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Involvement of p38 MAPK-mediated signaling in the calpeptin-mediated suppression of myogenic differentiation and fusion in C2C12 cells

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Abstract Calpeptin inhibits myoblast fusion by inhibiting the activity of calpain. However, the mechanism by which calpeptin inhibits myogenesis is not completely understood. This study examined how calpeptin affects the expression of the myogenic regulatory factors (MRFs) and the phosphorylation of p38 mitogen-activated protein kinase (MAPK) in differentiating C2C12 myoblasts. Consistent with previous reports, calpeptin inhibited the induction of μ-calpain and the formation of myotubes in these cells. In particular, calpeptin inhibited the expression of the early and mid differentiation markers including MyoD, Myf5, myogenin, and MRF4 as well as the expression of the late markers such as troponin T and myosin heavy chain (MyHC). Calpeptin also suppressed the phosphorylation of p38 MAPK in C2C12 cells. SB203580, a specific p38 inhibitor, prevented the expression of the muscle-specific markers and their fusion into myotubes in these cells, which was further accelerated in the presence of calpeptin. These findings suggest that calpeptin inhibits the myogenesis of skeletal muscle cells by down-regulating the MRFs and involving p38 MAPK signaling.

Keywords C2C12 skeletal muscle cells · Myogenesis · Calpeptin · p38 MAPK

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Introduction

Myogenesis is an ordered multistep process, in which myoblasts cease to divide, elongate, and fuse into multinucleated myotubes. These stages are driven by the expression of the MyoD family of transcription factors [1]. The differentiation of myoblasts is also stimulated by the expression of the myocyte enhancer factor (MEF2) [2, 3]. In contrast, the fusion of myoblasts and the formation of myotubes are Ca^{2+} dependent and are regulated mainly by the calpain–calpastatin system [4].

Calpain, a Ca^{2+}-dependent thiol protease, is widely distributed in many cells, along with its endogenous cysteine protease inhibitor, calpastatin [5]. Many studies reported that the degradation of membrane proteins contributes to the disorganization of cell membrane and cytoskeleton components in fusing cells, and that calpain plays an important role in the fusion-associated protein degradation [6–8]. Therefore, it has been suggested that the fusibility of myoblasts depends on the cellular calpain-to-calpastatin ratio. Indeed, pharmacological calpain inhibitors inhibit myoblast fusion [5].

Of the calpain inhibitors, calpeptin, a cysteine protease inhibitor, has long been used to examine the effects of calpastatin on the myoblast differentiation and fusion. Calpeptin inhibits myoblast fusion by inhibiting the activity of calpain without preventing calpastatin diminution [5, 9]. However, calpeptin is not a specific calpain inhibitor. This indicates that calpeptin can inhibit myoblast fusion by regulating the signaling pathways responsible for the expression and activity of calpain. However, the precise mechanisms involved in the calpeptin-mediated inhibition of myoblast fusion are still unclear.

The muscle regulatory factors (MRFs) such as MyoD, Myf5, myogenin, and MRF4 are skeletal muscle-specific transcription factors. These factors bind to the regulatory regions of the muscle-specific genes and then activate myogenic differentiation [10, 11]. In addition, p38 mitogen-activated protein kinase (MAPK) has long been implicated as a key signaling factor in the expression of muscle-specific genes as well as in the fusion of myoblasts [12–14]. It has also been suggested that calpeptin inhibits myoblastic differentiation and fusion by regulating the expression of MRFs, in which p38-mediated signaling is closely involved. In this study, we examined whether or not calpeptin influences the expression and activation of MRFs and p38 kinase in differentiating C2C12 myoblasts, and the mechanism for such a process.

Materials and methods

Chemicals and laboratory wares

Unless specified otherwise, all chemicals and laboratory wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively. Calpeptin (z-Leu-norleucinal) was purchased from ALEXIS® Biochemicals Corporation (San Diego, CA, USA) and dissolved in dimethylsulfoxide (DMSO) immediately before use. SB203580, a specific p38 MAPK inhibitor, was purchased from TOCRIS (MI, USA). The final DMSO concentration did not exceed 0.1% (v/v) in any of the experiments.

