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Research Paper

Honokiol attenuates vascular contraction through the inhibition of the RhoA/Rho-kinase signalling pathway in rat aortic rings

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Abstract

Objectives Honokiol is a small-molecule polyphenol isolated from the species *Magnolia obovata*. We hypothesized that honokiol attenuated vascular contractions through the inhibition of the RhoA/Rho-kinase signalling pathway.

Methods Rat aortic rings were denuded of endothelium, mounted in organ baths, and subjected to contraction or relaxation. Phosphorylation of 20 kDa myosin light chains (MLC_{20}), myosin phosphatase targeting subunit 1 (MYPT1) and protein kinase C (PKC)-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase (MLCP) of 17 kDa (CPI17) were examined by immunoblot. We also measured the amount of guanosine triphosphate RhoA as a marker for RhoA activation.

Key findings Pretreatment with honokiol dose-dependently inhibited the concentration–response curves in response to sodium fluoride (NaF) or thromboxane A_2 agonist U46619. Honokiol decreased the phosphorylation levels of MLC_{20}, $MYPT1_{Thr855}$ and $CPI17_{Thr38}$ as well as the activation of RhoA induced by 8.0 mM NaF or 30 nM U46619.

Conclusions These results demonstrated that honokiol attenuated vascular contraction through the inhibition of the RhoA/Rho-kinase signalling pathway.

Keywords $CPI17_{Thr38}$; honokiol; 20 kDa myosin light chains; $MYPT1_{Thr855}$; RhoA; Rho-kinase

Introduction

The small guanosine triphosphatase (GTPase) RhoA is a molecular switch on the signalling pathway for enhancement of Ca^{2+} sensitivity during smooth muscle contraction.[1] The activation of RhoA leads to the subsequent activation of the effector Rho-kinase.[2] Rho-kinase directly phosphorylates both CPI17 (protein kinase C (PKC)-potentiated inhibitory protein for heterotrimeric myosin light-chain phosphatase (MLCP) of 17 kDa) at Thr38 and the myosin phosphatase-targeting subunit 1 (MYPT1) at Thr855 of the MLCP, resulting in inhibition of the phosphatase activity.[3] The activity of MLCP is regulated by signalling pathways from vasoconstrictors and vasodilators, which inhibit and activate it, respectively. The RhoA/Rho-kinase pathway plays an important role in the development of cardiovascular diseases such as heart failure, coronary artery disease, arteriosclerosis, pulmonary hypertension, restenosis, and stroke since they cause excessive activation of the RhoA/Rho-kinase signalling pathway.[4–7] Recently, the RhoA/Rho-kinase pathway has received attention as genetic disruption of the pathway blocks elevation of blood pressure upon the hypertensive condition.[8]

Honokiol is a small-molecule polyphenolic compound isolated from the species *Magnolia obovata*, which has been used as a traditional medicine in Korea, China, and Japan.[9,10] Honokiol has many pharmacological activities including anxiolytic, antioxidative, anti-inflammatory, anti-angiogenic, antitumourigenic, and anti-osteoclastic actions.[10–13] Honokiol attenuated smooth muscle contractile responses through the blockade of intracellular calcium mobilization and stimulation of relaxing factor release.[14–16] Furthermore, long-term administration of honokiol had antihypertensive and vasorelaxant effects in spontaneously hypertensive rats (SHR).[16]

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Although the bark of *M. obovata* has been used for more than 1000 years as a folk medicine in Asia, there is no report to elucidate the molecular mechanism by which honokiol exerts vasorelaxation through modulation of the RhoA/Rho-kinase signalling pathway.[14] We have found that phytochemicals such as flavone, isoflavones, and glyceollin I inhibit vascular contractions through the inhibition of the RhoA/Rho-kinase signalling pathway.[17–19] Thus, we hypothesized that honokiol may attenuate vascular contraction through the inhibition of the RhoA/Rho-kinase signalling pathway. Therefore, we have investigated the inhibitory effects of honokiol on RhoA activation and the subsequent phosphorylation of MYPT1 or CPI17 and on vascular smooth muscle contraction induced by 9, 11-dideoxy-11α, 9α-epoxymethanoprostaglandin F2α (U46619) or sodium fluoride (NaF).

Materials and Methods

Chemicals

Honokiol was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Its chemical structure is shown in Figure 1. The drugs and chemicals were obtained from the following sources: U46619, NaF and KCl from the Sigma Chemical Co. (St Louis, MO, USA). Stock solutions of U46619 and of honokiol were prepared in dimethyl sulfoxide (DMSO). All other reagents were analytical grade.

