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Molecules and Cells

Inhibitory Effect of Genistein on Agonist-Induced Modulation of Vascular Contractility

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The present study was undertaken to determine whether treatment with genistein, the plant-derived estrogen-like compound influences agonist-induced vascular smooth muscle contraction and, if so, to investigate related mechanisms. The measurement of isometric contractions using a computerized data acquisition system was combined with molecular experiments. Genistein completely inhibited KCl-, phorbol ester-, phenylephrine-, fluoride- and thromboxane A_2-induced contractions. An inactive analogue, daidzein, completely inhibited only fluoride-induced contraction regardless of endothelial function, suggesting some difference between the mechanisms of RhoA/Rho-kinase activators such as fluoride and thromboxane A_2. Furthermore, genistein and daidzein each significantly decreased phosphorylation of MYPT1 at Thr855 had been induced by a thromboxane A_2 mimetic. Interestingly, iberiotoxin, a blocker of large-conductance calcium-activated potassium channels, did not inhibit the relaxation response to genistein or daidzein in denuded aortic rings precontracted with fluoride. In conclusion, genistein or daidzein elicit similar relaxing responses in fluoride-induced contractions, regardless of tyrosine kinase inhibition or endothelial function, and the relaxation caused by genistein or daidzein was not antagonized by large conductance K_{Ca}-channel inhibitors in the denuded muscle. This suggests that the RhoA/Rho-kinase pathway rather than K^+-channels are involved in the genistein-induced vasodilation. In addition, based on molecular and physiological results, only one vasoconstrictor fluoride seems to be a full RhoA/Rho-kinase activator; the others are partial activators.

INTRODUCTION

Genistein is a phytoestrogen that is structurally similar to 17 β-estradiol. It binds to both cytoplasmic and nuclear estrogen receptors more slowly than the endogenous hormone (Hsieh et al., 1998; Martin et al., 1978). Genistein binds to the β subtype of the estrogen receptor to trigger many of the same biological responses that are evoked by physiological levels of estrogens (Kuiper et al., 1998). In particular, significant reductions in humans in serum total cholesterol, low-density lipoprotein (LDL) cholesterol and triglyceride without changes in serum high-density lipoprotein (HDL) cholesterol concentrations were observed with soy protein, a rich source of genistein (Anderson et al., 1995; Hodgson et al., 1996; Mitchell and Collins, 1999). Genistein is a well-established and effective nonselective tyrosine kinase inhibitor (Akiyama et al., 1987), and thus may inhibit tyrosine kinase-mediated contraction of vascular smooth muscle (Liu and Sturek, 1996), in particular, the responses to 5-hydroxytryptamine (Watts et al., 1996). However, little is known about the relaxation mechanism exerted by estrogenic compounds such as genistein (5,7,4-trihydroxyisoflavone) or its inactive analogue daidzein.

It is generally accepted that the initiation of smooth muscle contractility is predominantly controlled by a Ca^{2+}-dependent increase in phosphorylation of a 20 kDa myosin light chain (MLC_{20}) (Somlyo and Somlyo, 1994). However, other pathways have now been described that may regulate the contractility of smooth muscle by regulating the phosphorylation of MLC_{20} independently of a rise in intracellular Ca^{2+} (Sakurada et al., 2003; Somlyo and Somlyo, 1998; Uehata et al., 1997). The phosphorylation of MLC_{20} promotes the interaction of actin and myosin II, and the contraction of smooth muscle. The degree of MLC_{20} phosphorylation or contraction does not always parallel the 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, a finding explained by so-called Ca^{2+} sensitization (Somlyo and Somlyo, 1994). Thus, an additional mechanism of regulation that modulates levels of the phosphorylated MLC_{20} and degree of contraction has been proposed. Subsequent studies have suggested that inhibition of MLC phosphatase by Rho-kinase (Kitazawa et al., 1991) or thin filament regulation including 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 the major pathway in Ca^{2+} sensitization.

In various smooth muscles, phenylephrine, phorbol ester, fluoride or a thromboxane A_2 mimetic has been shown to induce contractions, which may be due to enhancement of Ca^{2+} sensitivity. Especially, fluoride or thromboxane A_2 mimetics

