INTRODUCTION

Hypothermia which is defined as a body core temperature below 35.0°C, is characterized by shivering, mental confusion, muscle miscoordination, unstable physiological systems, and decreases in heart rate (Tankersley et al., 2003), respiratory rate, and blood pressure. Although hypothermia can be beneficial in cardiac arrest (Oh et al., 2015) and traumatic brain injury (Jensen et al., 2016), it is a risk factor for arrhythmia, bleeding, thrombosis, sepsis, and intraventricular hemorrhage. Hypothermia can induce nitric oxide-dependent vasodilatation, which can be prevented by atropine in isolated canine coronary, femoral, and renal arteries (Evora et al., 2007), suggesting that another pathway had a direct effect on vascular smooth muscle. Hypothermia significantly inhibited the fluoride-induced increase in pMYPT1 level and phorbol ester-induced increase in pERK1/2 level, suggesting inhibition of Rho-kinase and MEK activity and subsequent phosphorylation of MYPT1 and ERK1/2. These results suggest that the relaxing effect of moderate hypothermia on agonist-induced vascular contraction regardless of endothelial function involves inhibition of Rho-kinase and MEK activities.

Key Words: ERK1/2, fluoride, hypothermia, MYPT1, phorbol ester, Rho-kinase

Hypothermia Inhibits Endothelium-Independent Vascular Contractility via Rho-kinase Inhibition

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Abstract

The present study was undertaken to investigate the influence of hypothermia on endothelium-independent vascular smooth muscle contractility and to determine the mechanism underlying the relaxation. Denuded aortic rings from male rats were used and isometric contractions were recorded and combined with molecular experiments. Hypothermia significantly inhibited fluoride-, thromboxane A2-, phenylephrine-, and phorbol ester-induced vascular contractions regardless of endothelial nitric oxide synthesis, suggesting that another pathway had a direct effect on vascular smooth muscle. Hypothermia significantly inhibited the fluoride-induced increase in pMYPT1 level and phorbol ester-induced increase in pERK1/2 level, suggesting inhibition of Rho-kinase and MEK activity and subsequent phosphorylation of MYPT1 and ERK1/2. These results suggest that the relaxing effect of moderate hypothermia on agonist-induced vascular contraction regardless of endothelial function involves inhibition of Rho-kinase and MEK activities.

Key Words: ERK1/2, fluoride, hypothermia, MYPT1, phorbol ester, Rho-kinase
phorylation of the 20-kDa myosin light chain and subsequent enhanced contraction. Inhibition of MLCP in vascular smooth muscle is mediated by phosphorylation of the myosin phosphatase target subunit of MLCP via Rho-kinase, which leads to sustained phosphorylation of MLCP20. Subsequent studies have suggested that the inhibition of myosin phosphatase by Rho-kinase (Uehata et al., 1997; Sakurada et al., 2003; Akata, 2007) or thin filament regulation including the activation of protein kinase C (PKC), mitogen-activated protein kinase kinases (MEK), and extracelluar signal regulated kinase (ERK) 1/2, as well as phosphorylation of the actin binding protein caldesmon (Wier and Morgan, 2003) may precipitate Ca2+ sensitization.

Activation of ERK1/2, in addition to regulating vascular contractility, is also connected to pathologic hypertrophy, hyperplasia, hypertension, and atherosclerosis (Ruppert et al., 2013; Brietz et al., 2016). ERK1/2 is activated by threonine and tyrosine phosphorylation by the Raf-activated kinase MEK. Fluoride, phorbol esters, and thromboxane A2 mimetics have been shown to induce contractions of smooth muscles, which may be due primarily to enhanced Ca2+ sensitivity or partially to increased Ca2+ concentration. ERK1/2 activation was induced by thromboxane A2 mimetic (Gallet et al., 2003) or phorbol ester. Activation of ERK1/2 triggers ERK1/2-dependent cytoskeletal remodeling and formation of podosomes (Gu et al., 2007). However, it is possible that the contractions induced by fluoride or thromboxane A2 mimetics involve the RhoA/Rho-kinase pathway (Jeon et al., 2006). However, it is not known whether this pathway is inhibited during hypothermia-induced vascular smooth muscle relaxation in aortic rings contracted with a Rho-kinase activator (fluoride), a MEK activator (phorbol ester), or with dual activators of both Rho-kinase and MEK (thromboxane A2 or phenylephrine). Our aim in the present study was to investigate the possible roles of Rho-kinase or MEK inhibition on Ca2+ desensitization during hypothermia-induced relaxation of isolated, denuded rat aortas using Rho-kinase (fluoride) or MEK (phorbol ester) activators.

