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# PDE5 Inhibitor Promotes Melanin Synthesis Through the PKG Pathway in B16 Melanoma Cells

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## ABSTRACT

PDE inhibitors could increase cellular cGMP levels and are used to treat erectile dysfunction as well as pulmonary arterial hypertension. cGMP production was reported to be necessary for UVB-induced melanin synthesis, however, the effect of PDE5 inhibitor on melanin synthesis has not been examined. We found that PDE5 inhibitor (sildenafil or vardenafil) and the cGMP analog 8-CPT-cGMP stimulated CREB phosphorylation, leading to increased tyrosinase expression and melanin synthesis, which was counteracted by KT5823, a selective cGMP-dependent protein kinase (PKG) inhibitor. However, KT5823 did not affect cAMP-elevating agent-mediated melanin synthesis, indicating that KT5823 selectively inhibited cGMP-induced melanin synthesis. This is the first study to find that PDE5 inhibitor can promote melanin synthesis and reveal that PKG-dependent CREB phosphorylation and tyrosinase expression is involved in cGMP-induced melanin synthesis. Our results suggest that PDE5 inhibitor may be beneficial for the treatment of hypopigmentation diseases. J. Cell. Biochem. 113: 2738–2743, 2012. 

**KEY WORDS:** MELANIN SYNTHESIS; PDE5 INHIBITOR; PKG; TYROSINASE

Melanin is an important skin pigment in human and contributes significantly to the health of an individual [Gilchrest, 1989; Sturm, 2002]. Lack or decreased levels of melanin in human lead to many skin diseases, termed hypopigmentary disorders including vitiligo and gray hair [Hartmann et al., 2004; Dessinioti et al., 2009]. There are several treatments for hypopigmentation diseases, but there remain some problems, such as poor efficacy and severe side effects [Hartmann et al., 2004; Hercogova et al., 2007]. Thus the search for new types of treatments of hypopigmentation diseases with high efficacy and low toxicity is warranted.

Melanin is synthesized in melanocytes via a cascade of enzymatic reactions controlled by tyrosinase [Hearing, 1999; Kim et al., 2010]. Tyrosinase is the rate-limiting enzyme of melanin synthesis that catalyses the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to dopaquinone [Slominski et al., 2004]. Melanin synthesis is stimulated by a large variety of intrinsic and extrinsic factors, including cAMP-elevating agents, UVB, and a wide variety of growth factors and cytokines [Friedmann and Gilchrest, 1987; Yasumoto et al., 1997; Jang et al., 2009; Ho et al., 2010]. cAMP pathway plays a key role in the regulation of melanogenesis through activation of cAMP-dependent protein kinase (PKA) and cAMP response element-binding (CREB) transcription factor, which induced an up-regulation of tyrosinase expression and the stimulation of melanogenesis [Steingrimsson et al., 2004; Park et al., 2009].

For the first time in 1996, the second messenger cGMP was found to be required for melanin synthesis induced by UVB, which can increase cGMP content in melanocytes [Romero-Graillet et al., 1996]. Phosphodiesterase 5 (PDE5) is the predominant enzyme responsible for cGMP hydrolysis in various types of tissues or cells [Kass et al., 2007]. PDE5 inhibitors such as sildenafil and vardenafil

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have been approved for the treatment of erectile dysfunction, and recent clinical data show that they are also effective in the treatment for pulmonary arterial hypertension (PAH) [Evgenov et al., 2006; Ghofrani et al., 2006]. However, whether PDE5 inhibitor can promote melanin synthesis in melanocytes is unknown.

In the present study, using the well-characterized mouse melanoma cells B16, we examined the effect of PDE5 inhibitors sildenafil and vardenafil on melanin synthesis and elucidated the signaling pathway responsible for the beneficial effects of PDE5 inhibitors and cGMP.

## MATERIALS AND METHODS

### REAGENTS

MTT [-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide], L-DOPA (L-3,4-dihydroxyphenylalanine), IBMX (3-isobutyl-1-methylxanthine), 8-pCPT-cGMP [8-(4-chlorophenylthio)-cGMP] and KT5823 were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Hyclone. All primary antibodies except anti-tyrosinase antibody (Chemicon) were purchased from Cell Signaling Technology.

### CELL CULTURE

B16 melanoma cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences in China. The cells were cultured in DMEM with 10% FBS in a humidified atmosphere containing 5% $CO_2$ in air at 37°C.

