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Isoquinoline alkaloids from *Coptis japonica* stimulate the myoblast differentiation via p38 MAP-kinase and Akt signaling pathway

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ABSTRACT

To overcome the muscle atrophy, such as cachexia and sarcopenia, we tried to find myogenic agents from medicinal plants. From myogenic extract of *Coptis japonica*, we purified six isoquinoline alkaloids and evaluated their effects on transactivation of myoD and MHC expression in C2C12 cells during differentiation process. Among obtained compounds, magnoflorine most efficiently enhanced the myoblast differentiation by activating the p38 MAP kinase and Akt pathway, and also increased the number of multinucleated and cylinder-shaped myotubes. These results propose that magnoflorine from *Coptis japonica* might be a promising lead compound for the development of anti-muscle atrophy drug.

Muscle regeneration has attracted attention to overcome the degenerative diseases of skeletal muscle, such as muscle atrophy, cachexia, and sarcopenia. The progressive weakness and impaired function of muscle threaten the quality of life and reduce the survival rate of cancer patients. More than 30% of cancer patients lose their lives from muscle impaired weight loss. The loss of muscle function is commonly accompanied by the degradation of myo-proteins and the progressive decrease in muscle fiber cross-sectional area, muscle strength, nuclear number of myofibers and insulin responsiveness.[1]

Strategy for treatment of muscle degeneration is based on down-regulation of inflammatory molecules and myostatin, or up-regulation of cyclic AMP, proliferator-activated receptor gamma coactivator (PGC)-1α and insulin signaling pathway. Although a number of potential drugs have been developed, only megestrol acetate has been approved by the U.S. FDA for the treatment of skeletal muscle atrophy. Most of these drugs were designed to inhibit protein catabolism of muscle or elevate the satellite cell functions.

In normal conditions, quiescent satellite cells as a primary stem cell, continuously undergo proliferation and differentiation. Damaged muscle secrets various growth factors to activate the proliferating myogenic satellite cells, called myoblasts. Activated myoblasts induce various myogenic factors including MyoD, myogenic factor (Myf)-5, myogenin, and myogenic regulatory factor (Mrf)-4. MyoD and Myf-5 play an important role not only in the specification of myogenic lineage but also in the initiation of myoblast differentiation.[2] Especially, MyoD pivotally induces the expression of myogenic proteins such as myosin heavy chain (MHC) and myogenin through the interaction with non-muscle specific factors, including E proteins, myocyte enhance factor (Mef)-2 family, and transcriptional coeffectors.

Coptis japonica Makino (CJ) is a widely used medicinal herb in Korea. In traditional oriental medicine, the root of CJ has been applied to treat indigestion, acute and chronic gastritis, enteritis, diarrhea and severe skin disease.[3] The pharmacological effects of CJ alkaloids are attributed to the anti-inflammatory, anti-oxidative, anti-angiogenic, anti-hypertensive, or anti-Alzheimer's disease activities.[4] Isoquinoline type alkaloids, including berberine, epiberberine, magnoflorine and coptisine have been reported from CJ. Among them, berberine as a major constituent of CJ, has been reported to have multiple pharmacological effects. Berberine showed cardio-protective effect by attenuating the myocardial apoptosis via Notch1/Hes1-PTEN/Akt signaling[5] and by inhibiting excessive autophagy in cardiomyocytes through the regulation of AMPK and mTOR signaling.[6] Berberine also regulates glucose and lipid metabolism via activation of p38 MAPK-GLUT4, JNK pathway, and PI3K-Akt pathway.[4,7] Meanwhile, these pathways can promote the myoblast differentiation through the MyoD activation. The local and systemic chronic inflammation states were known to be associated with muscle degeneration and pro-inflammatory

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cytokine-induced proteasome pathway was reported to be responsible for muscle wasting.[8] CJ contains the anti-inflammatory compounds including berberine, epiberberine palmatine, jateorrhizine, and coptisine.[9] So, we anticipated to find stimulators of myoblast differentiation from *Coptis japonica*.

We reported several myogenic compounds such as tetrahydropalmatine, bakuchiol, and dehydrocorydaline from medicinal plants together with their mechanism of action.[10–12] In this study, we isolated isoquinoline alkaloids from the root of CJ and assessed their myogenic potential in C2C12 myoblasts.

