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Cilostazol promotes production of melanin by activating the microphthalmia-associated transcription factor (MITF)

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ABSTRACT

Cilostazol, a licensed clinical drug for the treatment of intermittent claudication, is a phosphodiesterase (PDE) inhibitor that selectively inhibits PDE3, a cAMP-degrading enzyme, thus elevating levels of intracellular cAMP. It has been reported that pigment production by melanocytes both tans the skin and protects against skin cancers. The effects of cilostazol in melanogenesis are as yet unknown. In this study, treatment with cilostazol was found to promote the production of melanin as well as increase both Tyrosinase enzymatic activity and expression of the Tyrosinase gene. Importantly, we also found that cilostazol led to increased expression of the microphthalmia-associated transcription factor (MITF), the "master regulator" of both melanocyte differentiation and pigment production. Interestingly, knockdown of MITF using siRNA abolished the effects of cilostazol in melanogenesis, thereby suggesting that MITF might play a critical role in melanogenesis. Increased expression of MITF was abolished by treatment with H-89, a specific protein kinase A (PKA) inhibitor, thereby suggesting that the PKA pathway plays a critical role in cilostazol-induced expression of MITF. Cilostazol in fact enhanced expression of p-CREB, which was inhibited by H-89. Moreover, this cilostazol-induced increase in expression of MITF was inhibited by downregulation of CREB using CREB siRNA. These data suggest that induction of MITF via the PKA/CREB pathway plays a critical role in cilostazol-induced production of melanin in B16-F10 melanoma cells.

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1. Introduction

Skin color and ease of tanning are the best known predictors of skin cancer [1]. Melanin plays an important role in shielding skin cells from ultraviolet (UV) radiation-induced damage [2]. In mammals, melanin is produced by melanocytes via an enzymatic cascade controlled by tyrosinase [3]. Tyrosinase is the rate-limiting enzyme involved in this process and humans with defective tyrosinase are albino. Tyrosinase is transcriptionally regulated by the microphthalmia-associated transcription factor (MITF). Current studies have considered MITF as the key transcriptional regulator of multiple enzymes involved in melanogenesis [4]. It has also been reported that MITF can regulate transcriptional activity of melastatin (TRPM1), an important functional protein involved in the regulation of melanocyte physiology and melanoma development [5]. Previous studies have shown that cAMP responsive element binding protein (CREB) in conjunction with the melanocyte-specific transcription factor SOX10 can activate MITF expression [6]. However, regulation of MITF is complex and is still not completely understood.

Cilostazol(6-[4-(1-cyclohexyl-1Htetrazol-5-yl)butoxy]-3,4-dihydro-2-(1*H*)-quinolinone) is a phosphodiesterase (PDE) inhibitor, which selectively inhibits PDE3, a 3′-5′-cyclic adenosine monophosphate (cAMP) degrading enzyme, thereby elevating levels of intracellular cAMP [7]. A previous study has demonstrated that cilostazol has the ability to activate phosphorylation of cAMP response element-binding protein (CREB) [8]. The effects of cilostazol in the expression of MITF and melanogenesis have not yet been thoroughly investigated. In this study, we determined the effects of cilostazol on melanin pigmentation. We found that cilostazol rendered B16-F10 melanoma cells darker and also increased expression of both the Tyrosinase gene and MITF.

2. Material and methods

2.1. Cell culture

B16-F10 melanoma cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin–streptomycin. MITF-m, CREB, and negative control small

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interfering RNAs (siRNAs) oligo were from Sigma, USA. Cells were treated with 10 μM cilostazol both with and without the addition of 10 μM PKA inhibitor H89 (Sigma, USA).

2.2. Real-time polymerase chain reaction (PCR)

For real-time quantitative PCR, total RNA was isolated from B16-F10 melanoma cells using TRIzol reagent following the manufacturer's protocol. After being treated with DNase I (GIBCO, USA), 2 μg of RNA were used for reverse transcription. Synthesized cDNA was added to 96-well microtiter plates and real-time PCR was assessed using TaqMan Reverse Transcription reagents (Applied Biosystems, USA) following the manufacturer's recommendations. The following primers were used in this study: MITF: forward, 5′-TTATAGTACCTTCTCTTTGCCAGTCC-3′; reverse, 5′-GTTTATTTGCTAAAGTGGTAGAAAGGTACT-3′; TRPM1: forward, 5′-CACCCAGAGCTACCCAACAGA-3′; reverse, 5′-CGGATATACATGGCTTTATTGGAA-3′; Ty rosinase: forward, 5′-GGCCAGCTTTCAGGCAGAGGT-3′; reverse, 5′-TGGTGCTTCATGGGCAAAATC-3′; GAPDH: forward, 5′-GGAGAAGGC TGGGGCTCAT-3′ (forward) and reverse, 5′-TGATGGCATGGACTG TGGTC-3′.