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have been known to induce contractions in blood vessel preparations and is a potent stimulator of Gs, Gi, Gq and transducin (Blackmore and Exton, 1986; Cockcroft and Taylor, 1987; Gilman, 1984; Kanaho et al., 1985; Tsai and Jiang, 2006). It is possible that contractions induced by fluoride or thromboxane A_2 mimetics involve the participation of the RhoA/Rho-kinase pathway (Jeon et al., 2006; Tsai and Jiang, 2006). However, there are no reports as to whether this pathway is inhibited in genistein- or daidzein-induced vascular smooth muscle relaxation in aortic rings precontracted with Rho-kinase activator fluoride or thromboxane A_2 mimetic. Also, it was reported that the mechanisms of the RhoA/Rho-kinase pathway are both Ca^{2+}-independent, involving MLC_{20} phosphorylation, and Ca^{2+}-dependent, involving ion channels such as the K^+-channel (Cachero et al., 1998; Jones, 2003; Luykenaar et al., 2004; Storey et al., 2002). Indeed, Ca^{2+}-activated K^+ (BKCa) channels are feasible targets and their activators or blockers are promising interventions for disease states characterized by an increased or decreased tonus of smooth muscles (Edwards and Weston, 1995; Nelson and Quayle, 1995). Therefore, the aim of the present study was to elucidate a possible role for Rho-kinase inhibition or additional Ca^{2+}-activated K^+ channel opening on Ca^{2+} desensitization in genistein or daidzein induced relaxation of isolated rat aortae using RhoA/Rho-kinase activators such as a full activator fluoride or a partial activator thromboxane A_2 mimetic and pharmacological inhibitors of Rho-kinase, tyrosine kinase or Ca^{2+}-activated K^+ channels.

MATERIALS AND METHODS

Tissue preparation

Male Sprague-Dawley rats weighing 320-350 g were anesthetized by sodium pentobarbital (50 mg/kg i.p.) followed by cervical dislocation, in agreement with procedures approved by the Institutional Animal Care and Use Committee of our institution. The thoracic aorta was 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). The aorta was cleaned of all adherent connective tissue, and the endothelium was removed by gentle abrasion with a cell scraper.

Contraction measurements

Care was taken to avoid rubbing the endothelial surface of the vessels that had intact endothelium. Two stainless-steel triangles were inserted through each vessel ring. Each aortic ring was suspended in a water-jacketed organ bath (20 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, USA). The rings were stretched passively by imposing the optimal resting tension, 2.0 g, which was maintained throughout the experiment. Each ring was equilibrated in the organ bath solution for 60 min before the experiment, which involved contractile responses to 50 mM KCl. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, ADInstruments, Australia).

To study pretreatment effects, genistein or daidzein was applied 30 min before the addition of fluoride or thromboxane A_2 mimetic U-46619. We also determined the direct effects of genistein, daidzein, iberiotoxin and Y27632 by addition of these agents after KCl (50 mM), phorbol ester (1 μM), phenylephrine (1 μM), fluoride (8 mM) or thromboxane A_2 mimetic U-46619 (0.1 μM) induced contractions had reached plateaus 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 to room temperature in a dry ice/acetone/TCA/DTT mixture. Then samples were 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 SDS-PAGE (Protogel, National Diagnostics), transferred to PVDF membranes, and subjected to immunostaining and densitometry, as above, using the appropriate antibody. The success of protein matching was confirmed by Naphthol Blue Black staining of the membrane and densitometry of the actin band. Any mismatch of lane loading was corrected by normalization to actin staining. Each set of samples from an individual experiment was run on 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, U-46619, KCl, acetylcholine, genistein, daidzein, iberiotoxin, phenylephrine, phorbol 12,13-dibutyrate and Y-27632 were purchased from Sigma Co. (USA). DTT, TCA and acetone were obtained from Fisher Scientific (USA). Enhanced chemiluminescence (ECL) was from Pierce (USA). The antibody against the phospho-myosin-targeting subunit of myosin light chain phosphatase (phospho-MYPT1) at Thr855 (1:5,000) and MYPT1 was purchased from Upstate Biotechnology (Lake Placid, USA) or BD Biosciences (USA) to check the level of RhoA/Rho-kinase activity (Wilson et al., 2005; Wooldridge et al., 2004). Anti-mouse IgM (goat) and anti-rabbit IgG (goat), conjugated with horseradish peroxidase, were used as secondary antibodies (1:2,000, 1:2,000, respectively, Upstate, Lake Placid, USA). Acetylcholine and iberiotoxin were dissolved in deionized water. Genistein and daidzein were prepared in dimethyl sulfoxide (DMSO) as a 100 mM stock solution and frozen at -20°C for later use. With DMSO alone, preliminary data showed that there were little effects of this vehicle at concentrations used in any of the protocols (data not shown).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Student's unpaired *t*-test was used to determine the statistical significance of the difference between means between two groups, and one-way analysis of variance (ANOVA) followed by Bonferroni's test was carried out for comparisons among three or more groups. Statistical analyses were done using SPSS 12.0 (SPSS Inc., USA). P values < 0.05 were regarded as statistically significant.

