Folic acid promotes the myogenic differentiation of C2C12 murine myoblasts through the Akt signaling pathway

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Abstract. Folic acid is a water-soluble vitamin in the B-complex group, and an exogenous intake is required for health, growth and development. As a precursor to co-factors, folic acid is required for one-carbon donors in the synthesis of DNA bases and other essential biomolecules. A lack of dietary folic acid can lead to folic acid deficiency and can therefore result in several health problems, including macrocytic anemia, elevated plasma homocysteine levels, cardiovascular disease, birth defects, carcinogenesis, muscle weakness and difficulty in walking. Previous studies have indicated that folic acid exerts a positive effect on skeletal muscle functions. However, the precise role of folic acid in skeletal muscle cell differentiation remains poorly understood. Thus, in the present study, we examined the effects of folic acid on neo-myotube maturation and differentiation using C2C12 murine myoblasts. We found that folic acid promoted the formation of multinucleated myotubes, and increased the fusion index and creatine kinase (CK) activity in a concentration-dependent manner. In addition, western blot analysis revealed that the expression levels of the muscle-specific marker, myosin heavy chain (MyHC), as well as those of the myogenic regulatory factors (MRFs), MyoD and myogenin, were increased in the folic acid-treated myotubes during myogenic differentiation. Folic acid also promoted the activation of the Akt pathway, and this effect was inhibited by treatment of the C2C12 cells with LY294002 (Akt inhibitor). Blocking of the Akt pathway with a specific inhibitor revealed that it was necessary for mediating the stimulatory effects of folic acid on muscle cell differentiation and fusion. Taken together, our data suggest that folic acid promotes the differentiation of C2C12 cells through the activation of the Akt pathway.

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Introduction

Folic acid, the fully oxidized monoglutamyl form of folate, is a water-soluble vitamin found mostly in green vegetables, peanuts, legumes, strawberries and orange juice, predominantly as polyglutamates (1). The mammalian system cannot synthesize folate *de novo*; therefore, an exogenous dietary supply of this vitamin is necessary to meet the daily requirements. Folic acid is the precursor to co-factors which act as one-carbon donors and are necessary for the synthesis of DNA bases (2). For this reason, folic acid is an essential dietary nutrient required for healthy cell growth and division. Folic acid deficiency has been linked to various human diseases, such as neural tube defects, atherosclerosis and cancers (3-5). In addition, Li *et al* (6) found that folic acid deficiency during pregnancy affects the skeletal muscle development of piglets.

Folic acid is able to regulate high levels of homocysteine, as 5-methyltetrahydrofolate, the predominant form of dietary folate, functions as a methyl-group donor in the conversion of homocysteine back to methionine. Elevated levels of plasma homocysteine have been linked to reduced mobility and muscle function (7). Betaine, another methyl donor which is also involved in homocysteine remethylation, has also been reported to regulate homocysteine levels (8). Recently, betaine was shown to promote muscle fiber differentiation and increase myotube size through insulin-like growth factor (IGF)-1 pathway activation (9). Hyperhomocysteinemia is associated with ischemic stroke and osteoporotic fractures in elderly men and women (10). A double-blind, randomized controlled study with elderly patients who had suffered a stroke demonstrated that oral treatment with folate and vitamin B12 decreased the incidence of hip fractures compared with a placebo control (11). This treatment may also improve postural stability and/or muscle function and strength, as folic acid regulates homocysteine levels. Overall, these aforementioned studies suggest that folic acid supplementation improves muscle function.

The differentiation of skeletal muscle cells is a highly organized process which is governed by muscle-specific transcription factors belonging to the MyoD family, such as MyoD and myogenin, as well as by the myocyte enhancer factor-2 (MEF2) family; cell differentiation involves highly complex processes, including withdrawal from the cell cycle, the expression of myotube-specific genes and cell fusion to form multinucleate myotubes (12-14). In addition, the activation of myogenic regulatory factors (MRFs), including MyoD, myogenic factor 5 (Myf5), MRF4 and myogenin, also regulates the expression of several muscle-specific genes, such as myosin heavy chain (MyHC, the major structural protein in myotubes) and creatine kinase (CK), in muscle fiber-type maturation (15,16). MyoD and Myf5 are essential for myoblast identity and act early in myogenesis to determine myogenic fate (17-19). Myogenin is essential for myoblast differentiation and acts at the late stages of myogenesis to control the fusion of myoblasts (19). Myoblasts, with controlled increases in the expression of MyoD, Myf5, myogenin and MRF4, and decreases in the activity of cell cycle regulatory factors, terminally differentiate into skeletal myocytes and fuse to form myotubes (20).