Organ bath study

The investigation was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Institutional Review Board, Kyungpook National University School of Medicine, Daegu, Republic of Korea). Male Sprague-Dawley rats (320–350 g) were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The thoracic aorta was immediately excised and immersed in an ice-cold, modified Krebs solution composed of (in mM) NaCl, 115.0; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; and dextrose, 10.0. 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 previously.[18] To investigate the direct effect of honokiol in vascular smooth muscle, the rings were denuded of endothelium by gently rubbing the internal surface with a forcep edge. Two stainless-steel triangles were inserted through each vessel ring. 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, Quincy, MA, 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 90 min before the experiment involving the contractile response to 50 mM KCl addition. Isometric contractions were recorded using a computerized data acquisition system (PowerLab/8SP, ADInstruments, Castle Hill, NSW, Australia). To determine the effect of honokiol on NaF or U46619, NaF or U46619 were added into organ baths to elicit tension 30 min after pretreatment with honokiol (10, 30, or 100 μM) or vehicle. The contractile responses to 30 nM U46619 or 8.0 mM NaF were recorded for 25 and 35 min, respectively.

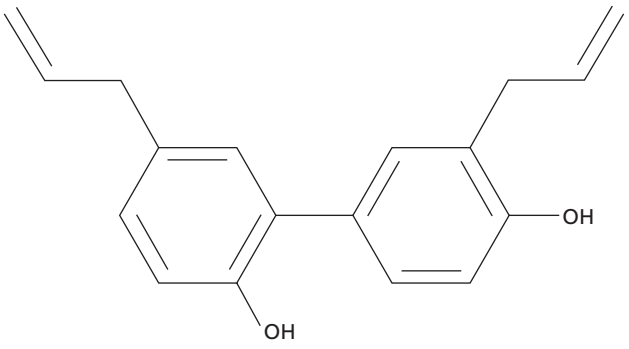

Figure 1 Chemical structure of honokiol $C_{18}H_{18}O_2$ (MW = 266.33).

Sodium dodecylsulfate-polyacrylamide gel electrophoresis for MYPT1 and CPI17

After completion of the functional study, muscle strips were quickly immersed in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (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 used. Previously stored samples were homogenized in a buffer as we described previously.[18,20] Protein-matched samples (Bradford assay) were electrophoresed sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes, and subjected to an immunoblot with a p-MYPT1$_{Thr855}$ antibody (1 : 4000, Upstate Biotechnology, Lake Placid, NY, USA) and a p-CPI17$_{Thr38}$ antibody (1 : 250, Santa Cruz Biotechnology, Inc, CA, USA) that detect phosphorylated MYPT1 and CPI17. Anti-rabbit IgG and anti-goat IgG, conjugated with horseradish peroxidase, were used as the secondary antibody (1 : 4000 (Sigma) and 1 : 1000 (Santa Cruz Biotechnology, Inc)). The nitrocellulose membranes were stripped of the p-MYPT1 and p-CPI17 antibody and reblotted with total MYPT1 antibody (1 : 4000 (BD Biosciences Pharmingen, San Diego, CA, USA)) and CPI17 antibody (1 : 500 (Upstate Biotechnology)). Anti-mouse IgG and anti-rabbit IgG conjugated with horseradish peroxidase were used as the secondary antibody (1 : 4000 (Sigma) and 1 : 1000 (Upstate Biotechnology)). The bands containing p-MYPT1, p-CPI17, t-MYPT1, and t-CPI17 were visualized with enhanced chemiluminescence (ECL) on films, and then analysed by a computer-assisted image analyser (Labworks, version 4.5; UVP Inc., Upland, CA, USA).

Urea-polyacrylamide gel electrophoresis for 20 kDa myosin light chains (MLC_{20}) phosphorylation

Muscle strips were quick frozen by immersion in acetone containing 10% TCA and 10 mM DTT precooled to −80°C and determined as described previously.[18,20] The aortic rings were washed in acetone containing 5 mM DTT to remove TCA, air dried, and stored at −80°C until used. Myosin was

extracted and analysed by urea-PAGE, followed by Western blotting using specific myosin light chain 20 antibody (1 : 2000 (Sigma)).[18,20]

Assay for RhoA activation

Muscle strips were quick frozen by liquid nitrogen, and kept at −80°C. The RhoA assay was performed as described previously.[18] The procedure followed the manufacture's protocol of G-LISA RhoA Activation Assay Biochem Kit (Cytoskelecton Inc, Denver, CO, USA).

Statistical analysis

Data are expressed as mean ± SEM and were analysed by repeated measures analysis of variance for tension measurement and one-way analysis of variance followed by Dunnett's test for biochemical studies. *P*-values less than 0.05 were regarded as significant.

Results

Effect of honokiol on vascular contraction induced by U46619 or NaF

Thirty minutes after the pretreatment with honokiol or vehicle, the concentration–response relationships to U46619 or NaF in endothelium-denuded aortic rings were obtained by means of a cumulative addition of the chemicals (Figure 2). Pretreatment with honokiol (10, 30, or 100 μM) inhibited the contractile responses to U46619 or NaF. Honokiol alone did not affect basal tension (data not shown).

