MENS-associated increase of muscular protein content via modulation of caveolin-3 and TRIM72

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Short title: MENS-associated increase in Cav-3 and TRIM72

Summary

Microcurrent electrical neuromuscular stimulation (MENS) is known as an extracellular stimulus for the regeneration of injured skeletal muscle in sports medicine. However, the effects of MENS-associated increase in muscle protein content are not fully clarified. The purpose of this study was to investigate the effects of MENS on the muscular protein content, intracellular signals, and the expression level of caveolin-3 (Cav-3), tripartite motif-containing 72 (TRIM72) and MM isoenzyme of creatine kinase (CK-MM) in skeletal muscle using cell culture system. C2C12 myotubes on the 7^{th} day of differentiation phase were treated with MENS (intensity: 10-20 μA, frequency: 0.3 Hz, pulse width: 250 ms, stimulation time: 15-120 min). MENS-associated increase in the protein content of myotubes was observed, compared to the untreated control level. MENS upregulated the expression of Cav-3, TRIM72, and CK-MM in myotubes. A transient increase in phosphorylation level of Akt was also observed. However, MENS had no effect on the phosphorylation level of p42/44 extracellular signal-regulated kinase-1/2 and 5'AMP-activated protein kinase. MENS may increase muscle protein content accompanied with a transient activation of Akt and the upregulation of Cav-3 and TRIM72.

Key words

Skeletal muscle cells, Microcurrent electrical neuromuscular stimulation, Protein content, Caveolin-3, Tripartite motif-containing 72.

Introduction

Skeletal muscle injury is a most common sport trauma during sports activities. Various stimuli are proposed to facilitate the regeneration of injured skeletal muscle (Assis *et al.* 2013, Fujita *et al.* 2014, Nagata *et al.* 2013, Richard-Bulteau *et al.* 2008, Shibaguchi *et al.* 2016). Especially, electrical stimulation of injured muscle is used for more than 70 years (Gutmann E and Gutmann L 1944), and then the positive effects of electrical stimulation on skeletal muscle have been obtained. Even though recent evidences demonstrate that microcurrent electrical neuromuscular stimulation (MENS), which is an electrical stimulation causing no muscle contraction, facilitates skeletal muscle regeneration (Fujiya *et al.* 2015, Ohno *et al.* 2013), the molecular mechanism of MENS-associated muscle regeneration remains unclear.

Increase in protein synthesis is the essential process during the regeneration of injured skeletal muscle (Jones 1982, Jones 1984, Kojima *et al.* 2007, Kubica *et al.* 2005), and is stimulated by mechanical stress induced by muscle contraction and stretch (Drummond *et al.* 2009, Morioka *et al.* 2008, You *et al.* 2012). On the other hand, protein synthesis in skeletal muscle cells is mediated by the intracellular signals, such as protein kinase B (Akt), p42/44 extracellular signal-regulated kinase-1/2 (ERK1/2), and 5'AMP-activated protein kinase (AMPK). The activation of Akt signaling, which is well known as a potent stimulator for protein synthesis (Rommel *et al.* 2001), induces muscle differentiation (Coolican *et al.* 1997) and hypertrophy (Bodine *et al.* 2001, Sugiura *et al.* 2005). ERK1/2 signaling also stimulates muscle cell proliferation (Coolican *et al.* 1997) and differentiation (Li and Johnson 2006). Furthermore, it has been reported that AMPK signaling, which is a mediator for cellular energy balance, negatively regulates muscle mass via suppression of

protein synthesis (Egawa *et al.* 2014, Sanchez *et al.* 2012). Although MENS stimulates the increase in protein content of atrophied and injured skeletal muscle (Fujiya *et al.* 2015, Ohno *et al.* 2013), the effects of MENS on Akt, ERK1/2, and AMPK in skeletal muscle cells are still unknown.

Caveolin-3 (Cav-3), which is an integral membrane protein, is expressed in muscle cells such as skeletal, cardiac, and smooth muscle cells (Song *et al.* 1996, Tang *et al.* 1996, Way and Parton 1996). Expression of Cav-3 is gradually upregulated during the differentiation of C2C12 cells (Fanzani *et al.* 2007, Galbiati *et al.* 1999). In addition, the upregulation of Cav-3 is observed in regrowing slow soleus muscle (Ohno *et al.* 2014), hypertrophied C2C12 cells, and fast muscles (tibialis anterior, extensor digitorum longus, and thigh muscles) of mice (Fanzani *et al.* 2007), whereas an atrophic agent dexamethasone downregulates Cav-3 expression in C2C12 cells (Fanzani *et al.* 2007). Furthermore, overexpression of Cav-3 enhances protein synthesis and myotube hypertrophy in C2C12 cells (Hadj Sassi *et al.* 2012). Therefore, the expression level of Cav-3 may be an indicator of protein synthesis that is regulated by hypertrophic and atrophic stimuli.

