Isoflavone Attenuates Vascular Contraction through Inhibition of the RhoA/Rho-Kinase Signaling Pathway

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ABSTRACT

Isoflavones decrease blood pressure, improve lipid profiles, and restore vascular function. We hypothesized that isoflavone attenuates vascular contraction by inhibiting RhoA/Rho-kinase signaling pathway. Rat aortic rings were denuded of endothelium, mounted in organ baths, and contracted with 11.9 epoxyxymethano-prostaglandin F2α (U46619), a thromboxane A2 analog, or KCl 30 min after the pretreatment with genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone), or vehicle. We determined the phosphorylation level of the myosin light chain (MLC20), myosin phosphatase-targeting subunit 1 (MYPT1), and protein kinase C-potentiated inhibitory protein for heterotrimeric myosin light-chain phosphatase of 17 kDa (CPI17) by means of the Western blot. We also measured the amount of GTP RhoA as a marker regarding RhoA activation. The cumulative additions of U46619 or KCl increased vascular tension in a concentration-dependent manner, which were inhibited by pretreatment with genistein or daidzein. Both U46619 (30 nM) and KCl (50 mM) increased MLC20 phosphorylation levels, which were inhibited by genistein and daidzein. Furthermore, both genistein and daidzein decreased the amount of GTP RhoA activated by either U46619 or KCl. U46619 (30 nM) increased phosphorylation of the MYPT1 Thr855 and CPI17 Thr38, which were also inhibited by genistein or daidzein. However, neither genistein nor daidzein inhibited phorbol 12,13-dibutyrate-induced vascular contraction and CPI17 phosphorylation. In conclusion, isoflavone attenuates vascular contraction, at least in part, through inhibition of the RhoA/Rho-kinase signaling pathway.

Isoflavones have been a subject of intensive researches that have evaluated their possible hypocholesterolemic effects, antioxidant effects, and estrogen-like effects on blood vessels (Kapiotis et al., 1997; Nestel et al., 1997). Investigations in regards to animals and in humans have suggested that dietary soy protein containing isoflavones reduce blood pressure, improve lipid profiles, and restore vascular function (Teede et al., 2001). The three major isoflavones found in soybeans are genistein, daidzein, and glycitein. Soy protein containing isoflavones has been observed to have several beneficial effects on cardiovascular health; a meta-analysis study showed that total cholesterol decreased by 9.3%, triglyceride by 10.5%, and low-density lipoprotein cholesterol by 12.9%, when an average of 47 g of soy protein was consumed daily (Anderson et al., 1995). Soybean isoflavones were suggested to inhibit proliferation of vascular smooth muscle and thus contribute to the prevention of atherosclerotic cardiovascular diseases (Pan et al., 2001). The small guanosine triphosphatase RhoA plays an important role as a molecular switch in the enhancement of Ca2+

ABBREVIATIONS: genistein, 4',5,7-trihydroxyisoflavone; daidzein, 4',7-dihydroxyisoflavone; glycitein, 6-methoxydaidzein; MLC20, myosin light chain; MYPT1, myosin phosphatase-targeting subunit 1; MLC, myosin light chain phosphatase; PKC, protein kinase C; CPI17, PKC-potentiated inhibitory protein for heterotrimeric MLC of 17 kDa; PDBu, phorbol 12,13-dibutyrate; Ro31-8220, bisindolylmaleimide IX, 3-[1-(3-amidinohydroxy)-propyl-1H-indol-3-yl]-3-(1-methyl-1H-indol-3-yl) maleimide; TCA, trichloroacetic acid; DTT, dithiothreitol; ANOVA, analysis of variance; GF109203X, 3-[1-[3-(dimethylaminopropyl)-1H-indol-3-yl]-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione monohydrochloride; RhoGDI, Rho GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; RhoGAP, Rho GTPase-activating protein; PD98, phosphatidylinositol 3-kinase; U46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α; Y27632, trans-4-[1(R)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride.
sensitivity of smooth muscle contraction (Hirata et al., 1992). Activated RhoA increases the Ca$^{2+}$ sensitivity of both MLCK phosphorylation and contractions in smooth muscle (Gong et al., 1995). Rho-kinase is an effector of the small GTP-binding protein RhoA and is a major cellular regulator for agonist-induced Ca$^{2+}$-sensitization in smooth muscle contraction. Rho-kinase directly phosphorylates the myosin phosphatase-targeting subunit 1 (MYP1) at Thr855 of myosin light-chain phosphatase (MLCP) and consequently inhibits phosphatase activity. Rho-kinase also phosphorylates protein kinase C (PKC)-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (CP117) (Eto et al., 1995), which directly inhibits the catalytic domain of MLCP. CP117 can be phosphorylated by both Rho-kinase and PKC.