Cell culture and induction of differentiation

C2C12 skeletal muscle cells were obtained from the American Type Culture Collection (ATCC, Rockwille, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (growth medium, GM) in 5% CO_2 at 37°C. When the cells had reached 80% confluence, cell differentiation was induced by changing the culture medium to a fresh DMEM medium supplemented with 2% horse serum (differentiating medium, DM) and culturing the cells at 37°C in an atmosphere containing 5% CO_2/95% air for various time periods.

Hematoxylin staining

In order to determine the level of myotube formation, C2C12 cells were fixed with 10% formalin for 30 min and stained with a hematoxylin solution (51275, Fluka Chemie GnbH, Steinheim, Switzerland) at room temperature for 5 min. The fusion index was calculated as the percentage of nuclei incorporated in the myocytes relative to the total number of nuclei. In addition, the cells were photographed using a Nikon E5400 digital camera mounted on an optical microscope (Nikon TS100, Nikon Corporation, Japan).

Western blot analysis

Cell lysates were made in an NP-40 lysis buffer (30 mM Tris–Cl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 1 mM PMSF, and a protease inhibitor mixture containing 1 μg/ml aprotinin and leupeptin), and protein content was quantified using the Bradford method [15]. Equal amounts of the protein samples (30 μg/sample) were separated by 12% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with primary antibodies and incubated with a horseradish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The polyclonal antibodies specific for p21 (SC-397, 1:200), Myf5 (SC-302, 1:200), MRF4 (SC-784, 1:200), MyoD (SC-760, 1:200), extracellular signal-regulated kinase (ERK; SC-94, 1:200), Troponin T (SC-8121, 1:200), and μ-calpain (SC-13990, 1:200); and the monoclonal antibodies specific for myogenin (SC-12732, 1:200) and p-p38 (SC-7973, 1:200) were purchased from Santa Cruz Biotechnology. The monoclonal antibodies specific to p53 (OP03, 10 μg/ml) and p38 (9217, 1:200) were purchased from Oncogene (Boston, USA) and Cell Signaling (MA, USA), respectively.

Immunostaining

C2C12 cells grown on separate coverslips were fixed with 4% paraformaldehyde for 30 min and washed with PBS. The cells were treated with monoclonal anti-myogenin

antibody, polyclonal anti-MyoD, anti-Myf5, or anti-MRF4 antibody at a 1:100 dilution for 1 h and then exposed to FITC-conjugated secondary antibodies. In addition, the cells were incubated in a 0.1 μg/ml DAPI solution for 1 min and observed using fluorescence microscopy (Axioskop 2, Carl Zeiss, Germany).

RNA preparation and reverse transcription–PCR (RT–PCR)

Total RNA was isolated from C2C12 cells according to the manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI, USA), and reverse transcription and PCR amplification were performed using the Access RT–PCR System (Promega) according to the manufacturer's protocol. PCR conditions used were 35 cycles of the following: 95°C for 30 s, 57°C for 30 s and 72°C for 1 min. The following PCR primers were used: myosin heavy chain (MyHC), sense 5′-AGA GCT GAC GTG CCT CAA TG′, and antisense, 5′-ATG CCT CTT CTT GCC CTT GT-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense 5′-CAC CAC CAT GGA GAA GGC CG-3′, and antisense, 5′-GAA CAC GGA AGG CCA TGC CA-3′. PCR products were analyzed on a 2% agarose gel and visualized by ethidium bromide staining. PCRs were repeated in at least three independent RNA preparations.

Statistical analyses

Data are expressed as a mean ± standard error (SE). One-way ANOVA on SPSS ver. 10.0 software was used for multiple comparisons. A $P < 0.05$ was considered significant.

Results

Calpeptin inhibits myoblast fusion and μ-calpain induction in the cultured C2C12 cells

C2C12 myoblasts grown in GM were induced to differentiate by changing the medium to DM as described in Materials and methods. The time for the change to DM was defined as 0 h. The cells were treated with 50 μM calpeptin at the time of the change from GM to DM, and the media were changed to fresh DM containing calpeptin every 2 days. The DM containing 0.1% DMSO was used as the control cultures for all the experiments. In the control cultures, myoblasts became confluent approximately 24 h after changing the medium to DM and then began to fuse. More than 50% of the myoblasts had fused to multinucleated myotubes at 84 h (Fig. 1a). In the calpeptin-treated cells, the fusion of myoblasts was inhibited in that only 20% fused cells were observed when treated with 50 μM calpeptin for the same time. In addition, most of the myoblasts were fused to myotubes at 120 h after changing to DM, but only 30% of the myoblasts were fused in the presence of calpeptin at the same time (Fig. 1b).