### CELL VIABILITY

Cell viability was determined by a colorimetric MTT assay. Briefly, $5 \times 10^3$ cells/well were plated into a 96-well culture plate. After 24 h, the culture medium was replaced with various concentrations of test compounds in DMEM with 10% FBS for 72 h. Then 10 μl of 5 mg/ml MTT was added to each well for another 4 h. The resulting formazan crystals were dissolved in the solution [10% SDS (sodium dodecyl sulfate), 5% isopropylcarbinol, 0.01 mol/l HCl (w/v/v)], and the absorbance was measured at 570 nm relative to 630 nm.

### MELANIN CONTENT MEASUREMENT

The melanin content was used as an index of melanin synthesis. Determine of melanin content was performed as described previously [Zhang et al., 2009]. In brief, $3 \times 10^4$ cells/well were plated into the 24-well culture plate and treated with test compounds. After 72 h of treatment, cells were washed twice with phosphate buffered saline (PBS) and dissolved in 200 μl of 1 N NaOH at 60°C for 1 h. The melanin content was detected spectrophotometrically by absorbance at 492 nm.

### TYROSINASE ACTIVITY ASSAY

Tyrosinase activity was examined as L-DOPA oxidase activity. Cells were washed twice with PBS, and then lysed in 50 mM sodium phosphate buffer (pH 6.8) containing 0.2% Triton X-100 and 0.8 mM L-DOPA. After 3 h of incubation at 37°C, the absorbance at 475 nm was measured.

In order to assess the direct effect of test compounds on tyrosinase activity, cells were solubilized with PBS containing 0.2% Triton X-100 and the lysate was clarified by centrifugation at 10,000*g* for 10 min. 90 μl of each lysate was aliquoted into each well of a 96-well plate, and 10 μl of 8 mM L-DOPA in the presence or absence of test compounds was then added to each well. After 20 min incubation at 37°C, the absorbance at 475 nm was measured.

### WESTERN BLOTTING

Cells were harvested and homogenized in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, 1 mM PMSF) with phosphatase and protease inhibitor cocktail (Sigma). Proteins were separated by 10% SDS–PAGE gels and transferred to PVDF membranes (Bio-Rad Laboratories). The membranes were then incubated with primary antibodies for phospho-CREB, tyrosinase, or β-actin. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were used as the secondary antibodies (Jackson ImmunoResearch Laboratories). The resulting immunoblots were visualized using Enhanced ChemiLuminescence substrate (Pierce) according to the manufacturer's instructions.

### STATISTICAL ANALYSIS

Data from all experiments are presented as the mean ± SD. Statistical analysis was performed by two-tailed unpaired Student's *t*-test. Differences were considered significant when the *P*-value was less than 0.05.

## RESULTS

### EFFECTS OF PDE5 INHIBITORS ON MELANIN CONTENT AND TYROSINASE ACTIVITY IN MELANOCYTES

In order to determine whether inhibition of PDE5 could promote melanin synthesis, B16 cells were incubated with PDE5 inhibitors sildenafil and vardenafil, respectively for 72 h. Compared with the untreated control, the melanin content was significantly increased by sildenafil and vardenafil in a dose-dependent manner (Fig. 1A). Figure 1B shows that sildenafil and vardenafil at the concentration of 5–20 μM did not affect cell viability, indicating that PDE5 inhibitors at their effective concentrations had no effect on cell proliferation. The cGMP analog 8-pCPT-cGMP and the cAMP-elevating agent IBMX were employed as the positive controls. Treatment of B16 cells with 8-pCPT-cGMP or IBMX resulted in a significant increase of melanin content (Fig. 1A), which was consistent with previous results [Romero-Graillet et al., 1996; Busca and Ballotti, 2000]. These results demonstrate that PDE5 inhibitors could promote melanin synthesis in B16 cells.

