Effects of walking combined with restricted leg blood flow on mTOR and MAPK signalling in young men

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Abstract
Walking combined with blood flow reduction (BFR-walk) elicits muscle hypertrophy. However, the skeletal muscle intracellular signalling behind this response is currently unknown.

Aim: To investigate the effects of BFR-walk on mechanistic target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signalling pathways in young men.

Methods: Six young men performed 20 min of treadmill walking at 55% of their predetermined maximum oxygen uptake. A pressure cuff belt was applied to the most proximal thigh of only one leg (BFR-Leg, external compression was 240 mmHg), whereas the other leg (CON-Leg) was without BFR during walking. Muscle biopsies were taken from the vastus lateralis of the CON-Leg before exercise and in both legs 3 h after exercise.

Results: Erk1/2 phosphorylation levels were significantly (P < 0.05) increased after exercise in both legs; however, only the BFR-Leg saw an increased phosphorylation of p38. For mTOR signalling, there were no changes in Akt, mTOR or S6K1 phosphorylation levels before or after walking. However, eEF2 phosphorylation level was significantly (P < 0.05) lower for the BFR-Leg 3 h after walking compared with CON-Leg.

Conclusion: BFR-walk exercise may activate some intracellular signalling cascades that are associated with muscle hypertrophy in young men.

Keywords cell signalling, low-intensity walking, muscle, occlusion.
et al. 2009b). These results suggest that activation of the mTOR signalling pathway plays a key role in increased muscle protein synthesis. Meanwhile, extracellular signal-regulated kinase (Erk)-MAPK and p38-MAPK signalling are also connected to the protein translational machinery by 90 kDa ribosomal S6 kinase (RSK) and mitogen-activated protein kinase-interacting kinase (Mnk) (Mahoney et al. 2009). RSK targets the same sites as S6K1, which provides redundancy between the MAPK and mTOR signalling pathways (Anjum & Blenis 2008).

In the past decade, a large body of evidence has shown that low-intensity (20–30%1RM) resistance training induces muscle hypertrophy when combined with blood flow restriction (BFR) to working muscle (Loenneke et al. 2012c). Although the mechanism responsible for muscle hypertrophy with low-intensity resistance training with BFR (LR-BFR) is less clear compared with high-intensity resistance training, some studies have demonstrated that a bout of LR-BFR increased muscle protein synthesis through the activation of both mTOR and MAPK signalling pathways (Fujita et al. 2007, Fry et al. 2010, Gundermann et al. 2012). Other proposed methods include acute muscle cell swelling (Yasuda et al. 2012), increased fiber type recruitment form metabolic accumulation (Yasuda et al. 2009, 2010), decreased myostatin (Laurentino et al. 2012), decreased atrogenes (Manini et al. 2011) and the proliferation of satellite cells (8 days into training intervention, 3 and 10 days after cessation of training) (Nielsen et al. 2012).

Meanwhile, this muscle hypertrophic effect is also observed with lower-intensity exercise such as walking in combination with BFR (BFR-walk), albeit to a smaller extent (Loenneke et al. 2012c). BFR-walk may be unique in that this stimulus may be able to simultaneously increase aerobic capacity and muscular size, which would be potentially beneficial to many (Ozaki et al. 2013). To illustrate, low aerobic capacity is a risk factor for developing cardiovascular disease in addition to being associated with an increased risk of disability, impaired gait, falls and osteoporosis caused by low muscle mass and strength (Ozaki et al. 2013). However, there is currently no evidence on the mechanisms of muscle hypertrophy with BFR-walk. Therefore, the purpose of this study was to investigate the effect of walking with BFR on the mTOR and MAPK signalling pathways.

Methods

Participants

Six young men (mean ± SE; age: 22 ± 1 years, body mass: 67.0 ± 2.4 kg, height: 1.73 ± 0.02 m, BMI: 22.4 ± 0.6 kg m\(^{-2}\)) volunteered to participate in this study. The subjects were recruited through printed advertisements and by word of mouth and had not participated in a regular exercise programme for at least the previous years. All subjects were non-smokers, were not taking any medication and were free of overt chronic diseases as assessed by self-report. All subjects were informed of the methods, procedures and risks and signed an informed consent document before participating in the study. The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of the Juntendo University (Japan).