Inhibitory effect of honokiol on vascular contractions and MLC_{20} phosphorylation induced by U46619 or NaF

To investigate whether honokiol relaxed vascular contraction through the regulation of thick filament, we determined the level of MLC_{20} phosphorylation. U46619 and NaF increased phosphorylation of MLC_{20} to 47.9 ± 2.3% and 52.0 ± 3.2%, respectively, in endothelium-denuded aortic rings. Treatment of rat aortic rings with 30 or 100 μM honokiol significantly decreased the phosphorylation level of MLC_{20} (Figure 3b and d) as well as vascular contraction (Figure 3a and c) induced by NaF or U46619 in endothelium-denuded rat aortic rings.

Inhibitory effect of honokiol on RhoA activation induced by U46619 or NaF

To determine whether honokiol inhibited the RhoA/Rho-kinase signalling pathway, we measured the level of GTP-RhoA. The addition of U46619 or NaF induced vascular contractions, which reached a plateau in 25 or 35 min, respectively. We freeze-clamped aortic smooth muscle contracted with NaF or U46619 and determined the amount of RhoA activation in it via G-LISA. NaF or U46619 increased the level of GTP-RhoA (1.96 ± 0.09-fold or 1.93 ± 0.13-fold) as compared with the basal level in endothelium-denuded aortic rings. As shown in Figure 4, honokiol (30 or 100 μM) nearly totally suppressed ($P < 0.01$) the level of GTP-RhoA induced by NaF or U46619. However, honokiol itself did not affect the basal level of GTP-RhoA (data not shown).

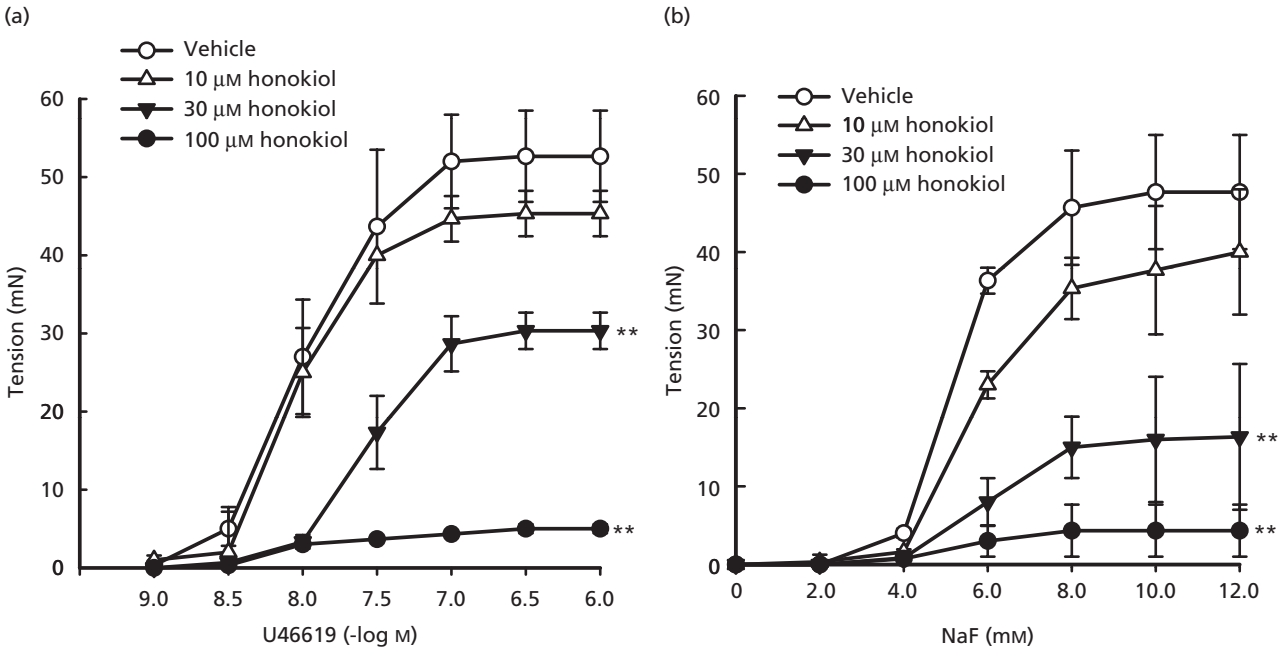

Figure 2 Effect of honokiol on U46619- or NaF-induced contractions in rat aorta. (a) U46619 (30 nM) or (b) NaF (8.0 mM) was added cumulatively to elicit tension 30 min after pretreatment with honokiol (10, 30, or 100 μM) or the vehicle (dimethyl sulfoxide) in denuded rat aortic rings. Developed tension is expressed as an absolute contractile force with mN. Data are expressed as means of four experiments with vertical bars showing SEM. $^{**}P < 0.01$ compared with vehicle.