Akt (also known as protein kinase B) is a serine/threonine Ser/Thr) kinase with key roles in the proliferation, survival, differentiation and viability of muscle cells (21,22). Akt controls both protein synthesis, through the mammalian target of rapamycin (mTOR) signaling pathway, and protein degradation through the Forkhead Box O transcription factors. mTOR has also been recognized as an important player in muscle cell differentiation. Akt/mTOR has been investigated in studies involving *in vivo* and *in vitro* models of skeletal muscle hypertrophy and atrophy (23,24). Definitive proof of the myogenic function of mTOR was provided by a study which revealed that a rapamycin-resistant mTOR mutant fully rescued C2C12 differentiation in the presence of rapamycin (25). When the phosphorylation of mTOR by Akt occurs, the activation of the 70-kDa ribosomal protein S6 kinase 1 (p70S6K1) and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) is then possible; this event is important for the promotion of muscle growth, as p70S6K1 and 4E-BP1 stimulate protein synthesis (26). Similar to the other members of the phosphatidylinositol kinase-related kinase family, mTOR is a Ser/Thr protein kinase that plays an important role in a nutrient-sensitive signaling pathway which regulates cell growth. It has been shown that myocytes isolated from *S6K1*$^{-/-}$ mice do not exhibit a hypertrophy response to IGF-1 stimulation, indicating that p70S6K1 is necessary for myotube hypertrophy (27).

The aim of the present study was to investigate the effects of folic acid on muscle cell differentiation using C2C12 murine myoblasts. We provide evidence that the supplementation of folic acid enhances myogenesis and induces the expression of MyoD, myogenin and MyHC in C2C12 cells. We also demonstrate that folic acid increases muscle differentiation through the activation of the Akt/mTOR pathway. Taken together, these data suggest that folic acid exerts a beneficial effect on muscle cell differentiation.

Materials and methods

Reagents. Folic acid, LY294002 and monoclonal antibody against β-actin (Cat. no. A5441) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folic acid was dissolved in 1 M NaOH to generate 100 mM stock solution and stored at -20˚C until use in the experiments; dilutions were made in culture medium. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Amresco (Solon, OH, USA). Antibodies against MyoD (sc-760), myogenin (sc-576), MyHC (sc-20641), phosphorylated (p-)Akt (Ser473; sc-7985-R) and Akt1/2/3 (sc-8312) were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies against p-mTOR (cat. no. 5536), mTOR (cat. no. 2983), p-p70S6K1 (cat. no. 9234), p70S6K1 (cat. no. 2708), p-4E-BP1 (cat. no. 2855) and 4E-BP1 (cat. no. 9452) were all purchased from Cell Signaling Technology (Danvers, 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 GE Healthcare Life Sciences (Logan, UT, USA). A Creatine kinase enzymatic assay kit (MaxDiscovery™ creatine kinase enzymatic assay kit) was purchased from Bioo Scientific Corp. (Austin, TX, USA).

Cell culture. Murine C2C12 myoblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). For the maintenance of the C2C12 myoblasts, the cells were cultured in growth medium consisting of DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37˚C. The growth medium was changed every 2 days.

Induction of myogenic differentiation. For the induction of myogenic differentiation, the C2C12 myoblasts at 80-90% confluence were transferred to differentiation medium composed of DMEM supplemented with 2% HS in order to initiate the differentiation of the myoblasts into myotubes. The medium was changed with fresh differentiation medium every 2 days.

Measurement of cell viability. Cell viability was determined by MTT assay. The cells were seeded in 6-well plates and incubated in culture medium until they reached 80-90% confluence. The medium was then switched to differentiation medium, and the cells were treated with or without folic acid (0, 10, 20, 50 and 100 μM) and observed after 6 days. The cells were incubated in the dark with MTT reagent (0.5 mg/ml) at 37˚C for 2 h. The medium was removed, the formazan was dissolved in dimethyl sulfoxide (DMSO), and the absorbance at 540 nm was measured using an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Fisher Scientific, Vantaa, Finland).

Measurement of 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 leupeptin and 100 μg/ml phenymethylsulfonyl fluoride (PMSF)] and complete protease inhibitor and stored at -70˚C until use. CK activity was determined using a CK enzymatic assay kit (Bioo Scientific Corp.), according to the manufacturer's instructions. Briefly, 250 μl CK reagent were added to 5 μl cell lysate in a microplate. CK activity was immediately measured 2 times at 5-min intervals at 340 nm. Each 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).