Study protocols

This study was composed of the following three tests: (i) a maximal exercise test to determine maximum oxygen uptake (VO\(_2\)max), (ii) a submaximal exercise test to determine a walking intensity and (iii) a main exercise test with muscle biopsies before and after walking at a predetermined intensity. In the main exercise test, BFR was randomly applied to only one leg (BFR-Leg), whereas the other leg was without BFR served as a control (CON-Leg) to compare the effects of walking with and without BFR on cell signalling. This unilateral model allows within-person comparisons under the same environment. Firstly, each subject visited the laboratory twice more than a week before the main exercise test and performed the two preparatory exercise tests described above (the maximal and submaximal exercise tests). For 3 days prior to the main exercise test, all subjects were prohibited from participating in vigorous physical activity, eating in excess, which means to consume their regular meals, ingestion of alcohol and taking a bath, which eliminated any effect of heat stress may have on the activation of cell signalling. On the day prior to the main exercise test, they consumed a standard dinner between 20:00 and 21:00 in the laboratory. After the dinner, subjects were permitted to drink only water until the end of the experiment. The next morning, the subjects visited the laboratory following an overnight fasting and rested quietly in the supine position. After 30 min of quiet resting, the first venous blood sample (Rest) was obtained from antecubital vain, and the first muscle biopsy was taken from a lateral portion of the vastus lateralis of the CON-Leg, which was used as the baseline data. The biopsy was performed using a disposable biopsy instrument (14 gauge, MaxCore; C.R. Bard, Covington, GA, USA) under sterile conditions and local anaesthesia (1% lidocaine). After 1 h of quiet resting, the subjects performed 20 min of
treadmill walking at 55% of predetermined VO$_2$max. Immediately after exercise, the subjects were escorted to a bed where the second venous blood sample was obtained. After that, blood samples were taken at 15, 30, 45, 90 and 120 min post-exercise in a supine position. Finally, 3 h post-exercise, another muscle biopsy (3H) was obtained from each leg. In the CON-Leg, the muscle sample was taken from an incision 2–3 cm away from the incision used in the first biopsy. The muscle biopsies were performed by a doctor proficient with the technique. All muscle tissues were frozen in liquid nitrogen and stored at −80 °C until analyses. Blood lactate and glucose levels were evaluated with single purpose metres immediately after blood sampling. Serum growth hormone (GH), cortisol, insulin, testosterone and insulin-like growth factor I (IGF-I) concentrations were measured immediately after blood sampling. Serum growth hormone levels were evaluated with single purpose metres immediately after blood sampling.

Preparatory exercise test

Maximal exercise test. The subjects performed a graded maximal running test on a treadmill to determine maximum oxygen uptake (VO$_2$max). VO$_2$ was measured using an automated metabolic monitor (Aeromonitor AE-300S, Minato Medical Science, Tokyo, Japan). The test started at 180 m min$^{-1}$ preceded by rest in a seated position for 2 min. The slope was set at 2 degrees and remained constant throughout exercise, and the speed was increased. The speed was increased by 10 m min$^{-1}$ every minute until exhaustion. Each subject’s electrocardiograph was monitored throughout, and heart rate (HR) at 1-min intervals. The criteria for VO$_2$max used in this study were (i) no increase in VO$_2$ despite increased running speed, (ii) HR > 90% of age predicted maximum HR (220-age), (iii) respiratory exchange ratio (RER) >1.10, (iv) blood lactate >10 mmol and (v) ratings of perceived exertion (RPE) >18. Of these criteria, at least three had to be met to be considered as a true VO$_2$max, and all subjects met this requirement.

Submaximal exercise test. All subjects performed a graded walking test to determine exercise intensity (35%VO$_2$max) of walking in the main exercise test because of the differences in energy efficiency between running and walking. VO$_2$ was measured throughout the exercise test. The test started at 100 m min$^{-1}$, and the speed remained constant throughout the exercise test. The slope was increased by 1 degree every 3 min until an exercise intensity for each subject reached approx. 70% VO$_2$max. Each subject’s electrocardiograph was monitored throughout, and HR and RPE were recorded every 3 min.

Exercise protocol and blood flow restriction

In the main exercise test, the subjects performed 20 min of treadmill walking at an exercise intensity of 55% VO$_2$max. The walking speed was 100 m min$^{-1}$ in all subjects, and the mean slope was 3.7 ± 0.2 degrees. Subjects wore elastic cuffs (5 cm wide) (Kaatsu-Master system, Sato Sports Plaza, Tokyo, Japan) on the most proximal portion of BFR-Leg during walking. Before walking, the subjects were seated on a chair, and the upper thigh-mounted cuff was applied with an initial compressive force of about 60 mmHg and firstly inflated at 120 mmHg (the approximate systolic blood pressure at heart level for each subject) for 30 s, and then the pressure was released. The air pressure was increased by 20 mmHg, held for 30 s and then released for 10 s before the next occlusive stimulation was performed. This process was repeated until 240 mmHg was reached. The cuff air pressure was released immediately upon completion of the session.