Equal amounts of the protein samples were separated electrophoretically and analyzed by immunoblotting using anti-μ-calpain antibody (Fig. 1c). In the control DM cultures, the level of μ-calpain increased to 1.4-fold of the initial level at 12 h of differentiation and was maintained to 120 h without any significant changes. In contrast, calpeptin reduced the μ-calpain level significantly in that there was a 50% decrease in the μ-calpain level when the cells were treated with 50 μM calpeptin for 72 h.

Calpeptin inhibits expression of MRFs in C2C12 myoblasts

Expression of MRFs is quite important for the successful progression of the myoblast differentiation program. Consistent with the previous reports [1, 10, 16, 17], MyoD, Myf5, and MRF4 proteins were presented in the control cultures at the time of the change from GM to DM, whereas myogenin was detectable only after inducing differentiation (Fig. 2, left panel). Marked inhibition of MRF expression was observed in calpeptin-treated cells, compared with the control cultures (Fig. 2, right panel). For example, the level of MyoD and MRF4 proteins was not increased between 0 and 24 h after treating the cells with 50 μM calpeptin but decreased at the later stages. Although an increase in myogenin and actin at protein levels was observed in the presence of calpeptin, there was an increase in the myogenic markers at the later stages with a further low level, compared with those of the control cultures.

The inhibitory effects of calpeptin on the expression of MRFs were also confirmed by staining the cells with the MRFs specific antibodies (Fig. 3). As shown in the figure, the expression of MRFs and their translocation into the nucleus were apparently suppressed by treating the cells with 50 μM calpeptin.

Calpeptin also affects the p21 expression and inhibits the troponin T level in the C2C12 myoblasts

Terminal cell cycle arrest is coupled to muscle differentiation and is essential for the activation of muscle-specific gene expression [18]. Differences in the protein expression levels of the cell cycle inhibitor p21 and of the late

