

Effect of Acute Interval Walking with Blood Flow Restriction on 4EBP1, ERK, p38, and Myostatin of Skeletal Muscle in Inactive Men

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Abstract

Background: There is mounting evidence that moderate- to high-intensity exercise training has a key role in skeletal muscle adaptation. Low-intensity exercise with Blood flow restriction (BFR) associated with unique effect on muscle hypertrophy. **Aims:** The aim of this study was to investigate the effect of acute interval walking with BFR on phosphorylation of 4EBP1, P38, ERK, and myostatin (MSTN) of skeletal muscle in inactive men. **Materials and Methods:** Five healthy inactive men were participated in the study. Training protocol includes five intervals 2-min walking with BFR at 50%–60% maximum heart rate and 1 min at rest. All samples were collected immediately before exercise and 3 h after BFR training. Phosphorylation of 4EBP1, P38, and ERK skeletal muscle was evaluated by Western blotting and MSTN by Elayza test. Dependent *t*-test was used to analyze the data after subtracting the posttest score from the pretest. **Results:** However, there was a significant difference between the pre- and post-test for 4EBP1 ($P = 0.001$), ERK ($P = 0.049$), and MSTN ($P = 0.0009$). There was no significant difference between the pre- and post-test of P38 ($P = 0/452$) ($P \geq 0.05$). **Conclusion:** As a result, acute interval walking with BFR activates mammalian target of rapamycin and mitogen-activated protein kinase pathways signaling in inactive men.

Keywords: Acute interval waking, blood flow restriction, hypertrophy, phosphorylation

INTRODUCTION

Resistance training with moderate- to high-intensity is associated with skeletal muscle adaptation. Exercise training with blood flow restriction (BFR) is a new method of training, which has a positive role in stimuli skeletal muscle growth factor. Regular resistance training is appropriate for increased strength and size in skeletal muscle^[1] whereas aerobic exercise improves maximum heart rate (MHR) and anaerobic threshold.^[2] Morphologic and physiologic different responses to aerobic and resistance training associated with an increase gene expression of myofibrillar protein, mitochondrial protein content, capillary density, and enzymes related to aerobic system for energy production and variety signaling pathways.^[2] Combination of aerobic and resistance training impaired development of strength and power aerobic in healthy controls.^[3] BFR training has been considered by the

researcher as a new method of training. Abe *et al.* suggested that BFR training can be used as an effective training method for simultaneous improvement in cardiopulmonary fitness and muscular conditioning.^[4] Restriction of blood flow in active skeletal muscle during low-intensity resistance training (15%–20% 1RM) has a similar effect in muscle hypertrophy compared with high-intensity resistance training.^[5,6] It has been shown that strength and hypertrophy significantly increase after low-intensity walking with BFR.^[5,7] In addition, Abe *et al.* and Park *et al.* reported that exercise training with BFR improves endurance capacity (increase oxidative enzyme, capillary

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density, stroke volume, glycogen stores, and reduce heart rate) and increases size and strength of skeletal muscle.^[4,8,9] Abe *et al.* demonstrated that 8 weeks' cycling with BFR compared to cycling without BFR increase MHR (6.4%), cross section area quadriceps, rectus femoris, and strength muscle (3.4, 4.6, and 7.7%, respectively). It suggests that BFR training can use as an effective training method for cardiopulmonary fitness in healthy and athlete participants.^[4]

Skeletal muscle adaptation-induced exercise training was including signaling mechanisms which associated with increase transient in different mRNA genes, arrived to peak at 3–12 h after exercise and after 24 h return to baseline levels.^[10] Chronic exercise training stimuli increase protein synthesis in skeletal muscle. The chronic exercise training stimulus with type of training leads to morphologic and metabolic adaptation within skeletal muscle and ultimately improves exercise capacity. It is well known that BFR training increases the strength and size of the muscle.^[11,12] Several studies demonstrated that Blood flow restriction resistance training through different molecular mechanisms induce adaptation.^[13,14] For muscle hypertrophy, protein synthesis pathways, such as mTOR and mitogen-activated protein kinase (MAPK), should be stimulated. Various factors can stimulate these paths, which are as follows: mechanical stress, metabolic stress, muscle injures, systematic an local hormones, reactive oxygen species production, heat-shock protein, insulin-like growth factor-I (IGF-I)/PI3K/Akt/mTOR signaling pathway, and satellite cell activity are listed as hypertrophy induce BFR resistance training.^[15] Myostatin (MSTN) is a negative potent factor that regulates skeletal muscle mass.^[16] Reduction of MSTN expression is associated with increased mTOR signaling pathway activity, protein synthesis, volume, and skeletal muscle mass.^[16] Although it is reported that BFR resistance training reduced MSTN expression, according to our knowledge, interval walking with BFR affects the amount of myostatin, which is likely to increase muscle mass.

