The combination of ursolic acid and leucine potentiates the differentiation of C2C12 murine myoblasts through the mTOR signaling pathway

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Received July 1, 2014; Accepted December 8, 2014

DOI: 10.3892/ijmm.2014.2046

Abstract. Aging causes phenotypic changes in skeletal muscle progenitor cells that lead to the progressive loss of myogenic differentiation and thus a decrease in muscle mass. The naturally occurring triterpene, ursolic acid, has been reported to be an effective agent for the prevention of muscle loss by suppressing degenerative muscular dystrophy. Leucine, a branched-chain amino acid, and its metabolite, β-hydroxy-β-methylbutyric acid, have been reported to enhance protein synthesis in skeletal muscle. Therefore, the aim of the present study was to investigate whether the combination of ursolic acid and leucine promotes greater myogenic differentiation compared to either agent alone in C2C12 murine myoblasts. Morphological changes were observed and creatine kinase (CK) activity analysis was performed to determine the conditions through which the combination of ursolic acid and leucine would exert the most prominent effects on muscle cell differentiation. The effect of the combination of ursolic acid and leucine on the expression of myogenic differentiation marker genes was examined by RT-PCR and western blot analysis. The combination of ursolic acid (0.5 μM) and leucine (10 μM) proved to be the most effective in promoting myogenic differentiation. The combination of ursolic acid and leucine significantly increased CK activity than treatment with either agent alone. The level of myosin heavy chain, a myogenic differentiation marker protein, was also enhanced by the combination of ursolic acid and leucine. The combination of ursolic acid and leucine significantly induced the expression of myogenic differentiation marker genes, such as myogenic differentiation 1 (MyoD) and myogenin, at both the mRNA and protein level. In addition, the number of myotubes and the fusion index were increased. These findings indicate that the combination of ursolic acid and leucine promotes muscle cell differentiation, thus suggesting that this combination of agents may prove to be beneficial in increasing muscle mass.

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Key words: ursolic acid, leucine, muscle cell differentiation, mammalian target of rapamycin signaling pathway

Introduction

Skeletal muscle development is a strictly regulated process involving the specification of mesodermal precursors into myoblasts, following the differentiation and fusion of these cells into multinucleated myotubes. Myogenic regulatory factors (MRFs) play a specific role in muscle fusion during differentiation (1,2). Among the MRFs, basic helix-loop-helix (bHLH) transcription factors, myogenic differentiation 1 (MyoD), myogenic factor 5 (Myf5), myogenin and MRF4 are critical to muscle formation. MyoD and Myf5 are required for the formation of skeletal muscle and play unique roles in the development of epaxial and hypaxial muscle, respectively (3,4). These two MRFs enable the differentiation of myogenic progenitors into myoblasts, which differentiate into myotubes by myogenin. Moreover, MyoD promotes myoblast differentiation by regulating the cell cycle (5). MRF4 is important for blocking the transcription of muscle-specific promoters, and enabling the growth and proliferation of skeletal muscle progenitors prior to differentiation. Myogenin activates MRF4 transcription independently, and synergistically stimulates the MRF4 promoter (6).

Ursolic acid, a natural pentacyclic triterpenoid carboxylic acid (7), is abundantly found in a number of fruits and plants, including apples (a major compound of apple peels), cranberries, basil, peppermint and rosemary (8). It has been used for pharmaceutical, cosmetic and food preparations. Of note, ursolic acid has garnered much attention as a therapeutic compound in a number of diseases, such as cancer (9), diabetes (10), Alzheimer's disease (11) and obesity (12), due to its anti-inflam-

matory and antitumor properties. Moreover, this triterpene has been reported to increase the insulin-induced phosphorylation of Akt, a serine/threonine-specific protein kinase, that plays a key role in multiple cellular processes, such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration (13). In skeletal muscle, Akt has been reported to inhibit muscle atrophy and promote muscle hypertrophy, which is linked to muscle protein synthesis. Recently, Kunkel *et al* (14) demonstrated that ursolic acid enhanced skeletal muscle insulin/insulin-like growth factor 1 (IGF-I) signaling, leading to Akt activation, muscle hypertrophy and reduced adiposity and blood glucose levels in mice. Ursolic acid has also been shown to increase muscle mass through Akt activation in fed a mice high-fat diet (15). In the same study, ursolic acid exhibited a beneficial effect by not only increasing skeletal muscle mass, but also increasing energy expenditure, leading to reduced obesity, improved glucose tolerance and decreased hepatic steatosis. These findings suggest the therapeutic potential for ursolic acid in muscle atrophy caused by aging and illnesses, including cancer and other metabolic diseases.

