Stimulation of myogenesis by ascorbic acid and capsaicin

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ABSTRACT

Myogenesis is a complex process regulated by several factors. This study evaluated the functional interaction between vitamin C and a high dose of capsaicin (a potential endoplasmic reticulum (ER) stress inducer) on myogenesis. After the induction of differentiation, treatment with ascorbic acid or ascorbic acid phosphate (AsAp) alone had minimal effects on myogenesis in C2C12 cells. However, treatment with capsaicin (300 μM) in undifferentiated C2C12 cells increased the expression levels of genes related to ER stress as well as oxidative stress. Myogenesis was effectively enhanced in C2C12 cells treated with a combination of capsaicin (300 μM) for one day before differentiation stimulation and AsAp for four days post-differentiation; subsequently, thick and long myotubes formed, and the expression levels of myosin heavy chain (MYH) 1/2 and *Myh1, Myh4,* and *Myh7* increased. Considering that mild ER stress stimulates myogenesis, AsAp may elicit myogenesis through the alleviation of oxidative stress-induced negative effects in capsaicin-pretreated cells. The enhanced expression of *Myh1* and *Myh4* coincided with the expression of *Col1a1*, a type I collagen, suggesting that the fine-tuning of the myogenic cell microenvironment is responsible for efficient myogenesis. Our results indicate that vitamin C is a potential stimulator of myogenesis in cells, depending on the cell context.

Keywords: myogenesis, capsaicin, endoplasmic reticulum stress, vitamin C

1. Introduction

Skeletal muscle is a specialized organ that generates force and movement and is composed of postmitotic, multinucleated muscle fibers. Appropriate skeletal muscle mass is also critical for maintaining glucose utilization and insulin sensitivity [1]. Furthermore, various bioactive molecules, termed myokines, are produced and secreted from skeletal muscle [2]. Skeletal muscle formation consists of a complex set of differentiation steps: commitment of mesenchymal stem cells to myoblast lineage cells, proliferation of myoblasts, and fusion of myoblasts into multinucleated myotubes with the expression of muscle-cell-specific proteins such as myosins [3, 4].

Myogenic differentiation is principally governed by the sequential expression of myogenic regulatory factors (MRFs), such as MYF5, MYOD, MYOG, and MRF4 [3-6], and many factors regulate myogenesis by modulating the expression levels of MRFs [7, 8]. Oxidative stress and hypoxia impair myogenesis by repressing the expression of *Myf5*, *Myod*, and *Myog* [9-11]. In contrast, endoplasmic reticulum (ER) stress stimulates myogenesis [12, 13]. Vitamin C, a generic term for all compounds exhibiting the biological activity of ascorbic acid (AsA), modulates myogenesis by upregulating MYOG expression [14, 15]. Dietary vitamin C has also been shown to improve skeletal muscle function in patients with facioscapulohumeral muscular dystrophy, an inherited skeletal muscle disease [16]. The present study examined the effect of vitamin C supplementation on the expression of genes related to myogenesis in C2C12 myogenic cells.

2. Materials and methods

2.1. Materials

AsA, AsA phosphate (AsAp), and capsaicin were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Mouse monoclonal antibodies against MYH1/2 (A4.1025) and

rabbit polyclonal antibody against MYOG were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, UK), respectively.

2.2. Cell Culture

C2C12 myoblasts [17] were obtained from the RIKEN BioResource Research Center. Cells were cultured in growth medium, that is, Dulbecco's modified Eagle's medium (DMEM) with heat-inactivated 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C under a humidified 5% CO_2 atmosphere. To induce differentiation from myoblasts to myotubes, the medium was replaced 1 d after confluence (day 0) with differentiation medium consisting of DMEM with 2% horse serum (HS) supplemented with antibiotics. Ascorbic acid (AsA: 100 μM or 200 μM) or ascorbic acid 2-phosphate (AsAp: 100 μM or 200 μM) was added from day 0 to day 4. High doses of capsaicin (200 μM or 300 μM) or dimethyl sulfoxide, as a vehicle control, were administered from day -1 to day 0.

2.3. RNA Isolation and real-time reverse transcription (RT)-quantitative (q) PCR

Total RNA isolation and real-time RT-qPCR were performed as previously described [18]. The nucleotide sequence of qPCR primers is given in Table S1. The ΔΔCt method was used to normalize the levels of target transcripts to *Hprt1* levels.

2.4. Western blot

Western blot analyses were performed as previously described [19]. The immunoreactive proteins were visualized using Chemi-Lumi One Ultra (Nacalai Tesque, Kyoto, Japan) following the manufacturer’s protocol.

2.5. Giemsa and immunofluorescence staining

Cells were fixed with methanol for 2 min and subsequently stained with Giemsa stain for

20 min. For immunofluorescence staining, cells were fixed with 10% formalin in phosphate-buffered saline (PBS). The fixed cells were treated with 0.1% Triton X-100 in PBS for permeabilization, followed by blocking with blocking solution (5% normal goat serum in PBS) for 20 min. The cells were subsequently incubated with the primary antibody against MHC1/2 in blocking solution overnight at 4 °C. After washing with 0.1% Tween-20 in PBS, cells were incubated with Alexa Fluor 488 in blocking solution for 1 h, followed by staining with 2.5 µg/ml Hoechst 33342 for detection of nuclei. Three independent images were randomly captured and analyzed morphometrically. The MYH1/2-positive myotube number and nuclear number were measured, and the number of nuclei was calculated using ImageJ software (http://rsbweb.nih.gov/ij/). The fusion index was calculated as the ratio of the number of nuclei incorporated into myotubes to the total number of nuclei [20]. In addition, the maturation index was defined as the number of nuclei in myotubes.

2.6. Statistical Analyses

Data are expressed as the mean ± SEM. Data on gene expression were log-transformed to provide an approximation of normal distribution before the analysis. Statistical analyses of gene expression were performed using the SAS statistical software (version 9.1; SAS Inst. Inc., Cary, NC). Data on the effect of AsA, AsAp, or capsaicin alone in C2C12 cells were analyzed by one-way analysis of variance (ANOVA). When the effect of a group was significant, comparisons among groups were performed using Dunnett's test. Data on the effect of combined treatment with capsaicin and AsAp were analyzed by two-way ANOVA including the effect of capsaicin, AsAp, and the interaction of capsaicin and AsAp. When at least one effect was significant, differences among treatments were compared using Tukey's test. Differences were considered significant at $P < 0.05$, and those $0.05 \leq P < 0.10$ were considered to demonstrate a tendency.