Genistein and daidzein prevented agonist-induced vascular contraction in isolated rat aortic rings (Vera et al., 2005). There are few reports exhibiting whether isoflavones exert vasorelaxation by modulating the RhoA/Rho-kinase signaling pathway or phosphorylation of MYP1 or CP117.

It was hypothesized that isoflavone attenuates vascular contraction by inhibiting the RhoA/Rho-kinase signaling pathway. Therefore, the purpose of present study was to investigate the inhibitory effects of isoflavones on RhoA activation and subsequent phosphorylation of MYP1 or CP117 and on vascular smooth muscle contraction induced by U66619 or KCl.

Materials and Methods
Materials. U66619, genistein, daidzein, phorbol 12,13-dibutyrate (PDBu), Ro31-8220, KCl, and phenylephrine were obtained from the Sigma-Aldrich (St. Louis, MO). Stock solutions of U66619, genistein, daidzein, PDbu, and Ro31-8220 were prepared in dimethyl sulfoxide. All other reagents were of analytical grade.

Tissue Preparation and Tension Measurement. The investigation is in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996). Male Sprague-Dawley rats, weighing 320 to 350 g, were used. With animals under anesthesia (50 mg/kg sodium pentobarbital i.p.), the thoracic aorta was immediately excised and immersed in an ice-cold, modified Krebs' solution composed of 115.0 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl$_2$, 1.2 mM MgCl$_2$, 25.0 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, and 10.0 mM dextrose. The aorta was cleaned of all adherent connective tissue on wet filter paper, soaked in the Krebs-bicarbonate solution, and cut into four ring segments (4 mm in length) as described by Seok et al. (2006). All rings were denuded of endothelium by gently rubbing the internal surface with the edge of forceps. Two stainless steel triangles were inserted through each vessel ring. Care was taken to avoid rubbing the endothelial surface of the vessels that had intact endothelium. Each aortic ring was suspended in a water-jacketed organ bath (22 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; Grass Instruments, Quincy, MA). 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 90 min before the experiment involving the contractile response to 50 mM of KCl. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP; ADInstruments Pty Ltd., Castle Hill, Australia). For the contractile response, aortic rings were pretreated with 10, 30, and 100 lM genistein or daidzein for 30 min and subjected to cumulative addition of U66619 or KCl.