Protein extraction

Approximately 10–15 mg of frozen muscle tissue was homogenized (1 : 9, wt/vol) in ice-cold buffer (50 mM HEPES, 4 mM EGTA, 10 mM EGTA, 50 mM β-glycerophosphate, 25 mM NaF, 5 mM Na$_3$VO$_4$, 1% Triton X-100, pH 7.4) with protease inhibitors (Complete tablet; Roche Diagnostics Corp., Indianapolis, IN, USA) and phosphatase inhibitors (phosSTOP tablet; Roche Diagnostics). The homogenates were centrifuged at 10 000 g for 10 min at 4 °C to removal the supernatants following rotation for 60 min at 4 °C. Total protein concentrations of the supernatants were determined using a bicinchoninic acid protein assay (Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as the standard. The supernatants were diluted (1 : 1) in a sample buffer (4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.004% bromophenol blue, 125 mM Tris-HCl, pH 6.8) following regulation of the protein concentrations of them to 2.6 mg mL$^{-1}$. All samples were boiled for 5 min at 95 °C.

SDS-PAGE and Western blot analysis

Samples were electrophoresed in a 7.5, 10 or 15% SDS polyacrylamide gel at 150 V for 50 min. Following electrophoresis, the gels were incubated in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 10 min, and then, the protein was transferred to a polyvinylidene difluoride (PVDF) membrane.
(Bio-Rad Laboratories, Hercules, CA, USA) at 100 V for 60 min on ice. After transfer, the membranes were washed (10 min x 3) in Tween Tris buffer saline (T-TBS: 137 mM NaCl, 20 mM Tris, 0.1% Tween 20) and then blocked in PDVF blocking reagent (Toyobo, Osaka, Japan) for 1 h. The membranes were subsequently washed in T-TBS and incubated in primary antibody solution (NKB-201; Toyobo) with a phospho-specific primary antibody (antibody concentrations are described below) for 2 h at room temperature. Following wash in T-TBS, The membranes were incubated in secondary antibody solution (NKB-301; Toyobo) with a rabbit horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After wash in T-TBS, enhanced chemiluminescence reagent (ECL plus; GE Healthcare, Piscataway, NJ, USA) was applied to each membrane. The phosphorylated proteins were visualized using an image analysis apparatus (Light Capture; ATTO, Bunkyo-ku, Japan) following a 5-min incubation. Each band was quantified by computerized densitometry using specialized software (CS Analyzer 3.0; ATTO).

To determine the total levels of each protein, the membranes were re-probed with appropriate antibodies as stated above. The phosphorylation of each protein was expressed relative to the total amount of it. The data were analysed as the fold change in phosphorylation compared with the level before exercise for each condition.

**Antibodies**

The primary and secondary antibodies used were all purchased from Cell Signalling Technology (Beverly, MA): phospho-Akt (Ser473; 1 : 2000), phospho-mTOR (Ser2448; 1 : 2000), phospho-S6K1 (Thr389; 1 : 500), phospho-eEF2 (Thr56; 1 : 2000), phospho-Erk1/2 (Thr202/Tyr204; 1 : 2000), phospho-p38 (Thr180/Tyr182; 1 : 1000), total Akt (1 : 2000), total mTOR (1 : 2000), total S6K1 (1 : 500), total eEF2 (1 : 2000), total Erk1/2 (1 : 2000), total p38 (1 : 1000) and anti-rabbit IgG horseradish peroxidase-conjugated antibody (1 : 10 000).

**Statistical analyses**

Results are expressed as means and standard error (SE) for all variables. The sample size was small, and some of the hormone concentration and protein phosphorylation data were not normally distributed. Therefore, the Wilcoxon signed rank test was used to identify differences in baseline vs. 3H values and in BFR and CON conditions for protein phosphorylation. Changes in hormone concentrations were examined with Friedman’s test. Post hoc testing was performed using the Dunn test when appropriate. Statistical significance was set at $P \leq 0.05$.