L-leucine is an essential amino acid that belongs to a group of branched-chain amino acids (BCAAs). BCAAs, including leucine, valine and isoleucine, are found mainly in skeletal muscle, and are thus essential in supporting muscle growth, comprising up to one-third of muscle (16). Among the BCAAs, leucine has been shown to directly activate the mammalian target of rapamycin (mTOR) signaling cascade which is closely associated with protein synthesis in muscle (17). Leucine has also been reported to stimulate muscle growth and repair through the accumulation of glycogen, which is a muscular energy source. β-hydroxy β-methylbutyrate (HMB), the most popular muscle enhancing agent and a metabolite of leucine is known to reinforce muscle (18). Several lines of evidence indicate that dietary supplements, such as leucine and HMB, promote body mass and strength (19,20). Due to their anabolic effects and ability to promote muscle recovery following injury and in some muscle diseases, the dietary supplementation of leucine or HMB is considered an attractive therapeutic agent for reducing muscle loss in aging populations (21,22).

The mTOR signaling pathway is one of the main signaling pathways controling protein synthesis and cell proliferation and is a serine/threonine protein kinase belonging to the phosphatidylinositol 3-kinase (PI3K)-related kinase protein family that regulates cell growth, cell proliferation, cell motility and cell survival (20). The mTOR signaling pathway is mediated by two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 and mTORC2 share several components and contain unique proteins. The activity of mTORC1 is stimulated by insulin, growth factors, amino acids (particularly leucine) and oxidative stress through the PI3K/Akt pathway. Activated mTORC1 kinase upregulates protein synthesis through the phosphorylation of key regulators of mRNA translation, including eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1), a binding partner of the cap-binding protein, eIF4E (23). 4E-BP1 is an important regulator of overall translational levels in cells (24). It has also been suggested to control the growth rate of tissue during development as a growth regulator (25). The ribosomal protein S6 kinase 1 (S6K1) is one of two mammalian p70 proteins, acting to converge growth factor, hormonal, nutrient and energy signals in order to maintain cellular homeostasis. In addition, the kinase activity of S6K1 leads to an increase in protein synthesis and cell proliferation (26). The aim of the present study was to investigate whether the combination of ursolic acid and leucine promotes greater myogenic differentiation compared to treatment with either agent alone in C2C12 murine myoblasts.

Materials and methods

Reagents. Ursolic acid, L-leucine and monoclonal antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ursolic acid was dissolved in dimethyl sulfoxide (DMSO) and stored at -20˚C prior to the experiments and dilutions were made in culture medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA). Antibodies against myosin heavy chain (MHC; sc-20641), MyoD (sc-760), myogenin (sc-576), phospho-Akt (Ser 473; sc-7985-R), and Akt1/2/3 (sc-8312) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against 4E-BP1 (cat. no. 9452), phospho-4E-BP1 (cat. no. 2855) and phospho-p70S6K (cat. no. 9234) were purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibody against p70S6K (bs-6370R) was obtained from Bioss (Woburn, MA, USA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Welgene Inc. (Daegu, Korea) and horse serum (HS) was from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) and penicillin-streptomycin were purchased from HyClone (Logan, UT, USA). The creatine kinase enzymatic assay kit (MaxDiscovery™ Creatine Kinase Enzymatic Assay kit) was purchased from Bioo Scientific Corp. (Austin, TX, USA).

Cell culture and cell viability assay. The murine myoblast cell line, C2C1,2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at 37˚C in humidified 95% air and 5% CO_2 in growth medium (GM), containing DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at up to 70-80% confluence. During proliferation, the cells were seeded at $2x10^5$ cells/well in 6-well culture plates and grown in GM. When the cells reached approximately 90-100% confluence, the GM was removed and replaced with differentiation medium (DM), containing DMEM supplemented with 2% horse serum (HS) and ursolic acid, leucine or a combination of ursolic acid and leucine. The medium was changed every 2 days until day 6. The effects of ursolic acid, leucine or their combination on cell viability were determined by MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes.

Creatine kinase (CK) activity. The cells were washed with phosphate-buffered saline (PBS) and then lysed with lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% NP-40, 2 μg/ml aprotinin, 2 μg/ml leupeptine and 100 μg/ml phenymethylsulfonyl fluoride (PMSF)], and complete protease inhibitor and stored at -70˚C until use. CK activity was determined by a CK enzymatic assay kit (Bioo Scientific Corp.), according to the manufacturer's instructions. Briefly, 250 μl of CK reagent were added to 5 μl of cell lysate in a microplate. Subsequently, CK activity was immediately measured 2 times with a 5-min time interval at 340 nm. The assay was performed in duplicate. The

average 5 min absorbance increase was multiplied by 2,186 (conversion factor) to obtain the CK activity (IU/l).