Cav-3 also plays a crucial role in repair of skeletal muscle membrane, interacting with tripartite motif-containing 72 (TRIM72) (Cai *et al.* 2009a, Cai *et al.* 2009b, Cai *et al.* 2009c). TRIM72 is expressed in the plasma membrane and intracellular vesicles of skeletal and cardiac muscle cells, and is one of TRIM family composed of a really interesting new gene finger domain, a B-box, two coiled coil domains, and a spla and ryanodine receptor domain (Cai *et al.* 2009a, Lee *et al.* 2010). TRIM72 is upregulated accompanying with myogenesis (Jung and Ko 2010, Lee *et al.* 2010, Yi *et al.* 2013). Recently, it was reported that TRIM72 was upregulated during regrowth of atrophied soleus muscle (Ohno *et al.* 2014). Even

though MENS has facilitating effects on the regrowth of atrophied muscle and the regeneration of injured muscle (Fujiya *et al.* 2015, Ohno *et al.* 2013), there is no evidence regarding the effects of MENS on the expression of Cav-3 and TRIM72 in skeletal muscle. Furthermore, it has been also reported that Akt plays a mediator to regulate the expression of Cav-3 and TRIM72 in C2C12 cells (Fanzani *et al.* 2007, Lee *et al.* 2010).

In the present study, the effects of MENS on the muscular protein content, intracellular signals (Akt, ERK1/2, AMPK), and the expression level of Cav-3 and TRIM72 in skeletal muscle were investigated using cultured C2C12 cells. In addition to the increases in Cav-3 and TRIM72 during muscle differentiation (Cai *et al.* 2009b, Fanzani *et al.* 2007, Lee *et al.* 2010), MM isoenzyme of creatine kinase (CK-MM) is known as a marker of myogenic differentiation (Chamberlain *et al.* 1985, Larraín *et al.* 1998). Therefore, we also investigated the change in CK-MM following MENS treatment.

Methods

Cell culture

Mouse myoblast C2C12 cells (6 x 10^4 cells/well) were cultured on 6-well culture plates with type I collagen-coated surface (Biocoat, Corning Inc., Corning, NY, USA). Cells were maintained in 2 ml of growth medium consisting of Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Yokohama, Japan) supplemented with 10% heat-inactivated fetal bovine serum containing high glucose (4.5 g/l glucose, 4.0 mM L-glutamine, without sodium pyruvate) for proliferation under a humidified atmosphere with 95% air and 5% CO_2. On the 3rd day of the proliferation phase (at ~80% confluence), the culture medium was then changed to same amount of differentiation medium consisting of DMEM supplemented with 2% heat-inactivated horse serum containing low glucose (1.0 g/l glucose, 4.0 mM L-glutamine, and 110 mg/l sodium pyruvate) for differentiation, as was described previously (Egawa *et al.* 2014). The differentiation medium was changed every 2 days and cultures were maintained for 8 days to form myotubes.

MENS

MENS using an electrical stimulator (Trio300, Ito Co., Ltd., Tokyo, Japan) was delivered to the culture medium through platinum electrodes, which placed on opposing inside walls of a well (Figure 1). The cells in a plate were simultaneously treated with MENS in a cell culture incubator (37°C, 5% CO_2). Although the electrodes were also set in the medium of the untreated control cells in the same manner, no MENS was applied.

Experiment 1: Effects of MENS on protein content in C2C12 cells

First of all, we investigated whether the condition of MENS have effects on increase in protein content of skeletal muscle cells. The myotubes on the 7th day of differentiation phase were treated with MENS, then the cells were collected 24 h after the application of MENS (Figure 2A). The condition for MENS (intensity: 10 or 20 μA, frequency: 0.3 Hz, pulse width: 250 ms, duration: 0, 15, 30, 60, and 120 min, n = 8 wells per group) was referred to the previous study that observed increase in muscular protein content in mouse skeletal muscle (Ohno *et al.* 2013). These conditions of MENS did not induce myotube contraction.