RESULTS

Effect of genistein on contractions of denuded or intact aortae induced by a full RhoA/Rho-kinase activator fluoride

The absence of endothelium was verified by lack of relaxation after the addition of acetylcholine (1 μM) to precontracted ring segments. The cumulative addition of fluoride (3-8 mM) produced concentration-dependent contractions (Fig. 1C). The vasoconstrictor used here proved to be a full RhoA/Rho-kinase activator (Fig. 6D). Genistein showed no significant effect on

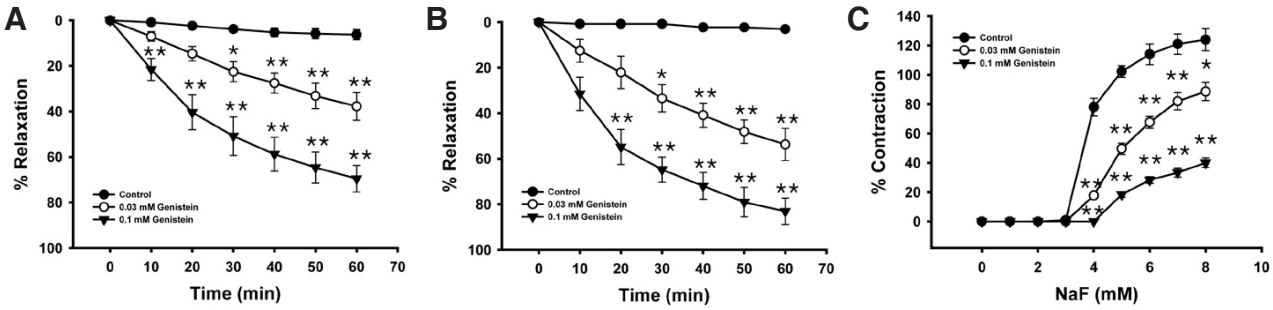

Fig. 1. Effect of genistein on NaF-induced vascular contraction. (A, B) Direct relaxing effect of genistein on 8 mM NaF-induced vascular contraction in the absence (A) or presence (B) of endothelium. (C) Effect of pretreated genistein on NaF-induced vascular contraction. NaF was added cumulatively to elicit tension in the presence or absence of genistein for 30 min in aortic rings with endothelium denuded. Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *$P < 0.05$, **$P < 0.01$, presence versus absence of genistein.

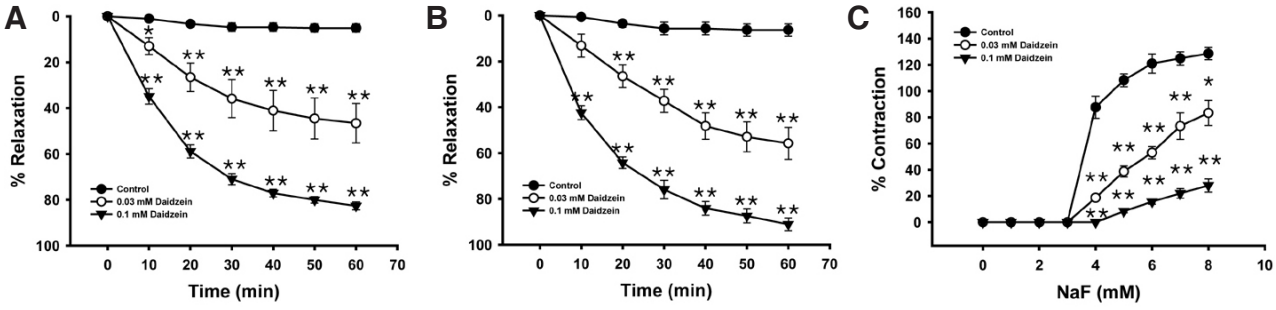

Fig. 2. Effect of daidzein on NaF-induced vascular contraction. (A, B) Direct relaxing effect of daidzein on 8 mM NaF-induced vascular contraction in the absence (A) or presence (B) of endothelium. (C) Effect of pretreated daidzein on NaF-induced vascular contraction. NaF was added cumulatively to elicit tension in the presence or absence of daidzein for 30 min in aortic rings with endothelium denuded. Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *$P < 0.05$, **$P < 0.01$, presence versus absence of daidzein.

basal tension (data not shown) but completely inhibited contraction by a full activator fluoride regardless of endothelial function (Fig. 1). This suggests that the relaxation mechanism might involve inhibition of Rho-kinase.