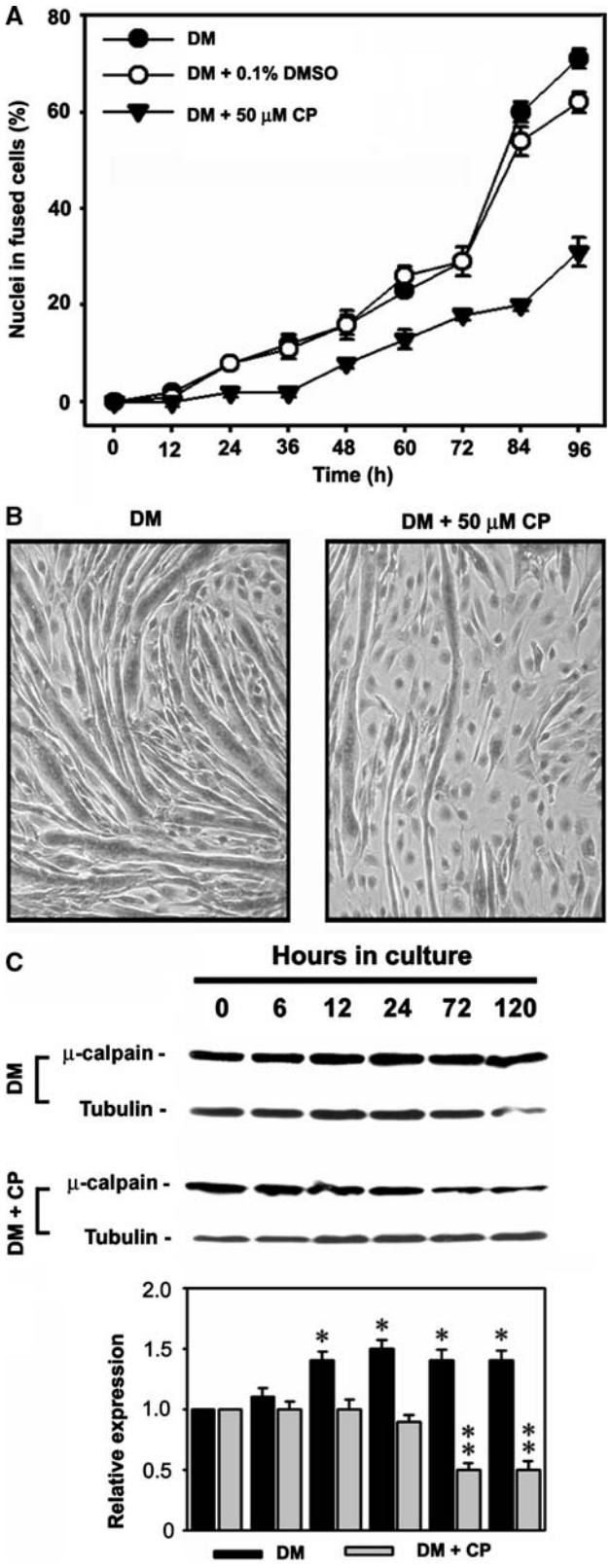

Fig. 1 Calpeptin inhibits myoblast fusion and μ-calpain induction in cultured C2C12 cells. (**a**) Fusion curves for the C2C12 cells. Percentage of nuclei in the multinucleated cells was calculated at different times by changing the media to DM in the presence of calpeptin. (**b**) The cells were cultured in DM with or without 50 μM calpeptin and stained with hematoxylin at 5 days of differentiation. (**c**) The cells were incubated in DM containing 50 μM calpeptin, and the levels of μ-calpain were detected by immunoblotting at the indicated times. The intensity of the bands was quantified using a Gel-Print System (Core Bio Corp., Korea) and is expressed as the relative expression of triplicates. The tubulin bands are shown to indicate loading condition of the gel. $*P < 0.05$ and $**P < 0.01$ versus control cultures

differentiation marker, troponin T, between the control and calpeptin-treated cells were also analyzed by immunoblotting (Fig. 4). In the control cultures, an increase in p21 expression started from day 1, peaked at 2 days, and decreased 5 days after changing to DM. On the other hand, the expression of troponin T reached a maximum at day 3 and decreased at day 5 of differentiation. In contrast, the calpeptin-treated cells showed the persistent and prolonged accumulation of p21 from the initially increased point at 2–5 days of differentiation, despite the cellular level of troponin T expression remaining very low throughout the whole differentiation process.

Calpeptin promotes the p38-mediated inhibition of the expression of MyoD and MyHC as well as of the myotube formation

Although the functional role of MAPKs in myogenic differentiation is not completely understood, accumulating evidences suggest that the induction of myoblast differentiation is positively controlled by the p38-mediated signaling pathway [12–14]. There is also report showing that activation of p38 kinase is essential for the myogenic differentiation of L6E9 rat skeletal muscle cells, whereas its inhibition almost completely prevents the progression of

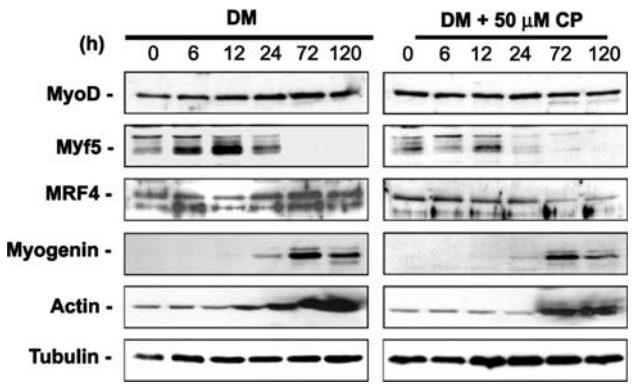

Fig. 2 Calpeptin inhibits MRF expression in differentiating C2C12 myoblasts. The cells were cultured in GM and transferred to DM at 80% confluence in the presence or absence of 50 μM calpeptin for the indicated times, and the cell lysates were analyzed by immunoblotting with the indicated antibodies. The experiments were repeated three times and representative data are shown. The tubulin bands are shown to indicate loading condition of the gel