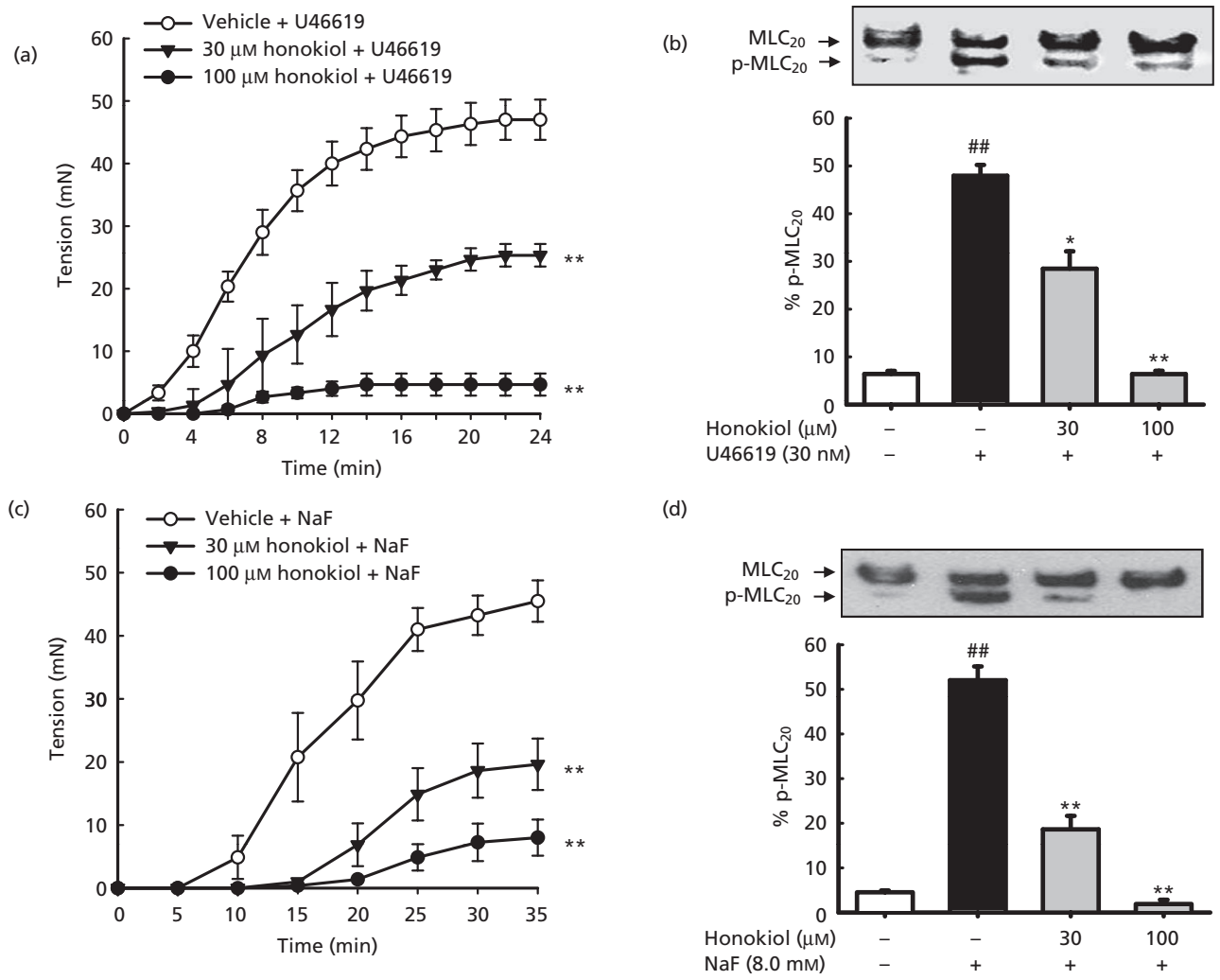

Figure 3 Inhibitory effect of honokiol on U46619- or NaF-induced phosphorylation of 20 kDa myosin light chains in rat aorta. (a) U46619 (30 nM) or (c) NaF (8.0 mM) was added to elicit tension 30 min after pretreatment with honokiol (30 or 100 μM) or the vehicle (dimethyl sulfoxide) to denuded aortic rings. Developed tension is expressed as an absolute contractile force with mN. When the tension reached a plateau, the phosphorylation level of 20 kDa myosin light chains (MLC_{20}) in response to U46619 (b) or NaF (d) was measured and expressed as a percentage of the total MLC_{20} (four aortic rings). p-MLC_{20}, phosphorylated MLC_{20}. Data are expressed as means with vertical bars showing SEM. For (a) and (c): $^{**}P < 0.01$ compared with vehicle. For (b) and (d): $^{\#\#}P < 0.01$ compared with control. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with U46619 or NaF alone.