Tyrosinase is the rate-limiting enzyme of melanin synthesis [Slominski et al., 2004]. Figure 1C,D shows that both sildenafil and vardenafil at the concentration of 5–20 μM induced a significant up-regulation of tyrosinase activity after 24 or 72 h of treatment. Likewise, tyrosinase activity was increased by 8-pCPT-cGMP and IBMX, respectively. In addition, sildenafil significantly increased tyrosinase activity in A375 human melanoma cells (Supplementary Fig. 1A), indicating that the effect is not limited to the mouse cell line (B16 cells) but can be observed in the human cell line. The

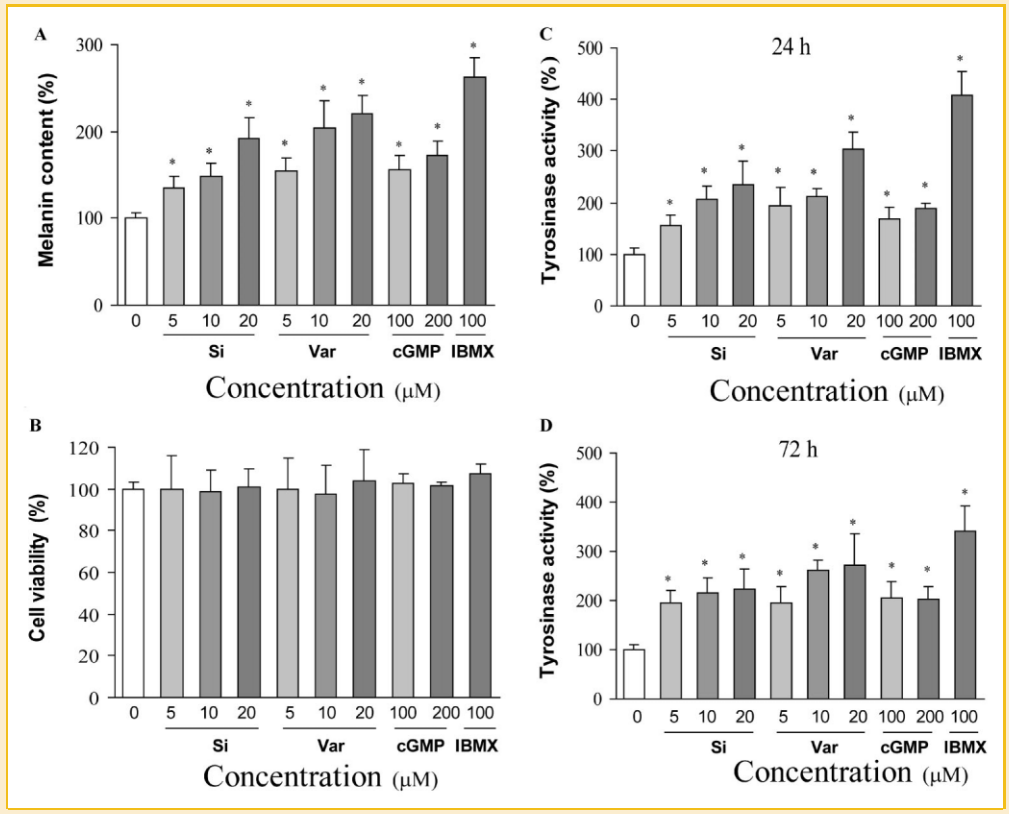


Fig. 1. PDE5 inhibitors increased melanin content and tyrosinase activity in B16 cells. Cells were treated with sildenafil, vardenafil, 8-pCPT-cGMP, or IBMX for 72 h, and then melanin content and cell viability were detected. A: Melanin content assay. B: MTT assay. Tyrosinase activity assay was performed after 24 (C) or 72 h (D) of treatment in B16 cells. Differences statistically significant from the untreated control: $^{*}P < 0.05$. Si, sildenafil; Var, vardenafil; cGMP, 8-pCPT-cGMP.

inhibitory effects of sildenafil and IBMX on cell proliferation may be due to the differentiation of A375 cells induced by them (Supplementary Fig. 1B), because up-regulation of tyrosinase acitivity is an important marker of A375 cell differentiation [Liu et al., 2006].

### EFFECT OF KT5823 ON SILDENAFIL-MEDIATED MELANIN SYNTHESIS AND TYROSINASE ACTIVITY IN B16 CELLS

PDE5 inhibitor exerts their biological effects mainly through the PKG pathway [Reffelmann and Kloner, 2003]. In order to confirm whether PDE5 inhibitor-mediated melanin synthesis was associated with the PKG pathway, B16 cells were pretreated with KT5823, a selective PKG inhibitor. Both 8-pCPT-cGMP and sildenafil-induced melanin synthesis (Fig. 2A) and up-regulation of tyrosinase activity (Fig. 2B) were significantly inhibited by KT5823. However, KT5823 did not inhibit the up-regulation of tyrosinase activity and melanin synthesis induced by IBMX in B16 cells. These findings indicate that PKG may be involved in PDE5 inhibitor-mediated up-regulation of tyrosinase activity and subsequent melanin synthesis.