2.3. Western blot analysis

B16-F10 melanoma cells were lysed with lysis buffer (1% (v/v) Triton X-100, 20 mM Tris–HCl, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 μM leupeptin hemisulfate salt, 0.2 U/ml aprotinin) for 30 min on ice. Protein lysates were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The resolved proteins were electroblotted onto Immobilon-P (Millipore, USA). After having been blocked with 5% w/v non-fat dried milk for 1 h at RT, membranes were sequentially incubated with primary antibodies and secondary antibodies. Chemoluminescence luminal reagents (Santa Cruz Biotechnology) were used to develop blots. The following antibodies were used in this study: mouse anti-MITF-m from Bioworld Technology, USA; mouse anti-β-actin, p-CREB (Ser 133) and total CREB from Cell Signaling, USA; and rabbit anti-TRPM1 from Abcam, USA.

2.4. Tyrosinase enzymatic activity and melanin contents assay

The rate of l-DOPA oxidation was used to measure Tyrosinase activity as described previously [9]. Following the indicated treatment, B16-F10 melanoma cells were washed with ice-cold PBS and lysed by incubation in cell lysis buffer [1nMPMSF] at 4 °C for 15 min. Lysates were centrifuged at 14,000 rpm for 15 min to obtain the supernatant for activity assay and melanin contents assay. 10 μg total proteins were added to each well in a 96-well plate and then mixed with 100 μl 0.1% l-DOPA in 0.1 M PBS (pH 6.8) (M/V). Following incubation at 37 °C for 0.5 h, absorbance at 475 nm was measured to calculate Tyrosinase enzymatic activity. The cell pellet was dissolved in 100 μl of 1 N NaOH/10% DMSO for 1 h at 80 °C and solubilized melanin was then measured at 405 nm.

2.5. Statistical analysis

All data are expressed as means ± SEM. Statistical comparisons were performed using one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

3. Results

When cilostazol was used to treat B16-F10 melanoma cells, we found that cilostazol rendered cells darker. As is shown in Fig. 1A, cilostazol treatment led to about a 2.5 times increase in melanin content. Melanoma cells originate from melanocytes, which produce the skin's dark melanin pigment. Tyrosinase converts tyrosine to DOPA and dopaquinone and is the rate-limiting enzyme involved in melanin synthesis. After having been treated with cilostazol, real time PCR results showed that expression of the Tyrosinase gene was significantly increased (Fig. 1B). Accordingly, Tyrosinase enzymatic activity markedly increased after treatment with 10 μM cilostazol (Fig. 1C).

Previous studies have reported that MITF, the "master regulator" of both melanocyte differentiation and pigment production, could regulate the expression of Tyrosinase and other melanogenic enzymes [10]. Thus, we investigated the effects of cilostazol in the expression of MITF. Indeed, results showed that cilostazol can increase expression of MITF at both RNA (Fig. 2A) and protein levels (Fig. 2B) in a dose dependent manner. Importantly, cilostazol treatment was also able to increase expression of TRPM1, a well-defined MITF target gene, which is involved in the regulation of melanocyte physiology and melanoma development, at both RNA (Fig. 2C) and protein levels (Fig. 2D).

In order to determine whether this increase in MITF expression was involved in the observed increase in melanin production and Tyrosinase enzymatic activity, we investigated the effects of MITF knockdown on cilostazol-induced melanin production. B16-F10 melanoma cells were transiently transfected with MITF siRNA. Our results demonstrated that knockdown of MITF significantly abolished the effects of cilostazol on production of melanin content (Fig. 3A). Correspondingly, Western blot results show that the cilostazol-induced increase in TRPM1expression can be inhibited by transfection with MITF siRNA (Fig. 3B). Moreover, inhibition of MITF also abolished the effects of cilostazol on Tyrosinase enzymatic activity (Fig. 3C). These data suggest that induction of MITF plays a casual role in cilostazol-induced melanogenesis.