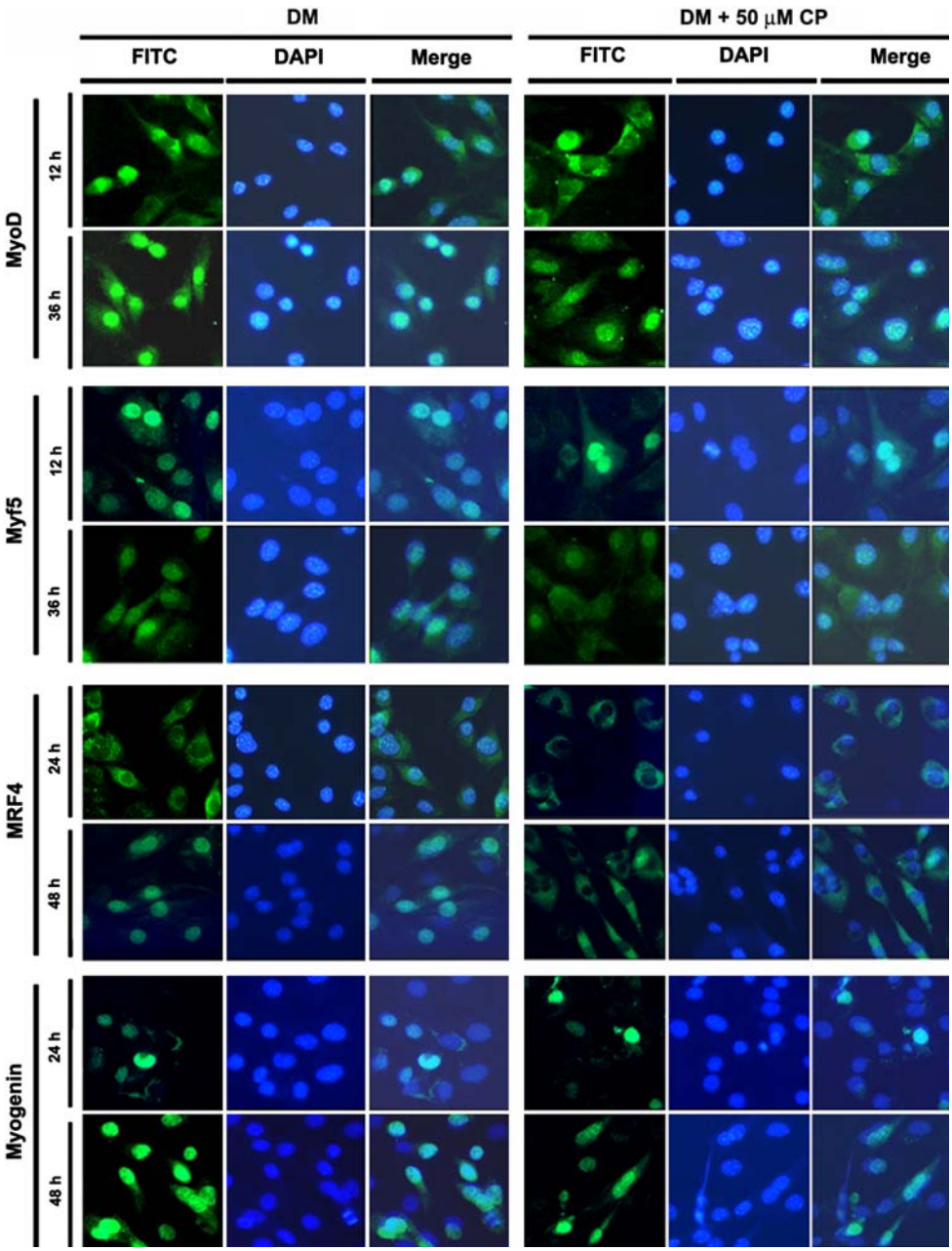

Fig. 3 Expression of muscle-specific proteins in differentiating C2C12 myoblasts. The cells were cultured in DM supplemented with or without 50 μM calpeptin. At the indicated times, cultures were fixed and stained with antibodies specific for the MRFs and counterstained with DAPI solution (magnification, 200×)

