Inhibition of Melanogenic Activity by 4,4′-Dihydroxybiphenyl in Melanoma Cells

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In our previous study, we showed that 4,4′-dihydroxybiphenyl (44'-BP) reduced melanin content via the inhibition of tyrosinase. In the current study, we utilized 44'-BP treated B16 melanoma cells (B16 cells) to measure several key cellular parameters known to be involved in melanogenic activity. Included in these measurements were tyrosinase and microphthalmia transcription factor (MITF) protein levels, cyclic AMP levels, protein kinase A (PKA) activation, and reduced glutathione (GSH) and oxidized glutathione (GSSG) levels. Results showed that 44'-BP effectively suppressed the amounts of tyrosinase and MITF proteins, cAMP levels, and PKA activation. In addition, 44'-BP enhanced the GSH/GSSG ratio. In conclusion, our data provide an evidence that 44'-BP suppressed several key parameters in the melanogenic pathway by downregulating the cAMP-dependent PKA signaling pathway and decreasing MITF gene expression (implied from the reduced protein levels), which in turn suppressed tyrosinase. We propose that the antimalanogenic action of 44'-BP is likely carried out by a combined effect of its anti-oxidant property and its ability to enhance intracellular GSH levels.

Key words 4,4′-dihydroxybiphenyl; cyclic AMP; reduced glutathione; microphthalmia transcription factor; protein kinase A; tyrosinase

It has been reported that the melanogenic activity can be inhibited by antioxidants, like α-tocopherol and hydrocoumarins,1) and that reduced glutathione (GSH), an important biological reductant, plays a role in the modulation of the melanogenic process.2)

Melanogenesis, which is catalyzed enzymatically by the key enzyme, tyrosinase, is also influenced by other non-enzymatic factors such as ultraviolet rays and α-melanocyte-stimulating hormone (α-MSH).3) In the case of α-MSH-induced melanogenesis, the enhancement of tyrosinase activity, tyrosinase mRNA, and intracellular cyclic AMP (cAMP) were observed.4)

The requirement of cAMP for melanogenesis is quite clear for pigmentation. cAMP also increases tyrosinase mRNA and promotes increased microphthalmia transcription factor (MITF) expression. MITF is a melanocyte-specific transcription factor crucial for melanocyte survival, development and differentiation through the activation of protein kinase A (PKA).5) PKA is a serine/threonine kinase that is an inactive tetramer consisting of two regulatory subunits and two catalytic subunits, and its activation occurs by the binding of cAMP to its regulatory subunits and then releasing its catalytic subunits from the regulatory subunits.6)

Recently, we reported that 4,4′-dihydroxybiphenyl (44′-BP) has a melanogenesis inhibitory effect through the inhibition of tyrosinase and reduction of melanin content.7) However, the underlying process by which tyrosinase activity and melanogenesis were inhibited by 44′-BP was not elucidated. In this current study, we attempted to investigate the effect of 44′-BP on major cellular regulators involved in melanogenesis in B16 melanoma cells (B16 cells). The analysis was carried out by quantifying amounts of tyrosinase and MITF proteins, cAMP levels, phosphorylated PKA, and GSH and oxidized glutathione (GSSG) levels.

MATERIALS AND METHODS

Materials 44′-BP, α-MSH, and all other chemical reagents were purchased from Sigma Chemical Co. Western blotting detection reagents were obtained from Amersham. RNAzolB was obtained from TEL-TEST, Inc. Polyclonal antibodies to MITF and pPKA were obtained from Santa Cruz Biotechnology. The antibody to β-actin was from Sigma. The rabbit polyclonal anti-tyrosinase (pep7) antibody was from Dr. V. Hearing (Bethesda, Maryland). Monoclonal sheep anti-mouse IgG antibody or donkey anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from Serotec (Oxford, U.K.). Polyvinylidene difluoride (PVDF) membranes were obtained from Millipore Corporation. cAMP assay kit was purchased from R & D system, Inc.

Cell Culture System B16 cells (from Korean Cell Line Bank) were cultured in DMEM with 10% fetal bovine serum (FBS; Gibco) and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO2 in air at 37°C. B16 cells were cultured in 24-well plates for each assay.

All the experiments were determined in triplicate and repeated three times to ensure reproducibility.

GSH and GSSG Assay Assays for GSH and GSSG were carried out by the method of Pandey and Katiyar.8) Twenty-five percent of the meta-phosphoric acid-added cell pellets were centrifuged at 12000 rpm for 10 min, and the supernatant was taken for assay. For GSH, 1 mM EDTA-50 mM phosphate buffer was added to the supernatant followed by o-phthalaldehyde. After 20 min at room temperature, the fluorescence was measured at excitation wavelength of 360 nm and emission wavelength of 460 nm. GSSG was assayed after preincubated with N-ethylmaleimide for 20 min and 0.1 M NaOH was substituted for 1 mM EDTA-50 mM phosphate buffer.