As aforementioned, previous studies reported that walking with BFR increases the size and muscle mass.^[7,11] Abe *et al.* for the first study shown that volume and isometric strength of rectus femoris increased after 3 weeks' walking with BFR (4%–7% and 8%–10%, respectively).^[11] Several studies confirm the Abe *et al.*'s results of walking with BFR.^[7,17] Hypertrophy mechanism-induced low-intensity aerobic exercise with BFR is unclear. However, the previous studies demonstrated that hypertrophy may be related to increase of growth hormone (GH), IGF-I, and other regulatory myogenic factor. Single-bout low-intensity aerobic exercise with BFR (50 m/min) increased GH circulatory, but IGF-I levels unchanged after single-bout and 3 weeks' walking training.^[7] Although this mechanism is related to increase strength and muscle volume after aerobic exercise with BFR, probably there are other mechanisms that play a role in hypertrophy.

mTOR complex 1 (mTORC1) and MAPK are major signaling pathways for protein synthesis and skeletal muscle

hypertrophy.^[17-19] It has been shown that mTORC1 activity is required for skeletal muscle protein synthesis stimulate because administration of mTORC1 inhibitor before resistance training suppressed protein synthesis induce resistance training.^[20] The hypertrophy mechanism inducing resistance training with BFR is unclear. Few studies demonstrated that single-bout resistance training with BFR through mTOR and MAPK increased protein synthesis.^[13,14,21] However, despite the increase in muscle volume after aerobic exercise with BFR, it is unclear whether mTOR and MAPK signaling pathways have the role in increase muscle volume after aerobic exercise with BFR or not. MAPK is a kind of protein kinase that consists of two main types of ERK and P38. MAPK is inactive in normal mode which is activated by various signals such as apoptosis and heat-shock proteins. Some exercises can also stimulate these paths.^[22] The present study investigates the effect of acute interval walking with BFR on phosphorylation of 4EBP1, P38, ERK, and MSTN rectus femoris of healthy inactive men.

MATERIALS AND METHODS

This is a semi-experimental study; five health inactive men (age = $33 \pm 1/5$ and body mass index = $26/24 \pm 4$ kh/h²) who did not participate in any physical activity daily after acquisition eligible conditions participated in this study. In human studies that require biopsy, research on a large number of people is less feasible. Drummond *et al* studies have been done on 6 people.^[20] Training protocol includes five intervals 2-min walking with BFR at 50%–60% MHR and 1 min at rest. In addition, participants were advised to maintain the food intake and might not be different from usual 48 h before the experimental period. One week before the study, each participant familiarized with the testing procedures, including exercise protocol and muscle biopsy samplings. All patients provided written informed consent before participating in the study.

Exercise protocol

BFR was undertaken by pressure belts by the Katsu machine made by Pooyesh Sanat Aria Company in Iran. Before exercise training, participants were seated on a chair and pressure belts were closed on the top of both legs. In the seated position, the belt was repeatedly set for 30 s and then released for 10 s. The pressure was increased from 120 mmHg to 200 mmHg. The pressure was increased during exercise training because belt air pressure is one of the intensity variables exercise and participants were adapted to the occlusion stimulus.^[11] After warming, participants walking on the treadmill (6 km/h with increment 5%). The acute interval walking program includes five intervals 2-min walking with BFR at 50%–60% MHR (maximum heart rate) and 1 min at rest.^[9,11] The BFR of leg muscle was maintained for the entire session training including the interval walking and 1-min rests period (15 min BFR). The belt pressure was released in lasting intervals and participants walking for 5–10 min for recovery period without BFR. Blood pressure, rate of perceived exertion, and heart rate were monitoring for safety. No adverse effects from the BFR

protocol (e.g., excessive fatigue or pain) were reported by any of the participants.