3. Results and discussion

We first examined the effect of treatment with AsA or AsAp, a stable AsA derivative, during myogenesis on the expression of *Myh*s and *Mrf*s in C2C12 cells (Fig. 1A, B). AsA (200 μM) increased the expression of *Myh1*, whereas *Myh4* expression decreased in culture containing AsA or AsAp (Fig. 1A). Treatment with AsA (100 μM or 200 μM) or AsAp (200 μM) significantly increased the expression of *Myog* (Fig. 1B).

ER stress induced by tunicamycin has been shown to stimulate myogenesis [12, 13]. However, the onset of ER stress can induce oxidative stress [21] which inhibits myogenesis [9-11]. Thus, we hypothesized that combination treatment with an ER stress inducer and vitamin C may enhance myogenesis. We first treated C2C12 myoblasts with tunicamycin to induce ER stress, but severe cell death was reproducibly detected (data not shown). We previously showed that a high dose of capsaicin (100 μM) induced relatively mild ER stress in brown preadipocytes [22]. Preliminary experiments showed that capsaicin at 100 μM after differentiation induction led to severe cell death, but capsaicin treatment prior to differentiation induction resulted in cell death within the acceptable range (data not shown).

We examined the expression levels of several genes related to ER stress in undifferentiated C2C12 cells treated with capsaicin (200 or 300 μM) (Fig. 2A). ER stress is sensed by three kinds of ER-resident transmembrane proteins: inositol-requiring enzyme (IRE) 1, activating transcription factor (ATF) 6, and PKR-like ER kinase (PERK), followed by modulation of downstream gene expression [23-25]. The activation of IRE1 increases the formation of the spliced form of X-box binding protein 1 (*sXbp1*). Additionally, activation of the IRE1 pathway increases ER degradation by inducing α-mannosidase-like protein (*Edem*) and homocysteine-induced endoplasmic reticulum

protein (*Herp*) genes. The stimulation of the ATF6 pathway also increases the expression of *Herp* as well as *p58*^{ipk}, ER protein (*Erp*) *72*, glucose-regulated protein (*Grp*) *78*, and C/EBP homologous protein (*Chop*). Phosphorylation of PERK in response to ER stress increases *Chop* and *Atf4* mRNA levels [23-25]. During this study, treatment with capsaicin at 300 μM increased the expression of *sXbp1*, *Herp*, *p58*^{ipk}, *Erp72*, *Grp78*, and *Chop*, whereas capsaicin at 200 μM decreased the expression of *Atf4* (Fig. 2A). These results suggest that treatment with capsaicin at 300 μM induces ER stress in C2C12 cells; in particular, it stimulates the ATF6 pathway and is likely to activate the IRE-1 pathway.

Hmox1 expression was significantly increased following capsaicin treatment at 300 μM (Fig. 2B). The expression levels of *Myf5* and *Myod* significantly increased in response to treatment with capsaicin at 200 μM, whereas *Myog* expression numerically increased following treatment with 200 μM capsaicin and significantly decreased with 300 μM capsaicin (Fig. 2C). These results suggest that treatment with capsaicin at 300 μM also induced oxidative stress, and that the expression levels of *Mrf*s did not increase under capsaicin-induced ER stress and oxidative stress. We also examined the expression of *Trpv1*, a receptor for capsaicin [26], but significant expression was not detected in C2C12 cells (data not shown).

We treated C2C12 cells with capsaicin (300 μM) for 1 day before the induction of differentiation, followed by treatment with vitamin C for 4 days after its initiation. Giemsa staining indicated that multinucleated myotube formation increased in response to combined treatment with capsaicin and AsAp (Fig. 3A). MYH1/2 expression was evaluated by immunofluorescence analysis. Treatment with either capsaicin or AsAp slightly, but significantly, stimulated myogenesis (Fig. 3B). Combination treatment with capsaicin and AsAp resulted in the formation of thick and long myotubes (Fig. 3B). The fusion index, measured as the ratio of nuclei in MYH1/2-expressing cells to the total

number of nuclei, increased significantly in response to treatment with either capsaicin or AsAp, and increased further after combined treatment with capsaicin and AsAp (Fig. 3C). Similar effects of capsaicin and AsAp were detected via the maturation index, which reflects the number of nuclei in a myotube (Fig. 3D).

Comparable results were also obtained using RT-qPCR and western blot analyses (Fig. 3E, F). The capsaicin-induced expression of *Myh1* was greatly increased by AsAp treatment (Fig. 3E). In contrast, neither capsaicin nor AsAp substantially affected *Myh2* expression. Treatment with capsaicin increased the expression of *Myh4* and *Myh7*, and AsAp decreased the expression of *Myh4* but not *Myh7* (Fig. 3E). Western blot analyses showed that MYH1/2 expression in cells treated with capsaicin prior to differentiation increased in response to treatment with AsAp after the induction of differentiation (Fig. 3F), which was consistent with the results gathered on *Myh1* mRNA levels (Fig. 3E).

To explore the mechanism underlying enhanced myogenesis induced by treatment with capsaicin and AsAp, we examined the expression of *Hmox1* (Fig. 4A), *Mrf*s (Fig. 4B), and genes related to extracellular matrix formation (Fig. 4C). Unlike on day 0 (Fig. 2B), capsaicin decreased the expression of *Hmox1* on day 4 (Fig. 4A); AsAp also decreased *Hmox1* expression (Fig. 4A). However, decreased *Hmox1* expression following the addition of 200 μM AsAp was not further decreased by capsaicin. Expression levels of *Myog*, but not *Myf5* and *Myod*, were increased by capsaicin or AsAp, and the combination treatment led to a synergistic increase in the expression of *Myog* (Fig. 4B). The regulatory changes in *Myog* mRNA levels coincided with those in the MYOG protein (Fig. 3F). Previous studies have shown that modulation of the extracellular matrix affects myogenesis [27-31]. Although capsaicin or AsAp significantly changed the expression of genes related to matrix metalloprotease (*Mmp*s) and its inhibitor (*Timp1*), except for *Mmp13*, the extent of modification of the expression levels was minimal (Fig. 4C). In

contrast, treatment with capsaicin or AsAp alone did not affect the expression of α-1 type I collagen (*Col1a1*), but the combination treatment significantly increased *Col1a1* mRNA levels. Expression levels of *Col1a1* were positively correlated with those of *Myh1* and *Myh7* (Fig. 3D).