Immunofluorescence staining and determination of the fusion index. The C2C12 myoblasts were cultured in 6-well plates and cell differentiation was induced with the use of differentiation medium with or without folic acid (0, 2.5, 5, 10 and 20 μM) for 6 days. For immunofluorescence microscopy, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. After the cells were blocked in 2% normal goat serum, they were then incubated with primary antibody at 4˚C for 24 h. mouse anti-MyHC antibody was used at a 1:200 dilution. MyHC was detected by incubating the cells with goat anti-mouse Rhodamine Red-X (1:1,000; Jackson ImmunoResearch, Bar Harbor, ME, USA) at 4˚C for 1 h, and 4'-6-diamidino-2-phenylindol (DAPI) was then used to label the nuclei. All images were taken using a confocal microscope (FV10C-W), using the same exposure time, and were analyzed in VS-FlexGrid Pro 8.0J (both from Olympus, Tokyo, Japan). Differentiated myotubes in a specific microscopic field were observed under x20 magnification. Either the total number of nuclei or the number of nuclei within MyHC-positive myotubes was counted in 5 fields/sample. The fusion index was calculated as follows: (%) = (number of nuclei within MyHC-stained myotubes/total number of nuclei) x100. All experiments were performed 3 times.

Western blot analysis. The C2C12 myoblasts were cultured in 6-well plates and differentiation was induced with the use of differentiation medium with or without folic acid (0, 2.5, 5, 10 and 20 μM) for 6 days. For western blot analysis, the myoblasts and differentiated myotubes were washed with PBS and homogenized in lysis buffer. Following centrifugation (14,240 x g) at 4˚C for 15 min, the supernatant was collected and the protein concentration was determined using protein assay reagents (Bio-Rad, Hercules, CA, USA). Equal amounts of protein extracts were denatured by boiling them at 100˚C for 5 min in sample buffer (Bio-Rad). The proteins were separated by 6-15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Berlin, Germany). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 buffer (TBST; 20 mM Tris, 100 mM NaCl, pH 7.5 and 0.1% Tween-20) for 1 h at room temperature followed by incubation with primary antibodies specific for each protein at 4˚C for 24 h: MyHC (1:800), MyoD (1:1,000), myogenin (1:1,000), β-actin (1:50,000), p-Akt (Ser473) (1:1,000), Akt1/2/3 (1:1,000), p-mTOR (1:3,000), mTOR (1:3,000), p-p70S6K1 (1:3,000), p70S6K1 (1:3,000), p-4E-BP1 (1:3,000) and 4E-BP1 (1:3,000). The blots were washed with TBST buffer and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:3,000; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Immunolabeling was carried out using an enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Statistical software (version 10.0; StatSoft, Inc., Tulsa, OK, USA) was used for statistical analysis. Data are presented as the means ± SD. Data were analyzed using the Student's t-test. A P-value <0.05 was considered to indicate a statistically significant difference.

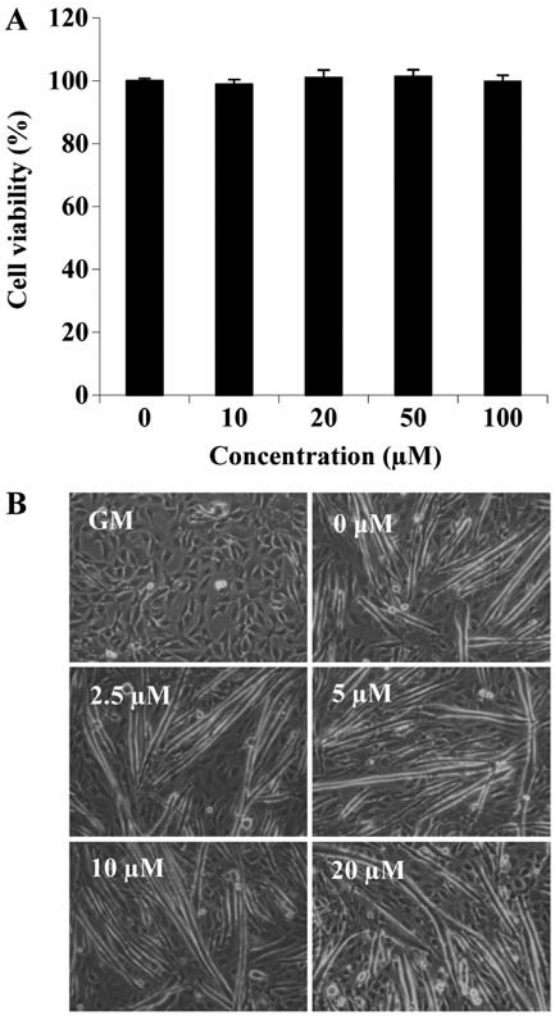

Figure 1. Effects of folic acid on the viability and morphology of C2C12 cells. Cells were cultured in differentiation medium with increasing concentrations of folic acid for 6 days. (A) Cell viability was assessed by MTT assay. No change was observed between the groups. Results are expressed as the means ± standard deviation (SD; n=3) and as a percentage of the untreated controls. (B) Images of morphological changes were obtained by phase contrast photomicrography at the end of the experiment (6th day). GM, growth medium.

