The effect of hot and cold temperature on the expression of nitric oxide related signal factors in the rat skeletal muscle during acute exercise

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ABSTRACT

The purpose of this study was to investigate the effect of hot and cold temperature on the expression of nitric oxide related signal factor in the rat skeletal muscle during acute exercise. Male Sprague-Dawley rats ($n = 60$) were randomly assigned to one of three temperature groups (normal = 20°C, hot = 34°C, cold = 4°C). The Rats ran the treadmill running for the intensity of 90min at 26M/min with slope degree 10°. Solues and extensor digitorum longus muscle were exercised during the given exercise(30, 60, 90min). The western blotting and ELISA analysis were performed for the expression of nitric oxide(NO), eNOS, GC, and PKG protein. The NO expression was not significantly decreased in cold and hot temperature. However, eNOS, GC, and PKG protein were significantly decreased in cold and hot temperature. In this study, eNOS, GC, and PKG protein expressions were affected by cold and hot temperature. Cold and hot temperature can affect the vasodilation of vascular muscle cells through the changes in NO related vasodilation signal factors.

Keywords: NO, eNOS, GC, PKG, Hot and cold temperature

INTRODUCTION

In modern society, lack of physical activities has been known to increase the risk of cardiovascular diseases such as hypertension, high cholesterol, obesity and insulin-resistance. Exercise has been recommended as an active method to prevent and treat such diseases. Despite unidentified its mechanism, exercise has been known to prevent cardiac diseases and to resolve adults diseases such as hypertension and obesity [3]. In particular, studies have been recently actively conducted on metabolic substances that play an important role in the prediction and prevention of cardiovascular diseases, of which nitric oxide (NO), which has been known to be produced in the endothelial cells and be involved in vasodilation, has been actively studied [16,17].

NO has been reported to be produced when L-arginine is converted into L-citrulline by Nitrite oxide synthase (NOS) in the endothelial cells [1], and subsequently to spread into the smooth muscle cell inside the vessel, leading to the activation of Guanylate cyclase (GC) which is a main receptor

of NO[9]. The activated GC has been reported to increase cyclic guanosine monophosphate (cGMP) production, and to activate angiotensin II (ANG II) signal pathway related to the expansion and relaxation of the muscle and vessel, and to inhibit endothelin-1 signal pathway by cellular calcium concentration via cGMP dependent protein kinase (PKG) [10,30].

Several studies have suggested that NO could regulate blood pressure and NO activity reduction could cause hypertension [3,13]. Vascular muscle cell (VMC), which constitutes the inner wall of the blood vessel and plays an important role in vasodilation, has been reported to sustain basal tone in an normal condition [30]. The basal tone can be achieved due to the existence of certain amount of Ca^{2+} level in the VMC. NO is produced in the vascular endothelial cells by stimulus like exercise, which causes PKG/Protein Kinase A (PKA) activation. Subsequently, phospholamban activates Ca^{2+} pump, resulting in vasodilation [21,30]. Therefore, reduction in NO activity has been reported to cause arterial stiffness and systolic hypertension. In addition, compared to

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healthy adults, hypertension patients have been reported to show higher blood pressure during exercise due to the impairment of arterial vasodilation caused by decreased functions of endothelium-derived relaxing factors (EDRFs) such as NO, epinephrine, acetylcholine, adenosine diphosphate (ADP), vasopressin, endothelin, and serotonin [13,21]. Therefore, as NO change conferred directly associated with hypertension, it is essential to investigate the relationship between NO and NOS related factors and the prevention and treatment of hypertension[17].

NO, which is a vascular relaxing factor, has been known to activate reactions related to vasodilation in the endothelial cells that are stimulated by mechanical shear stress, vascular stimulus due to muscle expansion by contraction, oxygen deficiency and neuronal stimulus, and then express NOS which causes NO production from L-arginine[28]. NOS has been known to exist as three cellular isoforms such as endothelial NOS (eNOS), neuronal NOS nNOS), and cytokine inducible NOS (iNOS), among which eNOS is closely associated with changes in EDRF involved in vasodilation in the skeletal muscle cells[13,21].