Inhibitory effect of honokiol on MYPT1$_{Thr855}$ phosphorylation induced by U46619 or NaF

We determined the level of the phosphorylated MYPT1$_{Thr855}$, a Rho-kinase substrate. U46619- and NaF-stimulated MYPT1$_{Thr855}$ phosphorylation increased significantly (2.23 ± 0.05 and 2.56 ± 0.29-fold, respectively) compared with the control. As shown in Figure 5, honokiol (30 or 100 μM) decreased the phosphorylation level of MYPT1$_{Thr855}$ induced by NaF or U46619.

Inhibitory effect of honokiol on CPI17$_{Thr38}$ phosphorylation induced by U46619 or NaF

We also determined whether honokiol decreased the phosphorylation of CPI17$_{Thr38}$ induced by U46619 or NaF in the same rat aortas for measurement of MYPT1 phosphorylation. U46619 and NaF increased the phosphorylation level of CPI17$_{Thr38}$ as compared with the control, which was decreased by pretreatment with honokiol (30 or 100 μM) (Figure 6).

Discussion

This study has demonstrated that honokiol attenuated vascular contraction through the inhibition of the RhoA/Rho-kinase signalling pathway. Honokiol decreased the activation of RhoA and subsequent Rho-kinase-dependent phosphorylation of MYPT1$_{Thr855}$ and CPI17$_{Thr38}$. Honokiol also inhibited the phosphorylation of MLC_{20} and the contraction in denuded rat aortic rings.

U46619, a thromboxane A_2 agonist, as well as NaF activated RhoA, increased phosphorylation of MLC_{20}, and induced contraction, which were inhibited by honokiol (Figures 2–4). This study showed that U46619 and NaF activated RhoA approximately 2.0-fold and 1.9-fold over the

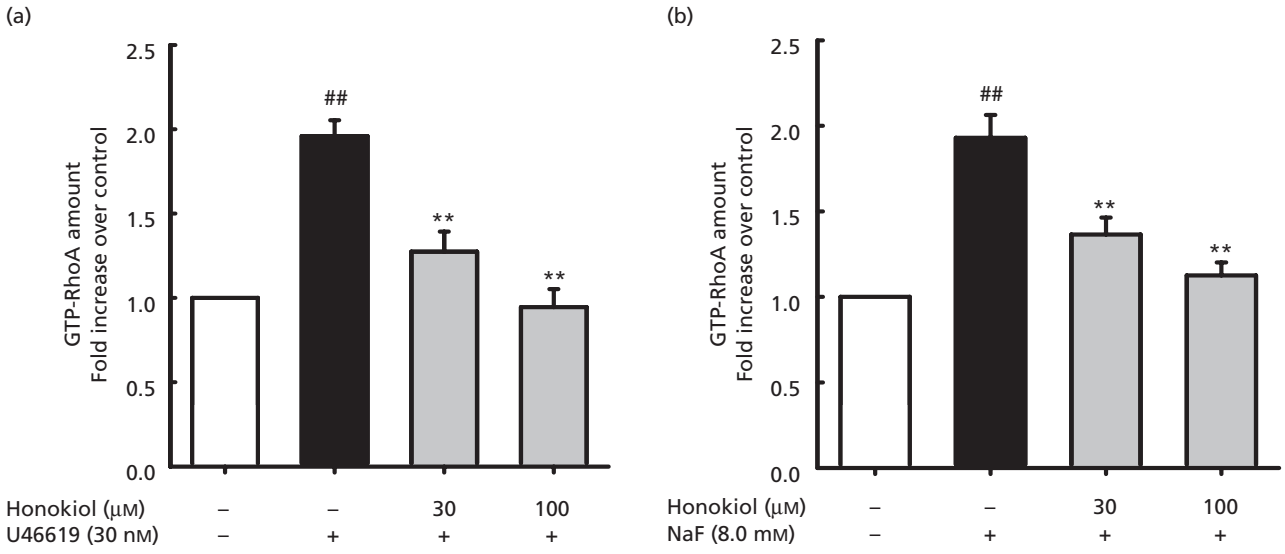

Figure 4 Inhibitory effect of honokiol on U46619- or NaF-induced RhoA activation in rat aorta. (a) U46619 (30 nM) or (b) NaF (8.0 mM) was added to elicit tension 30 min after pretreatment with honokiol (30 or 100 μM) or the vehicle (dimethyl sulfoxide) to denuded aortic rings. The amount of GTP RhoA was assayed by a RhoA G-LISA Activation Assay kit. Absorbance of the control (optical density of approximately 0.4 at 490 nm) was expressed as one arbitrary unit. Data are expressed as means of four experiments with vertical bars showing SEM. ##$P < 0.01$ compared with control. **$P < 0.01$ compared with U46619 or NaF alone.