Western Blot. After functional study, muscle strips were quickly immersed in acetone containing 10% trichloroacetic acid (TCA) and 10 mM diethiothreitol (DTT) precooled to −80°C. The aortic rings were washed in acetone containing 5 mM DTT to remove TCA, air-dried, and stored at −80°C until use. Previously stored samples were homogenized in a buffer containing 320 mM sucrose, 50 mM Tris, 1 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitors leupeptin (10 lM), trypsin inhibitor (10 lM), aprotinin (2 lM), phenylmethylsulphonyl fluoride (100 lM), and phosphatase inhibitor β-glycerophosphate (50 mM). Protein-matched samples (Bradford assay) were electrophoresed (SDS-polyacrylamide gel electrophoresis), transferred to nitrocellulose membranes, and subjected to an immunoblot with a pMYP1 antibody (1:4000; Upstate Biotechnology, Lake Placid, NY) and a CP117 antibody (1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that detects phosphorylated MYP1 and CP117. Anti-rabbit IgG and anti-goat IgG, conjugated with horseradish peroxidase, were used as secondary antibody (1:4000; Sigma-Aldrich; and 1:1,000, Santa Cruz Biotechnology, Inc.). The bands containing pMYP1 and pCP117 were detected with enhanced chemiluminescence visualized on films. The nitrocellulose membranes were stripped of the pMYP1 and pCP117 antibody and rebotted with total form of MYP1 antibody (1:4000; BD Biosciences, San Diego, CA) and CP117 antibody (1:500; Upstate Biotechnology). Anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were used as secondary antibody (1:4000; Sigma-Aldrich; and 1:1,000; Upstate Biotechnology).

MLC$_{20}$ Phosphorylation. Muscle strips were quick frozen by immersion in acetone containing 10% TCA and 10 mM DTT precooled to −80°C. Muscles were washed four times with acetone containing 5 M DTT for 15 min each to remove TCA and were soaked 1 h with frequent vortex in 100 lL of urea sample buffer containing 20 mM Tris base/23 mM glycine, pH 8.6, 8.0 M urea, 10 mM DTT, 10% glycerol, and 0.04% bromphenol blue. Polyacrylamide gels (12.5%) containing 40% glyceraldehyde were pre-electrophoresed for 30 min at 200 V. The running buffer consists of 20 M Tris base/23 M glycine, pH 8.6, 2.3 M thiglycolate, and 2.3 M DTT. The urea-extracted samples (12 lL) were electrophoresed at 300 V for 6 h, transferred to nitrocellulose membranes, and subjected to immunoblotting with a specific myosin light-chain 20 antibody (1:2000; Sigma-Aldrich). Anti-mouse IgG (goat), conjugated with horseradish peroxidase, was used as a secondary antibody (1:4000; Assay Designs, Inc, Ann Arbor, MI). The bands containing myosin light chains were visualized with enhanced chemiluminescence on films and then analyzed by NIH Image as described (Kim et al., 2004).

Assay for RhoA Activation. Muscle strips were quick frozen by liquid nitrogen and stored at −80°C. For RhoA activation assays, the procedure followed the manufacturer's instruction regarding RhoA G-LISA Activation Assay (Cytoskeleton Inc., Denver, CO). Briefly, previously stored samples were homogenized in a lysis buffer and were centrifuged at 13,200 rpm for 15 min at 4°C. The supernatant, containing 37 lM of proteins, was transferred into a plate, and equal volumes of ice-cold binding buffer mixtures were added into each well. The plate was shaken on a cold orbital microplate shaker (300 rpm) for 30 min at 4°C, flicked out of the solution from wells, and incubated with diluted anti-RhoA primary antibodies followed by secondary antibodies on a microplate shaker (300 rpm) at room temperature for 45 min each. The plate was incubated with a horseradish peroxidase detection reagent for 15 min at 37°C, and, after addition of an horseradish peroxidase stop buffer, the absorbance was immediately recorded at 490 nm.

Statistical Analysis. Data are expressed as mean ± S.E.M. and were analyzed by repeated measures ANOVA or one-way ANOVA followed by the post hoc Tukey's test to determine statistical significance. P values of less than 0.05 were regarded as significant.

Results
Effect of Pretreatment with Genistein or Daidzein on the U66619- or KCl-Induced Contractions. Thirty
minutes after the pretreatment with genistein, daidzein, or vehicle, the concentration-response relationships to U46619 or KCl in endothelium-denuded aortic rings were obtained by means of a cumulative addition of the chemicals (Fig. 1). The tension is expressed as a percentage to initial contractions in response to 50 mM KCl. Pretreatment with genistein or daidzein shifted U46619-induced concentration-response curves rightward (Fig. 1A). The contractile response to KCl was inhibited by pretreatment with either genistein or daidzein (Fig. 1B).