Here, we revealed that vitamin C alone had a minimal effect on myogenesis, but that combination treatment with capsaicin prior to differentiation and AsAp after differentiation induction enhanced myogenesis, resulting in thick and long myotubes, and increased MYOG and MYH1/2 expression. Thus, the effect of vitamin C on myogenesis is considered to be conditional, depending on the cell context. Currently, information on the involvement of vitamin C in regulatory myogenesis is limited. The present results suggest the importance of vitamin C as a potential and powerful stimulator of myogenesis.

The onset of mild ER stress in undifferentiated myogenic cells is required for the vitamin C-induced enhancement of myogenesis. To date, two functions of vitamin C have been identified, that is, its roles as an antioxidant and a stimulator of collagen maturation [32]. Because AsAp decreased the expression of *Hmox1* and increased the expression of *Col1a1* in cells pretreated with capsaicin, both functions of vitamin C are likely to be involved in enhanced myogenesis. Treatment with capsaicin induces ER stress, which leads to an increase in ROS production in some biological systems [22, 33, 34]. Treatment with AsAp after pre-treatment with capsaicin on stimulatory myogenesis may result from the alleviation of the negative effects resulting from mild ER stress. Stimulation of myogenesis is also related to increased collagen synthesis. Production of the extracellular matrix modulates myogenesis, and type I collagen stimulates myoblast differentiation and MYOG [31]. Murakami et al. [35] showed the presence of an unfolded protein response element, a *cis*-acting regulatory element identified in the promoters of genes induced by ER stress, in *Col1a1*, which is involved in tunicamycin-induced *Col1a1* transcription.

Treatment with capsaicin and AsAp may stimulate *Col1a1* gene transcription via regulatory elements in C2C12 cells.

ER stress has been suggested to remove vulnerable cells for myogenesis through induction of apoptosis, leading to increased formation of myotubes [12]. In addition, the ER-localized isoform of the USP19 deubiquitinating enzyme has been shown to inhibit myogenesis by suppressing ER stress [13]. Considering that treatment with capsaicin prior to differentiation increased the expression of ER stress markers, capsaicin in concert with AsAp may enhance myogenesis through these mechanisms.

Although a high dose of capsaicin (300 μM) enhanced myogenesis in C2C12 cells subsequently treated with AsAp, significant expression of *Trpv1*, a capsaicin receptor [26], was not expressed in C2C12 cells. We previously observed that capsaicin (100 μM)-induced Ca^{2+} influx was not inhibited by 5-iodo-resiniferatoxin, a TRPV1 antagonist, in brown preadipocytes [22]. Therefore, it is possible that capsaicin at 300 μM elicits cell responses through a TRPV1-independent mechanism. The detailed mechanisms should be clarified in future studies.

References

[1] C.S. Stump, E.J. Henriksen, Y. Wei, J.R. Sowers, The metabolic syndrome: role of skeletal muscle metabolism, Ann. Med. 38 (2006) 389-402. https://doi.org/10.1080/07853890600888413.

[2] P. Senesi, L. Luzi, I. Terruzzi, Adipokines, myokines, and cardiokines: the role of nutritional interventions, Int. J. Mol. Sci. 21 (2020) 8372. https://doi.org/10.3390/ijms21218372.

[3] J. Chal, O. Pourquié, Making muscle: skeletal myogenesis in vivo and in vitro, Development 144 (2017) 2104-2122. https://doi.org/10.1242/dev.151035.

[4] W. Chen, D. Datzkiw, M.A. Rudnicki, Satellite cells in ageing: use it or lose it, Open Biol. 10 (2020) 200048. https://doi.org/10.1098/rsob.200048.

[5] H. Weintraub, The MyoD family and myogenesis: redundancy, networks, and thresholds, Cell 75 (1993) 1241-1244. https://doi.org/10.1016/0092-8674(93)90610-3.

[6] P.S. Zammit, Function of the myogenic regulatory factors Myf5, MyoD, Myogenin and MRF4 in skeletal muscle, satellite cells and regenerative myogenesis, Semin. Cell. Dev. Biol. 72 (2017) 19-32. https://doi.org/10.1016/j.semcdb.2017.11.011.

[7] N. Zanou, P. Gailly, Skeletal muscle hypertrophy and regeneration: interplay between the myogenic regulatory factors (MRFs) and insulin-like growth factors (IGFs) pathways, Cell. Mol. Life Sci. 70 (2013) 4117-4130. https://doi.org/10.1007/s00018-013-1330-4.

[8] O. Hernández-Hernández, R.D. Ávila-Avilés, J.M. Hernández-Hernández, Chromatin landscape during skeletal muscle differentiation, Front Genet. 11 (2020) 578712. https://doi.org/10.3389/fgene.2020.578712.

[9] Y. Furutani, M. Murakami, M. Funaba, Differential responses to oxidative stress and calcium influx on expression of the transforming growth factor-β family in myoblasts and myotubes, Cell Biochem. Funct. 27 (2009) 578-582. https://doi.org/10.1002/cbf.1614.

[10] A.J. Majmundar, N. Skuli, R.C. Mesquita, M.N. Kim, A.G. Yodh, M. Nguyen-McCarty, M.C. Simon, O_2 regulates skeletal muscle progenitor differentiation through phosphatidylinositol 3-kinase/AKT signaling, Mol. Cell. Biol. 32 (2012) 36-49. https://doi.org/10.1128/mcb.05857-11.

[11] Y. Ikeda, A. Satoh, Y. Horinouchi, H. Hamano, H. Watanabe, M. Imao, M. Imanishi, Y. Zamami, K. Takechi, Y. Izawa-Ishizawa, L. Miyamoto, T. Hirayama, H. Nagasawa, K. Ishizawa, K.I. Aihara, K. Tsuchiya, T. Tamaki, Iron accumulation causes impaired myogenesis correlated with MAPK signaling pathway inhibition by oxidative stress, FASEB J. 33 (2019) 9551-9564. https://doi.org/10.1096/fj.201802724rr.

[12] K. Nakanishi, N. Dohmae, N. Morishima, Endoplasmic reticulum stress increases myofiber formation in vitro, FASEB J. 21 (2007) 2994-3003. https://doi.org/10.1096/fj.06-6408com.

[13] B. Wiles, M. Miao, E. Coyne, L. Larose, A.V. Cybulsky, S.S. Wing, USP19 deubiquitinating enzyme inhibits muscle cell differentiation by suppressing unfolded-protein response signaling, Mol. Biol. Cell 26 (2015) 913-923. https://doi.org/10.1091/mbc.e14-06-1129.