The MeOH extract of CJ was partitioned with diethyl ether, chloroform and n-BuOH to obtain each fraction. As we observed myogenic effect of n-BuOH fraction, we purified six alkaloids and identified their structures as follows, berberine (**1**), jatrohizzine (**2**), epiberberine (**3**), 8-hydroxy-7, 8-dihydrocoptisine (**4**), magnoflorine (**5**) and palmatine (**6**) by spectroscopic analysis (Fig. 1) and comparison with reported data.[13]

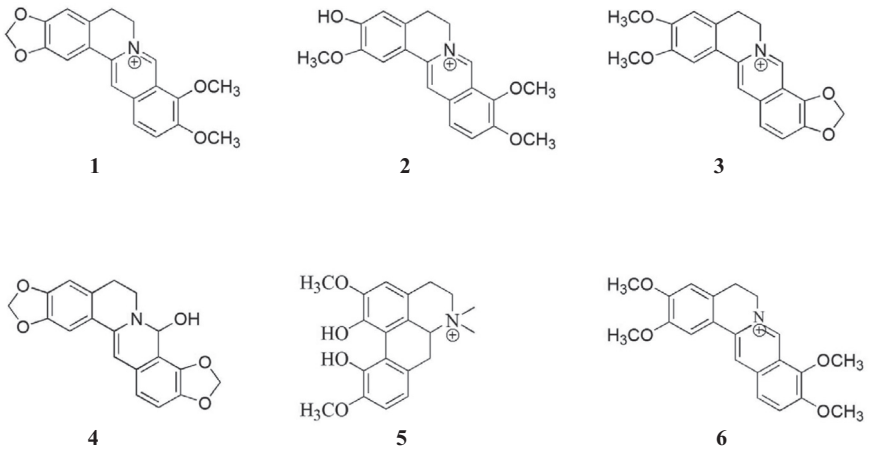
To investigate the myogenic effect of CJ compounds, we measured myoD transcriptional activity in C2C12 cells expressing the MyoD-responsive reporter 4RTK-luc.[14] The MyoD contains a basic helix-loop-helix (bHLH) motif that can recognize a core consensus sequence of E-box to transactivate the myogenic genes.[15] The MyoD-responsive reporter 4RTK-luciferase construct, which contains four E-box sites fused to a thymidine kinase promoter was used to analyze the MyoD transcriptional activity.

After 24 h of reporter gene transfection, cells were treated with test compounds and harvested to measure luciferase activity. CJ compounds (10 nM, 24 h) significantly increased the MyoD transcriptional activity (Fig. 2A). Magnoflorine (**5**) showed the highest MyoD transcriptional activity among the six isoquinoline alkaloids of CJ. All the compounds did not show any significant cytotoxicity in this experiment. As reviewed by Li and Wang,[16] magnoflorine has anti-hemolytic,[17] sedative and anxiolytic,[18] anti-atherosclerotic,[19] anti-cataractic[20] and anti-diabetic activities.[21] Previously we reported that an isoquinoline alkaloid, tetrahydropalmatine, from *Corydalis ternata* activated MyoD transcriptional activity.[10,22]

C2C12 cells, a primary line of murine myoblasts, is considered as a model cell for the study of skeletal muscle development.[23] The cell-cell contact in confluent cultures initiates the myoblasts differentiation and activates the MyoD expression, followed by MHC expression. C2C12 myoblasts were treated with test compounds for 2–3 days in differentiation medium (2% horse serum-containing DMEM). As differentiation progressed, the mononucleated cells became more elongated and cylinder-shaped myocytes and fused together into multinucleated myotubes.[24] The terminally differentiated myotubes express the muscle specific genes including myogenin, Mrf-4 and MHC, thus, the MHC expression is a late marker of myogenic differentiation. Treatment of CJ compounds (10 nM) up-regulated MHC expression in differentiated C2C12 cells (Fig. 2B). Consistent with the results of MyoD transcriptional activation, magnoflorine was the strongest inducer of MHC expression. These results show that CJ compounds stimulate the MyoD-induced myogenic differentiation and magnoflorine is the most promising myogenic agent among six isoquinoline alkaloids of CJ.

