Molecular and Cellular Biochemistry **275:** 103–107, 2005.

Involvement of calpain in melanogenesis of mouse B16 melanoma cells

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Received 1 November 2004; accepted 24 January 2005

Abstract

In the current study, the involvement of calpain, a cysteine proteinase in the regulation of melanogenesis was examined using mouse B16 melanoma cells. In response to α-melanocyte-stimulating hormone (α-MSH), B16 melanoma cells underwent differentiation characterized by increased melanin biosynthesis. The total calapain activity was decreased within 2 h following α-MSH-treatment, and restored to the initial level in 6–12 h. To further investigate the involvement of calpain in the regulation of melanogenesis, the effect of calpain inhibitors on α-MSH-induced melanogenesis was examined. Inhibition of calpain by either *N*-acetyl-Leu-Leu-norleucinal (ALLN) or calpastatin (CS) peptide blocked α-MSH-induced melanogenesis. The magnitude of inhibition of melanin biosynthesis was well correlated with a decrease in the activity of tyrosinase, a key regulatory enzyme in melanogenesis. Treatment of B16 cells with ALLN caused marked decrease in both tyrosinase protein and mRNA levels. These results indicate that calpain would be involved in the melanogenic signaling by modulating the expression of tyrosinase in mouse B16 melanoma cells. (Mol Cell Biochem **275**: 103–107, 2005)

Key words: calpain, differentiation, melanin, melanogenesis, tyrosinase

Abbreviation: ALLN, calpastatin, cAMP, cyclic AMP, CS, melanocortin 1 receptor, microphthalmia-associated transcription factor, α-MSH, α-melanocyte-stimulating hormone; MITF, MC1R, N-acetyl-leucyl-leucyl-norleucinal.

Introduction

Calpains are a family of Ca^{2+}-dependent cytosolic cysteine proteinases that consist of ubiquitous and tissue-specific isoforms in mammalian tissues [1, 2]. Two mammalian calpains, m- and μ-calpain, are ubiquitously expressed and have been extensively characterized. The μ- and m-isoforms differ in requirement for Ca^{2+} *in vitro*, with the half-maximal activity for μ- and m-calpain being 50 μM Ca^{2+} and 300 μM Ca^{2+}, respectively [1, 2]. Calpain is implicated in a variety of cellular processes including cell proliferation, apoptosis, and differentiation [3].

Melanogenesis is a principal parameter of differentiation of melanocytes and melanoma cells [4]. Synthesis of melanin starts from the conversion of the amino acid L-tyrosine to dopaquinone by tyrosinase, the enzyme catalyzing the rate-limiting step for the melanin biosynthesis [5]. In melanocytes or melanoma cells, melanogenesis is induced by α-melanocyte-stimulating hormone (α-MSH) and cyclic AMP (cAMP)-elevating agents [6]. α-MSH, which induces differentiation in melanocytes or melanoma cells, binds to its specific receptor (MC1R), resulting in the activation of stimulatory GTP-binding protein (Gs), which in turn stimulates adenylate cyclase to produce cAMP [7]. cAMP stimulates melanogenesis mainly via activation of microphthalmia-associated transcription factor (MITF), a melanocyte-specific transcription factor, thereby leading to induction of tyrosinase expression [8]. However, the mechanism(s) involved in the regulation of melanogenesis has not been fully understood. This study was designed to

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investigate the involvement of calpain in melanogenesis of B16 melanoma cells by using calpain inhibitors, *N*-acetyl-Leu-Leu-norleucinal (ALLN) and calpastatin (CS) peptide and it was for the first time demonstrated that calpain exerts a positive regulatory role in the melanogenic signaling by modulating expression of tyrosinase.