Studies on the correlation of exercise and NO with blood pressure have been mainly conducted on exercise type [1,19] and the effect of NO change on blood pressure during acute exercise and endurance training [4,16] to date. In addition, the mechanism of blood pressure change has been described based on EDRF change. However, for sudden death which has recently become an issue in sports, heart attack has been reported to be attributable to a rapid increase in exercise intensity and exercise in hot or cold environment. Therefore, local temperature change in hot or cold environment may exert burden on the heart and cause hypertension, thereby threatening health [15,18]. However, despite the significant importance of such factors, studies on changes in vascular relaxing substances that may significantly affect blood pressure in hot and cold environments have been insufficiently conducted.

Accordingly, this study was conducted to investigate

changes in NO, vasodilators, and EDRF signal factors such as eNOS, GC, and PKG during exercise in hot (34°C) and cold (4°C) environments, and to establish a theoretical evidence on the mechanism of blood pressure based on changes in exercise environments.

METHODS

Subject

Male Sprague-Dawley rats (n = 60) with the same age (10 weeks) were assigned to three groups (20 rats per group) <Fig 1>. The laboratory animals were bred in plastic cages (two animals per cage) with temperature 20-23°C, humidity 70-80%, and 12-hour dark and light cycle. The laboratory animals selected were fed with commercial hard food (protein 22.5%, lipid 3.5%, low fiber 7.0%, ash 9.0%, Ca 0.7%, P 0.5%; Korea animal) and water during the study.

Experimental methods

Before experiment, all laboratory animals were randomly assigned into the acute exercise group, and took an adaptive exercise in the treadmill (Omnipacer model LC-4, Omnitech columbus) for rodents for the intensity of 10-15 minutes at 5-20m/min with slope degree 0° for 2-3 days one week before the experiment. After adaptation period, the laboratory animals were placed at temperature at which the acute exercise was planned to be practiced (Fig. 1) for 15 minutes. Subsequently, the animal groups ran the treadmill running at the experimental temperature (Fig 1) with a velocity of 26m/min corresponding to approximately 75% of rodent VO_2max and slope degree 10° for 90 minutes [2]. The normal temperature group and the cold temperature group normally completed the exercise, whereas the hot temperature group was exhausted around 60 minutes, failing to complete 90-minute exercise. Thus, the hot temperature group was

Fig 1. subject and experimental protocol

excluded from the analysis of 90-minute exercise. After exercise, cervical dislocation was performed on all the experimental animals collected at each sampling time, and the soleus (SM) and extensor digitorum longus (EDL) were then taken and stored at -80°C before analysis.

Analysis methods

Protein extraction and western blotting

For protein extraction, protease inhibitor (Roche, Mannheim, Germany) containing solution was added to lysis buffer (Roche, Mannheim, Germany), and skeletal muscle homogenized and centrifuged at 14,000rpm for 30 minutes. After separating the supernatant in the centrifuged solution, the supernatant $2\mu\text{l}$ was added to protein assay solution (BioRad, CA, USA) 1ml and the protein content was then quantified using a spectrophotometer (Eppendorf, CA, USA) at 560 nm absorption. For western blotting, $30\mu\text{g}$ quantified protein was mixed with water and SDS-gel loading solution to make $20\mu\text{l}$, followed by denaturation at 100°C for 10 minutes. The denatured protein ($20\mu\text{l}$) was loaded on 8-12% SDS-polyacrylamide gels prepared by Mini-Protein II dual-lab apparatus (BioRad, CA, USA) along with SDS-PAGE molecular weight marker (BioRad, CA, USA), followed by electrophoresis at 100 Volt for 2 hours. After electrophoresis, for the transfer of the protein into $0.45\mu\text{m}$ PVDF membrane (BioRad, CA, USA), the SDS-gel with the protein, PVDF membrane and 3M paper were overlapped in transfer solution (190mM glycine, 50mM Tris-base, 20% methanol, .05% SDS) using Mini trans-module (BioRad, CA, USA), followed by transfer at 100 Volt for 2 hours. The membrane with the transferred protein was placed in phosphate-buffered saline (PBS-NaCl 8g, KCl 0.2g, Na_2HPO_4 1.44g, KH_2PO_4 0.24g, pH7.4) with 5% skim milk or 3% bovine serum, followed by blocking in a rocker platform (Fine PCR, Korea) for $4^{\circ}\text{C}/12$ hours. Then, the membrane was washed three times in PBS with 0.1% tween-20 (PBST) for 10 minutes. The washed membrane was placed in the solutions with primary antibodies such as polyclonal eNOS (Santacruz biotechnology, Santacruz, USA), guanylate cyclase (Santacruz biotechnology, Santacruz, USA), and PKG (Santacruz biotechnology, Santacruz, USA) for 2 hours, followed by washing-out in PBST for $1 \times 15\text{min}$ and $2 \times 5\text{min}$. After the reaction with the primary antibodies, the membrane was placed in the solution with mouse/rabbit anti-rabbit IgG conjugated secondary antibody (Santacruz biotechnology, Santacruz, USA) for 2 hours,