### EFFECTS OF SILDENAFIL ON TYROSINASE PROTEIN LEVELS IN B16 CELLS

The protein levels of tyrosinase in B16 cells were assessed by Western blotting. As the positive control, IBMX increased the protein levels of tyrosinase at 6 or 24 h (Fig. 3A). At the concentration of 3–30 μM, sildenafil dose-dependently increased the protein levels of tyrosinase at 6 or 24 h (Fig. 3A). Figure 3B shows that treatment with 50 or 100 μM of 8-pCPT-cGMP for 24 h also up-regulated the protein levels of tyrosinase in B16 cells. Actually, 8-pCPT-cGMP and sildenafil did not directly activate tyrosinase in a cell-free system for tyrosinase activity assay (Fig. 3C). Therefore, both cGMP and PDE5 inhibitor could promote melanin synthesis by up-regulating tyrosinase expression in B16 cells.

### EFFECTS OF SILDENAFIL ON CREB PHOSPORYLATION IN B16 CELLS

Next we evaluated the influence of PDE5 inhibitor on the CREB pathway, which regulates tyrosinase expression. As shown in Figure 3D, IBMX increased CREB phosphorylation as reported. We found that sildenafil treatment increased CREB phosphorylation. Also, CREB phosphorylation was enhanced in 8-pCPT-cGMP treated cells (Fig. 3D). These results suggest that both PDE5 inhibitor and cGMP could induce CREB phosphorylation, which may play an important role in PDE5 inhibitor-mediated melanin synthesis.

### EFFECT OF KT5823 ON SILDENAFIL-MEDIATED TYROSINASE EXPRESSION AND CREB PHOSPORYLATION IN B16 CELLS

To directly demonstrate the involvement of CREB in PDE5 inhibitor-mediated tyrosinase expression and melanin synthesis,

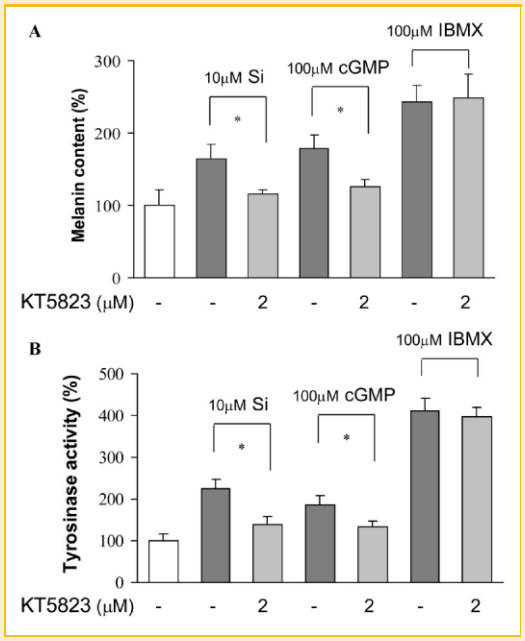


Fig. 2. KT5823 inhibited sildenafil-mediated melanin synthesis and up-regulation of tyrosinase activity. B16 cells were pre-incubated with or without KT5823 for 15 min. A: Melanin content was detected after 72 h of treatment with sildenafil, 8-pCPT-cGMP or IBMX. B: Tyrosinase activity was detected after 24 h of treatment. $^{*}P < 0.05$, compared with sildenafil or 8-pCPT-cGMP only. Si, sildenafil; cGMP, 8-pCPT-cGMP.

we examined the effect of the PKG inhibitor KT5823 in B16 cells. As shown in Figure 4A, the up-regulation of tyrosinase expression induced by sildenafil was reduced by KT5823. Furthermore, KT5823 could inhibit sildenafil-induced CREB phosphorylation. Similarly, 8-pCPT-cGMP-mediated tyrosianse expression and CREB phosphoylation were inhibited by KT5823 (Fig. 4B). As a selective PKG inhibitor, KT5823 did not affect the effect of IBMX on tyrosinase expression and CREB phosphoylation (Fig. 4C). These results reveal that CREB phosphoylation may be a critical pathway in PDE5 inhibitor-mediated melanin synthesis.