C2C12 cells were lysed in cell lysis reagent (CelLytic^{TM}-M, Sigma-Aldrich, St. Louis, MO, USA) with some modification of the previously reported method (Egawa *et al.* 2014) to extract total muscle protein from the cells. Briefly, the cells in each well were rinsed twice with 1 ml of ice-cold phosphate-buffered saline. Then, the cells of each well were scraped into 0.3 ml of cell lysis reagent on ice. The cell lysate was sonicated and centrifuged at 20,000 g at 4^{o}C for 10 min. The supernatant was collected for the determination of protein content. Protein content in the supernatant was determined by the Bradford technique (Protein Assay Kit, Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Sigma) as the standard. Protein content was expressed relative percentage to the value of untreated control group (0 min).

Experiment 2: Effects of MENS on intracellular signals in C2C12 myotubes

According to the results of Experiment 1 and the previous study that observed changes in intracellular signals in mouse skeletal muscle (Ohno *et al.* 2013) the myotubes on 7th days of differentiation phase were treated with MENS at 10 μA for 60 min (frequency: 0.3 Hz,

pulse width: 250 ms). The cells were collected 1, 3, and 6 h after the application of MENS (n = 8 wells per group, Figure 2B). In Experiment 2, we investigated the MENS-associated early responses (1, 3, and 6 h after the stimulation) of the protein expression levels (Cav-3 and TRIM72) and the intracellular signals involved in muscle protein synthesis (Akt, ERK1/2, AMPK).

Western blot analysis was performed with some modification of the previously reported method (Egawa *et al.* 2014, Ohno *et al.* 2014). The cells were lysed in 0.3 ml of cell lysis reagent (CelLytic^{TM}-M, Sigma) with 10% (v/v) Protease Inhibitor Cocktail (P8340, Sigma) and 1% (v/v) Phosphatase Inhibitor Cocktail (524625, Calbiochem, San Diego, CA, USA) and centrifuged. Then the supernatant was collected. After the determination of protein content in the supernatant, the supernatant samples were mixed with sodium-dodecylsulfate (SDS) sample buffer [30% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, 62.5 mM Tris-HCl, 0.05% (w/v) bromophenol blue and pH 6.8] and were boiled for 5 min. The SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 8-12% polyacrylamide containing 0.5% SDS at a constant current of 20 mA for 120 min. Equal amounts of protein (8 μg) were loaded on each gel. Molecular weight markers (Bio-Rad Precision Markers) were applied to both sides of gel as the internal controls for transfer process or electrophoresis.

Following SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) using a Bio-Rad mini trans-blot cell at a constant voltage of 100 V for 60 min at 4°C. After the transfer, the membranes were blocked for 1 h using a skim milk. Then, the membranes were incubated for 2 h with a primary antibody: Cav-3 (ab2912, Abcam, Cambridge, UK), TRIM72 (PAB18940, Abnova, Taipei, Taiwan), CK-MM

(ab198235, Abcam), phosphorylated Akt at Ser^{473} residue (p-Akt: 9271, Cell Signaling Technology Inc., Danvers, Mass., USA), total Akt (t-Akt: 9272, Cell Signaling), phosphorylated ERK1/2 at Thr^{202}/Tyr^{204} residue (p-ERK1/2: 9101, Cell Signaling), total ERK1/2 (t-ERK1/2: 9102, Cell Signaling), phosphorylated AMPKα at Thr^{172} residue (p-AMPK: 2531, Cell Signaling), total AMPKα (t-AMPK: 2532, Cell Signaling), GAPDH (G9545, Sigma) and then reacted with a secondary antibody: anti-goat or anti-rabbit immunoglobulin G conjugate to horseradish peroxidase for 2 h. After the final wash, protein bands were visualized using chemiluminescence (GE Healthcare, Buckinghamshire, UK), and signal density was measured using Light-Capture (AE-6971) with CS Analyzer version 2.08b (ATTO Corporation, Tokyo, Japan).

Statistical analysis

All values were expressed as means ± SEM. Statistical significance was analyzed by one-way analysis of variance followed by Dunnett test. The significance level was accepted at $p<0.05$.