Western blot analysis. Following treatment, the cells were harvested and washed with cold PBS and then lysed in lysis buffer. Following centrifugation, the supernatant was collected and the protein concentration was determined using protein assay reagents (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were denatured by boiling at 100˚C for 5 min in 2X Laemmli sample buffer (Bio-Rad Laboratories). The protein samples were separated on 6-15% SDS-PAGE gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBS-T; 20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature. The membranes were then incubated with various primary antibodies at 4˚C overnight. After being thoroughly washed with TBS-T buffer, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). Antigen-antibody complexes were detected by the Enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Immunocytochemistry. For immunofluorescence analysis to evaluate myogenic differentiation, the cells were cultured on gelatin-coated coverslips. For this, the coverslips were soaked in 1 M HCl for 24 h, rinsed 3 times with distilled water and then rinsed 3 times with 95% ethanol. The coverslips were submerged in gelatin solution (0.1 mg/ml) for 5 min, air-dried and placed into 6-well culture plates. Finally, the culture plates with coverslips were sterilized under a UV lamp for at least 2 h. The cells ($2x10^5$/well) were seeded and cultured overnight. The cells were then rinsed with PBS, fixed with 4% formalin and 0.3% glutaraldehyde for 20 min, washed with PBS, incubated in PBS supplemented with 0.2 % Triton X-100 and 2% normal goat serum for 1 h at 4˚C. The cultures were then incubated overnight with primary antibody against MHC (1:200), followed by rhodamine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). The samples were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) to prevent fading. The samples were examined under a confocal laser scanning microscope (Zeiss, Göettingen, Germany).

Fusion index. The images were randomly selected from at least 3 regions from each well. The fusion index was calculated based on the scoring of random regions with stained nuclei and myotubes.

Reverese transcription-polymerase chain reaction. Total RNA was prepared using TRIzol reagent (Invitrogen). cDNA for the PCR template was synthesized using TOPscript™ RT DryMIX(dT18) (Enzynomics, Daejeon, Korea). For PCR, specific primers were used for the analysis of the expression for the following molecules: MyoD forward, 5'-AGTGAATGAGGCC TTCGAGA-3' and reverse, 5'-CTGGGTTCCCTGTTCTG TGT-3' (514 bp); myogenin forward, 5'-ACCAGGAGCCCC ACTTCTAT-3' and reverse, 5'-ACGATGGACGTAAGGG AGTG-3' (583 bp); GAPDH forward, 5'-ACTCCACTCACGG CAAATTC-3' and reverse, 5'-CCTTCCACAATGCC AAAGTT-3' (370 bp). Subsequently, the PCR reactions were performed in a Mastercycler gradient unit (Eppendorf, Hamburg, Germany) using PCR PreMix (Bioneer Corp., Daejeon, Korea). Each PCR cycle was 94˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 1 min. The numbers of cycles of PCR were 26 for MyoD, 27 for myogenin and 25 for GAPDH. The amplified PCR products (7 μl) were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining.

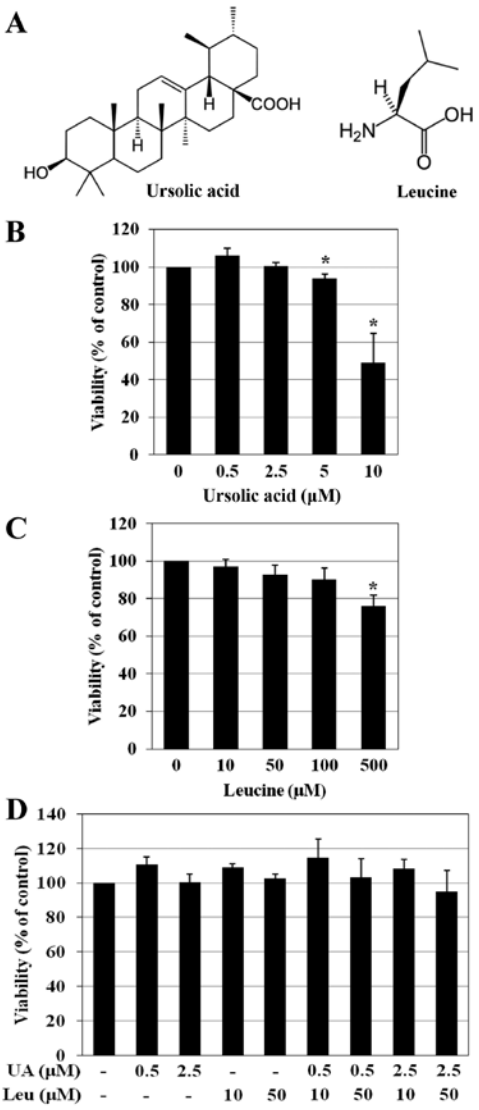

Figure 1. Chemical structures (A) and the effects of ursolic acid or/and leucine on the viability of C2C12 cells. One day after seeding, the cells were treated with various concentrations of (B) ursolic acid, (C) leucine and (D) various combinations of ursolic acid and leucine for 6 days and the percentage of cell survival was then measured by MTT assay. The results are expressed as the means ± SD (n=3). *P<0.05 compared with the control group. UA, ursolic acid; Leu, leucine.

Statistical analysis. The results were expressed as the means ± SD of 3 separate experiments and analyzed using the Student's t-test and ANOVA. Values of P<0.05 or P<0.01 were considered to indicate statistically significantly differences.