## DISCUSSION

cGMP production can be induced by UVB and plays an important role in its melanogenic effect [Romero-Graillet et al., 1996]. In the present study, we investigated the effect of inhibition of cGMP degradation by PDE5 inhibitors on melanin synthesis in B16 melanocytes. PDE5 inhibitors (sildenafil and vardenafil) increased melanin synthesis exhibited by increased melanin content in a dose-dependent manner, which was mimicked by the cGMP analog 8-pCPT-cGMP. As the positive control, IBMX, a cAMP elevating agent, also promoted melanin synthesis. The selective PKG inhibitor KT5823 specifically inhibited 8-pCPT-cGMP and sildenafil-induced melanin synthesis, however, it had no effect on IBMX-induced melanin synthesis. Our results indicate that PDE5 inhibitor-mediated melanin synthesis could be mediated through the PKG pathway at least partly.

To investigate the mechanism of PDE5 inhibitor-mediated melanin synthesis, we examined the effects of PDE5 inhibitor on

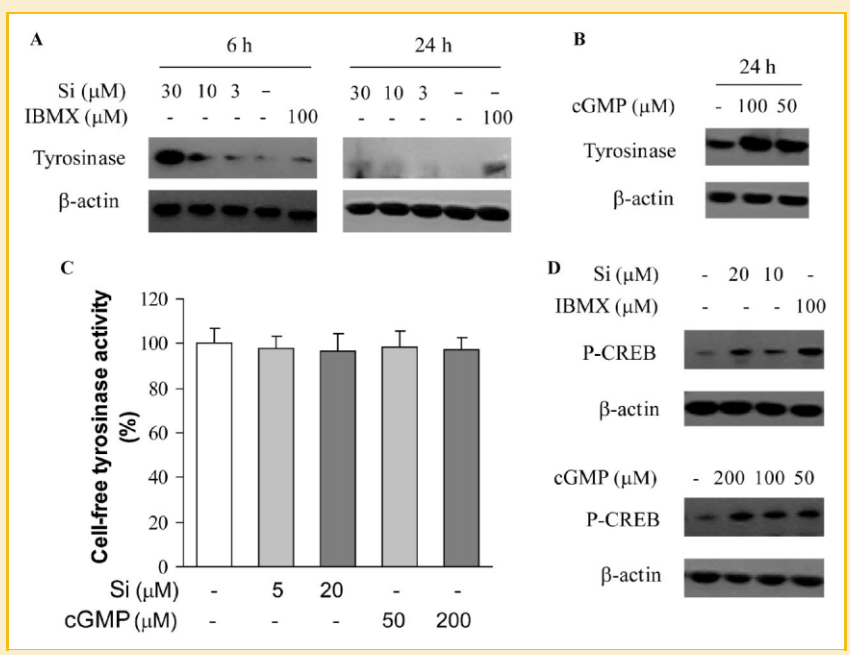


Fig. 3. Sildenafil up-regulated tyrosinase expression and increased CREB phosphorylation in B16 cells. A,B: Tyrosinase expression was detected by Western blotting in B16 cells treated with sildenafil, 8-pCPT-cGMP or IBMX. C: Tyrosinase activity in a cell-free system was performed as described in "materials and methods." D: After cells were treated with sildenafil, IBMX for 60 min or 8-pCPT-cGMP for 30 min, protein phoshorylation was detected by Western blotting. Si, sildenafil; cGMP, 8-pCPT-cGMP.