**MATERIALS AND METHODS**

**Tissue preparation**

Male Sprague-Dawley rats weighing 200-250 g were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and then subjected to cervical dislocation, in accordance with procedures approved by the Institutional Animal Care and Use Committee of Chung-Ang University, Korea, and Catholic University of Daegu, Korea (ICUC-2014-041). 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 10.0 dextrose (pH 7.4). They were then freed of all adherent connective tissue, and aortic endothelia were removed by gentle abrasion using a cell scraper if necessary.

**Contraction measurements**

Two stainless-steel triangles were inserted through each vessel ring, and each aortic ring was suspended in a water-jacketed organ bath (10 ml) that was adjusted from 37°C to 28°C (moderate hypothermia) using a thermostat and water baths and was 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, MA, USA). 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 or 1 µM phenylephrine were measured. Isometric contractions where the muscle length does not change during contraction (Ito et al., 2015), were recorded using one triangle anchored to a stationary support and the other connected to an isometric force transducer, an amplifier, and a computerized data acquisition system (PowerLab/8SP, AD Instruments, Castle Hill, NSW, Australia).

The indirect effect of hypothermia was determined by application of a temperature of 28°C using a thermoregulator and a water bath, 10 min before the application of fluoride (6 mM), thromboxane A2 mimetic (0.1 µM), phenylephrine (1 µM), or phorbol ester (1 µM) 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 diithiothreitol (DTT). Muscles were stored at -80°C until use. Tissues were brought up to room temperature in a dry ice/acetone/TCA/DTT mixture and then homogenized in a buffer containing 20 mM MOPS, 4% SDS, 10% glycerol, 10 mM DTT, 20 mM β-glycerophosphate, 5.5 µM leupeptin, 5.5 µM pepstatin, 20 kIU aprotinin, 2 mM Na3VO4, 1 mM NaF, 100 µM ZnCl2, 20 µM 4-(2-aminoethyl) benzenesulphonic fluoride (AEBSF), and 5 mEGTA. Protein-matched samples (modified Lowry protein assay, DC Protein Assay Kit, Bio-Rad, Hercules, CA, USA) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Protgel, National Diagnostics, Atlanta, Georgia, USA), transferred to polyvinylidene fluoride (PVDF) membranes, and subjected to immunostaining and densitometry using primary and secondary antibodies. 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 image.

**Chemicals and antibodies**

Sodium fluoride, KCl, phenylephrine, acetylcholine, U46619, Y-27632, 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 (Cell Signaling Technology, Danvers, MA, USA or Upstate Biotechnology, Lake Placid, NY, USA) were used to determine levels of RhoA/Rho-kinase activity (Woolridge et al., 2004; Wilson et al., 2005) or MEK activity. Anti-mouse IgM (goat) and anti-rabbit IgG (goat) conjugated with horseradish peroxidase were used as secondary antibodies (1:2,000 dilutions for both, Upstate). A specific MLCP20 antibody (1:1,500, Sigma) and anti-mouse IgG (goat) conjugated to horseradish peroxidase (1:2,000, Upstate) were used to determine the level of LC20 phosphorylation.

**Statistics**

Data are expressed as mean ± standard error of the mean.
**RESULTS**

**Effect of hypothermia on contractions of endothelium-denuded aortas induced by the full RhoA/Rho-kinase activator fluoride**

Endothelium was removed by gentle abrasion with a cell scraper to identify the direct effect of hypothermia on vascular smooth muscle. The absence of endothelium was confirmed by a lack of relaxation after treating contracted ring segments with acetylcholine (1 µM). Moderate hypothermia (28°C) had no significant effect on basal tension (data not shown), but significantly inhibited contraction induced by the Rho-kinase activator fluoride, regardless of the absence of endothelial nitric oxide synthesis (Fig. 1A) or intact (Fig. 1B) muscles. This suggests that the relaxation mechanism of hypothermia might involve the inhibition of Rho-kinase activity in addition to endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase.

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

To confirm the role of hypothermia on thick filament regulation of smooth muscle contractility, we measured levels of myosin phosphatase targeting subunit 1 (MYPT1) and phospho-MYPT1 in muscles quickly-frozen after 60 min exposure to hypothermia or normothermia for equilibration. Each relaxing ring was equilibrated in organ bath solution for 30-60 min before relaxation responses to hypothermia were measured. Data are expressed as the mean of 3-5 experiments with the vertical line representing SEM. *p<0.05, **p<0.01, presence versus absence of hypothermia.