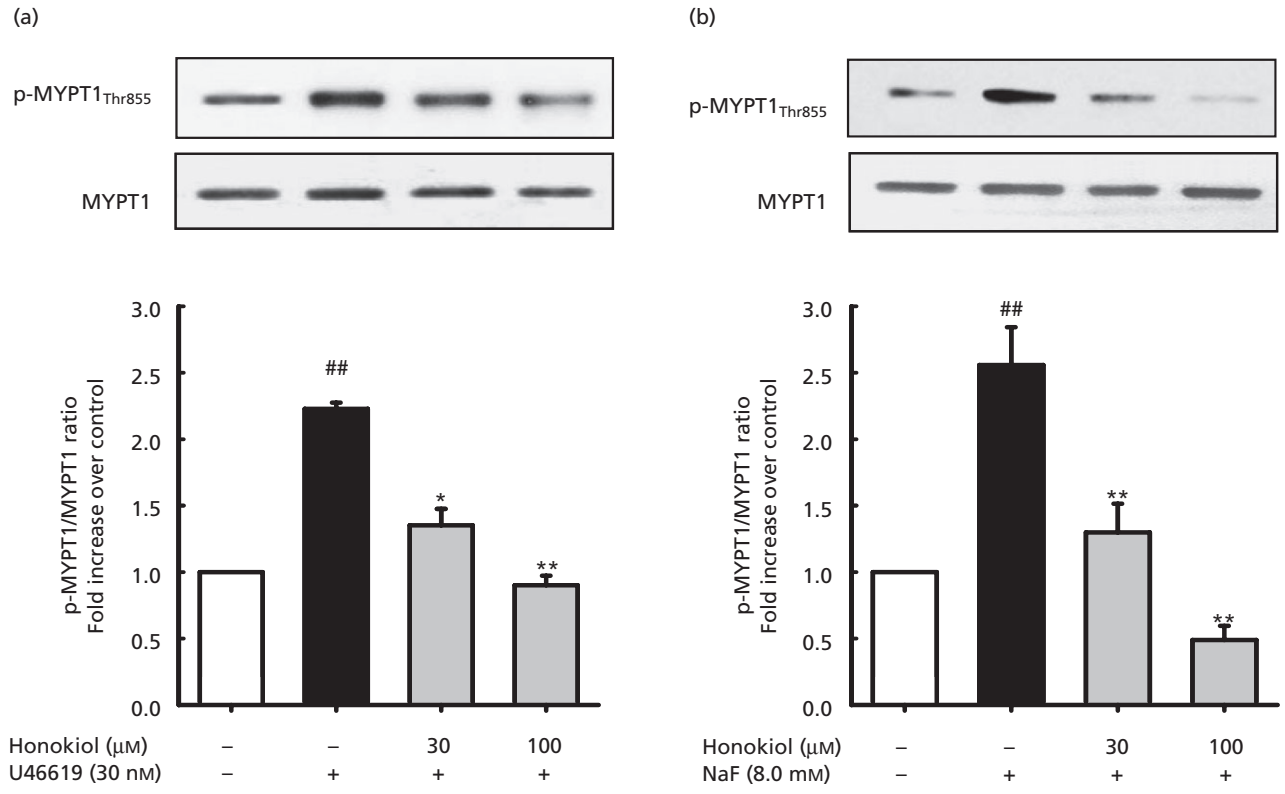

Figure 5 Inhibitory effect of honokiol on the phosphorylation level of $MYPT1_{Thr855}$ induced by U46619 or NaF in rat aorta. (a) U46619 (30 nM) or (b) NaF (8.0 mM) was added to elicit tension 30 min after pretreatment with honokiol (30 or 100 μM) or the vehicle (dimethyl sulfoxide) to denuded aortic rings. Myosin phosphatase targeting subunit 1 (MYPT1) phosphorylation at Thr855 was assessed by Western blot. Upper and lower bands in representative Western blots were probed with anti-pMYPT1 and anti-tMYPT1 antibodies, respectively. The ratio of density of phosphorylated MYPT1 (p-MYPT1; upper) to that of the total MYPT1 (lower) regarding the control was expressed as one arbitrary unit. Data are expressed as the means of four experiments with vertical bars showing SEM. ##$P < 0.01$ compared with control. *$P < 0.05$, **$P < 0.01$ compared with U46619 or NaF alone.