Results

Folic acid enhances the myogenic differentiation of C2C12 cells and CK activity. To investigate the role of folic acid in the process of muscle differentiation, we first determined the effects of folic acid on cell viability during differentiation. C2C12 cells cultured in differentiation medium were treated with folic acid (0, 10, 20, 50 and 100 μM) for 6 days. No significant differences were observed with the addition of up to 100 μM folic acid (Fig. 1A). Subsequently, we examined the effects of folic acid on the morphological changes of C2C12 cells (such as the loss of their typical triangular morphology and the cell shape gradually changed into a new elongated shape) that are associated with the differentiation process (Fig. 1B). On the 6th day of differentiation, myotube formation was increased by folic acid treatment in a concentration-dependent manner (Fig. 1B). These data suggested that folic acid promoted the myogenic differentiation of C2C12 cells. To confirm this, we examined the effects of folic acid on myogenic differentiation by measuring the fraction of nuclei incorporated into MyHC-stained myotubes on

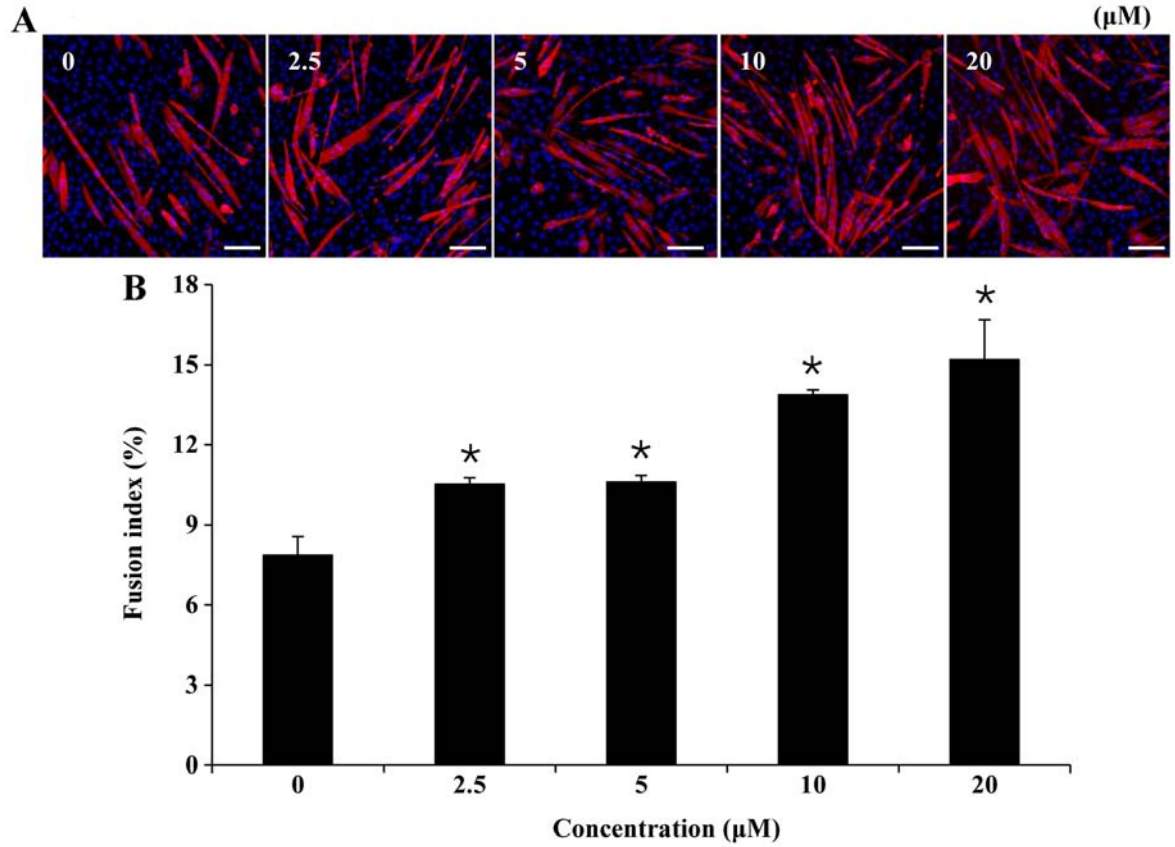

Figure 2. Effects of folic acid on the myogenic differentiation of C2C12 cells. (A) Immunocytochemical staining of the myotube marker, MyHC (red color), and the nuclei marker, DAPI (blue color), showed myotube formation in the C2C12 cells. The experiments were performed 3 times and a representative result is shown. (B) Fusion index was calculated by measuring the fraction of nuclei incorporated into MyHC-stained myotubes on the 6th day of differentiation from 5 random fields per treatment. The experiments were performed 3 times and representative results are shown. Bar graph represents the means ± SD. *P<0.05 compared with the untreated controls. Scale bar, 100 μm.