Quantitation of cAMP To measure intracellular cAMP levels, an enzyme immunoassay kit was used. In brief, B16 cells (7×105) were lysed in 0.1 M HCl to inhibit phosphodiesterase activity. Supernatants were collected, neutralized, and diluted. Following neutralization and dilution, a fixed

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amount of cAMP conjugate (alkaline phosphatase-labeled cAMP) was added to compete with cAMP present in the cell lysate. The cAMP substrate solution was added to the wells to determine binding enzyme activity. The color development was stopped and the absorbance was read at 405 nm. The intensity of the color was inversely proportional to the concentration of cAMP in the cell lysates.

**Protein Preparation and Western Blotting** The B16 cells (5×10⁴) were plated on 24-well multi-dishes and incubated in the presence or absence of 100 nm α-MSH. The cells were then incubated for 24 h with or without 44'-BP and washed twice in PBS at 4 °C. Total cell lysates were lysed in lysis buffer [40 mM Tris (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40 (NP-40), 0.1 mM sodium orthovanadate, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 100 μg/ml phenylmethylsulfonyl fluoride (PMSF)]. The supernatant was collected and protein concentrations were then determined with protein assay reagents (Pierce, Rockford, IL, U.S.A.). For the Western blotting, equal amount of proteins were boiled for 2 min and chilled on ice, subjected to 8—10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to a PVDF membrane. The proteins were visualized with the enhanced chemiluminescence (ECL) detection system (Amersham Life Science).

**Statistical Analysis** Data were collected as mean±standard error (n=3) and the biological significance p<0.05 was determined by the Student’s t-test.

**RESULTS**

**Effect of 44'-BP on GSH and GSSG Levels and GSH/GSSG Ratio** In the present study, the inhibitory action of 44'-BP on melanogenesis was examined by assessing GSH and GSSG levels and the GSH/GSSG ratio. Table 1 shows that intracellular GSH levels in B16 melanoma cells were enhanced by 44'-BP, while GSSG levels were suppressed. Consequently, the GSH/GSSG ratio was increased by 44'-BP, indicating an increased intracellular reducing power, which is expected to play a significant role in the regulation of melanogenesis.

**Downregulation of Tyrosinase and MITF by 44'-BP** Western blotting analysis revealed that tyrosinase protein levels were reduced by 44'-BP. To further investigate the involvement of transcription factors in the regulation of melanogenesis by 44'-BP, MITF protein levels were determined, finding that MITF protein levels were reduced in the B16 cells treated with 44'-BP, as shown in Fig. 1.

**Effect of 44'-BP on cAMP and PKA Activation** Since melanin biosynthesis requires cAMP, intracellular levels of cAMP was measured in the presence or absence of 44'-BP to investigate the effect of 44'-BP on cAMP levels. Data in Fig. 2 show that 44'-BP (25 μM) significantly reduced intracellular cAMP levels in α-MSH-untreated cells. This suppressive effect of 44'-BP on cAMP levels was also observed in α-MSH-treated cells, leading to the possibility that 44'-BP modulates the cAMP-dependent melanogenesis likely by reducing cAMP.

To investigate whether 44'-BP might affect PKA activation, the amount of phosphorylated Ser96 PKA regulatory type IIα subunit, pPKA (51 kDa) which is evidence of PKA activation was determined. The amount of phosphorylated PKA (pPKA) was measured in the presence or absence of 44'-BP. As shown by Fig. 3, 44'-BP effectively reduced pPKA, suggesting that α-MSH-induced pigmentation process was suppressed by 44'-BP in B16 cells.

**DISCUSSION**

In our previous work, we showed that 44'-BP reduced melanin content via the inhibition of tyrosinase, and in the same report, data on its non-cytotoxic nature were presented. In the current study, we attempted to identify those cellular factors involved in melanogenesis that are affected by the inhibitory action of 44'-BP. Our working hy-
cell lines and primary melanocytes.\(^5\) In the current study, the protein levels of both tyrosinase and MITF were shown to decrease, indicating melanogenesis suppression by 44′-BP through the MITF transcriptional mechanism, which is known to play an important role in melanogenesis. In this regard, a recent study of epigallocatechin-3-gallate on the inhibition of melanin and MITF\(^{15}\) is related to our present work.

In summary, the suppression of melanogenesis by 44′-BP in B16 cells was likely related to its antioxidant capacity, coupled with its ability to enhance intracellular GSH levels. Our current findings on reduced tyrosinase and MITF protein levels and the attenuation of cAMP and PKA are consistent with published data\(^9\) on the antimelanogenic activity. Based on data obtained from our investigations and the studies of others, we propose that the suppression of tyrosinase, MITF, cAMP, and PKA activation may be the major factors underlying 44′-BP’s antimelanogenic activity.

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**REFERENCES AND NOTES**