Effect of daidzein on contractions of denuded or intact aortae induced by a full activator fluoride

Daidzein completely inhibited contraction induced by a full activator fluoride regardless of endothelial function (Fig. 2) suggesting that Rho-kinase might be inhibited during the relaxation. Interestingly, fluoride-induced contraction was similarly inhibited by the inactive analog daidzein similarly to the effect of the tyrosine kinase inhibitor genistein. Moreover, these effects were seen regardless of endothelial function in either pretreatment or direct relaxation (Fig. 2).

Effect of iberiotoxin or Y-27632 on genistein or daidzein-induced relaxation of endothelium denuded aortae precontracted with fluoride

Iberiotoxin, a blocker of large-conductance calcium-activated potassium channels, had no significant effect on basal tension or fluoride-dependent regulation of contractility (data not shown) when used alone, and did not inhibit relaxation responses to genistein or daidzein in aortic rings denuded and precontracted with fluoride (Table 1). This suggests the role of the channels as none of the relaxation mechanisms at least in the denuded smooth muscle. On the other hand, it is not surprising that there was no additional relaxation effect of the Rho-kinase inhibitor Y-27632 coadministrated with genistein or daidzein compared to administration of genistein or daidzein alone (Table 1). This indicates that the relaxation mechanism of genistein and daidzein includes that of Y-27632, at least in contraction of denuded muscles induced by fluoride.

Effect of genistein on contraction of denuded or intact aortae induced by a partial RhoA/Rho-kinase activator-such as KCl-, phorbol ester-, phenylephrine- or thromboxane A_2

The cumulative addition of the thromboxane A_2 mimetic U-46619 (0.001-1 μM) produced concentration-dependent contractions (Fig. 3A). Interestingly, this response was not completely inhibited by inactive analogue daidzein but tyrosine kinase inhibitor genistein regardless of endothelial function (Figs. 3, 4A, 4B, 5A, and 5B). In addition, genistein also completely inhibited contraction induced by other vasoconstrictors including KCl, phorbol ester or phenylephrine (Figs. 6A, 6B, and 6C). The vasoconstrictors used here proved to be partial RhoA/Rho-kinase activators (Fig. 6D).

Effect of genistein on the level of phospho-MYPT1 at Thr-855

To confirm the role of genistein in thick filament regulation of smooth muscle contractility, we measured the levels of the myosin-targeting subunit of the myosin light chain phosphatase (MYPT1) and phospho-MYPT1 in muscles quick frozen after 30 min exposure to 0.1 μM of the thromboxane A_2 mimetic U-

Table 1. Time-dependent % relaxation effect of genistein and daidzein alone or coadministrated with iberiotoxin/Y-27632 on the contractile responses to fluoride of rat aortic rings with endothelium denuded

Drug	Time					
	10 min	20 min	30 min	40 min	50 min	60 min
Denuded endothelium						
G 0.03 mM	7.1 ± 2.1	14.7 ± 3.1	22.7 ± 4.1	27.6 ± 4.4	33.2 ± 5.6	37.5 ± 6.1
G + IBTX 3 nM	4.4 ± 1.3	9.2 ± 2.2	14.4 ± 2.3	17.5 ± 2.9	21.0 ± 2.9	24.0 ± 3.0
G + IBTX 10 nM	3.7 ± 0.8	7.5 ± 1.6	11.2 ± 1.5	14.2 ± 1.8	17.1 ± 2.3	19.4 ± 2.3
G + Y 0.1 μM	8.2 ± 2.7	13.3 ± 5.6	20.0 ± 6.9	25.2 ± 7.6	31.0 ± 6.8	36.2 ± 6.5
G + Y 1 μM	8.5 ± 2.6	15.6 ± 4.3	22.7 ± 7.2	31.1± 7.4	36.7 ± 7.8	39.5 ± 8.5
Denuded endothelium						
D 0.03 mM	13.0 ± 3.7	26.6 ± 6.2	35.9 ± 8.3	41.1 ± 8.8	44.5 ± 8.9	46.6 ± 8.6
D + IBTX 3 nM	6.5 ± 2.1	13.8 ± 2.0	19.5 ± 1.3	21.9 ± 1.3	23.5 ± 2.0	24.3 ± 2.2
D + IBTX 10 nM	5.9 ± 0.8	10.1 ± 1.5	16.9 ± 1.1	20.2 ± 1.8	21.1 ± 1.3	21.9 ± 1.2
D + Y 0.1 μM	7.3 ± 3.0	14.4 ± 3.9	22.7 ± 5.6	31.9 ± 5.0	40.3 ± 6.1	45.6 ± 5.7
D + Y 1 μM	12.6 ± 3.3	21.6 ± 3.8	31.2 ± 5.0	40.9 ± 6.3	47.3 ± 5.7	49.8 ± 6.6