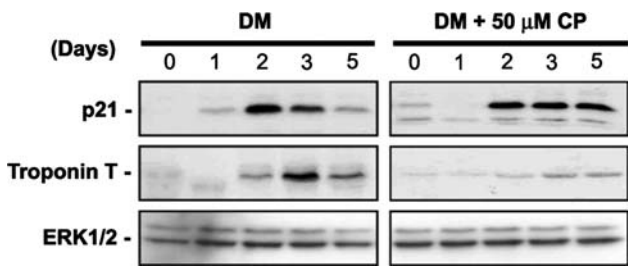

Fig. 4 Calpeptin affects the expression of p21 and reduces the troponin T level in differentiating C2C12 myoblasts. The cells were cultured in DM with or without 50 μM calpeptin. At the indicated times, the levels of p21 and troponin T were determined by Western blot analysis. ERK1/2 was used as control protein

differentiation with the attendant increase of ERK activity [19]. In addition, it is believed that p38 kinase but not JNK interacts with actin and modulates actin filament rearrangement during skeletal muscle differentiation [20]. Therefore, subsequent experiments were focused on the elucidation of whether or not the p38 signaling is involved in the calpeptin-mediated inhibition of myogenesis. To this end, the control and calpeptin-containing C2C12 cells were incubated in the presence or absence of SB203580, a pharmacological specific inhibitor of p38. Calpeptin inhibited the level of phosphorylated-p38 (p-p38) such that a very low level of p-p38 was observed after 48 h of differentiation, compared to that of the control cultures (Fig. 5a). Immunochemical

