A novel mechanism in control of human pigmentation by β-melanocyte-stimulating hormone and 7-tetrahydrobiopterin

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Abstract

The human skin holds the full machinery for pro-opiomelanocortin processing. The α-melanocyte-stimulating hormone (α-MSH)/melanocortin-1-receptor cascade has been implicated as a major player via the cAMP signal in the control of melanogenesis. Only very recently the β-endorphin/μ-opiate receptor signal has been added to the list of regulators of melanocyte dendricity and melanin formation. In this context it was reported that (6R)-1-erythro-5,6,7,8-tetrahydrobiopterin (6BH₄) can act as an allosteric inhibitor of tyrosinase, the key enzyme in melanogenesis, and this inhibition is reversible by both α- and β-MSH. It was also shown earlier that 7BH₄, the isomer of 6BH₄, is twice as active in this inhibition reaction. However, as yet it is not known whether 7BH₄ is indeed present in loco in the melanosome. We here provide evidence that this isomer is present in this organelle in a concentration range up to 50 × 10⁻⁶ M. Determination of β-MSH in melanosomal extracts yielded 10 pg/mg protein. Moreover, we demonstrate reactivation of the 7BH₄/tyrosinase inhibitor complex by β-MSH, whereas α-MSH failed to do so. Furthermore, we show intra-melanosomal t-dopa formation from dopachrome by 7BH₄ in a concentration range up to 134 × 10⁻⁶ M. Based on these results, we propose a new receptor-independent mechanism in the control of tyrosinase/melanogenesis by β-MSH and the pterin 7BH₄.


Introduction

Today there is compelling evidence that pro-opiomelanocortin (POMC) peptides are implicated in the regulation of skin colour in humans (Abdel-Malek et al. 1995). Moreover, it has been shown that the skin and the hair follicle are the local sources and targets for POMC-derived peptides including adrenocorticotrophin (ACTH), α- and β-melanocyte-stimulating hormone (MSH) and β-endorphin (Thody et al. 1983, Tsatmali et al. 1999, 2000, Kauser et al. 2003, 2005). The involvement of ACTH, and of α- and β-MSH in human skin pigmentation was first recognised upon systemic application into human volunteers where these peptides induced noticeable skin darkening (Lerner & McGuire 1961, 1964, Geschwind et al. 1972). Later, it was shown that ACTH, α-MSH and β-endorphin could stimulate melanogenesis and proliferation of epidermal and hair follicle melanocytes and modulate cell dendricity (Hunt et al. 1994, Abdel-Malek et al. 1995, Kauser et al. 2003, 2005). Both α-MSH and ACTH are proposed to be the key players in pigmentation via the melanocortin-1-receptor (MC1-R)/cAMP second messenger system (Tsatmali et al. 1999, 2000). Moreover, it was shown that β-MSH stimulates changes in melanocyte morphology, growth rates and melanin production (McLane & Pawelek 1988, Chakraborty et al. 1991). However, its mode of action is still not completely understood. Since the presence of both α-MSH and β-endorphin together with the complete POMC processing machinery, including prohormone convertases 1/2 (PC1, PC2) and 7B2, have been demonstrated inside the melanosome of epidermal and hair follicle melanocytes, it was expected that β-MSH is also present in this organelle (Peters et al. 2000, Kauser et al. 2003). In this context a receptor-independent stimulation of tyrosinase (EC 1·14·18·1) by α-MSH has been suggested where this peptide reacts as an activator of the (6R)-1-erythro-5,6,7,8-tetrahydrobiopterin (6BH₄)/tyrosinase inhibitor complex (Schallreuter et al. 1999, Peters et al. 2000). Here, it is noteworthy that epidermal melanocytes have the full capacity for autocrine de novo synthesis/recycling/regulation of 6BH₄ which, in turn, is mandatory for intracellular l-phenylalanine and l-tyrosine turnover via phenylalanine hydroxylase (EC 1·14·16·1; PAH) and tyrosine hydroxylase (EC 1·14·16·2; TH) respectively (Schallreuter et al. 1994b). The recycling of
6BH₄ is under the control of pterin-4a-carbinolamine dehydratase (EC 4·2·1·96; PCD) and dihydropteridine reductase (EC 1·6·99·7; DHPR) (Thöny et al. 2000). In the absence of a functionally active PCD enzyme, the 7-isomer of 6BH₄ is formed from non-enzymatic re-arrangement of the intermediate 4a-carbinolamine (Curtius et al. 1990a,b, Davis & Kaufman 1991, Davis et al. 1992). Elevated levels of 7BH₄ were first described by Curtius and colleagues in patients with a variant form of phenylketonuria (Curtius et al. 1988, 1990b). Later, this accumulation was explained by a mutation in the PCD gene (Adler et al. 1992). High 7BH₄ levels were also found in the epidermis of patients with the depigmentation disorder, vitiligo (Schallreuter et al. 1999). In the latter case, it was shown that these high levels are based on deactivation of the enzyme PCD due to accumulation of H₂O₂ in the 10⁻³ M range (Schallreuter et al. 2001).