The principal action of cilostazol is to elevate intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels [11]. cAMP can activate the transcriptional factor CREB via the PKA pathway.

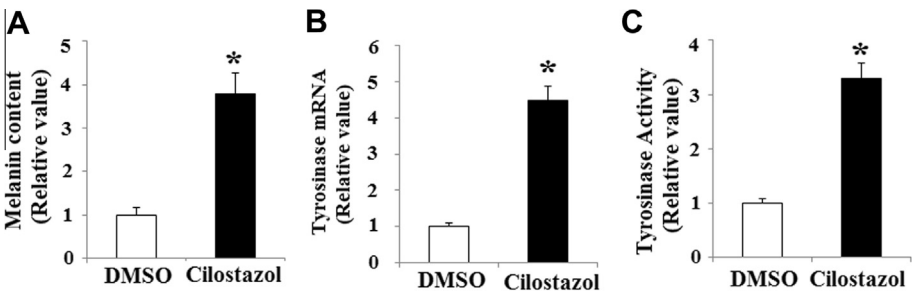

Fig. 1. Cilostazol induces melanogenesis in B16-F10 cells. (A) Cilostazol treatment leads to increased melanin content; (B) Real time PCR results displayed that cilostazol treatment results in increased relative mRNA expression of Tyrosinase; and (C) Cilostazol treatment leads to increased tyrosinase activity in extracts from B16-F10 cells (*, $p < 0.01$ vs. DMSO group, $n = 4$).

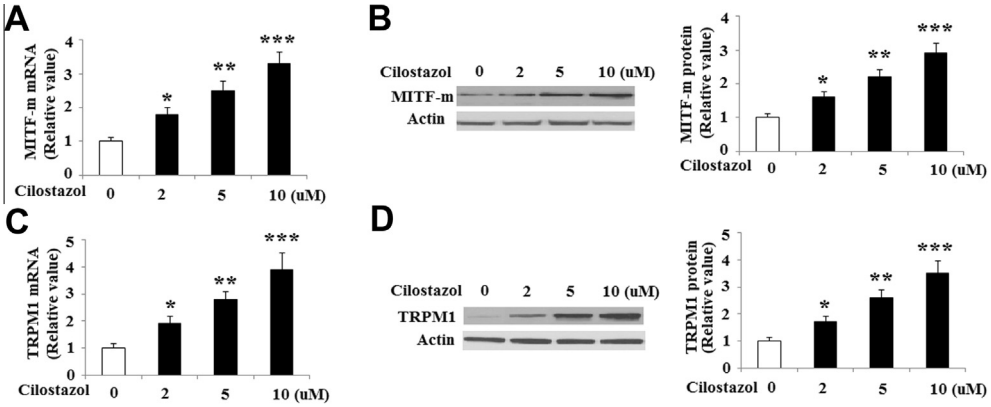

Fig. 2. Cilostazol induces MITF and TRPM1 expression. B16-F10 cells were stimulated with cilostazol at various concentrations for 48 h; (A) mRNA levels of MITF at various concentrations were determined by real-time PCR; (B) protein levels of MITF at various concentrations were determined by Western blot analysis; (C) mRNA levels of MITF target gene TRPM1 at various concentrations were determined by real-time PCR; and (D) protein levels of MITF target gene TRPM1 at various concentrations were determined by Western blot analysis (*, $p < 0.01$ vs. untreated control; **, $p < 0.01$ vs. 2 μM cilostazol treated group; ***, $p < 0.01$ vs. 5 μM cilostazol treated group, $n = 4$).

