulation of smooth muscle contractility, we measured levels...
Fig. 4. Effect of eupatilin on phorbol ester-induced vascular contraction. Each ring was equilibrated in the organ bath solution for 30∼60 min before relaxation responses to eupatilin were measured. Data are expressed as the means of 3∼5 experiments with vertical lines representing SEMs. Control respectively. *p < 0.05.

Fig. 5. Effect of eupatilin on fluoride-induced increases in phospho-MYPT1 levels. Phospho-MYPT1 protein levels were significantly decreased in quick frozen eupatilin-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. Eupa: 0.1 mM eupatilin; NaF: 8 mM sodium fluoride.

Fig. 6. Effect of eupatilin on phorbol ester-induced increases in phospho-ERK1/2 levels. Phospho-ERK1/2 protein levels were not decreased in quick frozen eupatilin-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, versus normal group respectively. Eupa: 0.1 mM eupatilin; PDBu: 1 μM phorbol 12,13-dibutyrate.

DISCUSSION

The present study demonstrates that eupatilin 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. Eupatilin has been previously recognized for its...
anti-inflammatory activity. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributes to eupatilin-induced vascular relaxation in rat aortas denuded and precontracted by a full RhoA/Rho-kinase activator fluoride or thromboxane A2 or by a partial activator phorbol ester.

The mechanism by which fluoride activates G-proteins has been established [9-11]. It has been reported that the effect of fluoride on heterotrimeric G protein is due to the formation of AlF4−, from fluoride and contamination of glassware [15,16], which mimics the effect of GTP [17]. Fluoride is also a classical Ser/Thr phosphatase inhibitor [18] 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 A2 have reported variable findings with regard to the contraction triggered by Rho-kinase activation [13,19]. These findings are consistent with the notion that eupatilin can decrease fluoride, phorbol ester or thromboxane A2 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 [20,21]. Rho-kinase has also been demonstrated to phosphorylate myosin light chains directly and independently of myosin light chain kinase and phosphatase activity [22]. Recently, Rho-kinase was found to be involved in vascular contractions evoked by fluoride, phorbol ester or thromboxane A2 [12,13,19].

The present study demonstrates that eupatilin ameliorates the maximal or submaximal contraction induced by vasoconstrictor fluoride, thromboxane A2 or phorbol ester endothelium-independently (Fig. 1 ~ 4), 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, eupatilin at a low concentration significantly inhibited fluoride-induced contraction regardless of endothelial function (Fig. 2), but not thromboxane A2- or phorbol ester-induced contraction (Fig. 3, 4). This suggests that the vascular contractions elicited by RhoA/Rho-kinase activators such as a full activator fluoride and a partial activator phorbol ester are achieved via different mechanisms (Fig. 5, 6). Therefore, we postulated that pathways other than the RhoA/Rho-kinase pathway might be involved in Ca2+ sensitization induced by the phorbol ester. Thus, eupatilin at a low concentration might not inhibit Ca2+ mobilization [23,24] or the phosphorylation of extracellular signal-regulated kinase (ERK), protein kinase C-potentiated inhibitory protein for protein phosphatase type 1 (CPI-17) or integrin-linked kinase (ILK) [25,26]. Furthermore, eupatilin decreased phosphorylation of MYPT1 at Thr855 induced by fluoride (Fig. 5), suggesting the inhibition of Rho-kinase activity as the major mechanism. However, eupatilin at the high concentration didn’t significantly decrease the phosphorylation of ERK1/2 induced by phorbol ester (Fig. 6) with partial relaxation (Fig. 4) suggesting the inhibition of MEK activity as a minor mechanism.

In summary, eupatilin at a low concentration significantly attenuates the contractions induced by a full activator fluoride regardless of endothelial function. In contrast, a partial activator phorbol ester-induced contraction was not significantly inhibited by eupatilin at this low concentration suggesting additional Ca2+ 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 eupatilin at a low concentration in fluoride-induced contractions involves the inhibition of Rho-kinase activity and not the inhibition of MEK activity. Interestingly, during phorbol ester-induced contraction, no inhibition of MEK activity and subsequent ERK1/2 phosphorylation induced by eupatilin at a high concentration suggest that MEK activity 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 eupatilin-induced vasorelaxation in denuded muscle.

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