**Inhibitory Effect of Genistein or Daidzein on U46619- or KCl-Induced MLC20 Phosphorylation.** To determine whether isoflavones affect U46619 (30 nM)- or KCl (50 mM)-induced MLC20 phosphorylation, aortic rings were pretreated with genistein, daidzein, or vehicle for 30 min before addition of U46619 or KCl. Both genistein (30 and 100 μM) and daidzein (30 and 100 μM) decreased U46619- or KCl-induced MLC20 phosphorylation (Fig. 2).

**Inhibitory Effect of Genistein or Daidzein on U46619-Induced MYPT1Thr855 Phosphorylation.** U46619 induced contractions in the rat caudal artery, accompanied by Rho kinase-dependent phosphorylation of MYPT1 at Thr855, without affecting the level of phosphorylation at Thr697 (Wilson et al., 2005; Tsai and Jiang, 2006; Jeon et al., 2007). It was examined...
Fig. 2. Inhibitory effect of genistein and daidzein on U46619 (A)- or KCl (B)-induced MLC\textsubscript{20} phosphorylation in rat aorta. U46619 (30 nM) or KCl (50 mM) were each added to elicit tension 30 min after pretreatment with genistein, daidzein, or vehicle to denuded aortic rings. Phosphorylation of MLC\textsubscript{20} was measured when the tension reached plateaus and was expressed as a percentage of the total MLC\textsubscript{20}. Data are expressed as the means of five experiments with vertical bars showing S.E.M. ##, $P<0.01$ as compared with the basal. *, $P<0.05$; and **, $P<0.01$ as compared with U46619 or KCl alone.

Fig. 3. Inhibitory effect of genistein and daidzein on U46619 (A)- or KCl (B)-induced RhoA activation in rat aorta. U46619 (30 nM) or KCl (50 mM) were each added to elicit tension 30 min after pretreatment with genistein, daidzein, or vehicle to denuded aortic rings. RhoA activation was assessed by RhoA G-LISA Activation Assay. Absorbance at the control (OD of around 0.4) was expressed as 1 arbitrary unit. Data were expressed as the means of four experiments, with vertical bars showing S.E.M. ##, $P<0.01$ as compared with the basal. **, $P<0.01$ as compared with U46619 or KCl alone.
whether U46619 induced phosphorylation of MYPT1<sub>Thr855</sub> in the rat aorta. Figure 4 shows that U46619 (30 nM) increased the phosphorylation level of MYPT1<sub>Thr855</sub>. If U46619-induced Rho-kinase activation is inhibited by isoflavone, the phosphorylation level of MYPT1<sub>Thr855</sub> should decrease. U46619 (30 nM) was applied 30 min after the pretreatment with 30 or 100 μM isoflavones (genistein or daidzein). As shown in Fig. 4, the phosphorylation level of MYPT1<sub>Thr855</sub> significantly decreased by 30 or 100 μM genistein (1.32 ± 0.29 and 0.39 ± 0.13, respectively) or 30 or 100 μM daidzein (1.55 ± 0.31 and 0.85 ± 0.19, respectively).

**Inhibitory Effect of Genistein or Daidzein on U46619-Induced CPI17<sub>Thr38</sub> Phosphorylation.** CPI17 can be phosphorylated at Thr38 in rat aorta (Fig. 5). The phosphorylation level of CPI17<sub>Thr38</sub> was significantly decreased by 30 and 100 μM genistein (0.55 ± 0.08 and 0.10 ± 0.04, respectively) and 30 and 100 μM daidzein (0.60 ± 0.13 and 0.08 ± 0.04, respectively) when compared with the phosphorylation level induced by U46619 alone.