**Effect of hypothermia or normothermia on thromboxane A2-induced vascular contraction in denuded muscles.** Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to hypothermia were measured. Data are expressed as the mean of 3-5 experiments with the vertical line representing SEM. *p<0.05, presence versus absence of hypothermia.

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**Fig. 1.** Effect of hypothermia or normothermia on fluoride-induced vascular contraction in denuded (A) or intact (B) muscles. Each ring was equilibrated in organ bath solution for 30-60 min before relaxation responses to hypothermia were measured. Data are expressed as the mean of 3-5 experiments with the vertical line representing SEM. *p<0.05, **p<0.01, presence versus absence of hypothermia.

**Fig. 2.** Effect of hypothermia on fluoride-induced increases in phospho-MYPT1 level. Phospho-MYPT1 protein level decreased in rapidly-frozen hypothermia-treated rat aorta in the absence of endothelium compared to normothermia-treated rat aortas contracted 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 mean of 3-5 experiments with the vertical line representing SEM. *p<0.05, **p<0.01, versus control or normal group respectively. Hypothermia: 28°C; Fluoride: 6 mM sodium fluoride.
ed with 6 mM fluoride. This work was conducted using quick frozen moderate hypothermia (28°C)-treated rat aortas in the absence of endothelium, and levels were compared to those of normothermia-treated rat aortas (Fig. 2). Interestingly, a significant decrease in fluoride-induced MYPT1 phosphorylation at Thr655 in response to hypothermia treatment was observed (Fig. 2). Thus, thick or myosin filament regulation, including myosin phosphatase activation via RhoA/Rho-kinase inactivation, might be involved in the reduced contractility of hypothermia-treated rat aortas.

Effect of hypothermia on contraction of denuded aortas induced by the dual Rho kinase and MEK activators thromboxane A₂, and phenylephrine

Moderate hypothermia (28°C) inhibited thromboxane A₂ mimetic U46619- (Fig. 3) or phenylephrine-induced contraction (Fig. 4) of denuded muscles, suggesting that dual activators (thromboxane A₂ mimetic and phenylephrine) act similarly to full activators targeting Rho-kinase.

Effect of hypothermia on contractions of denuded aortas induced by the MEK activator phorbol 12, 13-dibutyrate

Phorbol esters are primarily MEK activators and partial Rho-kinase activators (Goyal et al., 2009; Je and Sohn, 2009). Interestingly, phorbol 12,13-dibutyrate (PDBu)-induced contraction was inhibited by moderate hypothermia (28°C), regardless of endothelial nitric oxide synthesis in denuded muscles (Fig. 5), which suggested that thin or actin filament regulation including MEK/ERK was inhibited.

Fig. 4. Effect of hypothermia or normothermia on phenylephrine-induced vascular contraction in denuded muscles. Each ring was equilibrated in the organ bath solution for 30-60 min before relaxation responses to hypothermia were measured. Data are expressed as the mean of 3-5 experiments with the vertical line representing SEM. *p<0.05, presence versus absence of hypothermia.

Fig. 5. An original tracing of phorbol ester-induced vascular contraction in a normothermia (A) or hypothermia (B)-treated denuded rat aorta.
Effect of hypothermia on levels of ERK1/2 phosphorylated at Thr-202/Tyr-204

To confirm the role of hypothermia on thin filament regulation of smooth muscle contractility, we measured levels of ERK1/2 and phospho-ERK1/2 in muscles quick-frozen after 60 minutes of exposure to hypothermia for equilibration. Each relaxing ring was contracted with 1 µM phorbol 12,13-dibutyrate. As compared with normothermia-treated rat aortas, a significant decrease in ERK 1/2 phosphorylation at Thr202/Tyr204 was observed in moderate hypothermia (28°C)-treated rat aortas in the absence of endothelium (Fig. 6); full vasorelaxation (Fig. 5) and thin filament regulation were observed. These findings indicate that thin or actin filament regulation, including ERK1/2 phosphorylation via MEK activation, plays an important role in hypothermia-induced relaxation.

Effect of hypothermia on the level of LC20 phosphorylation

To investigate if hypothermia affected fluoride-induced MLC20 phosphorylation, aortic rings were treated with hypothermia 10 min before the addition of fluoride. Each relaxing ring was contracted with 6 mM sodium fluoride. This work was performed using quick-frozen hypothermia (28°C)-treated rat aortas in the absence of endothelium, and levels were compared to those of normothermia-treated rat aortas (Fig. 7). A significant decrease in fluoride-induced LC20 phosphorylation was found in response to hypothermia treatment (Fig. 7) or Y27632 (Rho kinase inhibitor) treatment. Thus, the reduced contractility of hypothermia-treated rat aorta may involve thick or myosin filament regulation via mechanisms including calcium immobilization and MLCK inactivation.