We further investigated the effect of magnoflorine on myoblast differentiation. C2C12 cells were differentiated in presence of magnoflorine (0.01–10 nM) and harvested for immunostaining and Western blotting analysis.[11] As shown in Fig. 3A, treatment of magnoflorine increased the level of MHC and MyoD in a dose dependent manner, while the control cells showed very low levels. The myogenic activity of magnoflorine was also accessed by immunofluorescence staining using anti-MHC antibodies and DAPI (Fig. 3B). The increased red-fluorescence indicated that magnoflorine dose dependently stimulated MHC expression in C2C12 cells. The cylinder-shaped multinucleated myotubes were observed by treatment of magnoflorine with DAPI counterstaining. The degree of myoblast differentiation was presented as the percentage of multinucleated MHC-positive cells. Magnoflorine increased number of cylinder-shaped myotube and higher rate of multinucleated myotubes. These results demonstrate that magnoflorine stimulates myoblast differentiation at nanomolar concentration to form myotubes.

The p38 mitogen-activated protein kinase (p38 MAPK) plays an important role in MyoD activation.[25] Phosphorylation of E proteins, Mef2 or SWI/SNF subunit BAF60 by p38MAPK promotes the dimerization of MyoD to induce the expression of myogenic factors.[26] Likewise, p38 MAPK indirectly activates the MyoD through the phosphorylation of binding partners of E proteins or Mef2.[26,27] The essential role of p38 MAPK in myogenesis has been proved using p38α MAPK deficient mice (p38α +/− heterozygote) and p38 MAPK chemical inhibitor.[28,29] To test whether p38 MAPK signaling pathway is involved in myoblast differentiation by magnoflorine, we analyzed the level of phosphorylated p38 MAPK after 2 days differentiation of C2C12 cells. By treatment of 0.1 nM magnoflorine, 6-fold increase of phospho-p38 MAPK was observed as



1: Berberine, **2**: Jatrohizzine, **3**: Epiberberine, **4**: 8-hydroxy-7, 8-dihydrocoptisine, **5**: Magnoflorine, **6**: Palmatine

Fig. 1. Structures of compounds **1**–**6** purified from *Coptis japonica*.

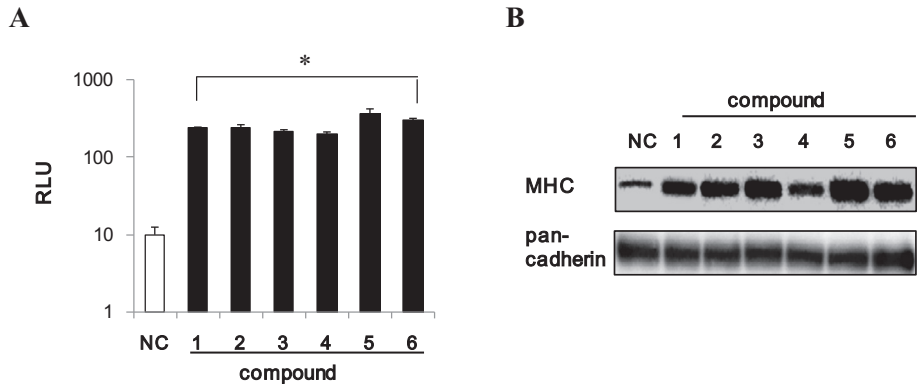


Fig. 2. Effect of compounds **1**–**6** on transactivation of myoD and myosin heavy chain (MHC) expression. (A) C2C12 cells were transiently transfected with plasmid mixture containing myoD expression vector and MyoD-responsive reporter 4RTK-luc. After 24 h transfection, cells were treated with each compound (10 nM) for an additional 24 h. Data are presented as relative luciferase activity (RLU) divided by the β-galactosidase activity and expressed as mean ± SD. NC; negative control; **1**–**6**, compounds **1**–**6**. ∗p < 0.001 vs. NC. (B) C2C12 myoblasts were differentiated for 2 days in the presence of each compound. Protein extracts (25 μg) were analyzed for MHC expression by Western blot analysis using pan-cadherin as a loading control.