%Relaxation is expressed as a percentage of the maximum contraction to 8 mM fluoride. The data are expressed as mean ± SEM of 3-7 experiments. D, daidzein; G, genistein; IBTX, iberiotoxin; Y, Y-27632

46619. Interestingly, there was a significant decrease in MYPT1 phosphorylation induced at Thr855 by U-46619. This was done using quick frozen genistein-treated rat aorta in the absence of endothelium and was compared to the vehicle treated rat aorta (Fig. 4C). Thr855 is the newly-highlighted site, and was used instead of Thr697 (Cockcroft and Taylor, 1987; Kanaho et al., 1985). Thus, thick or myosin filament regulation including myosin phosphatase activation through RhoA/Rho-kinase inactivation might be partly involved in decreased contractility in genistein-treated rat aorta. Furthermore, when the Rho-kinase inhibitor Y-27632 was coadministrated with genistein to rat aorta precontracted with thromboxane A_2 mimetic, there was no additional effect of Y-27632 compared to administration of genistein alone (Fig. 4C). This indicates that the relaxation mechanism of genistein includes that of Y-27632.

Effect of daidzein on contraction of denuded or intact aortae induced by a partial activator such as KCl, phorbol ester, phenylephrine or thromboxane A_2

The inactive analog daidzein was less effective in inhibiting contraction induced by a partial activator such as KCl, phorbol ester, phenylephrine or the thromboxane A_2 mimetic U-46619 compared to the tyrosine kinase inhibitor genistein; and this was true regardless of endothelial function in either pretreatment or direct relaxation (Figs. 3B, 5A, 5B, and 6). This suggested a difference between the relaxing mechanisms of genistein and its analogue daidzein.

Effect of daidzein on the level of phospho-MYPT1 at Thr-855

To confirm a role for daidzein in thick filament regulation of smooth muscle contractility, we measured levels of MYPT1 and phospho-MYPT1 in the muscles quick frozen after 30 min exposure to 0.1 μM of the thromboxane A_2 mimetic U-46619. Compared to the vehicle-treated rat aortae, there was a significant decrease in MYPT1 phosphorylation induced at Thr855 by U-46619 in quick frozen daidzein-treated rat aorta in the absence of endothelium (Fig. 5C). Therefore, thick or myosin filament regulation including myosin phosphatase activation through RhoA/Rho-kinase inactivation might be involved in slightly decreased contractility in daidzein-treated rat aorta.

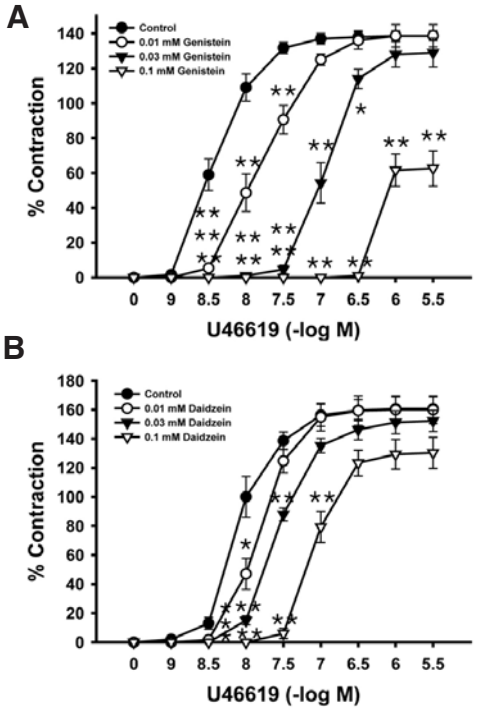

Fig. 3. Effect of pretreatment with genistein or daidzein on U46619-induced contraction. (A) Effect of genistein pretreatment on U46619-induced contractions. (B) Effect of pretreated daidzein on U46619-induced contractions. U46619 was added cumulatively to elicit tension in the presence or absence of genistein or daidzein for 30 min in aortic rings with endothelium denuded. Developed tension is expressed as a percentage of the maximum contraction to 50 mM KCl. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *P < 0.05, **P < 0.01, presence versus absence of genistein or daidzein.