followed by washing -out in PBST for $1 \times 15\text{min}$ and $2 \times 5\text{min}$. Then, the membrane was placed in ECL detection reagent (GE, CA, USA) solution (1ml) for 1 minute, and the relative intensity of immuno reactive bands was quantified using an imaging system, Bio-Rad chemidoc XRS (BioRad, CA, USA).

Analysis of blood nitric oxide

NO in the blood was analyzed using Griess assay used by Zago *et al.* (2010) and Ui Soo An *et al.* (2003). Griess reagent (Promega) was used for analysis. Sulfanilamide solution and NED solution were mixed with a ratio of 1:1 and was placed at room temperature (20°C) for 15 minutes, and was then mixed with $50\mu\text{l}$ serum separated blood sample. Then, the sample was placed at room temperature for 10 minutes in a dark condition, followed by measurement with ELISA (Bio-rad, CA, USA) reader at 530nm.

Statistical analysis

The mean and standard deviation were calculated using SPSS Win 12.0 software. T-test for differences by time period, and one-way ANOVA for validating the mean difference among the groups were applied. If $p < .05$, the hypothesis was accepted. A back-testing was conducted using Duncan method if a significant difference was found in the results of analysis.

STUDY RESULTS

Changes in blood nitric oxide by temperature difference during acute exercise

As shown in Fig 2, the change in blood nitric oxide was shown to gradually increase at normal temperature but to slightly decrease and then gradually increase at hot and cold temperature as the exercise time increased. However, the

Fig 2. Changes in NO concentration during acute exercise.

results were not statistically significant in all the groups.

Changes in eNOS in the extensor digitorum longus and the soleus by temperature difference during acute exercise

As shown in Fig 3, eNOS in the extensor digitorum longus was shown to significantly increase from 6.8 ± 1.1 to 9.61 ± 1.36 at normal temperature ($p < .05$) as the exercise time increased. Meanwhile, no significant increase was shown at hot temperature until the exercise was discontinued. eNOS in the extensor digitorum longus was shown to significantly decrease from 6.1 ± 0.46 to 4.98 ± 0.4 at low temperature 30 minutes after exercise initiation ($p < .05$) and then increase. However, no significant increase was shown until the exercise was finished compared to the control group.

eNOS in the soleus was shown to significantly increase from 6.25 ± 0.55 to 10.72 ± 1.2 at normal temperature as the exercise time increased ($p < .05$). Meanwhile, it was shown to significantly decrease from 6.77 ± 0.45 to 5.46 ± 0.55 and from 6.14 ± 0.15 to 4.35 ± 0.79 at hot and low temperature, respectively, 30 minutes after exercise initiation ($p < .05$).

*, **, ***: $p < .05$ vs same exercise time
a, b: $p < .05$ vs exercise time in normal temperature
E, f: $p < .05$ vs exercise time in Hot temperature
i, j: $p < .05$ vs exercise time in cold temperature

Fig 3. The expression of eNOS in the EDL and SOL in each group

Therefore, hot and low temperatures are likely to affect the expression of eNOS in the extensor digitorum longus and the soleus during the exercise.

Changes in GC in the extensor digitorum longus and the soleus by temperature difference during acute exercise

As shown in Fig 4, GC in the extensor digitorum longus was shown to significantly increase from 8.15 ± 0.4 to 10.45 ± 0.45 at normal temperature until 60 minutes after exercise initiation ($p < .05$) and then decrease to normal level as the exercise time increased. It was shown to slightly decrease at hot temperature 30 minutes after exercise initiation, which was not statistically significant. It was shown to significantly decrease from 7.32 ± 0.56 to 6.57 ± 0.15 at low temperature 30 minutes after exercise initiation ($p < .05$) and then increase as the exercise time increased.