Results

Experiment 1: Effect of MENS on protein content in C2C12 cells

In the present study, we examined the effects of MENS on the protein content of C2C12 myotubes. The cells were collected 24 h after the application of MENS (intensity: 10 or 20 μA, duration: 0-120 min, Figure 2A) for the evaluation of protein content. Figures 3A and B show the protein content in C2C12 myotubes 24 h after MENS treatment at 10 (A) and 20 μA (B), respectively. MENS with 10 μA for 15-60 min significantly increased the protein content of myotubes, compared to the untreated control level ($p < 0.05$). Overall response of protein content after MENS treatment at 20 μA was similar to that at 10 μA. MENS with 20 μA for 15-30 min significantly increased the protein content of the myotubes ($p < 0.05$). However, MENS with 10 μA for 120 min or 20 μA for 60 min had no impact on the protein content.

Experiment 2: Effect of MENS on intracellular signals

Since we confirmed that MENS-associated increase in the protein content of C2C12 myotubes was observed 24 h after the treatment, the effects of MENS with 10 μA for 60 min on the expression level of Cav-3 and TRIM72 were investigated in myotubes. The cells were collected 1, 3, and 6 h after the application of MENS (Figure 2B) for western blot analyses. Figure 4 shows the responses of Cav-3, TRIM72, and CK-MM to MENS in C2C12 myotubes. MENS increased the expression of Cav-3, TRIM72, and CK-MM in myotubes. The expression level of Cav-3, TRIM72, and CK-MM 6 h after MENS treatment was significantly increased, compared to the untreated control level ($p < 0.05$). In addition, we investigated the effects of MENS on the phosphorylated level of Akt,

ERK1/2, and AMPKα in myotubes (Figure 5). A transient increase in the relative expression level of p-Akt was observed 1 h after MENS treatment, compared to the untreated control level ($p < 0.05$). However, there was no significant change in the p-Akt level 3 and 6 h after MENS treatment. On the other hand, MENS had no effects on the level of p-ERK1/2 and p-AMPKα in myotubes.

Discussion

The present study demonstrated that MENS-associated increase of protein content in C2C12 myotubes accompanied with the upregulation of CK-MM, Cav-3, and TRIM72, and with a transient increase in phosphorylated form of Akt. On the other hand, MENS had no effects on the phosphorylation levels of ERK1/2 and AMPKα in C2C12 myotubes. This is the first report showing the effects of MENS on the total protein content, the expression level of Cav-3, TRIM72, and CK-MM proteins, and the intracellular signals in myotubes.

Upregulation of Cav-3, TRIM72, and CK-MM proteins in myotubes was observed following MENS treatment in the present study. It is reported that the upregulation of Cav-3 and TRIM72 is observed during differentiation of C2C12 cells (Cai *et al.* 2009b, Fanzani *et al.* 2007, Lee *et al.* 2010), and during reloading-associated muscle regrowth (Ohno *et al.* 2014), in hypertrophied myotubes induced by transfection of an activated form of Akt (Fanzani *et al.* 2007). Furthermore, MENS-associated upregulation of CK-MM, which is a maker of myogenic differentiation (Chamberlain *et al.* 1985, Larraín *et al.* 1998), was also observed. These observations indicate that MENS has a stimulating effect on myogenic differentiation.

In the present study, the protein content in myotubes was increased by MENS. This result is supported by the previous studies that MENS facilitated regrowth of atrophied and injured skeletal muscle in mice (Fujiya *et al.* 2015, Ohno *et al.* 2013). On the other hand, MENS with 10 μA for 120 min or 20 μA for 60 min had no impact on the protein content. Therefore, there is a possibility that the appropriate dosage (stimulation intensity x duration) of MENS may exist, although we cannot explain the difference in the response to stimulus parameter of MENS at present. Additional experiments are required to elucidate this issue.

It is suggested that the overexpression of Cav-3 shows the enhanced protein synthesis via the downregulation of myostatin in C2C12 cells (Hadj Sassi *et al.* 2012). Furthermore, in the present study, the transient and significant activation of Akt in myotubes was induced by MENS. This result is also consistent with the results from the previous study that MENS-associated upregulation of p-Akt expression was observed in mouse soleus muscle (Ohno *et al.* 2013). Since Akt plays a key role in intracellular signaling of protein synthesis in skeletal muscle (Bodine *et al.* 2001, Sugiura *et al.* 2005) as well as the expression of Cav-3 and TRIM72 in C2C12 cells (Fanzani *et al.* 2007, Lee *et al.* 2010), MENS-associated increase of protein content accompaying with the up-regulation of Cav-3 and TRIM72 in myotubes might be attributed to the activation of Akt. Further investigation is necessary to clarify precise role of Akt in muscle protein synthesis in response to MENS.