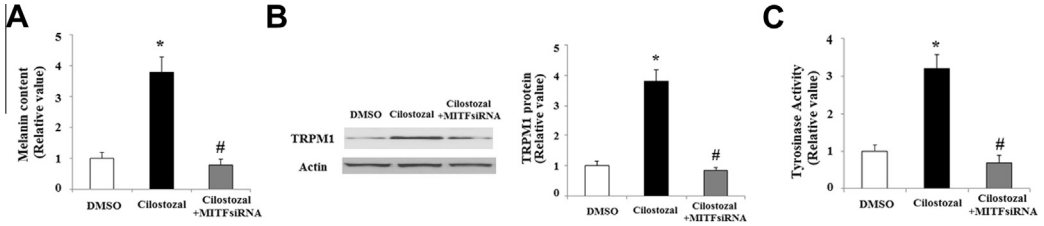

Fig. 3. Knockdown of MITF abolished the effects of cilostazol in melanogenesis in B16-F10 cells. Cells were transfected with MITF siRNA and treated with 10 μM cilostazol. (A) Quantification analysis revealed that inhibition of MITF abolished the effects of cilostazol in melanin content production; (B) representative Western blot and quantification analysis revealed that inhibition of MITF abolished the effects of cilostazol on expression of TRPM1; and (C) quantification analysis revealed that inhibition of MITF abolished the effects of cilostazol in tyrosinase activity (*, $p < 0.01$ vs. control group; #, $p < 0.01$ vs. cilostazol treated group).

Previous studies have shown that CREB is a main regulator of MITF expression [12]. Thus, we speculated that cilostazol could activate expression of MITF via the PKA/CREB axis. In order to confirm this hypothesis, we investigated the effects of PKA-specific inhibitor H-89 on the expression of MITF and sub sequential melanogenesis. As is shown in Fig. 4A, H-89 eliminated both the effects of

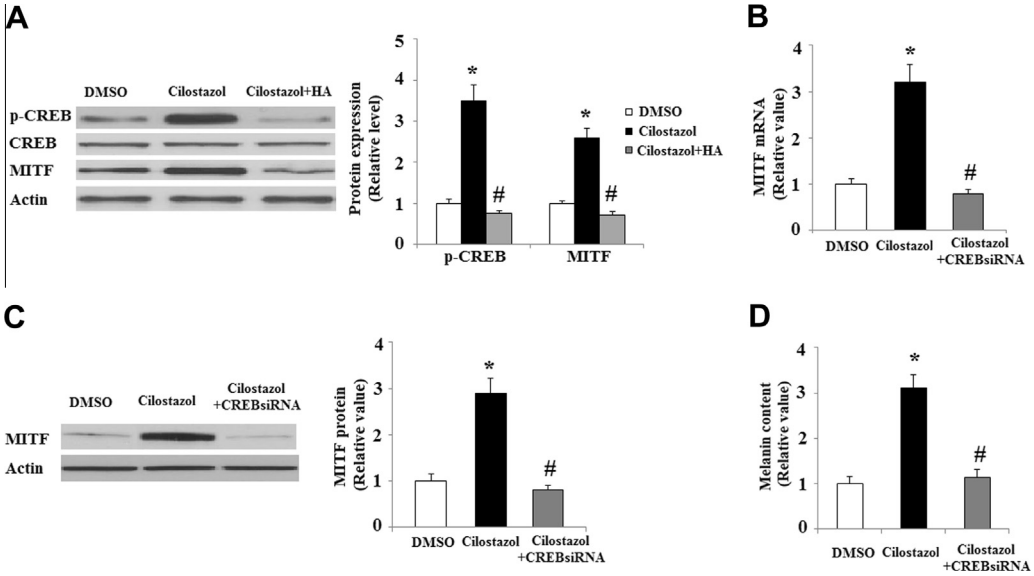

Fig. 4. Increased expression of MITF-M induced by cilostazol is regulated via the PKA/CREB pathway. (A) Representative Western blot and quantification analysis revealed that cilostazol could increase expression of p-CREB and MITF-M, which were significantly inhibited by PKA inhibitor H-89 (10 μM). (B) B16-F10 cells were transiently transfected with CREB siRNA 24 h prior to treatment with 10 μM cilostazol. Real time PCR results revealed that cilostazol-induced expression of MITF-M could be abolished by knockdown of CREB (*, $p < 0.01$ vs. control group; #, $p < 0.01$ vs. cilostazol treated group). (C) Representative Western blot and quantification analysis revealed that cilostazol-induced expression of MITF-M could be abolished by knockdown of CREB (*, $p < 0.01$ vs. control group; #, $p < 0.01$ vs. cilostazol treated group). (D) Quantification analysis revealed that inhibition of CREB abolished the effects of cilostazol in melanin content production (*, $p < 0.01$ vs. control group; #, $p < 0.01$ vs. cilostazol treated group).