[14] Y. Mitsumoto, Z. Liu, A. Klip, A long-lasting vitamin C derivative, ascorbic acid 2-phosphate, increases myogenin gene expression and promotes differentiation in L6 muscle cells, Biochem. Biophys. Res. Commun. 199 (1994) 394-402.

https://doi.org/10.1006/bbrc.1994.1242.

[15] A. Shima, J. Pham, E. Blanco, E.R. Barton, H.L. Sweeney, R. Matsuda, IGF-I and vitamin C promote myogenic differentiation of mouse and human skeletal muscle cells at low temperatures, Exp. Cell Res. 317 (2011) 356-366. https://doi.org/10.1016/j.yexcr.2010.11.001.

[16] E. Passerieux, M. Hayot, A. Jaussent, G. Carnac, F. Gouzi, F. Pillard, M.C. Picot, K. Böcker, G. Hugon, J. Pincemail, J.O. Defraigne, T. Verrips, J. Mercier, D. Laoudj-Chenivesse, Effects of vitamin C, vitamin E, zinc gluconate, and selenomethionine supplementation on muscle function and oxidative stress biomarkers in patients with facioscapulohumeral dystrophy: a double-blind randomized controlled clinical trial, Free Radic. Biol. Med. 81 (2015) 158-169. https://doi.org/10.1016/j.freeradbiomed.2014.09.014.

[17] D. Yaffe, O. Saxel, Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle, Nature 270 (1977) 725-727. https://doi.org/10.1038/270725a0.

[18] R. Kida, H. Yoshida, M. Murakami, M. Shirai, O. Hashimoto, T. Kawada, T. Matsui, M. Funaba, Direct action of capsaicin in brown adipogenesis and activation of brown adipocytes, Cell Biochem. Funct. 34 (2016) 34-41. https://doi.org/10.1002/cbf.3162.

[19] M. Funaba, M. Murakami, A sensitive detection of phospho-Smad1/5/8 and Smad2 in Western blot analyses, J. Biochem. Biophys. Methods 70 (2008) 816-819. https://doi.org/10.1016/j.jbbm.2007.10.001.

[20] Y. Furutani, T. Umemoto, M. Murakami, T. Matsui, M. Funaba, Role of endogenous TGF-β family in myogenic differentiation of C2C12 cells, J. Cell. Biochem. 112 (2011) 614-624. https://doi.org/10.1002/jcb.22953.

[21] S.H. Kim, D.Y. Kwon, J.H. Kwak, S. Lee, Y.H. Lee, J. Yun, T.G. Son, Y.S. Jung, Tunicamycin-induced ER stress is accompanied with oxidative stress via abrogation of sulfur amino acids metabolism in the liver, Int. J. Mol. Sci. 19 (2018) 4114. https://doi.org/10.3390/ijms19124114.

[22] R. Kida, T. Noguchi, M. Murakami, O. Hashimoto, T. Kawada, T. Matsui, M. Funaba, Supra-pharmacological concentration of capsaicin stimulates brown adipogenesis through induction of endoplasmic reticulum stress, Sci. Rep. 8 (2018) 845. https://doi.org/10.1038/s41598-018-19223-2.

[23] H. Malhi, R.J. Kaufman, Endoplasmic reticulum stress in liver disease, J. Hepatol. 54 (2011) 795-809. https://doi.org/10.1016/j.jhep.2010.11.005.

[24] B.M. Gardner, D. Pincus, K. Gotthardt, C.M. Gallagher, P. Walter, Endoplasmic reticulum stress sensing in the unfolded protein response, Cold Spring. Harb.

Perspect. Biol. 5 (2013) a013169. https://doi.org/10.1101/cshperspect.a013169.

[25] D. Kennedy, A. Samali, R. Jäger, Methods for studying ER stress and UPR markers in human cells, Methods Mol. Biol. 1292 (2015):3-18. https://doi.org/10.1007/978-1-4939-2522-3_1.

[26] M.J. Caterina, T.A. Rosen, M. Tominaga, A.J. Brake, D. Julius, A capsaicin-receptor homologue with a high threshold for noxious heat, Nature 398 (1999) 436-441. https://doi.org/10.1038/18906.

[27] J. Morgan, A. Rouche, P. Bausero, A. Houssaïni, J. Gross, M.Y. Fiszman, H.S. Alameddine, MMP-9 overexpression improves myogenic cell migration and engraftment, Muscle Nerve 42 (2010) 584-595. https://doi.org/10.1002/mus.21737.

[28] H. Lei, D. Leong, L.R. Smith, E.R. Barton, Matrix metalloproteinase 13 is a new contributor to skeletal muscle regeneration and critical for myoblast migration, Am. J. Physiol. Cell. Physiol. 305 (2013) C529-C538. https://doi.org/10.1152/ajpcell.00051.2013.

[29] C. Snyman, C.U. Niesler, MMP-14 in skeletal muscle repair, J. Muscle Res. Cell. Motil. 36 (2015) 215-225. https://doi.org/10.1007/s10974-015-9414-4.

[30] E. Nowak, M. Gawor, M.A. Ciemerych, M. Zimowska, Silencing of gelatinase expression delays myoblast differentiation in vitro, Cell Biol. Int. 42 (2018) 373-382. https://doi.org/10.1002/cbin.10914.

[31] X. Liu, Y. Gao, X. Long, T. Hayashi, K. Mizuno, S. Hattori, H. Fujisaki, T. Ogura, D.O. Wang, T. Ikejima, Type I collagen promotes the migration and myogenic differentiation of C2C12 myoblasts via the release of interleukin-6 mediated by FAK/NF-κB p65 activation, Food Funct. 11 (2020) 328-338. https://doi.org/10.1039/c9fo01346f.

[32] H. Padh, Vitamin C: newer insights into its biochemical functions, Nutr. Rev. 49 (1991) 65-70. https://doi.org/10.1111/j.1753-4887.1991.tb07407.x.

[33] S.S. Cao, R.J. Kaufman, Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease, Antioxid. Redox Signal. 21 (2014) 396-413. https://doi.org/10.1089/ars.2014.5851.

[34] H.M. Zeeshan, G.H. Lee, H.R. Kim, H.J. Chae, Endoplasmic reticulum stress and associated ROS, Int. J. Mol. Sci. 17 (2016) 327. https://doi.org/10.3390/ijms17030327.