Results

Ursolic acid and leucine have no effect on C2C12 cell viability. To determine the effects of ursolic acid and leucine on the

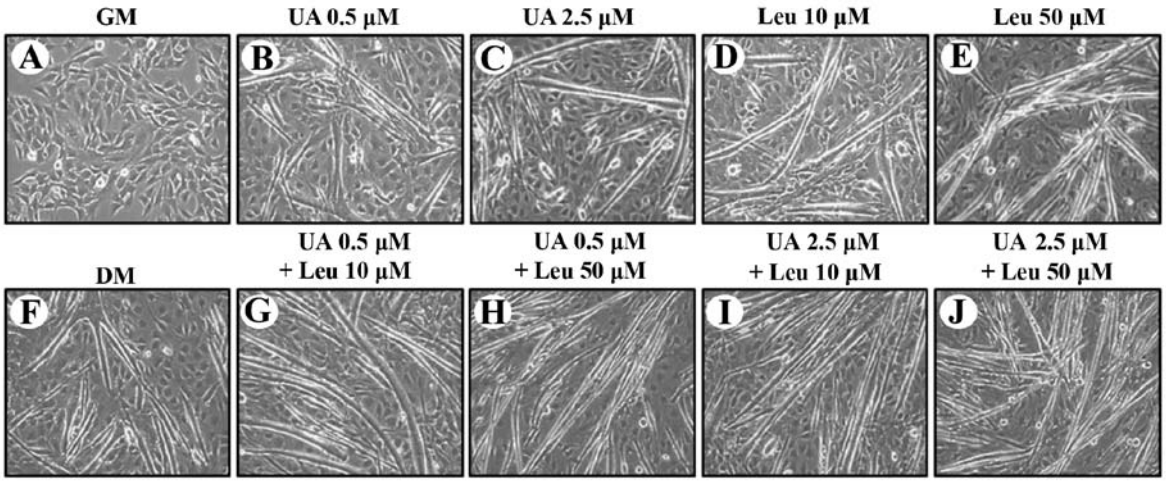

Figure 2. Effects of the administration of ursolic acid, leucine and the combination of ursolic acid and leucine on cell morphology. Cells were treated with ursolic acid, leucine of the combination of ursolic acid and leucine for 6 days, and were then photographed under a phase contrast microscope at x400 magnification. Both ursolic acid and leucine induced morphological changes in the C2C12 cells. The shape of the cells treated with ursolic acid and leucine differed greatly from those of the controls (GM and DM). The cells seemed to lose their typical triangular morphology and the cell shape gradually changed into a new elongated shape. In addition, the cells treated with the combinations of ursolic acid and leucine showed a more longer and stretched shape compared with the cells treated with either agent alone. The results are representative of 1 of 3 independent experiments with similar results. GM, growth medium; UA, ursolic acid; Leu, leucine; DM, differentiation medium.

viability of C2C12 cells, an MTT assay was performed. The C2C12 cells were cultured in DM and treated with ursolic acid (0-10 μM) or leucine (0-500 μM) for 6 days. Cell viability was not affected by treatment with 0-2.5 μM ursolic acid and 0-100 μM leucine. However, treatment with >2.5 μM of ursolic acid and >100 μM of leucine exerted cytotoxic effects (Fig. 1B and C). To investigate whether the combination of ursolic acid and leucine would promote the differentiation of C2C12 myoblasts, the cells were treated with various concentrations of ursolic acid and leucine (at concentrations that had not affected cell viability: ursolic acid, 0.5 and 2.5 μM; leucine, 10 and 50 μM). To confirm the effects of these combinations on cell viability, an MTT assay was conducted again. Treatment with various combinations of ursolic acid and leucine (Fig. 1D), similar to the treatments with either agent alone, had no effect on viability of the C2C12 cells.

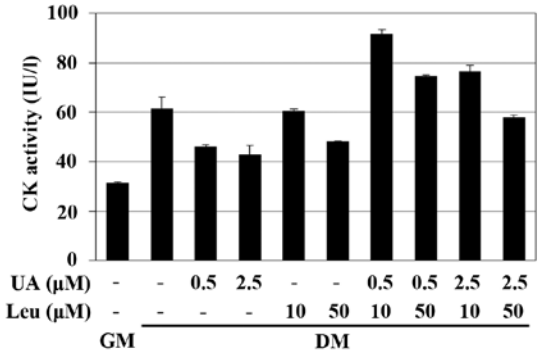

Figure 3. Creatine kinase (CK) activity of C2C12 cells treated with various combinations of ursolic acid and leucine. Analysis of CK activity from C2C12 myotubes induced to differentiate for 6 days by the administration of ursolic acid, leucine or the combination of ursolic acid and leucine. The results are representative of 1 of 3 independent experiments with similar results. UA, ursolic acid; Leu, leucine; GM, growth medium; DM, differentiation medium.