**Results**

Mean VO$_2$max and HRmax in the subjects were 3.4 ± 0.2 L min$^{-1}$, 51.3 ± 1.5 mL kg$^{-1}$ per min and 200 ± 3 bpm respectively.

Blood lactate (Fig. 1a) and serum GH (Fig. 2a) concentrations increased immediately after walking followed by a rapid fall towards the resting values. Similarly, serum insulin concentrations also increased rapidly to the peak values 15 min after walking, then promptly dropped towards the baseline (Fig. 2b). However, there were no significant changes in serum cortisol, testosterone or IGF-I and blood glucose concentrations (Figs 1 and 2).

With respect to MAPK signalling, Erk1/2 (Fig. 3a) and p38 (Fig. 3b) phosphorylation levels were significantly ($P < 0.05$) higher following exercise in the BFR-Leg, whereas only the Erk1/2 phosphorylation level increased

![Figure 1](image1.png)  
**Figure 1** Blood lactate and glucose concentration before and after walking. (a) blood lactate concentration. (b) blood glucose concentration. Data are shown as mean (±SE). $n = 6$. 

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significantly ($P < 0.05$) in the CON-Leg. For mTOR signalling, there were no changes in Akt and mTOR phosphorylation levels before or after walking (Fig. 4a,b). In addition, there was no significant difference in S6K1 phosphorylation between BFR and CON conditions although the increase in the BFR-Leg was about twice as high as that in CON-Leg (Fig. 4c). However, eEF2 phosphorylation level was significantly ($P < 0.05$) lower for BFR-Leg 3 h after walking than that of the CON-Leg (Fig. 4d).

Figure 2 Hormone concentrations before and after walking. (a) serum GH concentration. (b) serum insulin concentration. (c) serum IGF-1 concentration. (d) serum testosterone concentration. (e) serum cortisol concentration. Data are shown as mean (±SE). $n = 6$. GH, growth hormone; IGF-1, insulin-like growth factor-1.

Discussion

For the first time, we present data demonstrating that walking in combination with BFR significantly increased phosphorylation of Erk1/2 and p38 phosphorylation level, while eEF2 phosphorylation level was significantly lower for BFR-Leg than that of CON-Leg 3 h post-exercise. These signalling changes may contribute to the BFR-walk-induced muscle hypertrophy observed in the previous training studies.
Erk1/2 and p38 have been implicated as regulators of eEF2 kinase (eEF2K) (Knebel et al. 2001, Wang et al. 2001). The inhibition of eEF2K results in the dephosphorylation of eEF2, which accelerates translation elongation (Redpath et al. 1996). The increase in Erk1/2 and p38 phosphorylation level observed in the present study may result in dephosphorylation of eEF2 through the inhibition of eEF2K. Although not significant, there was a tendency for S6K1 phosphorylation to be higher in the BFR-Leg compared with the CON-Leg. This may also contribute to the inhibition of eEF2K activity as S6K1 regulated translation elongation via phosphorylation of eEF2K (Wang et al. 2001). Thus, the observation that eEF2 phosphorylation was lower in the BFR-Leg may suggest an enhancement in translation elongation. Furthermore, Erk1/2 and p38 phosphorylate the eukaryotic translation initiation factor 4E (eIF4E) through the activation of MAPK-interacting kinase (Mnk) 1 and Mnk2, which promotes translation initiation (Buxade et al. 2008, Mahoney et al. 2009). Thus, these changes in markers of both translation initiation and elongation would potentially have a positive effect on net protein balance.

The magnitude of phosphorylated cell signalling after a bout of BFR-walk appears to be lower compared with previously reported BFR resistance exercise studies (Fujita et al. 2007, Fry et al. 2010). The previous studies have shown that the phosphorylation of several selected proteins in both mTOR and MAPK signalling pathways increased 3 h after a bout of LR-BFR (Fujita et al. 2007, Fry et al. 2010). However, a bout of BFR-walk increased only two selected proteins in the MAPK signalling pathway at the same time following exercise. Furthermore, Erk1/2 phosphorylation level for LR-BFR was about six times as high as that of resting 3 h after exercise (Fry et al. 2010), whereas the increase in that for BFR-walk went no further than four times. These results appear to be in line with the notion that isotonic low-intensity resistance exercise with BFR produces greater muscle hypertrophy compared with walking in combination with BFR (Loenneke et al. 2012a,b,c). The contrast in the magnitude of change in muscle mass may result from inherent differences in the exercise mode and/or external load to the working muscle (Abe et al. 2012).