[35] T. Murakami, A. Saito, S. Hino, S. Kondo, S. Kanemoto, K. Chihara, H. Sekiya, K. Tsumagari, K. Ochiai, K. Yoshinaga, M. Saitoh, R. Nishimura, T. Yoneda, I. Kou, T. Furuichi, S. Ikegawa, M. Ikawa, M. Okabe, A. Wanaka, K. Imaizumi, Signalling mediated by the endoplasmic reticulum stress transducer OASIS is involved in bone

formation, Nat. Cell Biol. 11 (2009) 1205-1211. https://doi.org/10.1038/ncb1963.

Figure legends

Figure 1 Expression of *Myh*s and *Mrf*s in C2C12 myogenic cells treated with ascorbic acid or AsAp

One day after confluence (day 0), C2C12 myoblasts were differentiated into myotubes by culturing with 2% HS. Subsequently, AsA or AsAp at 100 μM or 200 μM was administered for 4 days (days 0-4). Expression levels of *Myh*s (A) or *Myf5*, *Myod*, and *Myog* (B) were quantified by RT-qPCR. The expression levels of the respective genes in control cells were set at 1. Data are presented as the mean ± SE (n = 4). * and **: $P < 0.05$ and $P < 0.01$ *vs.* control cell, respectively.

Figure 2 Expression of genes related to ER stress or oxidative stress and *Mrf*s in C2C12 myogenic cells treated with capsaicin

At confluence (day -1), C2C12 myoblasts were treated with or without capsaicin (200 μM or 300 μM) for 12 h (A) or 24 h (B, C). Expression levels of genes related to ER stress (A), oxidative stress (B), and *Mrf*s (C) were quantified by RT-qPCR. The expression levels of the respective genes in control cells were set at 1. Data are presented as the mean ± SE (n = 4). * and **: $P < 0.05$ and $P < 0.01$ *vs.* control cell, respectively.

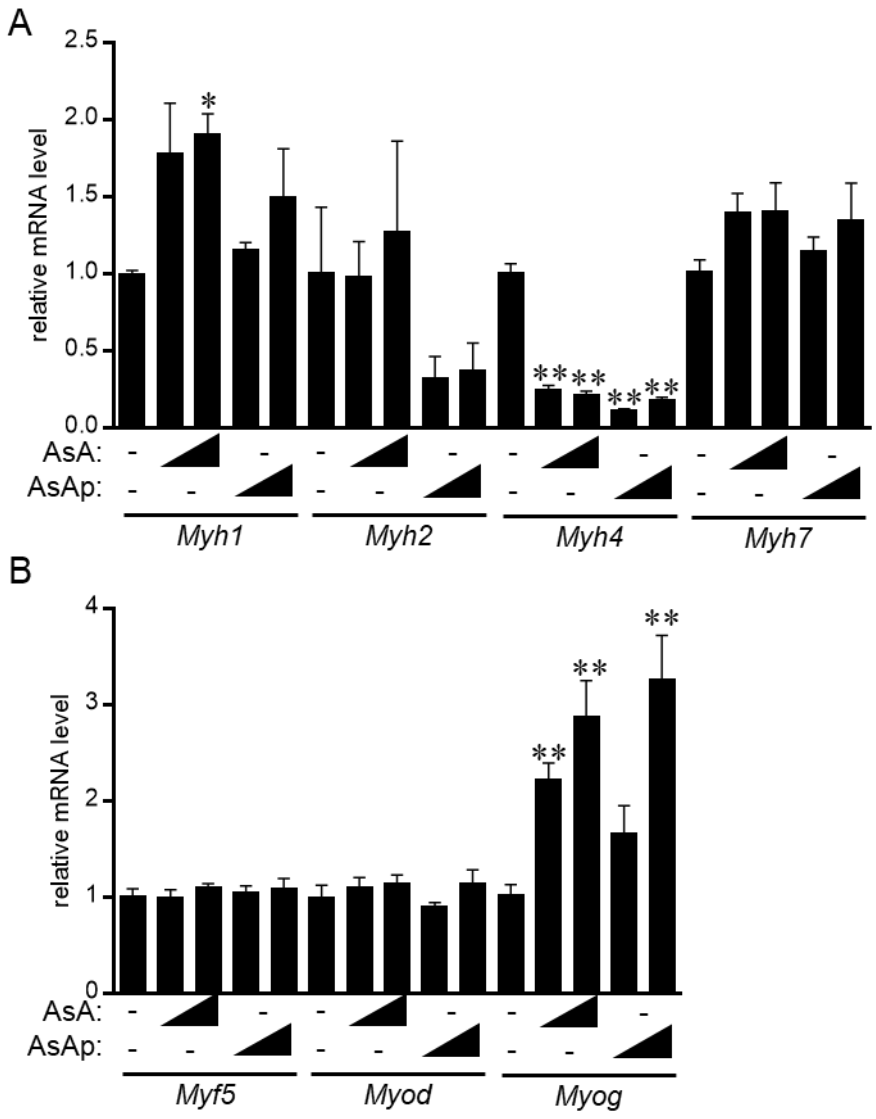
Figure 3 Myogenic differentiation in C2C12 myogenic cells treated with capsaicin and AsAp