Ursolic acid, leucine, and the combination of ursolic acid and leucine promote myotube formation. Our first hypothesis in the present study was that the combination of ursolic acid and leucine would promote greater myogenic cell differentiation than either ursolic acid or leucine alone. As shown in Fig. 2, both ursolic acid and leucine induced morphological changes in the C2C12 cells. When the ursolic acid- or leucine-treated groups were compared with the untreated groups (GM and DM), the cell shapes differed greatly (Fig. 2 A-F). After 3-4 days of differentiation, the cells seemed to lose their typical triangular morphology and the cell shape gradually changed into a new elongated shape. Furthermore, the differentiation-promoting effects of the different combinations of the agents were even more pronounced. The myotubes that formed in the cells treated with combinations of ursolic acid and leucine were more stretched and longer in shape than those in the cells treated with each agent alone (Fig. 2G-J). In particular, in the cells that were treated with the combination of 0.5 μM ursolic acid and 10 μM leucine, the myotubes showed a spindly ring pattern, indicating muscle hypertrophy and maturation (Fig. 2G).

The level of CK activity is increased by the combination of ursolic acid and leucine. CK activity was used as an indicator of differentiation. Compared to the GM control, CK activity levels in the cells that were treated with ursolic acid or/and leucine for 6 days were increased (Fig. 3). However, the CK activity level in the cells treated with either agent alone (ursolic acid or leucine) was lower than that in the cells treated with the various combinations of ursolic acid and leucine. The level of CK activity in the cells treated with the combination of 0.5 μM ursolic acid and 10 μM leucine was significantly increased by 50% compared to the DM control during differentiation, and this combination led to the most significant increase in CK activity. The second most significant increase in the level of CK activity was observed in the cells treated with the combination of 2.5 μM ursolic acid and 10 μM leucine.

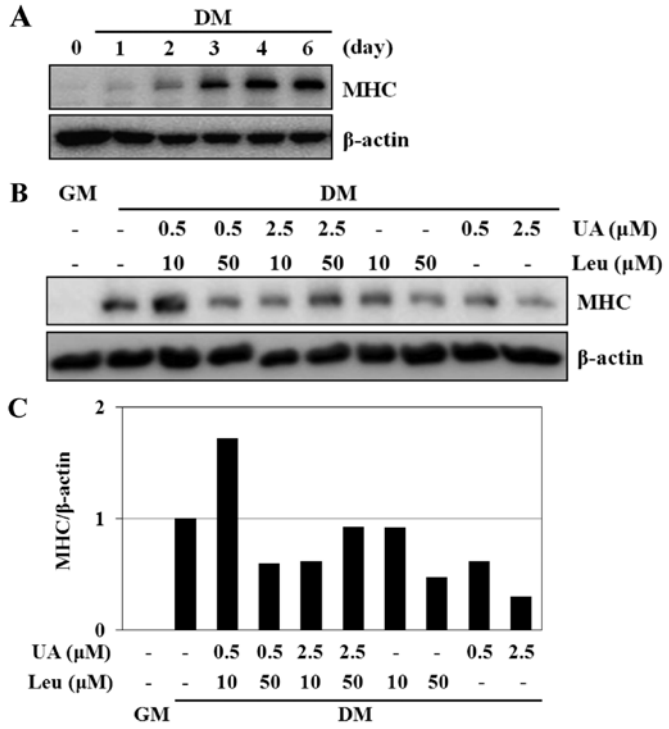

Figure 4. Myosin heavy chain (MHC) protein expression in C2C12 cells. (A) Western blot analysis was performed to detect the protein level of MHC in the C2C12 cells at 1, 2, 3, 4 and 6 days (time-dependent) of differentiation with no treatment. (B) The cells were treated with ursolic acid, leucine or various combinations of ursolic acid and leucine for 6 days, and the protein level of MHC was analyzed by western blot analysis. β-actin represents the quantity of protein loaded (loading control). (C) Densitometric analysis for the quantification of the results of western blot analysis as a relative value of the level of β-actin. Results are representative of 1 of 3 independent experiments with similar results. GM, growth medium; DM, differentiation medium; UA, ursolic acid; Leu, leucine.