Materials and methods

Materials

3,4-Dihydroxyphenylalanine (L-dopa), [Nle^4, D-Phe^7]-α-MSH, synthetic melanin were from Sigma. *N*-acetyl-Leu-Leu-norleucinal (ALLN) was from Roche. Calpastatin (CS) peptide was from Calbiochem. Calpain activity assay kit was from BioVision. Protease inhibitor mixture (Complete^{TM}) was from Roche. The antibody to tyrosinase was from Santa Cruz Biotechnology. Anti-rabbit and -mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from Amersham Pharmacia. Other reagents were of the highest quality available.

Cell culture

Mouse B16 melanoma cells were purchased from Riken Cell Bank (Tsukuba, Japan). B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% (v/v) fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin, at 37 °C in a humidified, CO_2-controlled (5%) incubator. Induction of melanogenesis was initiated by the addition of 1 μM [Nle^4, D-Phe^7]-α-MSH.

Melanogenic assays

For determination of melanin content, the cells were washed with PBS and dissolved in 1 N NaOH for 1 h at 60 °C. The absorbance at 470 nm was measured and melanin content was also measured using the authentic standard of synthetic melanin. For measurement of tyrosinase activity, the cells were washed with ice-cold PBS and lysed by incubating at 4 °C for 30 min in RIPA buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA) containing protease inhibitors (Complete^{TM} protease inhibitor mixture). The lysates were centrifuged at 15, 000 × g for 30 min to obtain the supernatant as source of tyrosinase. The reaction mixture contained 50 mM phosphate buffer, pH 6.8, 0.05% L-dopa and the supernatant (tyrosinase). After incubation at 37 °C for 20 min, dopachrome formation was monitored by measuring absorbance at wavelength 492 nm.

Assay of calpain activity

Calpain activity was measured by using calpain activity assay kit (BioVision) according to the manufacturer's protocol. This assay was based on fluorometric detection of cleavage of calpain substrate Ac-LLY-AFC. The cells were lysed by extraction buffer and centrifuged at 10, 000 × g for 1 min. The resulting cell lysate was transferred to a 96-well plate to which reaction buffer and calpain substrate were added. After incubation at 37 °C for 1 h, the samples were read in a fluorescence plate reader equipped with a 400 nm excitation filter and a 505 nm emission filter.

Electrophoresis and Western blot analysis

Cells were lysed in RIPA buffer containing protease inhibitors and centrifuged at 15, 000 × g for 30 min. The resultant supernatant (solubilized proteins) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gel for tyrosinase. Proteins were transferred electrophoretically onto a PVDF membrane. Blocking was performed in Tris-buffered saline containing 5% skim milk powder and 0.05% Tween-20. Western blot analysis using specific antibodies was performed as described previously [9]. The intensity of the bands was quantified by a densitometer.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from B16 melanoma cells using TRIZOL-Reagent according to manufacturer's protocol. RNA samples (2 μg/reaction) were reverse-transcribed with Superscript in the presence of oligo-dT, and RT reaction was used for amplification with Taq polymerase. The resulting cDNA was amplified using specific primers. The primers used were as follows; for tyrosinase 5′-GGCCAGCTTTCAGGCAGAGGT and 5′-T GGTGCTTCATGGGCAAAATC. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech) were added as a control for the same reverse transcriptase product. Amplification conditions were 94 °C (30 s), 60 °C (30 s), 72 °C (40 s) for 21 cycles (tyrosinase) or 18 cycles (GAPDH). The PCR product were electrophoresed on 1.3% agarose gel containing ethidium bromide.

Results and discussion

Calpain is implicated in a variety of cellular responses, which include regulated cell adhesion, cell motility, secretion, cytoskeletal arrangements, proliferation, and apoptosis [3]. In

addition, several lines of evidence support a role for calpain in cell differentiation of myoblasts [10], osteoblasts [11], chondrocytes [12], keratinocytes [13], and adipocytes [14, 15]. However, little information is available about the regulatory mechanism of calpain in cell differentiation. Mouse B16 melanoma cells have been known to undergo differentiation by stimuli such as α-MSH and cAMP-elevating agents [6]. The differentiated B16 melanoma cells were characterized by increased melanin biosynthesis. Thus, the B16 melanoma cells have widely been employed as a useful model for studying the molecular basis for melanogenesis during differentiation.