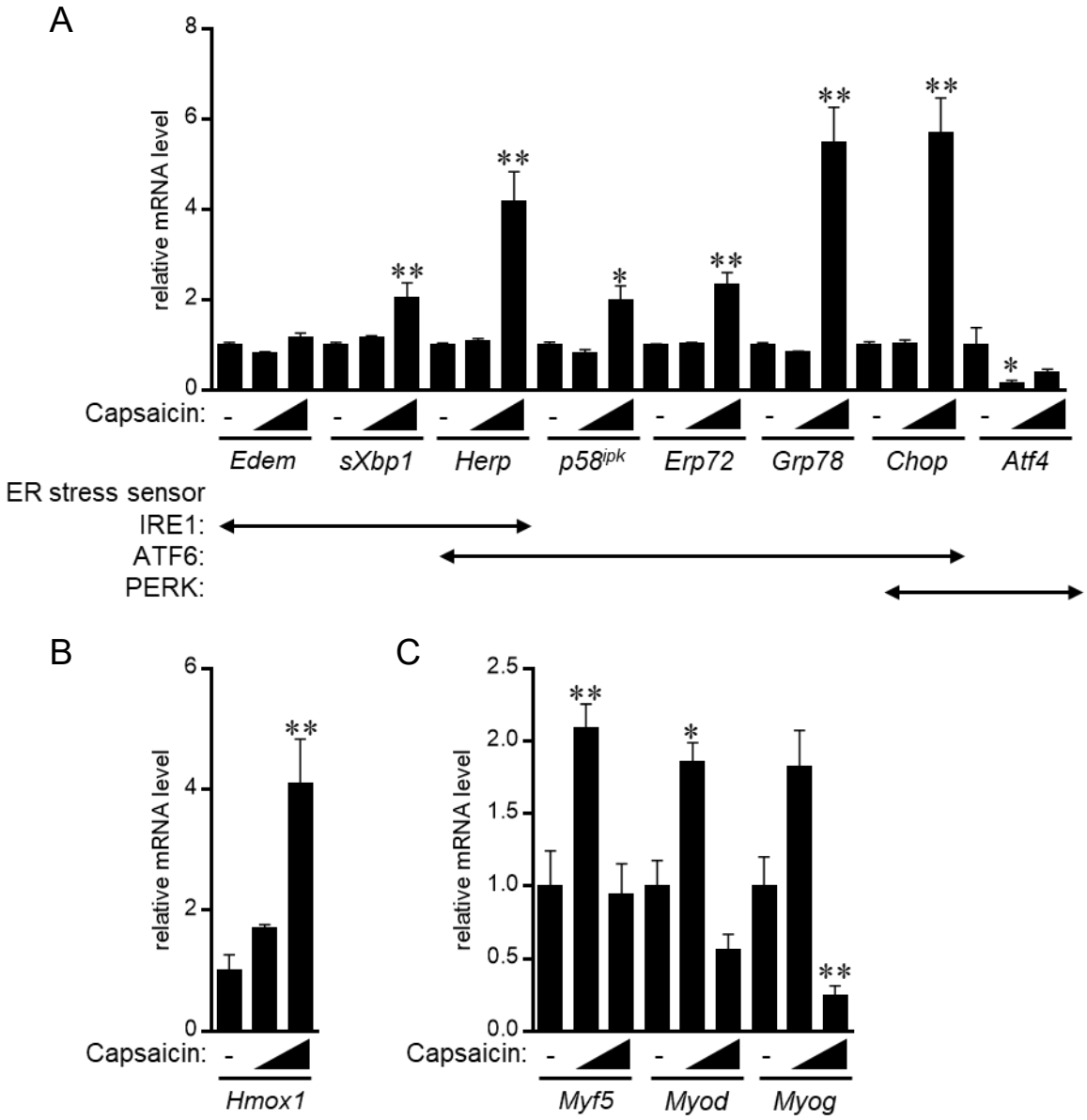
At confluence (day -1), C2C12 myoblasts were treated with or without capsaicin (300 μM) for 24 h, followed by culture in differentiation medium supplemented with AsAp (100 μM or 200 μM) for 4 days (A, E, F) or 8 days (B, C, D). (A) Representative image of Giemsa staining of C2C12 cells on day 4. Bar: 200 μm. (B) Representative image of immunostaining of myosin heavy chain in C2C12 cells on day 8. Green: MYH1/2, blue: nuclei. Bar: 100 μm. (C, D) Fusion index (the ratio of nuclei number in myotubes to total number of nuclei, C), and maturation index (number of nuclei in a myotube, D) are shown.

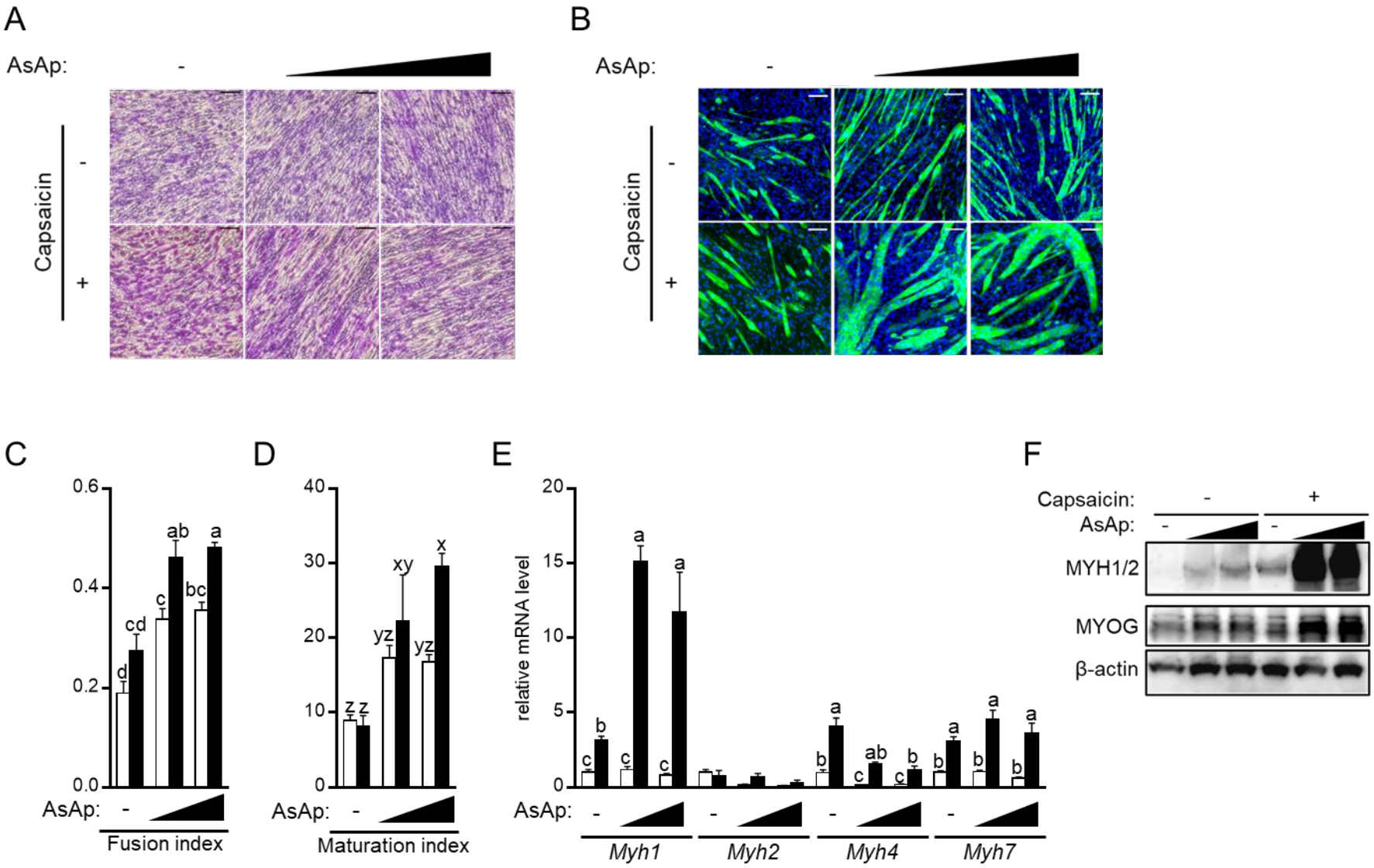
(E) Expression levels of *Myh*s were quantified by RT-qPCR. The expression levels of the respective genes in control cells were set at 1. Data are presented as the mean ± SE (n = 4). White bar: vehicle. Black bar: capsaicin. a, b, c: Means with a common letter on the bar did not differ significantly ($P < 0.05$). x, y, z: Means with a common letter on the bar did not differ significantly ($P < 0.10$). (F) Expression levels of MYH1/2 and MYOG were examined by western blot analysis. As a reference, the expression levels of β-actin were examined.