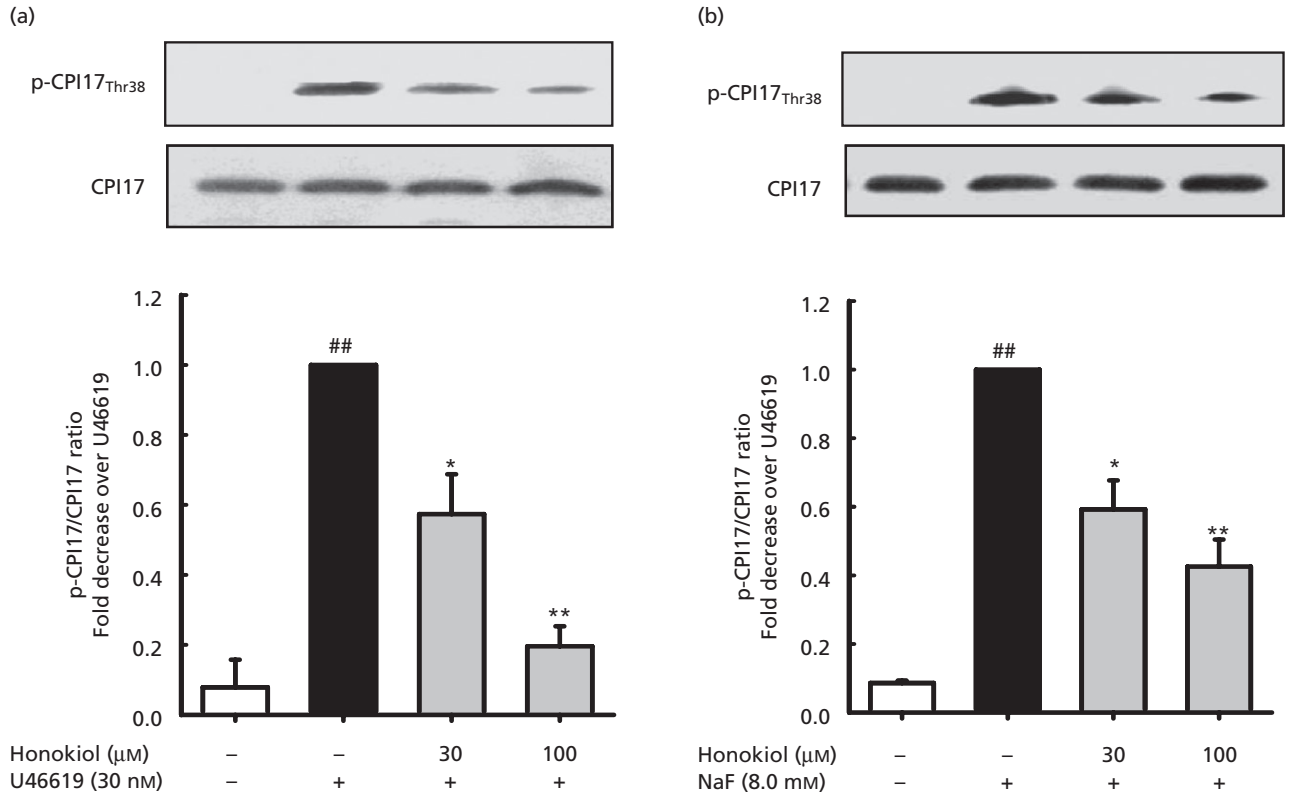

Figure 6 Inhibitory effect of honokiol on phosphorylation of $CPI17_{Thr38}$ induced by U46619 or NaF in rat aorta. (a) U46619 (30 nM) or (b) NaF (8.0 mM) was used to elicit tension 30 min after pretreatment with honokiol (30 or 100 μM) or the vehicle (dimethyl sulfoxide) to denuded aortic rings. Protein kinase C-potentiated inhibitory protein for heterotrimeric myosin light chain phosphatase of 17 kDa (CPI17) phosphorylation at Thr38 was assessed by Western blot. Upper and lower bands in the representative Western blots were probed with anti-pCPI17 and anti-tCPI17 antibodies, respectively. The ratio of density of phosphorylated CPI17 (p-CPI17; upper) to that of the total CPI17 (lower) regarding the positive control was expressed as one arbitrary unit. Data are expressed as means of four experiments with vertical bars showing SEM. ##$P < 0.01$ compared with control. *$P < 0.05$, **$P < 0.01$ compared with U46619 or NaF alone.