Furthermore, when the Rho-kinase inhibitor Y-27632 was coadministrated with daidzein to rat aorta precontracted with

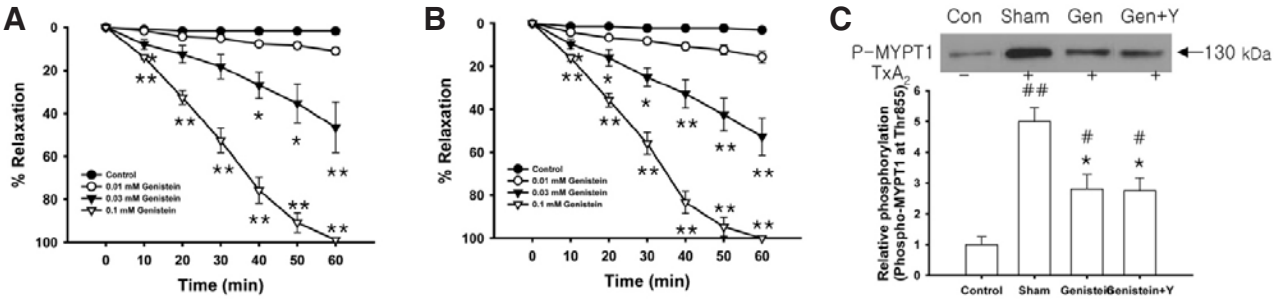

Fig. 4. Effect of genistein on U46619-induced contraction and related protein levels. (A, B) Direct relaxing effect of genistein on 0.1 μM U46619-induced vascular contraction in the absence (A) or presence (B) of endothelium. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *$P < 0.05$, **$P < 0.01$, presence versus absence of genistein. (C) Decreased phospho-MYPT1$_{Thr855}$ protein levels in quick frozen genistein-added rat aorta in the absence of endothelium compared to vehicle-added rat aorta precontracted with thromboxane A_2 mimetic U46619. Upper panel shows a typical blot and lower panel shows average densitometry results on the relative level of phospho-MYPT1. Data are expressed as means of 3-5 experiments with vertical bars showing SEMs. *$P < 0.05$, #$P < 0.05$, ##$P < 0.01$, versus sham or control respectively. Gen, 30 μM genistein; TxA_2, 0.1 μM thromboxane A_2; Y, 0.1 μM Y-27632.

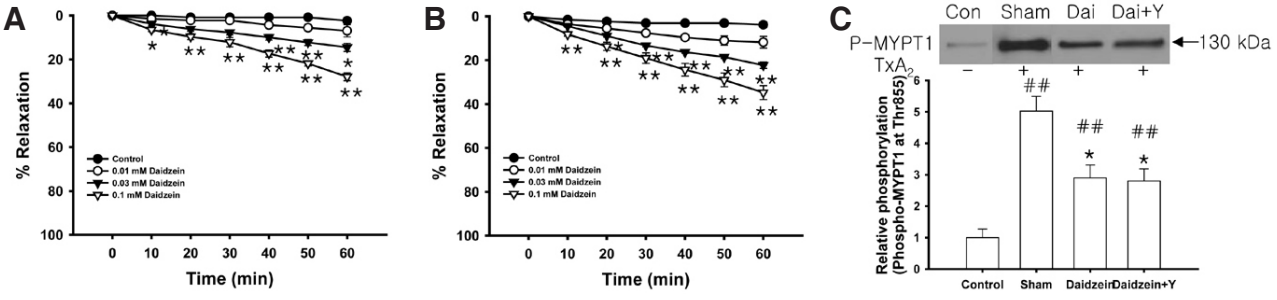

Fig. 5. Effect of daidzein on U46619-induced contractions and related protein levels. (A, B) Direct relaxing effect of daidzein on 0.1 μM U46619-induced vascular contraction in the absence (A) or presence (B) of endothelium. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *$P < 0.05$, **$P < 0.01$, presence versus absence of daidzein. (C) Decreased phospho-MYPT1$_{Thr855}$ protein levels in quick-frozen daidzein-added rat aorta in the absence of endothelium compared to vehicle-added rat aorta precontracted with thromboxane A_2 mimetic U46619. Upper panel shows a typical blot and lower panel shows average densitometry results on relative levels of phospho-MYPT1. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. *$P < 0.05$, ##$P < 0.01$, versus sham or control respectively. Dai, 30 μM daidzein; TxA2, 0.1 μM thromboxane A_2; Y, 0.1 μM Y-27632.

thromboxane A_2 mimetic, there was no additional effect of Y-27632 compared to daidzein alone (Fig. 5C) indicating that the relaxation mechanism of daidzein is similar to that of Y-27632.