GC in the soleus was shown to significantly increase from 7.88 ± 0.17 to 9.35 ± 0.11 at normal temperature 60 minutes after exercise initiation ($p < .05$). Meanwhile, it was shown to significantly decrease from 8.03 ± 0.15 to 7.11 ± 0.2 , and

*, **, ***: $p < .05$ vs same exercise time
a, b: $p < .05$ vs exercise time in normal temperature
E, f: $p < .05$ vs exercise time in Hot temperature
i, j, k: $p < .05$ vs exercise time in cold temperature

Fig 4. The expression of GC in the EDL and SOL in each group

from 8.31 ± 0.31 to 6.61 ± 0.2 ($p < .05$) at hot and low temperatures, respectively, 30 minutes after exercise initiation and then increase. Therefore, in the extensor digitorum longus and the soleus, temperature change during the exercise is likely to inhibit GC increase during the early phase of the exercise.

Changes in PKG in the extensor digitorum longus and the soleus by temperature difference during acute exercise

As shown in Fig 5, PKG in the extensor digitorum longus was shown to significantly increase from 9.19 ± 0.1 to 10.45 ± 0.31 at normal temperature 60 minutes after exercise initiation ($p < .05$) and then decrease to the normal level. Meanwhile, it was shown to significantly decrease from 9.51 ± 0.35 to 7.96 ± 0.65 at hot temperature 30 minutes after exercise initiation ($p < .05$) and continue to decrease until the exercise was finished. PKG in the extensor digitorum longus was shown to significantly decrease from 8.55 ± 0.3 to 3.15 ± 0.67 30 minutes after exercise initiation ($p < .05$) and then continued to decrease until the exercise was finished.

PKG in the SM was shown to significantly increase from 8.96 ± 0.15 to 10.35 ± 0.5 at normal temperature until 60 minutes after exercise initiation ($p < .05$) and then decrease. It was shown to slightly increase at hot temperature 30 minutes after exercise initiation, which was not statistically significant. It was shown to significantly decrease from 9.01 ± 0.1 to 7.08 ± 0.1 60 minutes after exercise initiation ($p < .05$). Meanwhile, it was shown to significantly decrease from 8.55 ± 0.3 to 5.64 ± 0.72 at cold temperature 30 minutes after exercise initiation ($p < .05$) and then continued to decrease. Based on these results, changes in temperature during exercise is likely to affect PKG activity. In particular, cold temperature is likely to inhibit PKG increase.

DISCUSSION

NO is an important factor of vascular regulation. In particular, the activities of NO and eNOS are closely associated with the development and regulation of hypertension in vascular relaxation [3,17]. In this study, among the EDRFs, important factors of vasodilation, NO and NO-related signal factors were shown to have differences in their expression according to exercise thermal environments. Therefore, this result suggests that the thermo-dependent change in signal factors related to eNOS in the skeletal muscle could become a potential factor to restrict vascular relaxation during exercise.

In general, an exposure to cold or hot temperature for a certain period has been reported to increase blood pressure, which is attributable to EDRF change [24,28,29]. Short- or long-term cold condition has been reported to affect sympathetic nerve system and renin-angiotensin system and to increase heart rate [5,28,31]. However, due to skeletal muscle use during exercise, blood flow caused by an increased metabolic oxygen requirement and shear stress has been reported to increase, which activated eNOS in the endothelial cells in the skeletal muscle, thereby increasing NO production [21].

Studies on the correlation of NO production with body temperature during exercise reported that NO production was reduced in animals exposed to cold environment [29]. In this study, NO was generally shown to tend to decrease cold environments compared to normal environment although it was not significant. Cold temperature has been known to suppress sympathetic nerve system and renin-angiotensin system [13,29], whereas hot temperature has been known to cause dehydration and to increase endothelin-1 (ET-1) [20]. ET-1 increase has been reported to suppress eNOS activity

Fig 5. The expression of PKG in the EDL and SOL in each group

and inhibit NO, causing vascular contraction and blood pressure increase [31]. However, ET-1 increase in the blood and muscle tissue has been reported to be caused by continuous temperature change with more than one week [10,29]. Furthermore, changes in vascular contracting factors continuously exposed to cold environment are not suitable for elucidating the methods and results of this study on acute exercise. Therefore, reduced vasodilation capacity due to rapid temperature change as shown in this study is likely to be more attributable to decreased NO production or change in other vascular relaxing factors rather than ET-1 [12,20].