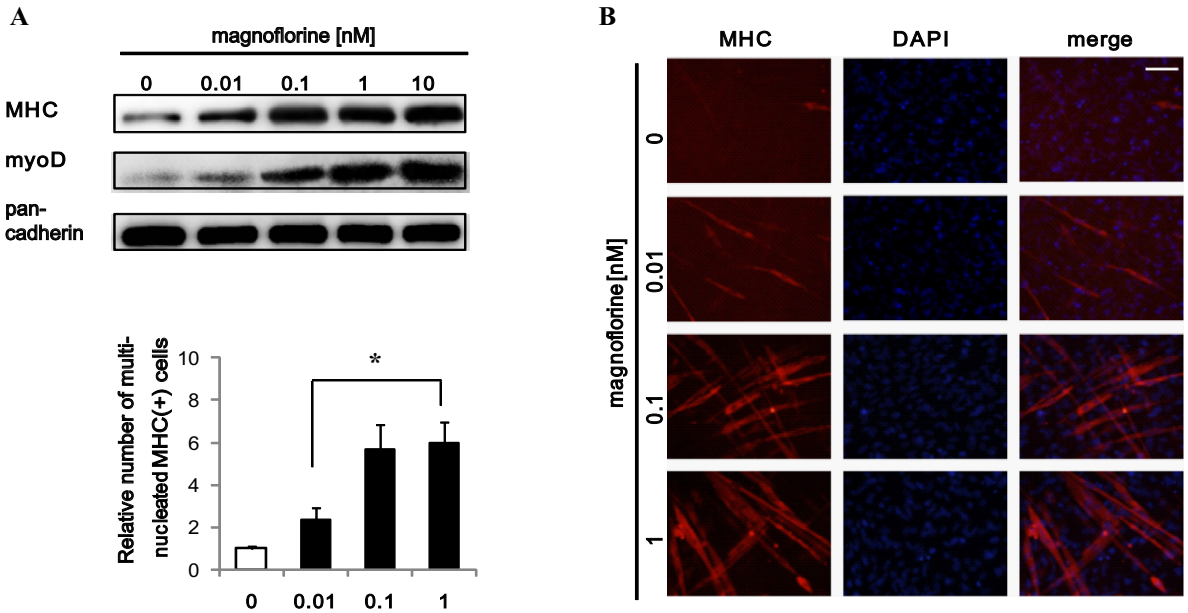


Fig. 3. Effect of magnoflorine on MHC and myoD expression. C2C12 myoblasts were differentiated for 2 days in the presence of different concentrations of magnoflorine. (A) MHC and myoD expression were assessed by Western blot analysis. (B) The differentiated myoblasts were immunostained with mouse anti-MHC and anti-mouse IgG2b Alexa-fluor 568. Immunofluorescence of MHC-positive myotubes was visualized in red, and DAPI-labeled nuclei were in blue. Multinucleated (⩾5) MHC-expressing myotubes were counted from randomly chosen areas. Scale bar = 200 μm. Data are expressed as mean ± SD. ∗p < 0.05 vs. control.

compared with control (Fig. 4). Our data suggest that activation of p38 MAPK signaling is an important contributor to the magnoflorine-stimulated myoblast differentiation. Akt (protein kinase B, PKB) signaling pathway along with p38 MAPK is influential mechanism for myoblast differentiation.[30] Constitutively active Akt isoform enhanced a muscle-specific gene expression,[31] and dominant-negative and kinase-mutant form of Akt inhibited myogenic gene expression in chicken embryonic myoblasts.[32] Cabane et al. reported that p38 directly increases the mRNA and protein level of Akt and Akt activation during myogenic differentiation.[33] On the basis of results showing p38MAPK activation by magnoflorine, we checked the Akt activation in differentiated myocytes. Magnoflorine increased the level of phospho-Akt as compared with control (Fig. 3). Taken together, p38 MAPK and Akt signaling are responsible for the magnoflorine-induced myoblast differentiation.

Throughout the last two decades, many trials were conducted to discover the therapeutic compounds for the treatment of skeletal muscle wasting. Most of the suggested potential compounds including eicosapentanoic acid, β-hydroxy-β-methylbutyrate, ghrelin, and resveratrol, act by inhibiting the inflammation and muscle protein catabolism. Resveratrol enhances insulin sensitivity via up-regulation of insulin signaling, AMPK, and SIRT1 signaling.[1] We have screened our house made chemical library by using MyoD reporter gene assay system in order to find new myogenic compounds from medicinal plants. Here, we report a promising candidate that can be applied to the drug development for muscle atrophy treatment, such as cachexia and sarcopenia. Isoquinoline alkaloids from *Coptis japonica* stimulate myoblast differentiation by promoting MyoD transcriptional activity. Especially magnoflorine increases expression of myogenic factors (MyoD and MHC) via the activation of p38 MAPK and Akt signaling.