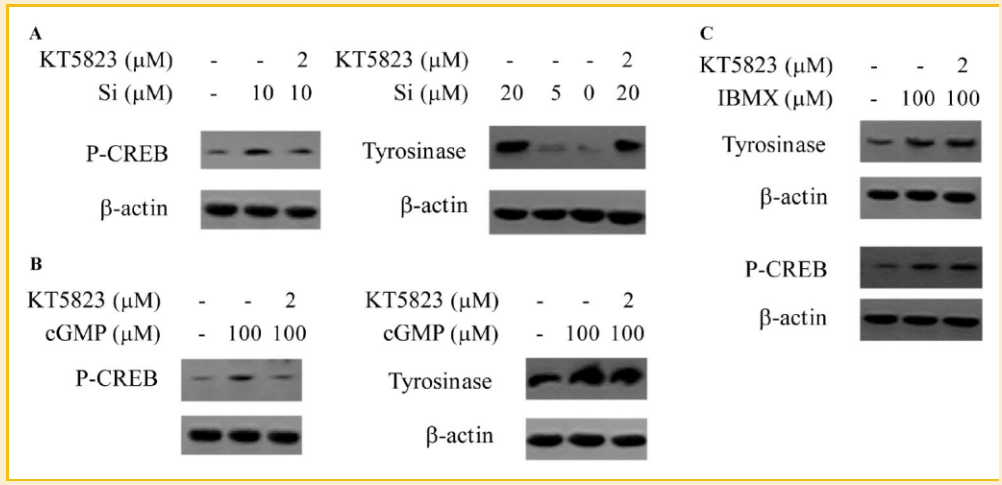


Fig. 4. KT5823 inhibited sildenafil-mediated CREB phosphoylation and tyrosinase expression. B16 cells were pre-incubated with KT5823 for 15 min, and then treated with sildenafil (A), 8-pCPT-cGMP (B), or IBMX (C) for different time. Western blotting was used to detect CREB phosphorylation (sildenafil and IBMX treatment for 60 min or 8-pCPT-cGMP treatment for 30 min) and tyrosinase expression (24 h treatment). Si, sildenafil; cGMP, 8-pCPT-cGMP.

the expression and activity of tyrosinase. Tyrosinase controls the rate-limiting step for melanin synthesis [Slominski et al., 2004], which can be regulated by either direct activation/inhibition or alteration of expression [Slominski et al., 2004; Guan et al., 2008; Zhang et al., 2009]. cGMP has been found to increase tyrosinase activity [Romero-Graillet et al., 1996], however, the mechanism responsible for that has not been reported. In our study, a significant up-regulation of tyrosinase activity in B16 cells was observed after 24 or 72 h of treatment with sildenafil or 8-pCPT-cGMP, but they did not directly affect tyrosinase activity in a cell-free system. Thus we further examined the effects of sildenafil and 8-pCPT-cGMP on tyrosinase expression by Western blotting at or before 24 h. As we expected, sildenafil and 8-pCPT-cGMP increased the protein levels of tyrosinase, implying that they up-regulated tyrosinase expression. Furthermore, KT5823, the selective PKG inhibitor, inhibited sildenafil and 8-pCPT-cGMP-induced up-regulation of tyrosinase expression, however, it did not inhibit the expression of tyrosinase induced by IBMX. Our results indicate that PDE5 inhibitor and cGMP could promote tyrosinase expression in melanocytes through a PKG pathway.

Although cGMP plays an important role in melanin synthesis induced by UV, how cGMP regulates melanin synthesis remains unknown. The transcription factor CREB is activated by a diverse array of extracellular signals through phosphorylation of the trans-activation domain and CREB is critical in a variety of cellular processes such as cell proliferation, differentiation and survival [Shaywitz and Greenberg, 1999; Benito and Barco, 2010; Xiao et al., 2010]. In melanocytes, CREB activation can induce a significant up-regulation of tyrosinase expression thereby increasing its activity, which is believed to be the most important reason responsible for cAMP-elevating agent-mediated melanin synthesis [Haddad et al., 1999; Busca and Ballotti, 2000]. In the present study, both sildenafil and cGMP increased CREB phosphorylation. KT5823 could inhibit the effect of sildenafil and cGMP on CREB phosphorylation but it did not inhibit IBMX-mediated CREB phosphorylation, suggesting that sildenafil and cGMP activated CREB through a PKG-dependent pathway. PKG has been demonstrated to directly phosphorylate CREB in vitro, but its effect is less than PKA [Gudi et al., 2000]. In intact cells, such as neuronal cells and baby hamster kidney cells, elevation of intracellular cGMP levels leads to increased CREB phosphorylation through the PKG pathway [Gudi et al., 2000; Sauzeau et al., 2003]. Therefore, sildenafil-mediated melanin synthesis may be attributable to activate CREB through the PKG pathway.

In conclusion, we found for the first time that PDE5 inhibitor promoted melanin synthesis by up-regulating tyrosinase expression through the PKG pathway in melanocytes. Additionally, PDE5 inhibitor and cGMP can phosphorylate CREB in a PKG-dependent manner, revealing that CREB activation may play a crucial role in PDE5 inhibitor and cGMP-mediated melanin synthesis. Our results support that PDE5 maybe a potential target for the treatment of hypopigmentary disorders.

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