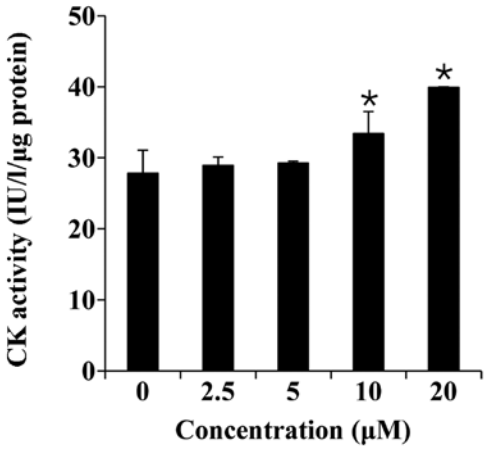

Figure 3. Effects of folic acid on creatine kinase (CK) activity in C2C12 cells. Cells were cultured in differentiation medium with increasing concentrations of folic acid for 6 days, lysed and subjected to creatine kinase (CK) activity assay. The experiment was performed 3 times and a representative result is shown. Bar graph represents the means ± SD. *P<0.05 compared with the untreated controls.

the 6th day of myogenic differentiation (Fig. 2A). The results, as indicated in Fig. 2B, demonstrated that the fusion index for each group was, in increasing order: control (7.9±0.7%), 2.5 μM folic acid (10.5±0.2%), 5 μM folic acid (10.6±0.2%), 10 μM folic acid (13.9±0.2%) and 20 μM folic acid (15.2±1.5%). Thereafter, we measured CK activity, which is a well-described marker of C2C12 cell differentiation (28). As shown in Fig. 3, folic acid (0-20 μM) significantly increased CK activity in a concentration-dependent manner.

Folic acid increases the expression of MyoD, myogenin and MyHC. Since myotube formation was affected by folic acid, we wished to determine the effects of folic acid on the expression of myogenic markers. MyoD is a muscle-specific transcription factor that is expressed in myogenic cells during late proliferation and early differentiation. It is commonly assumed that MyoD is upstream of myogenin during the differentiation process (29,30). The C2C12 cells were cultured in growth medium until they reached 80-90% confluence, and the medium was then switched to differentiation medium and the cells treated with or without folic acid; the cells were then harvested after 1 day of differentiation in order to measure the MyoD and myogenin expression levels. To determine the effects of folic acid on the late-stage differentiation marker, MyHC, the cells were cultured for 6 days. The results revealed a marked increase in MyoD expression in the cells treated with folic acid compared to the cells not treated with folic acid (Fig. 4). Myogenin is part of a second wave of regulators expressed in cells that have ceased mitosis and are committed to terminal differentiation (31). As expected, the expression of myogenin was also increased in a concentration-dependent

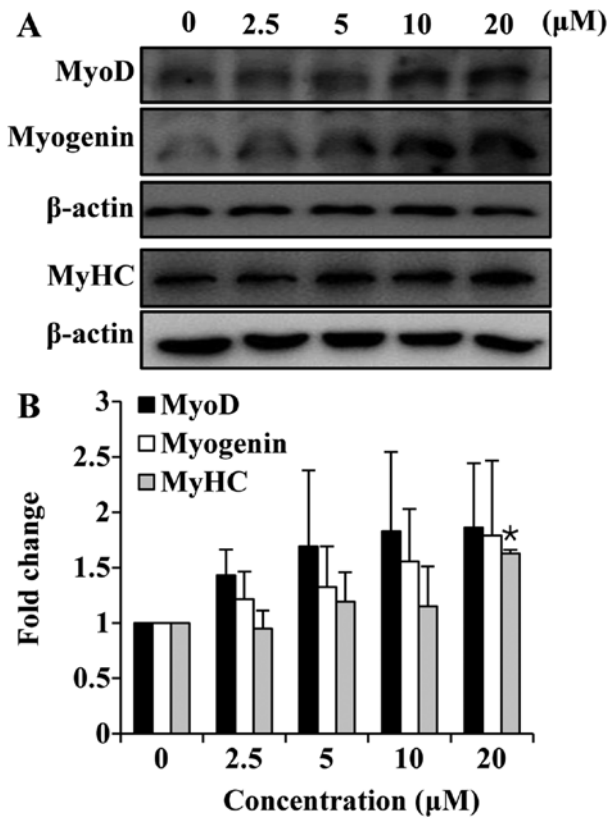

Figure 4. Effects of folic acid on MyoD, myogenin and myosin heavy chain (MyHC) expression. C2C12 cells were cultured in differentiation medium with folic acid. Cells were harvested on day 1 (MyoD, myogenin) and day 6 (MyHC) of differentiation. (A) Protein expression levels of MyoD, myogenin and MyHC were measured by western blot analysis. The experiments were performed 3 times and a representative result is shown. (B) Densitometric analysis of 3 independent experiments. The values were normalized to β-actin and the fold change relative to untreated cells is presented. *P<0.05 compared with the untreated controls.