In this study, we have examined the involvement of calpain in the regulation of melanogenesis using B16 melanoma cells. Melanogenesis was initiated by the addition of 1 μM α-MSH and assessed by determination of intracellular melanin content. After stimulation with α-MSH, melanin content increased in a time-dependent manner, reaching the maximal levels at 72 h (Fig. 1). Then we have examined the changes in calpain activity in B16 melanoma cells treated with α-MSH. As shown in Fig. 2, the calpain activity was markedly reduced within 2 h following addition of α-MSH, but it showed a progressive increase reaching to the initial level at 6–12 h after α-MSH treatment, followed by a gradual decrease (Fig. 2). The mechanism (s) for the changes of calpain activity by α-MSH treatment should be clarified, but the restoration of calpain activity at 6–12 h following the α-MSH treatment is suggested to participate in the subsequent melanogenic process.

To further investigate the involvement of calpain in the regulation of melanogenesis, the effect of a calpain inhibitor, ALLN on α-MSH-induced melanogenesis was examined. As shown in Fig. 3, ALLN remarkably inhibited the normalization of calpain activity at 6 h after addition of α-MSH. Whereas there was a time-dependent increase in melanin content after treatment with α-MSH, melanogenesis was almost completely repressed when cells were pretreated with ALLN (Fig. 4A). These observations led us to assume that calpain exerts a positive regulatory role in melanogenesis in B16 melanoma cells stimulated with α-MSH.

Melanogenesis is regulated by the activity of tyrosinase, a rate limiting enzyme in melanin biosynthesis, and we examined the effect of inhibition of calpain on α-MSH-mediated increase in tyrosinase activity. Although the tyrosinase activity in the absence of ALLN was enhanced nearly 6-fold by the treatment with α-MSH for 72 h, the activity was not increased in ALLN-treated cells (Fig. 4B).

ALLN has been reported to inhibit also other proteases than calpain [16] and it is not very specific for calpain. Therefore, we used another more specific calpain inhibitor, CS peptide [17] to examine it effect on melanin content and tyrosinase activity. As shown in Fig. 5A and B, this cell-permeable peptide significantly prevented melanogenesis induced by α-MSH. This findings support data with ALLN,

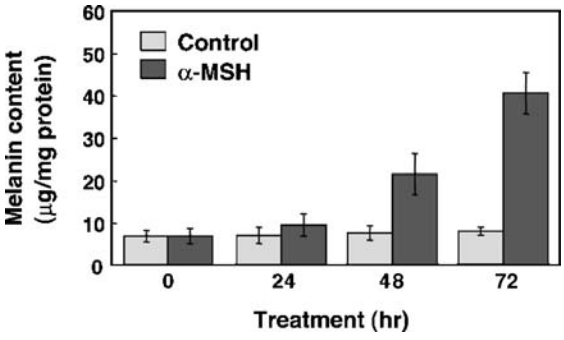

Fig. 1. Melanogenesis of B16 melanoma cells induced by α-MSH. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for the indicated times and the melanin content was determined as described under Materials and Methods. Data represent the mean ± S.D. of two different experiments each carried out in duplicate.

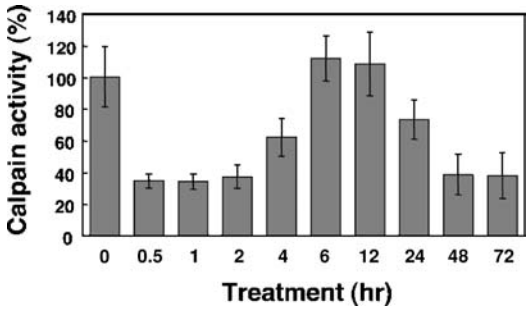

Fig. 2. Effects of α-MSH on calpain in B16 melanoma cells. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for the indicated times and the calpain activity was determined as described under Materials and Methods. Data represent the mean ± S.D. of two different experiments each carried out in duplicate.