resting level, respectively. These data were consistent with our previous reports that U46619 and NaF activated RhoA.[20,21] The activation of receptors coupled to trimeric G proteins ($G_{\alpha 12,\ 13}$) leads, through the activity of guanine nucleotide exchange factors (GEFs), to the exchange of GTP for GDP on RhoA. GTP-RhoA translocates to the plasma membrane, where it interacts with Rho-kinase to initiate signalling cascades. When Rho GTPase-activating proteins (RhoGAPs) catalyse hydrolysis of GTP bound to RhoA, GDP RhoA reassociates with Rho GDP-dissociation inhibitor (RhoGDI).[3] NaF activates RhoA through activation of G proteins.[22] Furthermore, NaF binds and inhibits RhoGAPs, thereby resulting in Rho activation.[23] U46619 activates GEF upon binding to the thromboxane A_2 receptor, which is coupled to $G_{\alpha 12/13}$.[24] Furthermore, honokiol reduced the activation of the RhoA induced by U46619 or NaF (Figure 4). Honokiol may have suppressed GTP RhoA activation, presumably through interaction with GEFs, RhoGAPs, or RhoGDI. However, the exact molecular mechanism by which honokiol inhibited RhoA activation remains to be elucidated.

The activity of myosin phosphatase decreased when the PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (CPI17) or MYPT1 was phosphorylated.[25] MLCP is a heterotrimer consisting of a catalytic subunit PPIcδ, a 130 kDa regulatory subunit MYPT1 and a 20 kDa subunit of unknown function.[26] The level of phosphorylated $MYPT1_{Thr855}$, which is an inhibitory site, significantly increased in response to agonist stimulation via the Rho-kinase pathway in smooth muscle.[27] Activation of Rho-kinase by U46619, NaF, or phenylephrine, an α_1-adrenergic agonist, phosphorylated MYPT1 at Thr855, but not MYPT1 at Thr697.[21,24,28] The phosphorylation of $MYPT1_{Thr697}$ was independent of the stimulation of G proteins, Rho-kinase, or PKC.[27,29] We observed that honokiol inhibited $MYPT1_{Thr855}$ phosphorylation induced by U46619 or NaF (Figure 5). These observations suggested that honokiol inhibited the phosphorylation of $MYPT1_{Thr855}$ induced by Rho-kinase.

CPI17 is another downstream effector of Rho-kinase, which was first isolated from the porcine aorta and identified as a substrate of PKC at Thr38.[30] The phosphorylation of CPI17 at Thr38 enhanced its potency for inhibiting the catalytic subunit of myosin phosphatase.[31] U46619-induced $CPI17_{Thr38}$ phosphorylation was abolished by a Rho-kinase inhibitor Y27632, suggesting that Rho-kinase was involved in U46619-induced CPI17 phosphorylation and contractions.[32] Those reports showed that U46619 caused CPI17 phosphorylation through the activation of the RhoA/Rho-kinase pathway. Our data also showed that U46619 or NaF

significantly increased $CPI17_{Thr38}$ phosphorylation, which was inhibited by honokiol (Figure 6).

The activity of MLCP, a constitutively active enzyme, is a major determinant of smooth muscle contraction under physiological and pathophysiological conditions.[33] One of the main substrates of Rho-kinase is MLCP, which is physiologically responsible for the dephosphorylation of the light chains of myosin II (MLC_{20}).[34] Furthermore, the activation of Rho-kinase by U46619 or NaF inhibited MLCP through phosphorylation of $MYPT1_{Thr855}$, leading to an increased MLC_{20} phosphorylation and contraction.[18,20,24] In this study, honokiol reduced not only vascular contraction, but also the phosphorylation of MLC_{20} induced by NaF or U46619 in endothelium-denuded rat aortic rings (Figure 4).

Recently, the oral administration of honokiol to SHR not only decreased systolic blood pressure, but also enhanced the aortic relaxant response to acetylcholine through the increasing nitric oxide production, ameliorating aorta remodelling, and antioxidant properties.[16] These suggested that chronic treatment with honokiol exerted an antihypertensive effect in SHR through its vasorelaxant action. The Rho-kinase pathway plays a crucial role in the regulation of arterial blood pressure.[35] The activity of Rho-kinase in the mesenteric artery and other vessels of SHR at the hypertensive stage were elevated with concomitant increase of the phosphorylation of MYPT1, as compared with normotensive controls.[36] Arterial hypertension is one of the most common cardiovascular disorders characterized by altered vascular tone and increased vascular contractility resulting in high blood pressure.[37,38] In our study, honokiol decreased vascular contraction induced by NaF or U46619 through the inhibition of the RhoA/Rho-kinase pathway. Therefore, honokiol may be a potential lead in the development of therapeutic drugs.

Conclusions

Honokiol attenuated vascular contraction through the inhibition of the RhoA/Rho-kinase signalling pathway. In vascular strips, honokiol directly relaxed vascular contraction induced by U46619 or NaF in the absence of endothelium. Honokiol inhibited vascular contractions in response to U46619 and NaF by inhibiting the activation of RhoA and the subsequent phosphorylation of $MYPT1_{Thr855}$, $CPI17_{Thr38}$, and MLC_{20} through the disinhibition of MLCP.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

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