**Effect of Ro31-8220, Genistein, or Daidzein on PDBu-Induced Contraction and CPI17<sub>Thr38</sub> Phosphorylation.** Thirty minutes after the pretreatment with Ro31-8220 (1.0 μM), genistein, daidzein, or vehicle, aortic rings were challenged with an addition of 0.1 μM PDBu. The tension is expressed as a percentage to initial contractions in response to 50 mM KCl. Pretreatment with Ro31-8220, a PKC inhibitor, inhibited the vascular contractility and phosphorylation of CPI17<sub>Thr38</sub> in response to PDBu. However, both genistein and daidzein had no effect on the PDBu-induced vascular contraction and CPI17 phosphorylation (Fig. 6).

**Discussion**

The present study demonstrates that isoflavones attenuate vascular contraction through inhibition of the RhoA/Rho-kinase signaling pathway. The isoflavones attenuated the activation of RhoA and subsequent Rho-kinase-dependent phosphorylation of MYPT1 and CPI17. However, isoflavones did not affect phosphorylation of CPI17 induced by an activation of PKC.

U46619, a thromboxane A<sub>2</sub> mimetic, and KCl activated RhoA, increased phosphorylation of MLC<sub>20</sub>, and induced contraction, which were inhibited by both genistein and daidzein (Figs. 1–3). RhoA/Rho-kinase activation plays an important role in the tonic component of KCl- or U46619-induced contraction and MLC<sub>20</sub> phosphorylation (Mita et al., 2002; Wilson et al., 2005). Both genistein and daidzein also inhibited U46619-induced phosphorylation of MYPT1<sub>Thr855</sub> and CPI17<sub>Thr38</sub> (Figs. 4 and 5), which are substrates of Rho-kinase. These results indicate that isoflavones suppress the U46619- or KCl-induced RhoA/Rho-kinase signaling pathway and are in agreement with reports that genistein strongly inhibited U46619- or vanadate-induced RhoA activation in the smooth muscles of rabbits or guinea pigs (Sakurada et al., 2001; Mori and Tsushima, 2004).

The activity of myosin phosphatase decreases when PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (CPI17) or MYPT1 is phosphorylated (Feng et al., 1999; Ito et al., 2004). We examined the level of phosphorylated MYPT1<sub>Thr855</sub>, which is an inhibitory site and is phosphorylated by the Rho-kinase. Activation of Rho-kinase by U46619 or phenylephrine, an α<sub>1</sub>-adrenergic agonist, phosphorylates MYPT1 at Thr855, but not MYPT1 at Thr697 (Wilson et al., 2005; Tsai and Jiang, 2006; Jeon et al., 2007). The phosphorylation of MYPT1<sub>Thr697</sub> is independent of the stimulation of G-proteins, Rho-kinase, or PKC (Kitazawa et al., 2003; Niño et al., 2003). We observed that both genistein and daidzein inhibited MYPT1<sub>Thr855</sub> phosphorylation induced by U46619 (Fig. 4) or by phenylephrine (data not shown). Altogether,

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**Fig. 4.** Inhibitory effect of genistein (A) and daidzein (B) on U46619-induced MYPT1 phosphorylation in rat aorta. U46619 (30 nM) was added to elicit tension 30 min after pretreatment with genistein, daidzein, or vehicle to denuded aortic rings. MYPT1 phosphorylation at Thr855 was assessed by Western blots. Top and bottom bands in representative Western blots were probed with anti-p-MYPT1 and anti-total MYPT1 antibodies, respectively. The ratio of density of phosphorylated MYPT1 (Top) to that of the total MYPT1 (bottom) regarding the control was expressed as 1 arbitrary unit. Data are expressed as the means of five experiments, with vertical bars showing S.E.M. ##, P < 0.01 as compared with the basal. *, P < 0.05; and **, P < 0.01 as compared with U46619 alone.
These results suggest that Rho-kinase mediates MYPT1-Thr855 phosphorylation in response to the activation of receptors coupled to G12/13 or Gq/11.