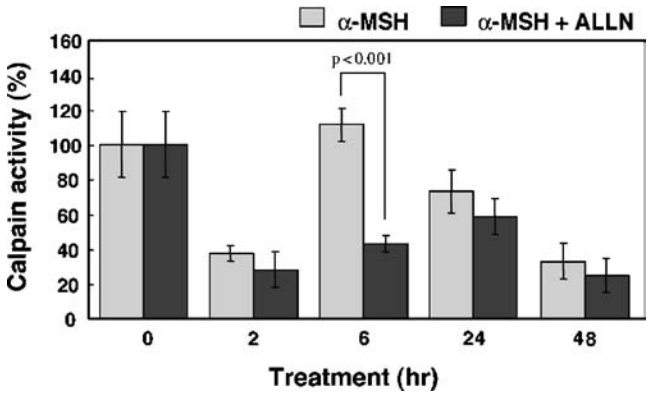

Fig. 3. Effect of ALLN on calpain activity in B16 melanoma cells. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for the indicated times with or without 10 μM ALLN and the calpain activity was determined as described under Materials and Methods. Data represent the mean ± S.D. of two different experiments each carried out in duplicate. Statistical analysis was performed using Student's *t*- test.

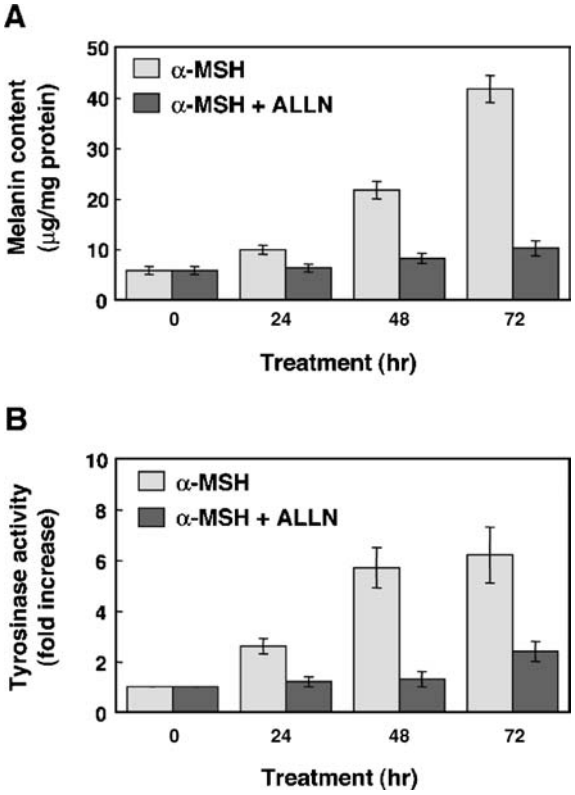

Fig. 4. Effect of ALLN on α-MSH-induced melanogenesis and tyrosinase activation in B16 melanoma cells. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for the indicated times with or without 10 μM ALLN. (A) The melanin content was determined as described under Materials and Methods. (B) Tyrosinase activity was determined by measuring the formation of dopachrome as described under Materials and Methods. Data represent the mean $\pm$ S.D. of two different experiments each carried out in duplicate.

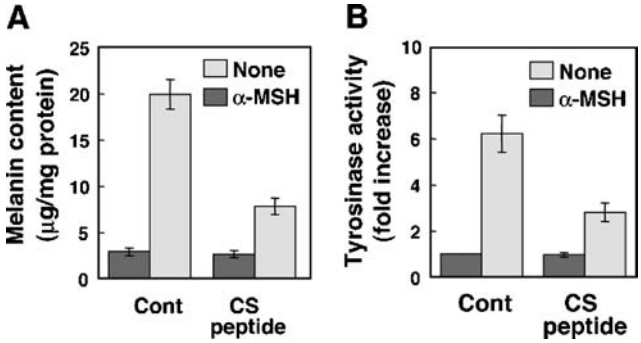

Fig. 5. Effect of calpastatin peptide on α-MSH-induced melanogenesis and tyrosinase activation in B16 melanoma cells. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for 48 h with or without 50 μM calpastatin (CS) peptide. (A) The melanin content was determined as described under Materials and Methods. (B) Tyrosinase activity was determined by measuring the formation of dopachrome as described under Materials and Methods. Data represent the mean $\pm$ S.D. of two different experiments each carried out in duplicate.