On the other hand, there were no significant changes in p-ERK1/2 and p-AMPKα in response to MENS in the present study. The phosphorylation level of ERK1/2 and AMPKα is upregulated after electrically-induced muscle contraction (Treebak *et al.* 2006, Wojtaszewski *et al.* 1999). As MENS induces no muscle contraction, MENS might have no effects on the signaling pathways of ERK1/2 and AMPKα. Additional experiments should be needed to explain this issue.

In conclusion, MENS may have a stimulating effect on muscle protein content in accompany with the upregulation of Cav-3 and TRIM72, and with a transient activation of Akt. Evidences from this study suggest that MENS stimulates not only protein synthesis but also membrane repair in the regeneration of injured skeletal muscle. MENS could be a potentially useful tool for injured athletes in sports medicine as well as rehabilitation.

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Conflict of interest

The authors have declared that no conflicts of interest exist.

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Figure captions

Figure 1. An illustration of the setup for a 6-well plate treated with microcurrent electrical neuromuscular stimulation (MENS) treatment. MENS using an electrical stimulator (Trio300, Ito Co., Ltd., Tokyo, Japan) was delivered to the culture medium through platinum electrodes, which placed on opposing inside walls of a well. Cells on 4 wells of a 6-well plate were stimulated by MENS. Other 2 wells of the plate were not used for MENS-treatment group.

Figure 2. (A) Protocol for Experiment 1. MENS with 10-20 μA for 15-120 min were delivered to C2C12 myotubes on the 7^{th} day of differentiation phase. Thereafter, the cells were collected 24 h after MENS treatment. (B) Protocol for Experiment 2. MENS at 10 μA for 60 min was delivered to C2C12 myotubes on the 7^{th} day of differentiation phase. Thereafter, the cells were collected 1 (M1), 3 (M3), and 6 h (M6) after MENS treatment.

Figure 3. Protein content in C2C12 myotubes after MENS at 10 μA (A) and 20 μA (B). Values are expressed as relative percentage to the value of 0 min (untreated control). Values are means ± SEM. n = 8 wells per group. *: significant different from 0 min, $p < 0.05$.

Figure 4. Effects of MENS at 10 μA for 60 min on the expression level of Cav-3, TRIM72, and CK-MM in C2C12 myotubes. Con: untreated control group; Cav-3: caveolin-3; TRIM72: tripartite motif-containing 72; CK-MM: MM isoenzyme of creatine kinase;

GAPDH: glyceraldehyde-3-phosphate dehydrogenase. See Figure 2 for other abbreviations. Values are expressed relative to the value of Con. Values are means ± SEM. n = 8 wells per group. *: significant different from Con, $p < 0.05$.

Figure 5. Effects of MENS at 10 μA for 60 min on the phosphorylation level of Akt, ERK1/2, and AMPK in C2C12 myotubes. p-Akt: phosphorylated Akt; t-Akt: total Akt; p-ERK1/2: phosphorylated p42/44 extracellular signal-regulated kinase (ERK)-1/2; t-ERK1/2: total ERK1/2; p-AMPK: phosphorylated 5'AMP-activated protein kinase (AMPK)α; t-AMPK: total AMPKα. See Figures 2 and 4 for other abbreviations. Values are expressed relative to the value of Con. Values are means ± SEM. n = 8 wells per group. *: significant different from Con, $p < 0.05$.