CPI17 is another potential mediator of Ca2+ sensitization that is independent of MYPT1 phosphorylation. CPI17 is a 17-kDa peptide, first isolated from porcine aorta (Eto et al., 1995), in which phosphorylation enhances its potency for inhibiting the catalytic subunit of myosin phosphatase (Seko et al., 2003; Pang et al., 2005). Pang et al. (2005) have shown that the U46619-induced CPI17 phosphorylation was not affected by the PKC inhibitor GF109203X but was abolished by a Rho-kinase inhibitor Y27632, suggesting that Rho-kinase is involved in U46619-induced CPI17 phosphorylation and contractions. These reports showed that U46619 caused CPI17 phosphorylation through activation of the RhoA/Rho-kinase pathway. Our data also showed that U46619 significantly increased CPI17-Thr38 phosphorylation, which was inhibited by both genistein and daidzein (Fig. 5). Daidzein inhibited CPI17-Thr38 phosphorylation induced by phenylephrine (data not shown).

Genistein and daidzein are structurally very similar, differing only in the substitution at position 5 (a hydroxyl group absent in daidzein) (Nakashima et al., 1991). The difference shows that genistein is a well known inhibitor of a broad
range of tyrosine kinase, whereas daidzein lacks inhibitory activity regarding the tyrosine kinase. However, both genistein and daidzein inhibited Rho-kinase and vascular contractions, suggesting that the inhibitory action of genistein or daidzein is independent of tyrosine kinase inhibition.

Isoflavones inhibited vascular contractions and MLC$_{20}$ phosphorylation induced by U46619 or KCl. Genistein and daidzein rightward shifted concentration-response curves to U46619 and to KCl in endothelium-denuded aortic rings. These data are consistent with previous reports that have used several vascular beds from male Wistar Kyoto rats and spontaneously hypertensive rats (Vera et al., 2005) and normotensive animals (Martínez et al., 2000; Janssen et al., 2001).

Flavonoids, including genistein, not only antagonized thromboxane A$_2$ receptors in platelets and smooth muscle cells but also impaired U46619-induced calcium mobilization in a concentration-dependent manner (Guerrero et al., 2007). In Fig. 1A, daidzein shifted the concentration-response curves rightward with similar $E_{\text{max}}$, whereas genistein shifted the concentration-response curves rightward, with $E_{\text{max}}$ being decreased. The inhibitory patterns imply that daidzein, rather than genistein, competitively antagonizes binding of U46619 to the receptor. A radioligand displacement study may be needed to elucidate whether daidzein is a competitive antagonist to U46619. However, the flavone, similarly to genistein, shifted the concentration-response curves rightward, with a decrease in $E_{\text{max}}$ (Jeon et al., 2007), suggesting that flavonoids may have two or more mechanisms to inhibit the Rho-kinase pathway.

To figure out the target sites of isoflavone regarding the Rho-kinase signaling pathway, we contracted aorta with KCl, which activates Rho-kinase by means of a different mechanism. KCl induces membrane depolarization and the subsequent influx of extracellular Ca$^{2+}$ (Mita et al., 2002). Isoflavones also inhibited vascular constriction induced by KCl. The fact that there were dose-dependent genistein- and daidzein-induced inhibition of KCl-mediated contractions supports the notion that the inhibitory targets of isoflavones with respect to vascular contractions are located somewhere in the Rho-kinase signaling pathway besides thromboxane A2 receptors.