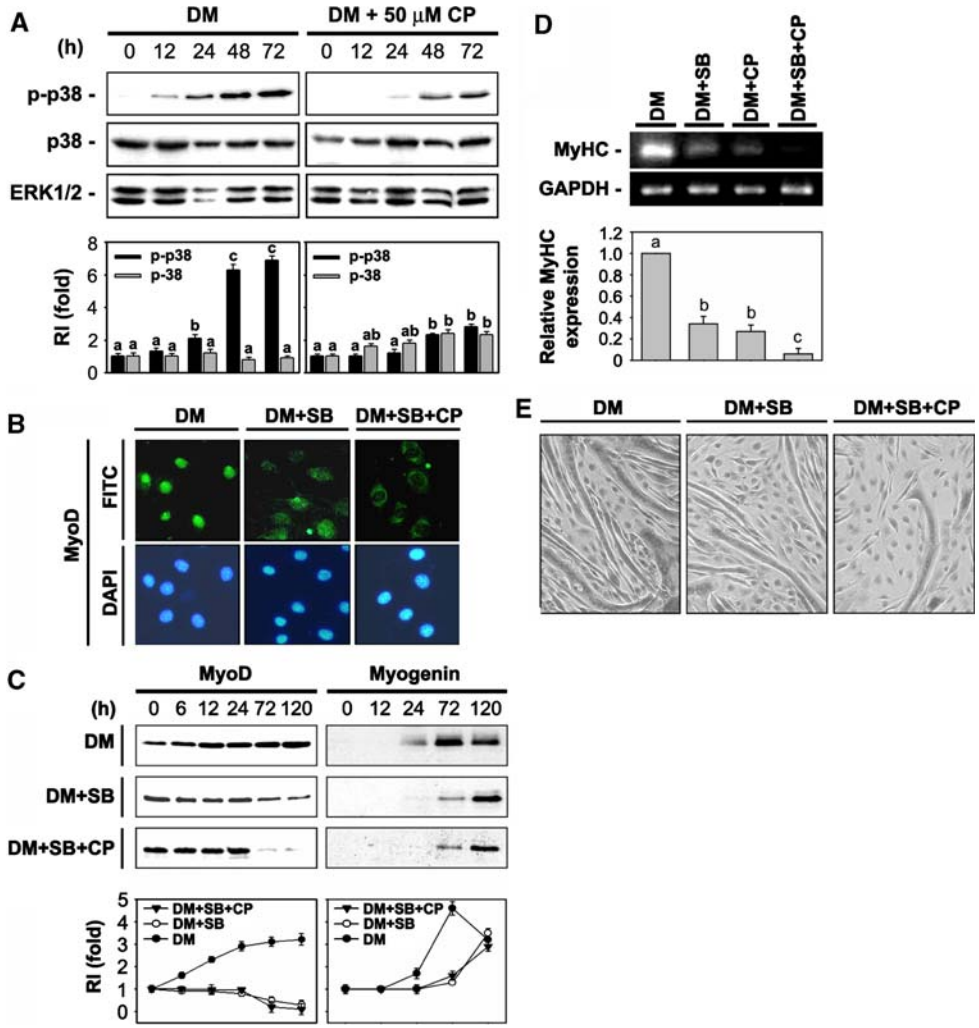

Fig. 5 Calpeptin promotes the p38-mediated inhibition of MyoD and MyHC expression as well as myotube formation in differentiating C2C12 myoblasts. (**a**) The cells were cultured in DM containing 50 μM calpeptin and at the indicated times the level of phosphorylated p38 were detected by immunoblotting. ERK1/2 was used as control protein. The intensity of the bands was quantified and is expressed as the relative intensity (RI) of triplicates. The different superscripts represent significant differences ($P < 0.05$) between the groups (Duncan's multiple range test). (**b**) The cells were cultured in DM containing 10 μM SB203580 (SB) and/or 50 μM calpeptin (CP) for 72 h, and the expression of MyoD was then determined by immunostaining with the specific antibodies along with counterstaining with DAPI (magnification, 200×). (**c**) The cells were also incubated in DM with 10 μM SB203580 and/or 50 μM calpeptin for the indicated times, and processed for the analysis of MyoD and myogenin expression using immunoblotting. A representative result from triplicate experiments is shown and the band intensities quantified are expressed as the relative intensity of triplicates. (**d**) The cells were cultured in DM with 10 μM SB203580 and/or 50 μM calpeptin for 72 h and then processed for RT–PCR to analyze the expression of MyHC. The intensity of the bands was quantified and is expressed as the relative expression of triplicates. The different superscripts represent significant differences ($P < 0.05$) between the groups according to Duncan's multiple range test. (**e**) The cells were cultured in DM with 10 μM SB203580 and/or 50 μM calpeptin and stained with hematoxylin after 5 days of differentiation

analyses also showed that SB203580 inhibited both the translocation of MyoD to the nucleus and its cellular expression, which became quite apparent when the cells were co-treated with calpeptin (Fig. 5b). The calpeptin and/or SB203580-mediated inhibition of MyoD expression was also confirmed by Western blot analysis (Fig. 5c). As shown in the figure, the cellular level of MyoD had almost completely disappeared 72 h after changing to DM containing both 10 μM SB203580 and 50 μM calpeptin. The cellular level of myogenin in the control DM culture increased from 24 h, peaked at 72 h, and was maintained to 120 h of differentiation. In contrast, in the presence of SB203580 and/or calpeptin, the increase of myogenin began from 72 h and was further augmented to a level similar to that of control culture at 120 h after changing to DM.

Figure 5d shows the involvement of p38-mediated signaling in the calpeptin-mediated inhibition of myogenesis. As shown in the figure, the level of MyHC mRNA was

significantly inhibited by either treating C2C12 cells with 10 μM SB203580 or 50 μM calpeptin, whereas its expression was almost completely abolished when treated with both inhibitors for 72 h. This inhibition perfectly correlated with the inhibition of myotube formation observed by hematoxylin staining of the cells exposed to SB203580 and/or calpeptin (Fig. 5e). Overall, these findings suggest that calpeptin inhibits the expression of the early and late myogenic markers as well as the myotube formation in C2C12 myoblasts, which is partially related to the inhibitory effect of calpeptin on the phosphorylation of p38 MAPK.

Discussion

Myogenesis is a naturally occurring process in skeletal muscle development, which is tightly regulated by numerous endo- and exogenous factors. Calpain has been suggested to play an important role in myoblast fusion, along with its endogenous inhibitor, calpastatin. Calpastatin interacts with calpain to inhibit the translocation and binding of calpain to the cell membrane and inhibits the calpain activity. Therefore, the inhibition of myotube formation is achieved through the inhibition of calpain activity and/or the accumulation of calpastatin. With this regard, several chemicals such as TGF-β, EGTA, and cytochalasin B have been shown to inhibit myoblast differentiation and fusion by modulating the balance of cytoplasmic calpain/calpastatin levels [5, 21, 22].