cilostazol on MITF expression as well as alterations in levels of phosphorylated CREB (Ser133), thereby suggesting that PKA plays a critical role in the increased expression of p-CREB and MITF in cilostazol treated B16-F10 cells. In order to further investigate whether CREB mediated cilostazol-induced expression of MITF, CREB expression was inhibited using siRNA. Effects of cilostazol on MITF expression were investigated in B16-F10 following knockdown of CREB. Indeed, knockdown of CREB completely abolished the increase in MITF expression induced by cilostazol in B16-F10 melanoma cells at both mRNA (Fig. 4B) and protein levels (Fig. 4C). These findings suggest that the effect of cilostazol on MITF is mediated by CREB. Importantly, knockdown of CREB significantly abolished cilostazol-induced production of melanin content (Fig. 4D).

4. Discussion

In the past several decades, melanogenesis has attracted increasingly more attention. It has been demonstrated that melanin is packaged in melanosomes, secreted, and internalized by adjacent keratinocytes where it is positioned on the sun-exposed side of the nucleus, thereby protecting cells from damaging UV radiation [13]. The ability to tan plays a critical role in primary protection from basal and squamous cell carcinomas, melanoma carcinogenesis, vitamin D homeostasis, and cosmesis [14].

Although melanin biosynthesis is a complex pathway, increasing evidence has shown that MITF is a critical factor in melanocytes' pigmentation process. Importantly, MITF regulates expression of enzymes (i.e. Tyrosinase) and structural proteins involved in melanin production, such as TRPM-1. Cilostazol is a phosphodiesterase (PDE) inhibitor which elevates intracellular levels of cAMP. However, its role in regulating melanogenesis has not yet been reported. Our data show that treatment with cilostazol enhances expression of MITF as well as its downstream factors TRPM-1 and Tyrosinase, thereby leading to an increase in tyrosinase activity and melanin content in B16-F10 melanoma cells. These observations suggest that cilostazol augments the functionality of melanin biosynthesis in B16-F10 melanoma cells.

Here, we highlight the existence of a link between phosphorylation of CREB and regulation of MITF in response to cilostazol treatment. Multiple signaling cascades participate in the regulation of MITF. CREB has been reported as an important regulator of MITF [15]. Moreover, a recent study demonstrated that PPAR-γ coactivator (PGC)-1α and PGC-1β work as critical components in this melanogenic system by activating the MITF promoter [16]. These findings are consistent with previous studies that show that the PGC-1α protein is regulated by CREB [17]. Importantly, Zuo and colleagues reported that cilostazol can promote mitochondrial biogenesis via activation of PGC-1α which is mediated by phosphorylation of CREB [18]. Although we did not investigate the role of PGC-1α in the effects of cilostazol in melanogenesis, it still provides support for the critical role of CREB in the regulation of MITF expression. We speculated that cilostazol activates CREB and that this activated CREB increases expression of MITF in multiple ways. It can directly regulate MITF transcription of by binding to the MITF promoter region, or it can indirectly regulate MITF transcription by activating PGC-1α and, thereby, initializing transcription of MITF. Interestingly, acupuncture has been reported to activate ERK-CREB [19], suggesting a potential role of acupuncture in regulating MITF and the production of melanin. In addition, natural food products and Chinese traditional medicine have been reported to regulate MITF expression [20]. However, the underlying mechanism is still unknown. Therefore, further studies in future will provide a complete picture of the effects of acupuncture and Chinese traditional medicine in MITF and the production of melanin.

MITF also plays a critical, though complex and incompletely understood, role in melanoma biology that may be separate from its role in protection from sun exposure [21]. In this study, knockdown of MITF was found to abolish the effects of cilostazol in melanogenesis, indicating the critical role of MITF in cilostazol-induced pigmentation. Cilostazol has been used in a recent study involving dermal fibroblasts. Long-term exposure to Ultraviolet B (UVB) irradiation leads to connective tissue damage through an increase in production of matrix metalloproteinase-1 (MMP-1) during skin photoaging. A recent study reports that cilostazol can inhibit MMP-1 expression and increase type I procollagen synthesis in UVB-irradiated human dermal fibroblasts, suggesting that cilostazol might be useful in the prevention and treatment of skin photodamage caused by UVB-irradiation. [22]. The combination of these findings suggests that cilostazol can potentially be applied in human pigmentation and melanoma treatment.

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