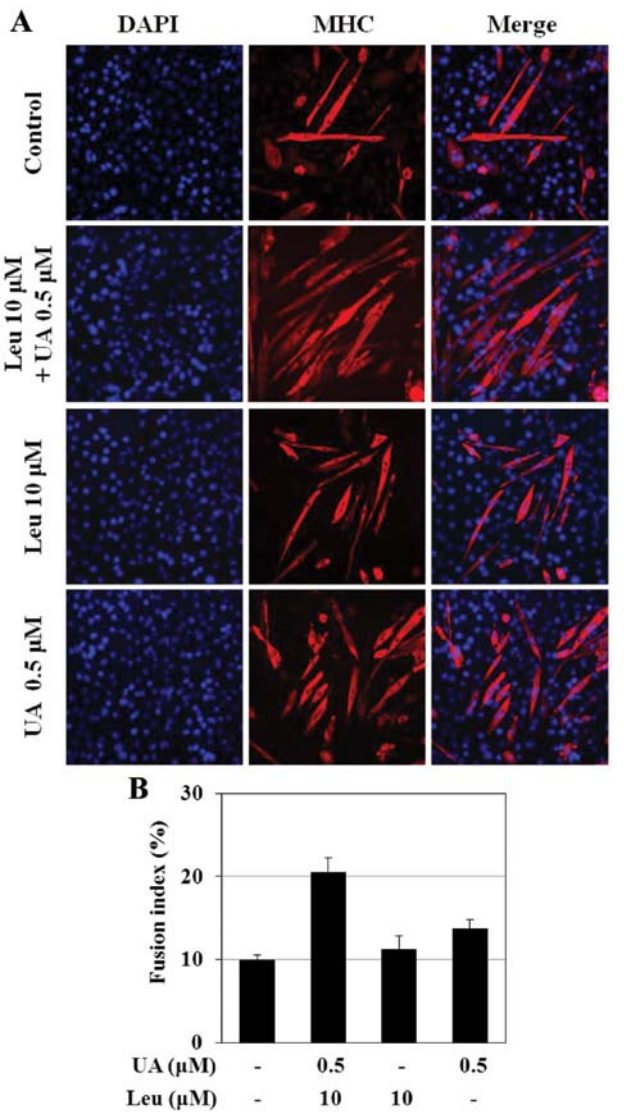

Figure 5. Effects of combination treatment with ursolic acid and leucine on myotubes numbers and fusion index. (A) Myotubes were allowed to differentiate for 6 days, in the absence (control) or presence of 0.5 μM ursolic acid or/and 10 μM leucine (single treatments and combination), and then stained with anti-MHC and DAPI (scale bar, 100 μm). (B) The fusion index was calculated from cells that were immunostained for MHC. Fusion index was defined as the number of nuclei present in myotubes in comparison to the total number of nuclei present in the observed field. Data were selected from 3 different and randomly selected microscopic fields. UA, ursolic acid; Leu, leucine.

The combination of ursolic acid and leucine enhances MHC protein expression. MHC is considered as a marker of the differentiated state, particularly in the late differentiation of myoblasts. First, western blot analysis was performed to detect the changes in the protein expression levels of MHC over time. The protein levels of MHC gradually increased over 6 days, with the highest levels being observed on day 6 (Fig. 4A). Subsequently, in order to confirm whether the combination of ursolic acid and leucine promotes the differentiation of C2C12 cells as opposed to treatment with each agent alone, the protein level of MHC was evaluated. As expected, treatment with the combination of 0.5 μM ursolic acid and 10 μM leucine induced a significant increase in the expression level of MHC (Fig. 4B). Compared to the DM control, MHC expression increased by almost 2-fold. In addition, compared to the cells treated with the agents alone (0.5 μM ursolic acid or 10 μM leucine), the protein expression of MHC increased by approximately 3- and 2-fold in the cells treated with the combination of 0.5 μM ursolic acid and 10 μM leucine, respectively (Fig. 4C).

The combination of ursolic acid and leucine increases the number of myotubes and the fusion index. To analyze the changes occurring in the nuclear arrangement of myotubes during the late phase of differentiation, immunofluorescence staining using antibody against MHC was performed. As shown in Fig. 5A, after 6 days of differentiation, most of the untreated cells (controls) that expressed MHC were mononuclear; however, in the presence of 0.5 μM ursolic acid or 10 μM leucine, myotubes containing 2 and more nuclei were observed. The number of myotubes containing 4 or more nuclei was highest in the cells treated with the combination of 0.5 μM ursolic acid and 10 μM leucine. In the cells treated with the combination treatment, the myotubes were characterized by a particular arrangement of the nuclei, forming a ring, a morphological marker of muscle maturation. With the combination treatment, the highest density of MHC-expressing cells and a greater number of elongated cells were observed, some of which fused into myotubes (Fig. 5A). The fusion index was also found to be significantly higher (>2-fold) in the cells treated