In contrast to the prevention of calpastatin loss in myoblasts, which are inhibited from fusing by TGF-β and EGTA, calpeptin does not prevent the loss of the calpastatin but inhibits other cysteine proteases such as cathepsin B, H, and L [5, 23]. However, cathepsin B has been shown to be only up-regulated during early myoblast fusion but down-regulated in multinucleated myotubes [24]. Moreover, calpeptin does not inhibit the loss of most of the calpastatin in differentiating myoblasts [5]. Therefore, it is believed that other regulatory factors such as MRFs rather than calpeptin-sensitive or insensitive proteases play an important role in the calpeptin-mediated inhibition of myogenesis.

As demonstrated previously, the two major steps in the early and late differentiation of skeletal muscle cells are the withdrawal of myoblasts from the cell cycle and the subsequent expression and activation of muscle-specific genes. In addition to the MRFs, p21 accumulates in post-mitotic myoblasts and induces an irreversible commitment to the terminal differentiation of myoblasts via the cooperation with the mid and late MRFs such as myogenin and MRF4 [25, 26]. This study showed that calpeptin strongly inhibited skeletal muscle differentiation by inducing a marked decrease in the MRFs levels. In addition, calpeptin suppressed the late differentiation markers, troponin T and MyHC, as well as the myotube formation, even though there was the prolonged accumulation of p21 in the calpeptin-treated C2C12 myoblasts.

The expression of p21 is a key event in triggering cell cycle withdrawal and myoblast differentiation. Similar to a previous report [27], p21 expression in the control culture was detectable after 1 day in DM, but increased markedly after 2 days and further decreased after 5 days albeit less dramatically. Particularly, however, the calpeptin treatment led to a persistent and sustained accumulation of p21 without a prominent induction of the late differentiation markers (Fig. 4). Although the precise mechanism by which calpeptin regulates accumulation of p21 can not be explained in the present study, we suggest that the prolonged maintenance of accumulated p21 level is not essential in the terminal myogenic process [13, 27]. This is because the expression of p21, a key event triggering cell cycle withdrawal, is a prerequisite to myogenesis in the early differentiating stage [28]. It is also suggested that p21 expression is affected by the time course of p38 kinase activation. This is because that although p38 signaling is absolutely necessary to the early differentiation stage, it also acts to inhibit the premature progression of muscle cells. Indeed, inhibition of p38 MAPK signaling promoted late stages of myogenesis in limb myoblasts [29]. In our preliminary study, the high level of p-p38 after 5 days of differentiation was also observed in calpeptin-treated cells but not in untreated control cells (data not shown), although the myotube formation was still higher in the control cultures at the same time (Fig. 5). This led us to postulate that calpeptin inhibits myogenic differentiation through two different pathways, i.e., regulation of p38-mediated signaling and of calpain–calpastatin system. Further detailed experiments will be needed to explain how calpeptin induces the persistent and prolonged accumulation of cellular p21.

The molecular mechanisms involved in the induction of myoblast differentiation and fusion into myotubes have been investigated, and the evidence provided emphasizes the potential role of p38 MAPK signaling on the regulation of muscle-specific transcription factors and of myogenesis in the whole differentiation processes [12–14, 27]. Consistent with these reports, our present data show that the p38-mediated signaling pathway controls the myogenic differentiation of skeletal muscle cells, and the expression of muscle differentiation markers is down-regulated by inhibiting the p38 pathway. In particular, these results show that calpeptin suppresses the phosphorylation of p38 in C2C12 myoblasts and further facilitates the SB203580-mediated inhibition of muscle-specific marker as well as myotube formation. Our data shown in this study suggest

for the first time that calpeptin inhibits myogenic differentiation by down-regulating the MRFs with the involvement of the p38 MPAK signaling. There is a report showing that myostatin inhibits myoblast differentiation by down-regulating MyoD [30]. This indicates that the early and mid differentiation marker can be a specific target of calpeptin. However, the present findings do not explain the exact mechanisms for how calpeptin inhibits the myogenesis of C2C12 cells. Further studies will be needed to better define the involvement of MRFs and p38 in the calpeptin-induced inhibition of myoblast differentiation. In particular, it is important to clarify how calpeptin inhibits the activation of p38 and whether calpeptin regulates the expression of MRFs directly in the differentiation process. In this regard, it is also important to examine the effect of calpeptin on upstream activators of p38 MAPK such as MKK3, MKK4, and MKK6.

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