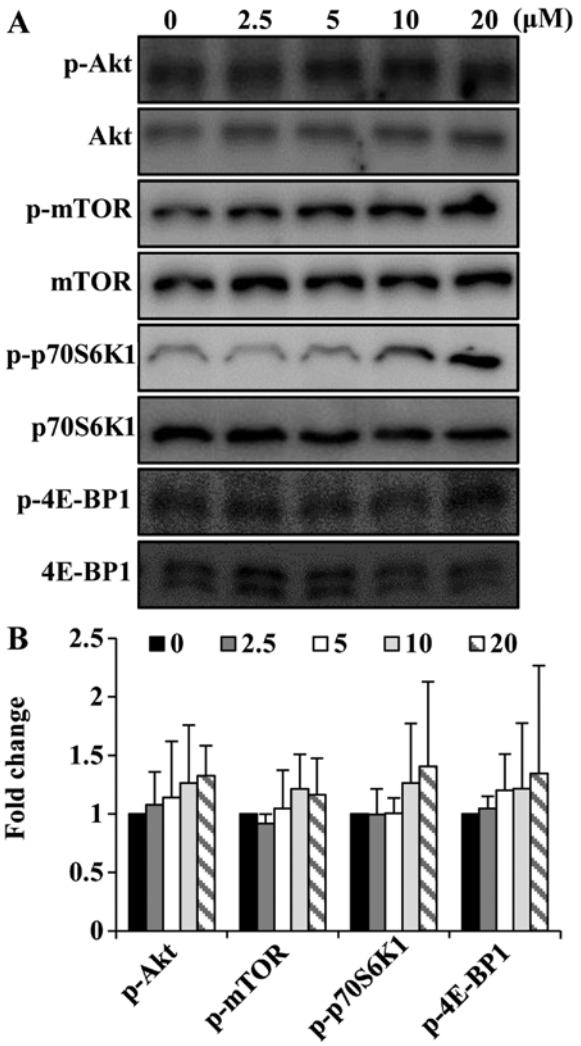

Figure 5. Effect of folic acid on Akt/mammalian target of rapamycin (mTOR) pathways. Cells were cultured in differentiation medium with folic acid for 8 h. (A) Protein expression levels of Akt signaling molecules were analyzed by western blot analysis. The experiments were performed three times and a representative result is shown. (B) Densitometric analysis of 3 independent experiments. The values were normalized to those of total protein and the fold change relative to untreated cells is presented.

manner following treatment with folic acid (Fig. 4). Finally, the expression of MyHC, a specific marker of myotubes, was also increased by treatment with folic acid (Fig. 4). Taken together, these data demonstrate that treatment with folic acid increases the expression of myogenic markers in C2C12 cells.

Folic acid activates the Akt/mTOR pathway in C2C12 cells. As Akt and mTOR are required for skeletal muscle development (23,24), we wished to determine whether folic acid modulates the activation of the Akt/mTOR pathway in C2C12 cells. To determine the ability of folic acid to affect the Akt/mTOR signaling pathway, short-term (8 h) stimulation experiments were performed. Folic acid increased the phosphorylation of Akt and mTOR in the C2C12 cells (Fig. 5). In addition to increasing the phosphorylation of Akt and mTOR, folic acid activated p70S6K1 and 4E-BP1, which are key downstream targets of the Akt/mTOR signaling cascade.

Akt activation is required for the induction of C2C12 cell differentiation by folic acid. To confirm whether Akt is necessary for the folic acid-induced differentiation of C2C12 cells, the cells were cultured in the presence or absence of the Akt inhibitor, LY294002, and then treated (or not) with folic acid. Treatment with folic acid for 8 h induced the phosphorylation of Akt; however, LY294002 reversed the effects of folic acid on Akt (Fig. 6A). In addition, pre-treatment with LY294002 abolished the effects of folic acid on MyoD and myogenin expression (Fig. 6B). This result was further confirmed by examining C2C12 myoblast differentiation. The results revealed that the blockade of Akt completely inhibited the differentiation of C2C12 myoblasts into myotubes in both the presence and absence of folic acid, as shown by the decrease in MyHC expression (late-stage differentiation marker) in the cells pre-treated with LY294002 (Fig. 6C). Under differentiation conditions, treatment with LY294002 did not alter cell viability (data not shown). We also conducted western blot analyses to confirm the effects of LY294002 on the folic acid-induced expression of MyHC. Consistent with the morphological changes of the C2C12 cells, the induction of MyHC expression by folic acid was markedly reversed in the LY294002-treated cells (Fig. 6D). Taken together, these data demonstrate that folic acid enhances the myogenic differentiation of C2C12 cells through the Akt signaling pathway.