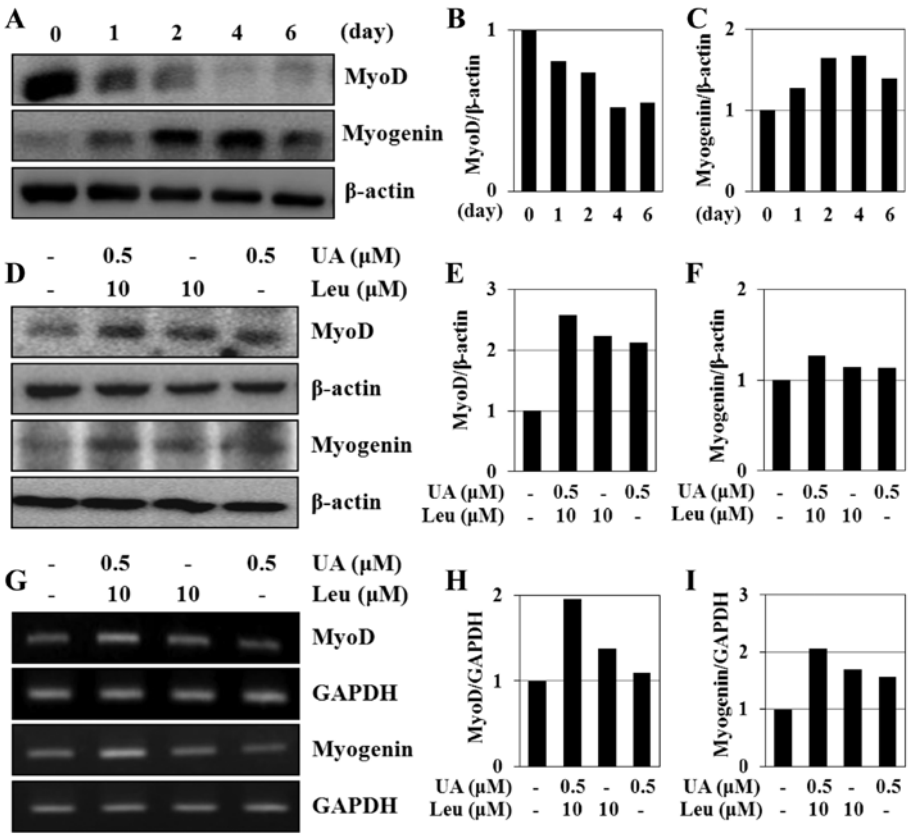

Figure 6. Effect of the combination of ursolic acid and leucine on myogenic regulatory factor (MRF) expression at the protein level and mRNA level. (A) Cells were cultured in differentiation medium (DM) for 0, 1, 2, 4 and 6 days. The protein level of MyoD, myogenin and β-actin was measured by western blot analysis. β-actin represents the quantity of protein loaded. (B and C) The blots were quantified by densitometry as a relative value of the level of β-actin. (D) Cells were treated with ursolic acid, leucine or the combination of ursolic acid and leucine for 1 day and 2 days each, and the protein levels of MyoD and myogenin were analyzed by western blot anlaysis. β-actin represents the quantity of protein loaded. (E and F) The blots were quantified by densitometry as a relative value of the level of β-actin. (G) Cells were treated ursolic acid 0.5 μM and/or leucine 10 μM for 6 days, and the mRNA levels of MyoD and myogenin were analyzed by PCR. (H and I) Each bar represents the relative levels of mRNA normalized to GAPDH. The blots were quantified by densitometry as a relative value of the level of GAPDH. Results are representative of 1 of 3 independent experiments with similar results. UA, ursolic acid; Leu, leucine.

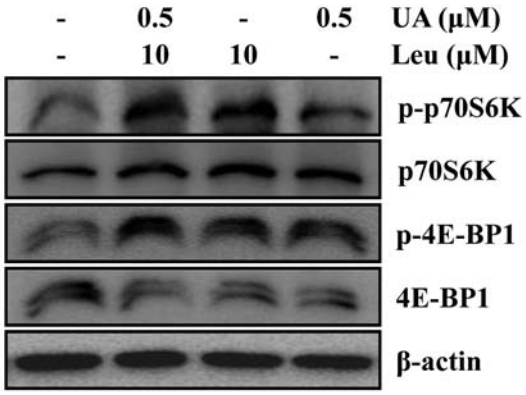

Figure 7. Effect of the combination of ursolic acid and leucine on the mTOR signaling pathway. Cells were cultured in differentiation medium (DM for 6 days with no treatment, or were treated with ursolic acid, leucine or the combination of both (0.5 μM ursolic acid and 10 μM leucine). The protein levels of phosphorylated (p-)p70S6K, p70S6K, p-4E-BP1 and 4E-BP1 were measured by western blot analysis. β-actin represents the quantity of protein loaded. Results are representative of 1 of 3 independent experiments with similar results. UA, ursolic acid; Leu, leucine.

with the combination of 0.5 μM ursolic acid and 10 μM leucine, compared to the untreated cells (Fig. 5B).

The combination of ursolic acid and leucine increases the expression levels of MyoD and myogenin. In our experiments, MHC was used as a marker of late differentiation. In order to determine the effects of ursolic acid and leucine on early differentiation, MyoD and myogenin were used as biomarkers. First, quantitative analysis of the results from western blot analysis further revealed time-dependent (on day 1, 2, 4 and 6) changes in the expression of the early differentiation markers, MyoD and myogenin. As shown in Fig. 6A and B, MyoD was predominantly expressed on day 1 (Fig. 6B), while myogenin was predominantly expressed on day 2 (Fig. 6C). Compared with the untreated controls, the protein expression of MyoD in the cells treated with the combination of 0.5 μM ursolic acid and 10 μM leucine increased by >2.5-fold (Fig. 6D and E). The epxression of myogenin was also increased, although the increase was not as notable as that of MyoD (Fig. 6D and F). In addition, the mRNA levels of the muscle regulatory factors, MyoD and myogenin, were elevated in response to the combination treatment (Fig. 6G). A sharp increase in the mRNA level of MyoD was observed in the cells treated with the combination of ursolic acid and leucine (Fig. 6H). The mRNA levels of myogenin were also increased, although the increase was not as notable as that of MyoD (Fig. 6I).