Several related studies suggested that eNOS is activated by the physical stimulation of the endothelial cells due to mechanical stimulus caused by skeletal muscle use or rapidly increasing blood flow in the skeletal muscle after exercise initiation and that subsequent eNOS activation causes NO increase, playing an important role in vasodilation during exercise [7,31]. In addition, this tendency can be various depending on changes in the utilization level of the skeletal muscle by continuous exercise [7,27]. In particular, in this study, NO and eNOS were shown to gradually increase at normal temperature as the exercise time increased, whereas they were shown to significantly decrease at hot and cold temperatures at the early phase of the exercise. In particular, such tendency was more significantly seen in the extensor digitorum longus that contains more fast-twitch muscle fiber used in the early phase of the exercise. Therefore, cold environment is likely to reduce NO production by inhibiting eNOS activity in the requirement of blood flow increase due to change in the utilization rate of the skeletal muscle, and become an important factor of decreased activity of GC and PKG related to vasodilation.

NO produced via exercise spreads into the vascular muscle cell (VMC) of the vessels, and activates guanylate cyclase (GC), a cellular NO receptor [9]. The activated GC increases cyclic GMP (cGMP) production and cGMP dependent protein kinase (PKG) activates Ca^{2+} pump via phospholamban, which results in the relaxation of the smooth muscle cell by releasing Ca^{2+} ions inside the VMC [21,31]. In addition, the activation of K channel pump due to the production of GC or oxygen free radical is also involved in such relaxation process [30,31].

In this study, no significant increase/decrease was shown in the blood NO at normal temperature. Meanwhile, eNOS, GC and PKG were shown to not only significantly increase as the exercise time increased, but also significantly decrease/increase depending on temperature. This result suggests that the exercise at normal temperature causes vasodilation via the sequential activation of $\text{eNOS} \rightarrow \text{NO} \rightarrow \text{GC}$

$\rightarrow \text{PKG}$ and these signal factors are important in the mechanism of vasodilation [25,31]. In particular, compared to NO changes at hot and cold temperatures, the signal factors were shown to significantly decrease in the early phase of the exercise in this study. Therefore, it is anticipated that the measurement of blood GC or cGMP level is more accurate than NO level which has a short half-life or is relatively unstable for the elucidation of vasodilation [25].

However, until now, studies on the relationship among exercise, vascular contraction and vasodilation, and temperature have been mainly conducted on ET-1 and ANG II expressions, but insufficiently conducted on vasodilation related to changes in GC and PKG [6,9]. A rapid temperature change in a short time causes negative effects such as increase in heart rate and blood pressure [28]. Therefore, changes in signal factors such as GC or PKG by temperature change can suggest results important for the mechanism of blood pressure change. In particular, GC can not be observed in the proteins in the skeletal muscle but be only observed in the vascular pericyte or plasma [9,25]. Therefore, the result obtained from the skeletal muscle in this study is likely to be attributable to changes in VMC contained in the skeletal muscle, and to be used as more accurate marker for vasodilation. In addition, GC and PKG are closely associated with thermoregulation according to temperature [8,23,26], and Ca^{2+} in the VMC plays an important role in vascular relaxation via PKG signal regulated by the transfer of ROS or electrolyte during exercise, as well as NO [11,30,32]. Therefore, it is necessary to investigate factors related to calcium signal factor in the early phase of exercise or continuous exercise. However, in this study, the expression patterns of Ca-related signal factors, such as cAMP and K_{ATP} channel, which may affect Ca metabolism during exercise like ROS or 3-phospho inositol dependent kinase (PKC), and ET-1 and ANG II, which are suggested as important factors of vascular contraction, were not directly investigated. [3,6,11,32] Therefore, a further study on the effect of changes in ROS and antioxidant enzymes on signal factors according to temperature change is required [33]. In addition, a further study is required to investigate pattern changes in a training program [10] rather than in a short-term exercise.

CONCLUSION

In this study, changes in exercise environment (temperature) were shown to affect EDRF signal factors. In particular, as GC and PKG showed decreased activities due to temperature change, they are likely to act as factors that inhibit vasodilation.

Therefore, changes in GC and PKG levels in the blood and tissues according to temperature will be able to be used as important markers for predicting hypertension risk caused by exercise, and further used for elucidating the mechanism of sudden death due to exercise. In addition, a further study is required to investigate the association of Ca signal factors related to vasodilation with increasing factors in the early phase of exercise and factors related to continuous exercise.

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