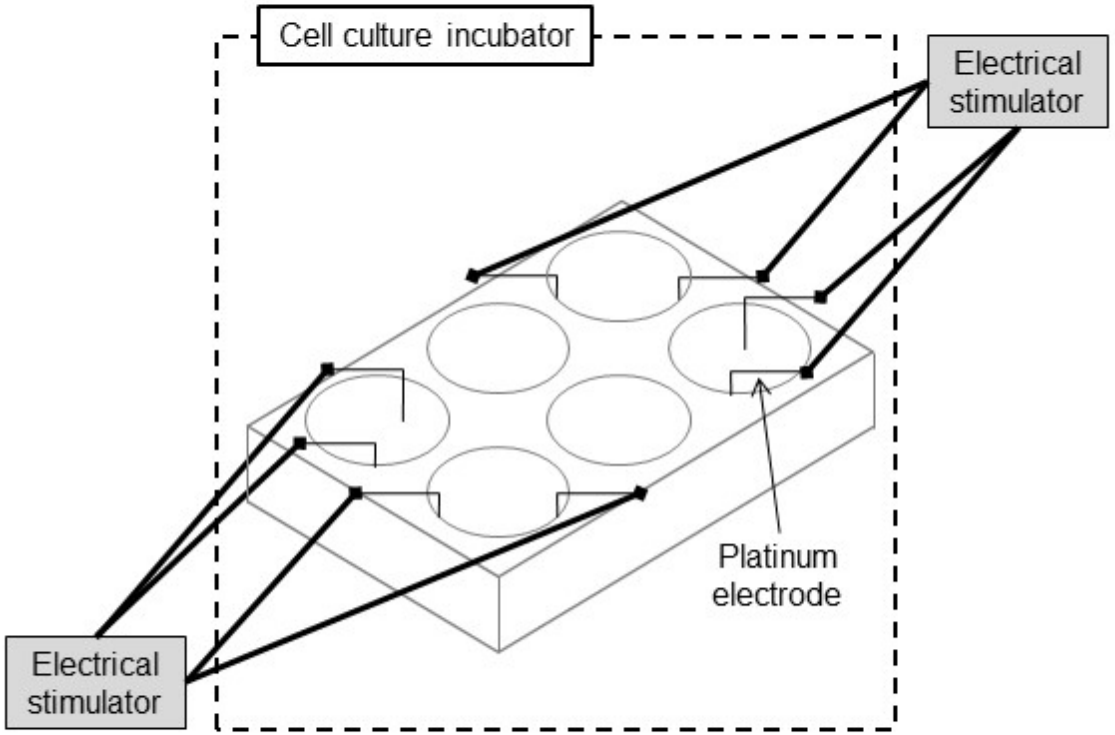
Cell culture incubator
Electrical stimulator
Electrical stimulator
Platinum electrode

Figure 1

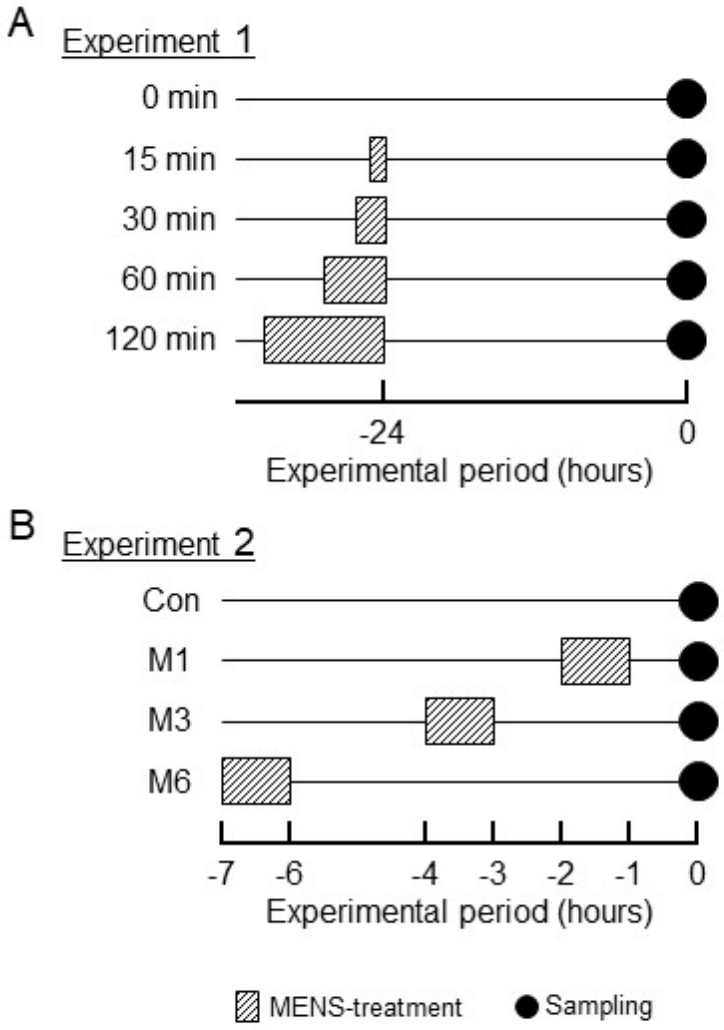
A
Experiment 1
0 min
15 min
30 min
60 min
120 min
-24
0
Experimental period (hours)
B
Experiment 2
Con
M1
M3
M6
-7
-6
-4
-3
-2
-1
0
Experimental period (hours)
MENS-treatment
Sampling