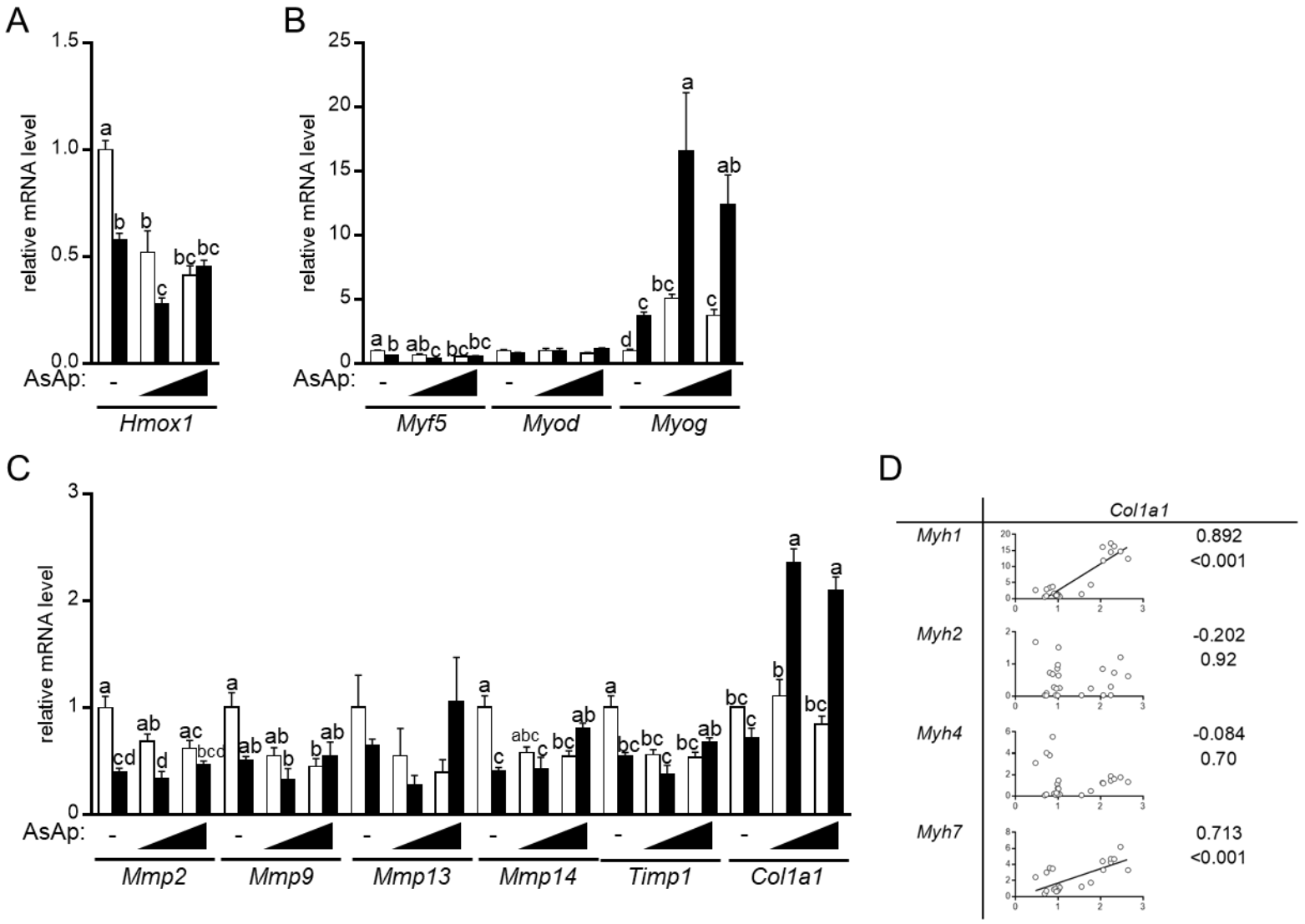
Figure 4 Expression of possible genes affecting modulated myogenesis in C2C12 myogenic cells treated with capsaicin and AsAp

At confluence (day -1), C2C12 myoblasts were treated with or without capsaicin (300 μM) for 24 h, followed by culture in differentiation medium supplemented with AsAp (100 μM or 200 μM) for 4 days. (A) Expression levels of *Hmox1* (A), *Myf5*, *Myod*, and *Myog* (B), and genes related to extracellular matrix formation (C) were quantified by RT-qPCR. The expression levels of the respective genes in control cells were set at 1. Data are presented as the mean ± SE (n = 4). White bar: vehicle. Black bar: capsaicin. a, b, c, d: Means with a common letter on the bar did not differ significantly ($P < 0.05$). (D) The relationship between expression levels of *Col1a1* and myogenic regulatory factors was evaluated using the Pearson correlation coefficient. *Upper*: correlation coefficient; *Lower*: *P-value.* The relationship is also illustrated in graphical form. The solid line indicates a significant regression line ($P < 0.05$).