The combination of ursolic acid and leucine promotes C2C12 myoblast differentiation through the mTOR signaling pathway. Protein synthesis occurs during embryonic development, cell growth, cell differentiation and aging. In previous studies, signaling pathways, such as the IGF-1, PI3K/Akt, and mTOR signaling cascades, have been shown to play a major role in protein synthesis by targeting several components of the translation machinery (27,28). To confirm the effects of the combination of ursolic acid and leucine on the activation of the mTOR signaling pathway, western blot analysis of S6K1 and 4E-BP1, which are two key downstream targets of the mTOR signaling pathway (29), was conducted. In addition, the phosphorylation of 4E-BP1 and S6K1 are crucial activation steps necessary for the downstream event (30). Treatment with the combination of ursolic acid and leucine increased the phosphorylation of mTOR and that of the downstream targets, 4E-BP1 and S6K1, as shown in Fig. 7.

Discussion

Skeletal muscle plays important roles in initiating movement, supporting respiration and maintaining homeostasis; the loss of skeletal muscle mass or function is associated with aging and a variety of diseases, such as cancer and diabetes (31). Several studies have assessed the potential use of ursolic acid or leucine as an effective material to stimulate myotube maturation and muscle differentiation in C2C12 cells (15,32,33). In the present study, we examined whether the combination of ursolic acid and leucine is more effective in inducing muscle cell differentiation when compared with the use of either agent alone.

The process of the formation of myofibers is controlled by a set of MRFs, such as MyoD and its relatives, Myf-5, MRF4 and myogenin. MRFs have different roles during myogenesis, and their expression levels also differ. While MyoD and Myf5 determine the myogenic lineage of muscle progenitor cells (3,34), myogenin and MRF4 drive the terminal differentiation and fusion of myoblasts into myotubes, the developing myofibers (35,36). MyoD is regulated by several biochemical modifications and interactions, such as ubiquitination (37), acetylation (38), phosphorylation (39) and is negatively regulated by methylation (40). In addition, it can interact with cell cycle regulators, such as retinoblastoma protein (pRB) (41) and cdk4 (42) to induce cell cycle withdrawal directly during myogenic differentiation.

In this study, combination treatment with ursolic acid and leucine exerted a stimulatory effect on myoblast differentiation. The combination of ursolic acid and leucine stimulated and enhanced the differentiation of myoblasts into myotubes, as evidenced by the increased expression of myogenic differentiation markers (protein levels of MHC, MyoD and myogenin) and (mRNA levels of MyoD and myogenin). In addition, the combination treatment enhanced myotube formation, as indicated by the morphological analysis, CK analysis and the analysis of MHC expression during the late differentiation phase. Combination treatment stimulated differentiation, as evidenced by the increased fusion rate and the increased average number of nuclei per myotube. As evidenced by the number and size of myotubes formed, the myoblasts treated with the combination treatment differentiated more rapidly in culture than the control myoblasts and the myoblasts treated with either agent alone (data not shown).

Secondly, we also found that the promoting effect of the combination treatment on muscle differentiation, stimulating muscle protein synthesis, was related to the activation of the mTOR signaling pathway. This results was in accordance with the data of previous studies indicating that the mTOR pathway mediates skeletal muscle differentiation, eventually determining protein synthesis and increasing muscle mass (20,43). The level of phosphorylation of mTOR and its downstream targets, 4E-BP1 and p70S6K, was increased by the combination treatment (Fig. 7). Based on the stimulatory effect observed on muscle protein synthesis through this pathway, combination treatment with ursolic acid and leucine may well have an additive or potentiating effect, and not a synergistic effect, on the differentiation of muscle cells.

In conclusion, our data demonstrate that combination treatment with ursolic acid and leucine has the potential to directly alter protein synthesis and enhance the differentiation of murine C2C12 myoblasts. The cells treated with the combination of ursolic acid and leucine exhibited a particular arrangement of the nuclei, forming a ring pattern, which is a morphological marker of muscle hypertrophy and maturation. In addition, the expression of differentiation markers, such as MHC, MyoD and myogenin increased at both the mRNA and protein level. The combination treatment enhanced the differentiation of C2C12 cells through the mTOR pathway. Overall, our data demonstrate that nutritional interventions, such as the supplementation of ursolic acid in combination with leucine, may prove to be beneficial in conditions such as muscle atrophy (sarcopenia), where there is a deficiency in muscle-building amino acids, such as BCAAs, and may help potentiate muscle fiber protein synthesis.

Acknowledgements

The present study was supported by the R&D Program of MOTIF/KEIT (10040391, the Development of Functional Food Materials and Device for Prevention of Aging-associated Muscle Function Decrease). This study was also supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP; (no. 2009-0083538). The authors would also like to thank the Aging Tissue Bank for providing research information and Mr. Kyoung-Pil Lee (Pusan National University) for assisting with confocal microscopy.

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