Figure 2

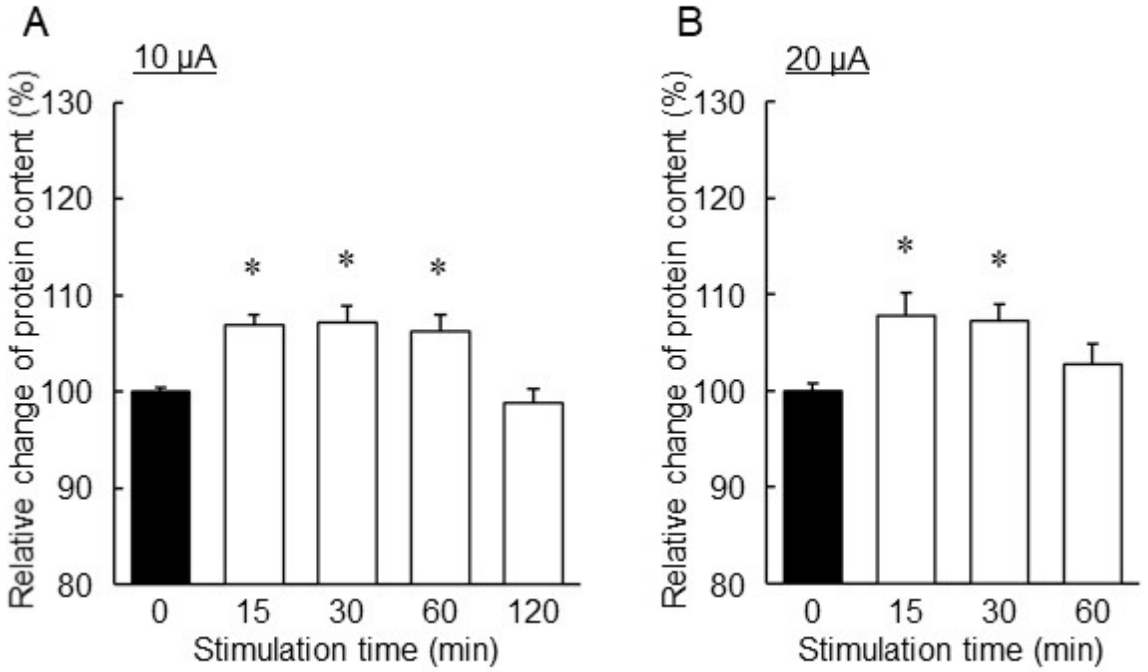
A
10 μA
Relative change of protein content (%)
130
120
110
100
90
80
*
*
*
0
15
30
60
120
Stimulation time (min)
B
20 μA
Relative change of protein content (%)
130
120
110
100
90
80
*
*
0
15
30
60
Stimulation time (min)