DISCUSSION

It was reported that the health benefits of the protein tyrosine kinase inhibitor genistein or its inactive analogue daidzein include relief of menopausal symptoms, improvement in blood cholesterol levels (Anderson et al., 1995), reduction of the risk of certain hormone-related cancers, enhancement of endothelium-dependent vasorelaxation (Räthel et al., 2005; Squadrito et al., 2002) and prevention of cardiovascular disease (Anthony, 2002). Although these beneficial actions of genistein indicate a potential for protection against vascular disease, recent clinical trials using genistein had shown no effect on the incidence of coronary artery disease (Sacks et al., 2006). Moreover, warnings against the excessive consumption of genistein have been issued, because some adverse effects of genistein have been reported. For example, perinatal and neonatal exposure to genistein caused abnormalities in the ovary and vagina, reduced size of the testis and prostate, and caused the suppression of sexual behavior in rodents (Delclos et al., 2001; Kyselova et al., 2004; Wisniewski et al., 2003). Therefore, this study has investigated whether the inhibition of RhoA/Rho-kinase activity or additional opening of K^+ channels contributes to genistein or daidzein-induced vascular relaxation in rat aortae pretreated or precontracted with a full RhoA/Rho-kinase activator fluoride or a partial RhoA/Rho-kinase activator such as KCl, phorbol ester, phenylephrine or thromboxane A_2.

The mechanism by which fluoride activates G-proteins has been clearly established (Blackmore and Exton, 1986; Cockcroft and Taylor, 1987; Kanaho et al., 1985). It has been reported that the effect of fluoride on heterotrimeric G protein is the result of formation of AlF_4^- from fluoride and trace amounts of aluminum, which can come from contamination of glassware (Chabre, 1990; Zeng et al., 1989), and the structural similarity of AlF_4^- to PO_4^{3-} enabling it to interact with GDP situated on the α-subunit of the G proteins, where it can mimic 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 dephosphorylation of proteins on Ser and Thr residues by endogenous phosphatases. On the other hand, previous studies examining the mechanisms underlying arterial contractions induced by the thromboxane A_2 mimetic U46619 have reported variable findings with regard to the contraction related to Rho-kinase activation (Nobe and Paul, 2001; Tasaki et al., 2003; Wilson et al., 2005). Therefore, it was consistent with the possibility that genistein can decrease fluoride- or thromboxane A_2 mimetic-induced contraction by inhibit-

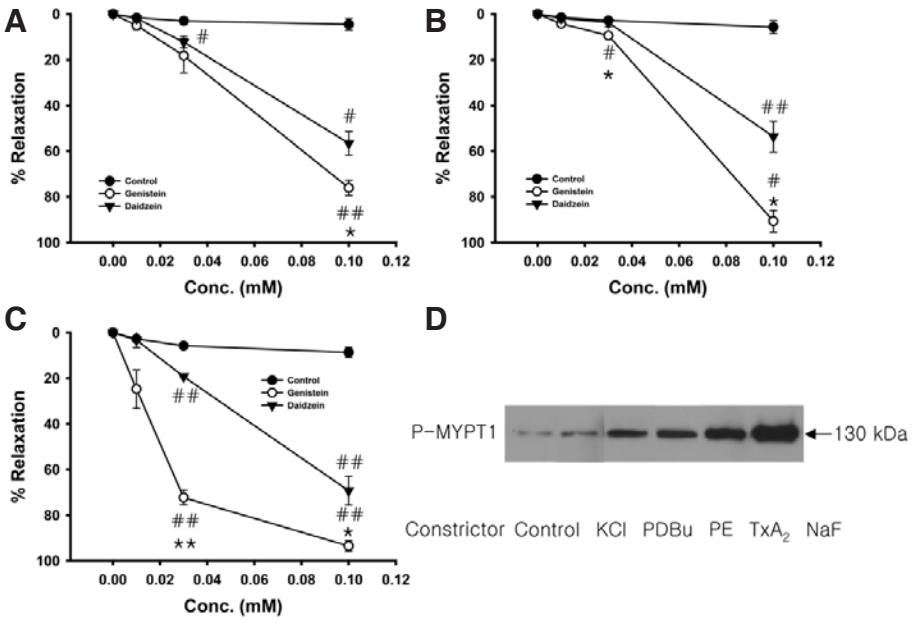

Fig. 6. The effect of genistein or daidzein on contractions induced by other vasoconstrictors and the related protein levels. (A, B, and C) Relaxing effect of genistein or daidzein on KCl- (A), phorbol ester- (B) or phenylephrine- (C) induced vascular contraction. Data are expressed as means of 3-5 experiments with vertical bars showing SEM. # < 0.05, *P < 0.05, versus control or daidzein respectively. (D) The phospho-MYPT1$_{Thr855}$ protein levels in quick frozen rat aorta denuded and precontracted with KCl, phorbol ester, phenylephrine, NaF or the thromboxane A_2 mimetic U46619 in a typical blot. KCl, 50 mM KCl; PDBu, 1 μM phorbol 12,13-dibutyrate; PE, 1 μM phenylephrine; NaF, 8 mM NaF; TxA_2, 0.1 μM thromboxane A_2; Gen, 30 μM genistein; Dai, 30 μM daidzein.