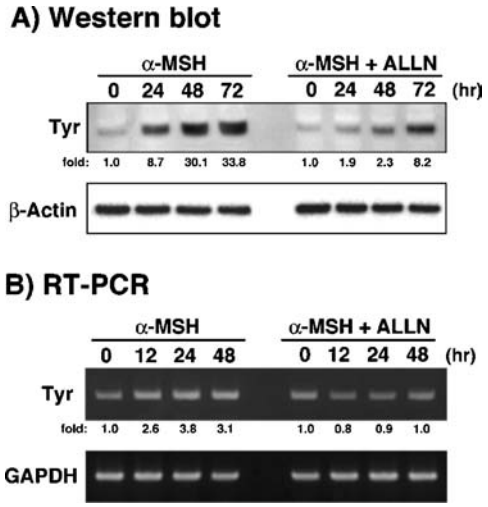

Fig. 6. Effect of ALLN on α-MSH-induced tyrosinase expression in B16 melanoma cells. The cells were treated with 1 μM [Nle^4, D-Phe^7]-α-MSH for the indicated times with or without 10 μM ALLN. (A) The expression level of tyrosinase protein was examined by Western blot analysis using specific antibody as described under Materials and Methods. (B) The level of tyrosinase mRNA was performed by RT-PCR analysis as described under Materials and Methods.

but the possible involvement of other intracellular proteases cannot be excluded, because the inhibition of melanogenesis by this peptide was remarkable but not complete (Fig. 5A and B). Nevertheless, it is conceivable that calpain is involved in the α-MSH-induced melanogenesis.

Next, the expression levels of tyrosinase protein were examined by Western blot analysis. Although the expression of tyrosinase protein was time-dependently enhanced after the treatment with α-MSH, the expression was greatly repressed in the presence of ALLN (Fig. 6A). Further, we examined the levels of tyrosinase mRNA by RT-PCR. mRNA levels of tyrosinase were shown to increase after addition of α-MSH (Fig. 6B). However, α-MSH-induced increase in tyrosinase mRNA levels was no longer observed in ALLN-treated cells (Fig. 6B). Thus, this decrease in tyrosinase activity was considered to be due to the decreased expression of the enzyme, as assessed by Western blot and RT-PCR analysis.

Several intracellular signaling pathways have been reported to involve transcriptional activation of tyrosinase gene. The activation of MITF, a transcription factor that regulates tyrosinase gene expression, is known to be a critical event during melanogenesis [8]. Extracellular signal-regulated kinase (ERK) phosphorylates MITF and promotes its degradation, thereby resulting in inhibition of tyrosinase expression and melanogenesis [18]. In addition, previous reports have demonstrated that the phosphatidylinositol 3-kinase (PI3K) pathway might be involved in the regulation of tyrosinase gene expression [19, 20]. Our recent study has shown that phospholipase D1 (PLD1) is an important

factor in the melanogenic signaling through modulating the expression of tyrosinase [21]. Although it is likely that calpain may be implicated in these proposed signaling pathways, further investigations are required to disclose the precise role of calpain, which are currently under progress in our laboratory.

In summary, the results presented here have demonstrated evidence to suggest that calpain plays a positive regulatory role in melanogenesis through modulating the expression of tyrosinase.

Acknowledgments

This work was supported in part by Grant-in-Aid for Young Scientists (B) 16790180 from the Ministry of Education, Science, Sports, and Culture of Japan.

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