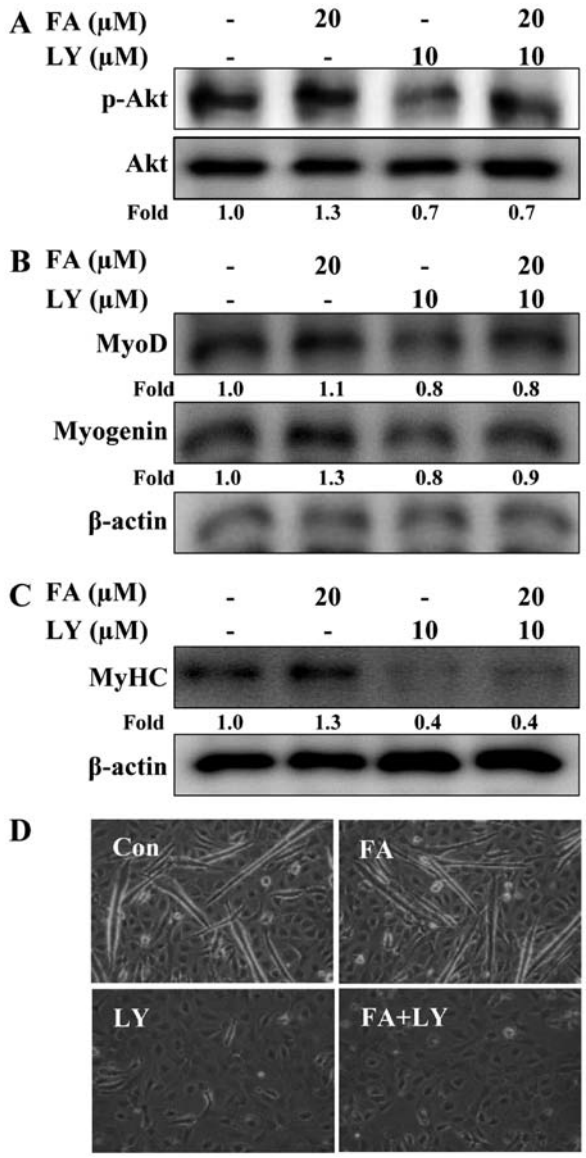

Figure 6. Effects of Akt inhibition on folic acid-induced C2C12 cell differentiation. Cells were cultured in differentiation medium with or without folic acid FA following pre-treatment with or without LY294002 for 30 min. Cells were incubated for a further (A) 8 h (p-Akt and Akt), (B) 1 day (MyoD and myogenin) and (C) 6 days [myosin heavy chain (MyHC)]. The expression of p-Akt, Akt, MyoD, myogenin and MyHC measured by western blot analysis. Densitometric analysis of 3 independent experiments is shown. The values were normalized to total Akt (for p-Akt) and those of β-actin (for MyoD, myogenin and MyHC), and the fold change relative to the untreated cells is presented. (D) Representative phase contrast photomicrographs showing myotube formation after 6 days. The experiments were performed 3 times, and a representative result is shown. FA, folic acid; LY, LY294002; Con, control.

Discussion

C2C12 cells are murine myoblasts derived from satellite cells, which can spontaneously differentiate into myotubes when moved from high-serum medium to low-serum medium. The C2C12 cells are a useful tool for studying the differentiation of myoblasts, the expression of various proteins, and also exploring mechanistic pathways (32). In the present study, we used C2C12 cells to examine the effects of folic acid on myogenic differentiation.

First, we observed that folic acid increased neo-myotube formation, compared to the untreated control cells, without affecting cell viability. The results revealed that folic acid increased the fusion of myoblasts into multinucleated myotubes, as determined by examining cell morphology and by MyHC staining. No significant difference in cell viability was observed in the cells treated with 10 and up to 100 μM folic acid (Fig. 1). Therefore, following this result, we expected folic acid to increase myogenic differentiation.