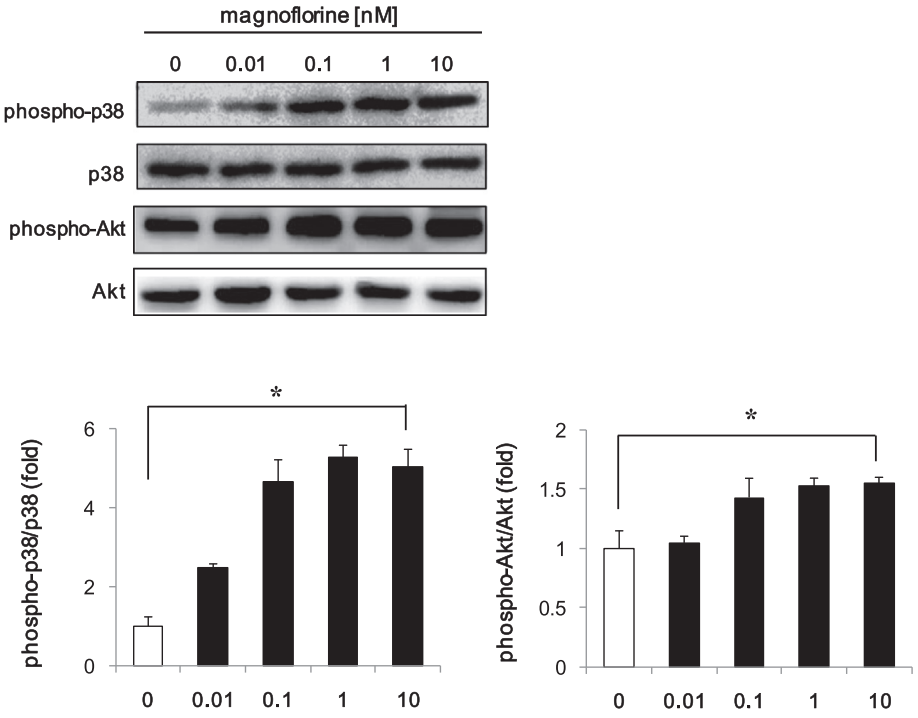


Fig. 4. The effect of magnoflorine on p38 MAP kinase and Akt signaling pathway activation. The lysates of differentiated myotubes were analyzed by Western blot. Data are expressed as mean ± SD. ∗p < 0.001 vs. control.

Magnoflorine might have therapeutic potential for muscular diseases associated with muscle wasting.

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13. Extraction and purification of isoquinoline alkaloids of *Coptis japonica*: The dried roots of CJ were purchased from the Kyungdong Oriental Drug Market in 2015 (Seoul, Korea) and have been deposited as voucher specimen (No. SPH 1502) in the herbarium of Sookmyung Women's University. The root was extracted (5 kg) for 9 h at 80 °C with methanol. The extract (600 g) was suspended in 2 M HCl aqueous solution and continuously partitioned with diethyl ether, $CHCl_3$ and n-BuOH. The n-BuOH soluble fraction (150 g) of CJ was subjected to silica gel column chromatography eluting with CH_2Cl_2/MeOH gradient system (10:1 → 5:1) to obtain 10 fractions. Fraction F9 was further chromatographed with a silica gel with CH_2Cl_2/MeOH gradient system (200:1→ 10:1) to afford nine sub-fractions. The fraction F9-4 was re-chromatographed on silica gel using EtOAc/MeOH/water (21:4:3) as eluent to afford compound **1** (berberine, 20.9 mg). Fraction F9-9 was chromatographed on silica gel using EtOAc/MeOH/water (15:3:1 → 15:10:1) to afford compound **2** (jatrohizzine, 7.6 mg) and compound **3** (epiberberine, 31.6 mg). Fraction F10-5 was subjected on silica gel chromatography with EtOAc/n-BuOH/formic acid/water (9:3:1:1 → 7:3:1:1) to afford compound **4** (8-hydroxy-7,8-dihydrocoptisine, 13.6 mg). Fraction F10-12 was subjected to silica gel chromatography with EtOAc/MeOH/water (20: 3:1) to afford compound **5** (magnoflorine, 11.6 mg). Fraction F10-3 was subjected to silica gel chromatography with EtOAc/n-BuOH/formic acid/water (9:3:1:1 → 5:3:1:1) to afford compound **6** (palmatine, 16.3 mg).
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