Although isoflavones suppressed GTP RhoA activation induced by U46619 and KCl, the molecular mechanism by which isoflavones inhibit RhoA activation remains to be elucidated. GDP dissociation inhibitors (RhoGDIs), guanine nucleotide exchange factors (GEFs), Rho GTPase-activating proteins (RhoGAPs), and GTP RhoA are all upstream of the Rho-kinase pathway. In a resting state, GDP RhoA resides in cytosol with its prenylated, hydrophobic tail buried within its partner, known as RhoGDI. Activation of receptors coupled to certain trimeric G proteins (G$_{q}$, G$_{12,13}$) leads, through the activity of GEFs, to the exchange of GTP for GDP on RhoA. GTP RhoA associates with the membrane, where it interacts with Rho-kinase to initiate signaling cascades. When RhoGAPs catalyze hydrolysis of GTP bound to RhoA, GDP RhoA reassociates with RhoGDI (Somlyo and Somlyo, 2003). In addition, a report has suggested that a phosphatidylinositol 3-kinase (PI3K) is essential for the Ca$^{2+}$-dependent Rho activation in vascular smooth muscles (Wang et al., 2006). Several studies implied that PI3K class II α-isofrom plays a role in the Ca$^{2+}$-dependent Rho activation (Stein and Waterfield, 2000; Azam et al., 2007). Isoflavone may suppress GTP RhoA activation, induced by U46619 or KCl, through interaction with GEFs, RhoGAPs, RhoGDIs, or PI3K.

The phosphorylation of CPI17$_{Thr38}$ was shown to be mediated by both Rho-kinase and PKC in vascular and other smooth muscle tissues (Eto et al., 2001; Niiro et al., 2003). PDBu-induced contraction and CPI17$_{Thr38}$ phosphorylation were suppressed by Ro31-8220, an inhibitor of PKC, but insensitive to genistein or daidzein (Fig. 6). These results are consistent with previous reports showing that the PKC inhibitor GF109203X, but not the Rho-kinase inhibitor Y27632, abolished only PDBu-induced CPI17$_{Thr38}$ phosphorylation. In addition, GF109203X did not inhibit thrombin- or U46619-induced CPI17$_{Thr38}$ phosphorylation (Pang et al., 2005; Azam et al., 2007). These observations suggest that genistein and daidzein inhibited the phosphorylation of CPI17$_{Thr38}$ induced by Rho-kinase, but not PKC.

MLC$_{20}$ is known to be phosphorylated by myosin light-chain kinase, Rho-kinase (Somlyo and Somlyo, 2003), and some of the Ca$^{2+}$-independent kinases including zipper-interacting protein kinase, integrin-linked kinase, and citron kinase (Deng et al., 2001). Zipper-interacting protein kinase and integrin-linked kinase contract smooth muscles in a Ca$^{2+}$-independent way (Deng et al., 2001) and are also able to diphosphorylate MLC$_{20}$, all of which were not inhibited by the Rho-kinase inhibitor (Kiss et al., 2002).

Activation of Rho-kinase by U46619 or KCl inhibits myosin light-chain phosphatase through phosphorylation of MYPT1$_{Thr555}$, leading to an increased MLC$_{20}$ phosphorylation and contraction (Sakurada et al., 2003; Wilson et al., 2005). U46619 or KCl each monophosphorylated MLC$_{20}$, which was inhibited by genistein or daidzein. This result is in accordance with the finding that genistein decreased MLC$_{20}$ phosphorylation induced by either vanadate (Mori and Tsushima, 2004) or angiotensin II (Rattan et al., 2003).

The soybean isoflavones, genistein, daidzein, and glycitein, may have potential benefits in regards to human health maintenance because of their biological effects (Pan et al., 2001). Isoflavones (phytoestrogens) have gained considerable attention for their potential role in improving risk factors regarding cardiovascular diseases (Sacks et al., 2006). Genistein, daidzein, and glycitein inhibit the growth and DNA synthesis of aortic smooth muscle cells in stroke-prone spontaneously hypertensive rats (Pan et al., 2001). It has been proven recently that activation of the RhoA/Rho-kinase signal transduction pathway is one of the principal mechanisms of vasoconstriction in arterial hypertension (Johns et al., 2000).

In conclusion, isoflavone attenuates vascular contraction by inhibiting RhoA/Rho-kinase signaling. These results suggest that isoflavone inhibited vascular contractions in response to U46619 or KCl, through disinhibition of MLCP, by inhibiting activation of RhoA and the subsequent phosphorylation of MYPT1$_{Thr555}$ and CPI17$_{Thr38}$.

References


Sequence Reader


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