DISCUSSION

The present study demonstrates that moderate hypothermia can modulate vascular contractility in an endothelium-independent manner. Interestingly, the mechanism involved seems not only to be endothelium-dependent, but also to involve the inhibition of MEK and Rho-kinase activity. Hypothermia resulting from extreme exposure to cold, alcohol intoxication, low blood sugar, anorexia, and advanced age has been previously recognized for its sympathetic nervous system excitation and hepatic dysfunction. Therefore, we investigated whether the inhibition of RhoA/Rho-kinase or MEK activity contributed to hypothermia-induced vascular relaxation in denuded rat aortas contracted by a Rho-kinase activator (fluoride), a MEK activator (phorbol 12,13-dibutyrate), or a dual activator (thromboxane A$_2$ and phenylephrine).

The mechanism by which phorbol esters or thromboxane A$_2$ mimetics activate MEK/ERK has been established (Gu et al., 2007). In contrast, previous studies that examined the mechanisms underlying arterial contractions induced by fluoride, thromboxane A$_2$, or phenylephrine have reported conflicting findings with regard to contractions triggered by Rho-kinase activation (Wilson et al., 2005; Tsai and Jiang, 2006). These findings are consistent with the notion that hypothermia can decrease fluoride-, thromboxane A$_2$, mimicetic-, and phorbol ester-induced contraction by inhibiting MEK or Rho-kinase activity.

The mechanisms by which MEK or Rho-kinase activation cause vascular contraction are being studied intensively, and several possibilities exist. Rho-kinase phosphorylates myosin light chain phosphatase, which decreases phosphatase activity and causes a buildup of phosphorylated myosin light chains (Pfitzer, 2001; Akata, 2007). Rho-kinase has also been dem-
onstrated to phosphorylate myosin light chains directly and independently of myosin light chain kinase and phosphatase activity (Amano et al., 1996). Recently, Rho-kinase was found to be involved in vascular contractions evoked by fluoride, phorbol esters or thromboxane A2 (Wilson et al., 2005; Jeon et al., 2006; Tsai and Jiang, 2006).

The present study demonstrated that hypothermia ameliorated contraction induced by vasoconstrictors (fluoride or phorbol ester) in an endothelium-independent manner (Fig. 1, 3, 4, 5), and that the mechanism involved the MEK/ERK and RhoA/Rho-kinase pathways. Previously, most vasodilation was believed to be caused by endothelial nitric oxide synthesis and the subsequent activation of guanylyl cyclase (Taubert et al., 2002; Ajay et al., 2003; Zou et al., 2015). In the present study, moderate hypothermia significantly inhibited fluoride- or phorbol ester-induced contraction, regardless of endothelial function (Fig. 1, 5). Furthermore, hypothermia significantly decreased the phosphorylation of MYPT1 at Thr205 induced by fluoride (Fig. 2) with full relaxation (Fig. 1) and ERK 1/2 phosphorylation at Thr202/Tyr204 induced by a phorbol ester (Fig. 6), suggesting that inhibition of MEK or Rho-kinase activity is a major mechanism underlying the effects of hypothermia on smooth muscle contractility. MLC20 is known to be phosphorylated by both MLCK and Rho-kinase (Somlyo and Somlyo, 2003). Activation of Rho-kinase by phorbol ester or fluoride inhibits the activity of myosin light chain phosphatase through phosphorylation of MYPT1, leading to an increase in MLC20 phosphorylation as well as contractions (Sakurada et al., 2003; Wilson et al., 2005; Akata, 2007). Both phorbol ester and fluoride induced phosphorylation of MLC20, and this was inhibited by Y27632 (Ferland et al., 2016) and hypothermia (Fig. 7).

In summary, moderate hypothermia significantly attenuated contractions induced by the RhoA/Rho-kinase activator fluoride independent of endothelial function. Furthermore, moderate hypothermia significantly inhibited phorbol ester-induced contraction due to MEK activation. Thus, the mechanism underlying the relaxation induced by moderate hypothermia of fluoride- or phorbol ester-induced contractions involves inhibition of MEK and Rho-kinase activity. Inhibition of Rho-kinase activity and subsequent MYPT1 phosphorylation induced by hypothermia during fluoride-induced contraction suggests that Rho-kinase inactivation is required for relaxation. In conclusion, in addition to endothelial nitric oxide synthesis in intact muscle, both MEK and Rho-kinase inhibition contribute synergistically to hypothermia-induced vasorelaxation of denuded smooth muscle.

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