Figure 3

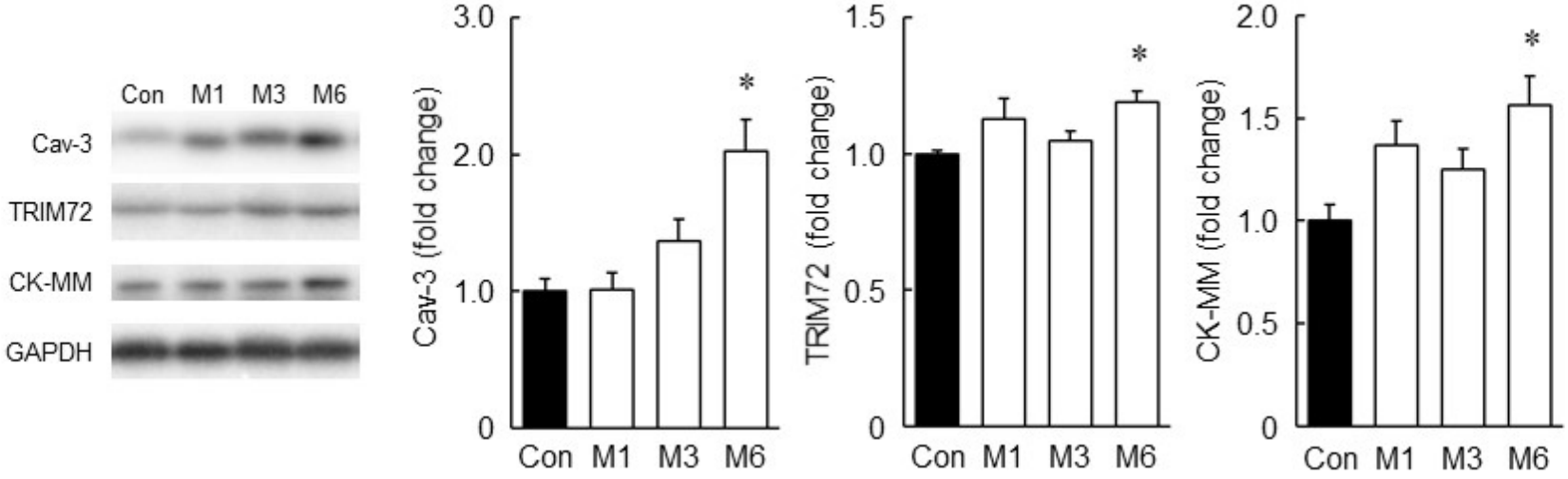
Con
M1
M3
M6
Cav-3
TRIM72
CK-MM
GAPDH
Cav-3 (fold change)
3.0
2.0
1.0
0
*
Con M1 M3 M6
TRIM72 (fold change)
1.5
1.0
0.5
0
*
Con M1 M3 M6
CK-MM (fold change)
2.0
1.5
1.0
0.5
0
*
Con M1 M3 M6

Figure 4

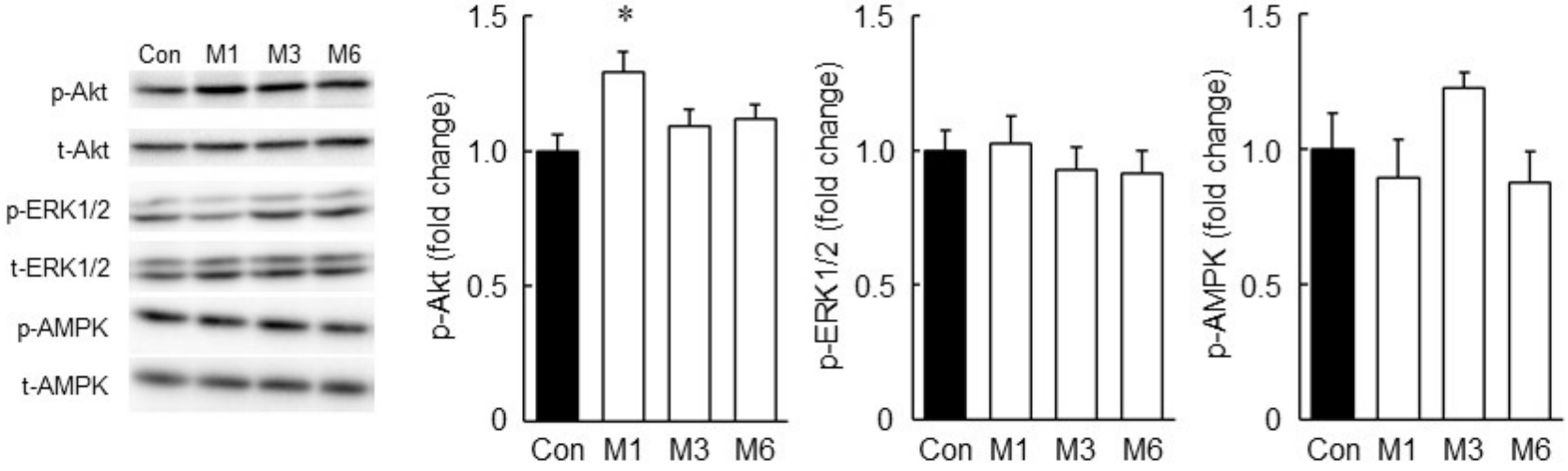
Con
M1
M3
M6
p-Akt
t-Akt
p-ERK1/2
t-ERK1/2
p-AMPK
t-AMPK
p-Akt (fold change)
p-ERK1/2 (fold change)
p-AMPK (fold change)
1.5
1.0
0.5
0
*
Con
M1
M3
M6

Figure 5