In this study, GH concentration reached about 16 ng mL$^{-1}$ after a bout of walking, and this value was higher compared with that observed in the previous studies with BFR-walk (Abe et al. 2006) and LR-BFR (Fujita et al. 2007) for young men. The exercise intensity (55%VO$_2$max) set in the present study was higher than that in the previous studies, and GH concentration can elevate significantly during normal walking alone when exercise intensity is more than 40%VO$_2$max (Godfrey et al. 2003). Furthermore, it is known that BFR to working muscles would enhance GH response to exercise (Abe et al. 2006, Fujita et al. 2007). It has been suggested that the exercise-induced elevations in GH contribute to increases in muscle protein synthesis and eventual muscle hypertrophy (Kraemer et al. 1990, Rooyackers & Nair 1997). However, pharmacological administration of GH appears to stimulate collagen synthesis not myofibril muscle protein synthesis (Doessing et al. 2010). Furthermore, the increase in lean body mass observed following GH treatment is not accompanied by increases in muscle strength and appears to increase solely due...
to an expansion of extracellular water volume (Birzniece et al. 2011). Additionally, recent studies have demonstrated that the exercise-induced elevation in GH did not enhance the activation of cell signalling pathways, the increase in muscle protein synthesis or the eventual muscle hypertrophy induced by high-intensity resistance training in humans (West et al. 2009, 2010). Therefore, the GH elevation observed in this study is unlikely to have had a large effect on the activation of cell signalling pathways and may instead be more related to fuel mobilization.

The reasons for the BFR-walk-induced activation of the signalling pathways are unclear, but a few possibilities exist. For example, muscle cell swelling, which is likely due to a fluid shift from the plasma into the muscle cell, may be one of the factors involved in activating mTOR and MAPK signalling (Loenneke et al. 2012a). To illustrate, a previous study found that a bout of BFR-walk exercise transiently increased thigh muscle thickness (Ogawa et al. 2012). Additionally, the higher intensity (55%VO₂max) used in the present study may also contribute to the activation of mTOR and MAPK signalling pathways compared with the previous studies which used lower intensities (Abe et al. 2006, Ozaki et al. 2011, Ogawa et al. 2012), because an increase in exercise intensity may have a positive effect on the activation of these signalling pathways (Widegren et al. 2000, Burd et al. 2010). However, it is clear from the paucity of data that more research is needed to better determine the mechanisms responsible for the signalling pathways activated by BFR-walk.
Interestingly, the present study has also presented the first scientific evidence that normal walking increases Erk1/2 phosphorylation, and this increase was about five times higher than resting. Previous research has observed increases in Erk1/2 phosphorylation during other forms of endurance exercise. For example, Yu et al. (2001) have shown that Erk1/2 phosphorylation increased about 8-fold following a bout of marathon running (Yu et al. 2001). Furthermore, Widegren et al. (2000) have demonstrated that 30 min of cycling at 40% VO2max and 75% VO2max resulted in about 12-fold and 40-fold increases in Erk1/2 phosphorylation (Widegren et al. 2000). In addition to exercise intensity, the activation of Erk1/2 also appears to be sensitive to the number of contractions performed during exercise (Williamson et al. 2003, Burd et al. 2010). Therefore, considering that Erk1/2 phosphorylation appears to increase with increased exercise intensity and the number of contractions in human skeletal muscle, it is plausible that the lower increase in the present study may be due to the mode of exercise. Given that there were no differences between conditions, our results seem to suggest that Erk1/2 phosphorylation may be closely related to the volume of contractions completed during the exercise bout. This is based on previous research showing an ‘intensifying’ effect of BFR on exercise when volume is matched.

Noted limitations of the current study include the use of an arbitrary pressure for each subject as well as the small sample size. For example, some research suggests that the pressure applied in the lower body should be individualized to the size of the leg to which the pressure is applied (Loenneke et al. 2012b). In addition, it is acknowledged that phosphorylation markers of anabolic pathways by themselves do not necessarily predict changes in muscle protein synthesis. Future research could investigate this further by measuring muscle protein balance.

In conclusion, the present study is the first to observe that BFR in combination with walking increased Erk1/2 and p38 phosphorylation levels in young men. Furthermore, eEF2 phosphorylation level is lower for BFR-walk than for normal walking. These changes in cell signalling pathways might be related to the muscle hypertrophic effects with BFR-walk exercise.

Conflict of interest

No conflict of interests to disclose for any authors.

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