Secondly, we observed that folic acid increased the expression of MyoD and myogenin and of the muscle-specific structural genes, MyHC and muscle CK during the differentiation phase (Figs. 3 and 4). These data suggest that treatment with folic acid increased the differentiation of C2C12 cells. The myogenic differentiation program is known to be largely controlled by the myogenic basic helix-loop-helix family of transcription factors (MyoD, myogenin, Myf5 and MRF4) and MEF2, which regulate the expression of several muscle-specific genes, such as MyHC, and p21, a cyclin-dependent kinase inhibitor (33,34). Notably, similar transcriptional programs regulate skeletal muscle satellite cell proliferation/differentiation *in vivo* and the maturation of isolated myoblasts *in vitro*. Expression during myogenesis is distinct among the MRF members; undifferentiated myoblasts express Myf5 and MyoD, but not myogenin (35). By contrast, previous studies have demonstrated that multinucleated myotubes are all positive for Myf5, MyoD and myogenin, although the expression level of Myf5 is higher in mononuclear myoblasts and MyoD and myogenin is predominantly expressed in myotubes (36,37). MyoD directly binds to MEF2 and enhances MEF2-dependent transcription through its own transcriptional activities, which results in the cooperative enhancement of MEF2- and MyoD-dependent myogenic differentiation (38). Myogenin has been proven to play a critical role in the transcriptional regulation of the multiple epidermal growth factor-like-domains 10 (MEGF10) gene, a well-known myogenic regulator of satellite cells (39). Both MyoD and myogenin bind to E-box elements in the promoter region of muscle-specific genes and regulate their expression in skeletal muscle (40). Cornelison *et al* (41) reported that MyoD deficient (*MyoD*$^{-/-}$) satellite cells displayed aberrant morphology during the later phases of proliferation and differentiation, and exhibited major differences in myogenic gene expression and efficiency of terminal differentiation compared to wild-type satellite cells. Taken together, these data indicate that MyoD and myogenin regulate myogenic differentiation through transcriptional activities and protein-protein interactions. Our results suggest that folic acid promotes myogenic differentiation by increasing the expression of muscle-specific factors, such as MyoD, myogenin and MyHC.

Finally, we found that treatment with folic acid activated the Akt/mTOR signaling pathway (Figs. 5 and 6). By inhibiting the Akt pathway with a specific inhibitor (LY294002), we demonstrated that this pathway is required for the stimulatory effects of folic acid on muscle cell differentiation. MyHC expression displayed a differential sensitivity toward Akt inhibition, revealing an Akt activity-dependent induction by folic acid. The crucial role of Akt in myogenic differentiation and hypertrophy has been previously demonstrated (42,43). In skeletal muscle, Akt signaling plays a role in hypertrophy and contributes to increase in the size of C2C12 myotubes (24).

In dystrophic muscle, elevated Akt signaling has been associated with advanced dystrophy and peak stages of muscle hypertrophy (44). Izumiya *et al* (45) demonstrated that muscle-specific Akt transgene expression led to muscle hypertrophy, whereas decreasing adipose mass was due to the growth of type IIb muscle fibers, which was accompanied by an increase in strength. Furthermore, the IGF-phosphoinositide 3-kinase (PI3K)-Akt signaling pathway has been shown to stimulate myogenic differentiation by inducing myogenin gene expression (46). Also, the IGF-PI3K-Akt signaling pathway can target MyoD and MEF2 by enhancing the transcriptional activity of MyoD and MEF2 in normal myogenic cells (47). Taken together, these data support the theory that the activation of Akt in muscle plays a role as a key mediator of the hypertrophic response by promoting myogenic gene expression. Therefore, we hypothesize that folic acid promotes myogenic differentiation by increasing myogenin gene expression through Akt signaling.

The loss of skeletal muscle mass and reduced contractive force are common and disabling features of various human diseases, including neuromuscular disorders, cancer, AIDS and diabetes (48,49). The decrease in muscle function has also been shown to be associated with aging, a sedentary lifestyle and immobilization (50). Furthermore, aging- and disease-related skeletal muscle wasting has been shown to be associated with inflammatory and homocysteine levels (50). Folic acid was found to exert an anti-inflammatory effect (51) and to inhibit inflammation-related signaling molecule nuclear factor-κB (NF-κB) and its upstream mitogen-activated protein kinases (52); thus folic acid may exert beneficial effects on skeletal muscle function. Moreover, the metabolism of homocysteine and folic acid are closely linked to skeletal muscle weakness through mitochondrial dysfunction that involves epigenetic changes (53,54).

In conclusion, the findings of our study indicate that treatment with folic acid promotes skeletal muscle myoblast differentiation, particularly affecting the progression of the differentiation process and myotube morphology. This suggests that the Akt-dependent regulation by folic acid controls the expression of MyoD, myogenin and MyHC.

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