ing 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, resulting in decreased phosphatase activity and a buildup of phosphorylated myosin light chains (Pfitzer, 2001; Somlyo and Somlyo, 2000). Rho-kinase has also been demonstrated to directly phosphorylate myosin light chains independently of myosin light chain kinase and phosphatase activity (Amano et al., 1996). Recently, a role for Rho-kinase in vascular contractions evoked by sodium fluoride or thromboxane A_2 was established (Jeon et al., 2006; Tsai and Jiang, 2006; Wilson et al., 2005). It is likely that Rho-kinase and tyrosine kinase may be in the same signaling pathway, but it is unclear which is activated first. While some studies have found that tyrosine kinases function as an upstream effector of Rho-kinase (Nakao et al., 2002; Nobes et al., 1995), others place tyrosine kinases downstream of Rho (Finn and Ridley, 1996).

The present study demonstrates that daidzein causes submaximal relaxation compared to genistein in contractions induced by various vasoconstrictors except fluoride suggesting two different mechanisms between the two types of vasoconstrictors. One mechanism primarily involves RhoA/Rho-kinase pathway; the other only partially involves this pathway (Fig. 6). Genistein completely inhibited KCl-, phorbol ester-, phenylephrine-, fluoride- or thromboxane A_2-induced contraction regardless of endothelial function (Figs. 1, 3A, 4A, 4B, 6A, 6B, and 6C), and daidzein decreased fluoride-induced vasoconstriction more than it did to KCl-, phorbol ester-, phenylephrine- or thromboxane A_2 mimetic-induced contraction (Figs. 2, 3B, 5A, 5B, 6A, 6B, and 6C). This suggests a difference between the mechanisms of RhoA/Rho-kinase activators such as a full activator fluoride and a partial activator thromboxane A_2 (Fig. 6D). Therefore, we postulated that pathways other than the RhoA/Rho-kinase pathway might be involved in Ca^{2+} sensitization induced by the thromboxane A_2 mimetic U-46619. This suggests that genistein might inhibit Ca^{2+} entry (Davis et al., 2001; Low, 1996) or the phosphylation of extracellular signal-regulated kinase (ERK) (Shimizu and Weinstein, 2005; Wang et al., 2004), 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). Moreover, when the Rho-kinase inhibitor Y-27632 was coadministrated with genistein or daidzein to rat aorta precontracted with fluoride, there was no additional relaxation effect of Y-27632 compared to administration of daidzein or genistein alone (Table 1). This indicates that the relaxation mechanism of genistein or daidzein includes that of Y-27632. Furthermore, genistein and daidzein decreased phosphorylation of MYPT1 at Thr855 induced by thromboxane A_2 mimetic (Figs. 4C and 5C), suggesting inhibition of Rho-kinase activity. Also, when Y-27632 was coadministrated with genistein or daidzein to rat aorta precontracted with thromboxane A_2 mimetic, there was no additional effect of Y-27632 compared to administration of daidzein or genistein alone (Figs. 4C and 5C). This indicats that the relaxation mechanism of genistein or daidzein includes that of Y-27632. Additionally, the closing of K^+-channels including K_{Ca}-channels on the arterial smooth muscle cell membrane decreases K^+-efflux and depolarization induced opening of Ca^{2+}-channels and vasoconstriction (Nelson and Quayle, 1995). However, the relaxation caused by genistein or daidzein was not antagonized by the large conductance K_{Ca}-channel inhibitor iberiotoxin, at least in the denuded muscle (Table 1).

In summary, the tyrosine kinase inhibitor genistein completely attenuates the contractions in rat aortic rings induced by most vasoconstrictors regardless of endothelial function. In contrast, daidzein completely inhibited only fluoride-induced contraction. This suggests additional Ca^{2+} entry or the phosphorylation of ERK, CPI-17 or ILK in addition to effects on the RhoA/Rho-kinase pathway in the partial activator-induced contractions. The possible relaxing mechanism of genistein or daidzein in fluoride-induced contractions of denuded muscle does not include inhibition of Ca^{2+}-activated K^+ channels opening but inhibition of Rho-kinase activity. This suggests that tyrosine kinase activity is less important for relaxation in this case. In conclusion, Rho-kinase inhibition is a major mechanism in daidzein and genistein-induced vasorelaxation, at least in the denuded and fluoride-contracted muscle. Only one vasoconstrictor, fluoride, seems to be a full RhoA/Rho-kinase activator; the others are partial activators.

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