A
relative mRNA level
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
**
**
AsA:
AsAp:
Myh1
Myh2
Myh4
Myh7
B
relative mRNA level
4
3
2
1
0
**
**
**
AsA:
AsAp:
Myf5
Myod
Myog


A
relative mRNA level
Capsaicin:
Edem
sXbp1
Herp
p58ipk
Erp72
Grp78
Chop
Atf4
ER stress sensor
IRE1:
ATF6:
PERK:
B
relative mRNA level
Capsaicin:
Hmox1
C
relative mRNA level
Capsaicin:
Myf5
Myod
Myog


A
AsAp:
-
Capsaicin
-
+
B
AsAp:
-
Capsaicin
-
+
C
0.6
0.4
0.2
0.0
d
cd
c
bc
ab
a
AsAp:
-
Fusion index
D
40
30
20
10
0
z
z
yz
xy
yz
x
AsAp:
-
Maturation index
E
relative mRNA level
20
15
10
5
0
AsAp:
-
Myh1
Myh2
Myh4
Myh7
F
Capsaicin:
-
+
AsAp:
-
-
MYH1/2
MYOG
β-actin


A
B
C
D
relative mRNA level
AsAp:
Hmox1
Myf5
Myod
Myog
Mmp2
Mmp9
Mmp13
Mmp14
Timp1
Col1a1
Myh1
Myh2
Myh4
Myh7
0.892
<0.001
-0.202
0.92
-0.084
0.70
0.713
<0.001

Supplementary information

Stimulation of myogenesis by ascorbic acid and capsaicin

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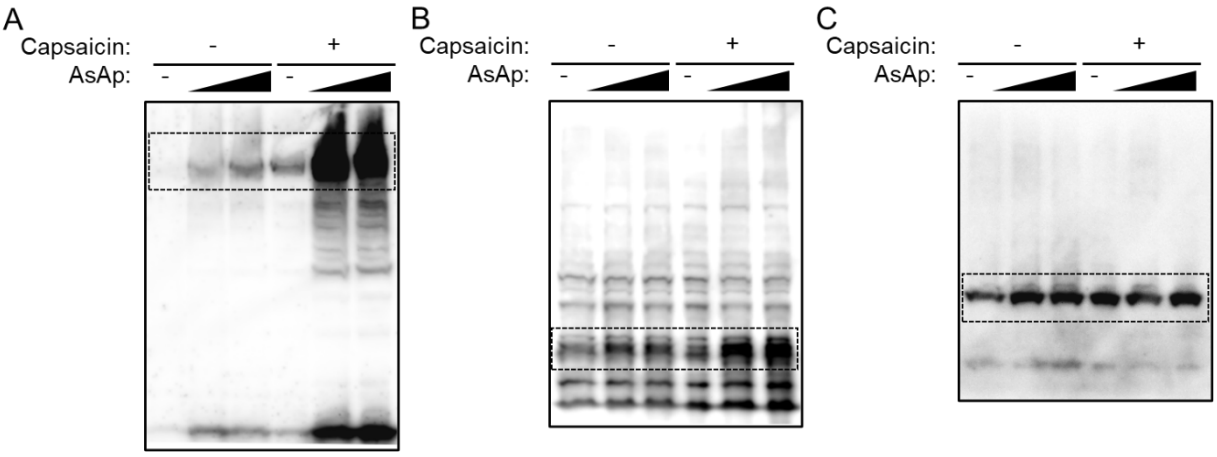

Figure S1. Images of full-length blot shown in Fig. 2F

Western blot analysis was visualized by chemical luminescence-based method. The raw results of Western blot analysis are shown. Dashed squares were cropped and shown in Fig. 6B.

Table S1. Oligonucleotide PCR primers for RT-qPCR

	5’-primer	3’-primer
Atf4	5’-GGCTGGTCGTCAACCTATAAA-3’	5’-CAGGCACTGCTGCCTCTAAT-3’
Chop	5’-GCCAGAATAACAGCCGGAAC-3’	5’-GACCAGGTTCTGCTTTCAGGT-3’
Col1a1	5’-CATGTTCAGCTTTGTGGACCT-3’	5’-GCAGCTGACTTCAGGGATGT-3’
Creb3l1	5’-GTGTCTATGCAGCCAGTCAGA-3’	5’-GTTTGAGCTCCCAGCCTTCT-3’
Edem	5’-GGTCTTCGAAGCTACGATAAGG-3’	5’-GGGCTGTTTGGAATCAGTTATTA-3’
Erp72	5’-GCTACTGGAGTTCTATGCACCA-3’	5’-CCTTCAAAGTACTGGCAATTTTC-3’
Grp78	5’-CTGAGGCGTATTTGGGAAAG-3’	5’-TCATGACATTCAGTCCAGCAA-3’
Herp	5’-GCAGCGACGTTTTCTGTTTT-3’	5’-CTCTGTCTGAACGGAAACCAC-3’
Hmox1	5’-TGAAGGAGGCCACCAAGGAGGT-3’	5’-GGTACAAGGAGGCCATCACCAGCTT-3’
Hprt1	5’-GTTATGACCTAGATTTGTTTTGTA-3’	5’-CTTCATGACATGTCGAGCAAGTCT-3’
Mmp2	5’-GATGTCGCCCCTAAAACAGA-3’	5’-TTTCAGCACAAAGAGGTTGC-3’
Mmp9	5’-AGACGACATAGACGGCATCC-3’	5’-TCGGCTGTGGTTCAGTTGT-3’
Mmp13	5’-GCCAGAACTTCCCAACCAT-3’	5’-TCAGAGCCCAGAATTTTCTCC-3’
Mmp14	5’-GAGAACTTCGTGTTGCCTGA-3’	5’-CTTTGTGGGTGACCCTGACT-3’
Myh1	5’-GGACCCACGGTCGAAGTTG-3’	5’-GGCTGCGGGCTATTGGTT-3’
Myh2	5’-CAGCTGCACCTTCTCGTTTG-3’	5’-CCCGAAAACGGCCATCT-3’
Myh4	5’-CCGAGCAAGAGCTACTGGA-3’	5’-TGTTGATGAGGCTGGTGTTC-3’
Myh7	5’-CATCCCCAATGAGACAAAGTC-3’	5’-CGGAAGTCCCCATAGAGAATG-3’
Myf5	5’-CTGCTCTGAGCCCACCAG-3’	5’-GACAGGGCTGTTACATTCAGG-3’
Myod	5’-AGCACTACAGTGGCGACTCA-3’	5’-GGCCGCTGTAATCCATCAT-3’
Myog	5’-ACAGGCCTTGCTCAGCTC-3’	5’-CGCTGTGGGAGTTGCATT-3’
$p58^{ipk}$	5’-AAGAAGTCCTCTCAGACCCAGA-3’	5’-GAGTTCCAACTTCTGTGGAAGG-3’
sXbp1	5’-TGCTGAGTCCGCAGCAGGTG-3’	5’-GCTGGCAGGCTCTGGGGAAG-3’
Timp1	5’-GCAAAGAGCTTTCTCAAAGACC-3’	5’-AGGGATAGATAAACAGGGAAACACT-3’