Muscle biopsy

A unilateral muscle sample was obtained from the lateral point of the vastus lateralis (15–25 cm from mid-patella) of the participant's dominant leg using the percutaneous biopsy technique with suction (TSK ACECUT Biopsy Needle, TSK CO, Japanese). Biopsy is a less invasive method for tissue sampling which has been used by several studies. In this study, a 14 × 11G needle was used for muscle biopsy. After local anesthetic using 2 ml lidocaine 2%, the skin was hold by CO-axial guide needle and inserted to muscle tissue.^[23] Immediately after the procedure, the muscle sample was removed from the needle and frozen in liquid nitrogen, for further storage at –80°C. The muscle sampling was performed immediately before the exercise protocol (30 min after rest) and 3 h after training protocol.^[20]

Western blotting

A complete protease and phosphatase inhibitor was used to homogenize small pieces of the skeletal muscle tissues in cell lysis buffer. Protein phosphorylation was measured using nanodrop. Samples were denaturized at 95°C for 5 min, and then 30 ug of total protein was separated by 8%–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred on to polyvinylidene fluoride membrane. To block nonspecific sites, membranes were immersed in 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h. Then, membranes were incubated with primary antibodies (dilution 1:1000) for 2 h, and after washing in phosphate-buffered saline (PBS), they were exposed to a secondary antibody conjugated by peroxidase for 1 h and they were visualized by Enhanced Chemiluminescence reagent.

Elayza test

A volume of 100 µl of specific antibodies to the protein were added to the wells of 96 individual *Escherichia coli* plates and the plates were placed in the refrigerator at room temperature for one night. The contents of the wells were drained and rinsed once with a PBS washer buffer. Then, 300 µl of the buffer blocker was added to the wells, and the plates were incubated for 1 h at room temperature and on a rotor (rotator) with a gentle round. The contents of the wells were evacuated and washed 3 times with washing buffer. After the third wash with successive tapping on the paper, the remaining fluid in the wells was also discharged. The supernatant was removed from the freezer and after that it was completely melted, and it was used in the next step. In this way, the standards in the kit were prepared.

All statistical analyses were performed using the SPSS statistical software (version 19.0; SPSS Inc., Chicago, IL, USA), and the result is expressed as mean ± standard deviation, unless otherwise stated, with a significance level of $P < 0.05$ two-tailed. The Shapiro–Wilk's test was used for evaluating the normality of distribution. Dependent *t*-test was used to evaluate the difference between groups.

RESULTS

According to the results of this research, acute interval walking with BFR increases phosphorylation of 4EBP1 and ERK and decreases phosphorylation of P38 and concentration of MSTN. These differences were significant for 4EBP1 ($P = 0.001$) [Figure 1] and ERK ($P = 0.049$) [Figure 2] and MSTN ($P = 0.0009$) [Figure 3] and no significant for P38 ($P = 0.452$) [Figure 4].

DISCUSSION

The main finding of this study is that acute interval walking with BFR affects the signaling pathway of protein synthesis. The present study shows that phosphorylation of 4EBP1 and ERK in vastus lateralis muscle is increased due to acute interval walking with BFR, which is a significant increase. Increased levels of 4EBP1 and ERK phosphorylation (especially in 4EBP1) indicate the effect of this exercise on the signaling pathway of the protein and muscle stimulation for hypertrophy. In 1995, McCarthy reported increased strength and hypertrophy by combining cycling and BFR, while cycling exercises alone did not induce these adaptations.^[24] Rognmo *et al.* also indicated in their 2004 study that low-level exercise, such as walking, when accompanied by blood flow restriction, could significantly improve the thigh muscle cross section and knee joint strength in young and old participants.^[25] In 2010, Abe *et al.* also showed that the combination of short-term cycling activities with BFR improves hypertrophy and aerobic capacity in men,^[7] all of which are consistent with the results of the present study.

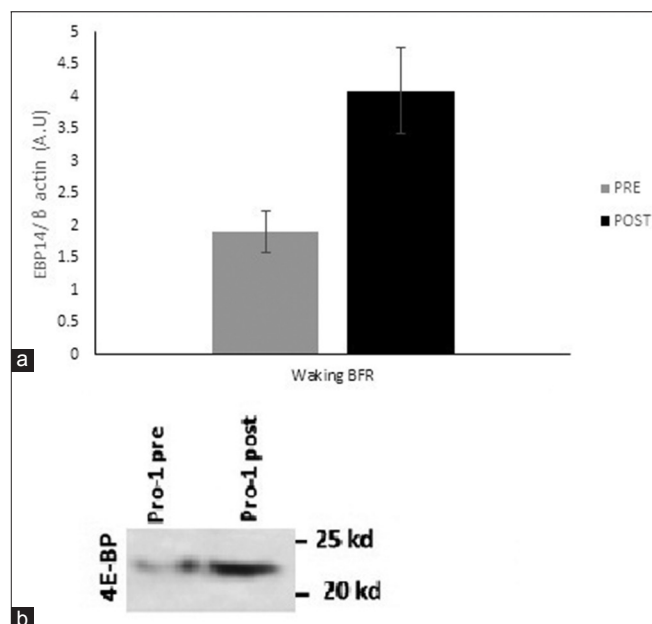


Figure 1: The vastus lateralis protein level of 4EBP1 in scheme, W-B at pre- and post-acute interval walking with blood flow restriction by Western blotting ($n = 5$). (a) Analysis histogram and (b) Representative Western blots. Data were presented. Data are expressed as mean ± standard deviation. W-B, acute interval walking with blood flow restriction

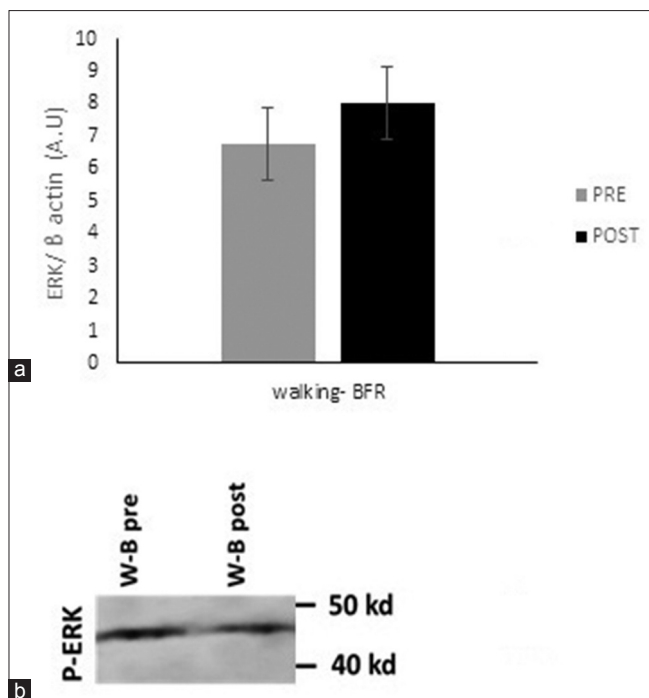


Figure 2: The vastus lateralis protein level of ERK in scheme, W-B at pre- and post-acute interval walking with blood flow restriction by Western blotting ($n = 5$). (a) Analysis histogram and (b) Representative Western blots. Data were presented. Data are expressed as mean \pm standard deviation. W-B, acute interval walking with blood flow restriction

According to Abe *et al.*, mTOR signaling is associated with a muscle protein translation machine,^[26] which is accompanied by the induction of muscle hypertrophy. Long-term exercise training has been shown to rarely accompany significant muscular hypertrophy in young and middle-aged specimens. On the other hand, resistance exercises lead to increased strength and muscle hypertrophy, and however, the combination of low aerobic exercise with BFR is associated with muscle hypertrophy.^[7,13]

Based on the findings of this study, the phosphorylation of P38 after acute interval walking with BFR did not significantly decrease. The results of studies by Ozaki *et al.* indicate that walking with BFR did not significantly change the phosphorylation of some of the various proteins in the mTOR pathway.^[17] The present study showed that the goal of the mechanism of MAPK, which consists of (Erk1)-MAPK and p38-MAPK, can be accompanied by incremental and decreasing changes in acute interval walking with BFR in vastus lateralis muscle. It has been shown in this study that Erk1 was significantly increased, but p38 was nonsignificant. Hence, it can be said that a training protocol may have a different effect on different parts of the MAPK. It seems that part of the activation of this pathway is due to increased secretion of the GH,^[27] the exchange of fluid between blood and active muscle tissue,^[28] and hypoxia induced by BFR.^[15]

Another result of this study was that there was a significant decrease in the amount of MSTN phosphorylation; Fujita in

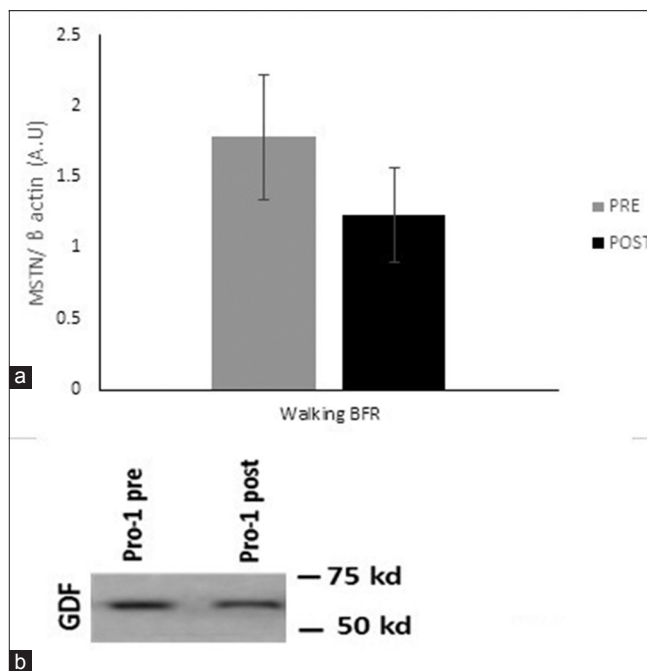


Figure 3: The vastus lateralis protein level of myostatin in scheme, W-B at pre- and post-acute interval walking with blood flow restriction by Western blotting ($n = 5$). (a) Analysis histogram and (b) Representative Western blots. Data were presented. Data are expressed as mean \pm standard deviation. W-B, acute interval walking with blood flow restriction

2008 showed that acute resistance training is associated with a reduction in the amount of MSTN, which in the long term could lead to muscle growth.^[20]

In the same vein, Roth *et al.* reported that in response to resistance training, the levels of MSTN decreased by 37%, independently of age and sex. Although this hypothesis cannot be accepted definitively, it seems that the response of MSTN to exercises with BFR in long-term periods is expected.^[29] Since a number of researchers reported a definite and significant decline after a training period. For example, Laurentino *et al.*, in their study reduced the expression of Myostatin gene by 8 weeks of resistance training with Blood flow restriction.^[30]

According to the findings of the present study on the reduction of MSTN levels, it can be said that acute periodic walking with BFR can stimulate the signal pathway of protein synthesis. Since previous studies have reported that the deletion of MSTN gene in mice increases the number and size of the muscle fibers. The findings suggest that MSTN may have two distinct regulatory functions: One is the adjustment of the final number of muscle fibers during growth and the second is the regulation of the growth of postnatal muscle fibers. For example, it has been shown that increasing the expression of various inhibitors of Myostatin, including follistatin, both increases the number and size of the muscle fibers in mice.^[31] Similarly, in the study of Lee and Ochi in 2015, the use of MSTN neutralizing antibodies induced significant increases in muscle growth in adult mice, and this growth seems to be related only to the increase in the size of the muscle fibers.^[32]

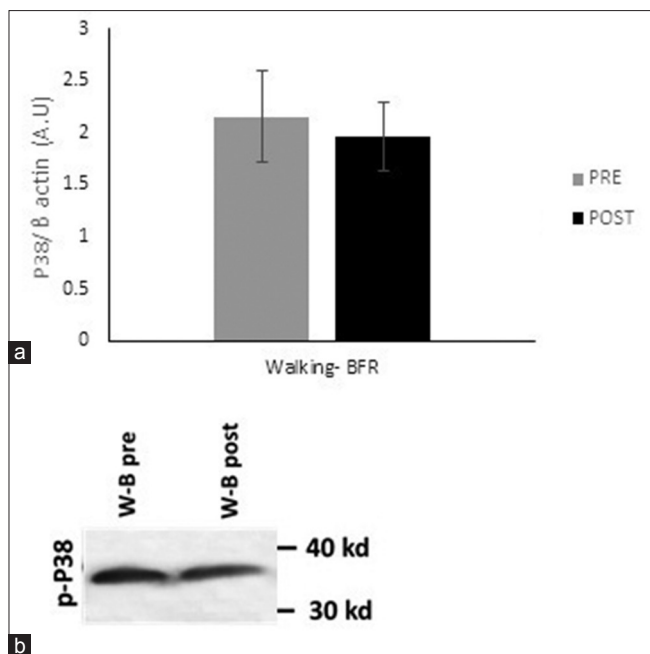


Figure 4: The vastus lateralis protein level of P38 in scheme, W-B at pre- and post-acute interval walking with blood flow restriction by Western blotting ($n = 5$). (a) Analysis histogram and (b) Representative Western blots. Data were presented. Data are expressed as mean \pm standard deviation. W-B, acute interval walking with blood flow restriction

The exact mechanism for reducing MSTN is not clear. When performing obstructive exercises, active muscle cells develop hypoxia and increase the amount of metabolites and calling of fast-twitch fibers.^[33]

CONCLUSION

According to the findings of this study, acute interval walking with BFR effects of phosphorylation of some of regulatory factors in the signaling pathway of protein synthesis. This training method can be offered to people who are not able to perform resistance and endurance exercises.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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