Taken together, a role for 7BH₄ in the pathophysiology of these two diseases has been proved. However, to the best of our knowledge a physiological role for this isomer has never been documented.

Earlier in vitro studies reported that 7BH₄ is a potent competitive inhibitor of PAH (Curtius et al. 1990a,b, Davis et al. 1992), whereas this isomer was a potent uncompetitive inhibitor of tyrosinase (Wood et al. 1995). In fact it was shown that 7BH₄ was twice as potent at inhibiting tyrosinase activity compared with 6BH₄ (Wood et al. 1995). Unlike the activation of the 6BH₄/tyrosinase inhibitor complex by α-MSH, 7BH₄ cannot be removed from its inhibitor complex by this peptide (Moore et al. 1999, Schallreuter et al. 1999).

Since β-MSH can also activate the 6BH₄/tyrosinase inhibitor complex under in vitro conditions (Marles et al. 2002), we asked the question whether this peptide has the capacity to bind 7BH₄ from its inhibitor complex. In order to substantiate this hypothesis, it was mandatory to show the presence and functionality of β-MSH and 7BH₄ in melanocytes and in its specific organelle, the melanosome. Therefore, we applied various in situ and in vitro techniques: immunofluorescence labelling, immunogold-electron microscopy, Western blot analyses, radio-immunoassay, enzyme kinetics and HPLC analysis. Our data demonstrate that β-MSH is present in keratinocytes and in melanocytes and that both β-MSH and 7BH₄ are found in the melanosome. Based on these results we propose that 7BH₄ can regulate human melanogenesis via β-MSH by a receptor-independent mechanism. Moreover, our observation suggests, for the first time, a physiological function for the 7-isomer of 6BH₄.

Materials and Methods

Cell culture

Individual epidermal melanocytes and keratinocytes were established from 3 different healthy donors (skin phototype III, Fitzpatrick classification) (Fitzpatrick et al. 1967) obtained after informed consent from plastic surgery (n = 1) or alternatively from suction blister roof (n = 2) using the method of Kiustala and Mustakallio (1964). This study was approved by the local Ethics committee and was in agreement with the principles of the Helsinki declaration.

The human melanoma cell lines FM55 and FM94 and murine B16 melanoma cells were cultured in RPMI 1640 (Gibco Invitrogen, Paisley, Strathclyde, UK) medium containing 10% FBS (Gibco Invitrogen) and 1% penicillin/streptomycin (Gibco Invitrogen). Cell lines required a change of medium every 1–2 days. Cultures were passaged after the population reached 70–80% confluence.

In situ and in vitro immunofluorescence labelling of β-MSH in melanocytes

Normal human skin from five different healthy donors (skin phototype III, Fitzpatrick classification) (Fitzpatrick et al. 1967) was obtained via 3-mm punch biopsies and mounted using OCT embedding medium (Raymond A Lamb, Eastbourne, East Sussex, UK). Cryosections, 5 µm thick, were cut onto poly-t-lysine (Sigma, Poole, Dorset, UK)-coated slides. The slides were air-dried at room temperature and fixed in ice-cold acetone for 10 min at −20 °C and rehydrated in PBS for 5 min.

Sections were blocked in 10% normal donkey serum (NDS) in PBS for 90 min at room temperature and rinsed briefly in PBS. This was followed by incubation with the first primary melanocyte/melanosome antibody NKI/beteb (gp100) (Monosan, Bradsure Biologics Ltd, Loughborough, Leics, UK) diluted 1:20 in 1% NDS, for 18 h at 4 °C. After washing 4 times in PBS for 20 min, the sections were incubated with a fluorescein-conjugated donkey anti-mouse secondary antibody (Jackson Immunoresearch Laboratories, Inc., Pennsylvania, PA, USA) at a dilution of 1:100 with 1% NDS, for 1 h at room temperature. Following incubation, sections were washed as before and blocked with 10% NDS at room temperature for 1 h. After a brief rinse in PBS, sections were incubated with the second primary β-MSH polyclonal antibody (Bachem Ltd, St. Helens, Merseyside, UK), diluted 1:200, for 2 h at room temperature, followed by a short washing. A rhodamine-conjugated donkey anti-rabbit antibody (Jackson Immunoresearch Laboratories, Inc,) was added at a dilution of 1:100, and left for incubation at room temperature for 1 h. After a final wash, sections were carefully dried and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories Ltd, Peterborough, UK).

Sections were viewed with a Leica DMI83B/E fluorescence microscope (Wetzlar, Germany) and photo-documented using a computer-assisted 3-CCD colour video camera and the Image Grabber PCI graphics program (both from Optivision, Osset, West Yorkshire, UK). For staining of cultured epidermal melanocytes, cells


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were seeded into 8-well Laboratory-Tek chamber slides (ICN Biomedicals, Inc., Costa Mesa, OH, USA) and allowed to attach. Before fixation in ice-cold methanol for 10 min at –20 °C, cells were rinsed briefly in PBS. The staining procedure was followed as described above with cells incubated for 2 h at room temperature with β-MSH at a dilution of 1:100.

**Immunogold transmission electron microscopy for detection of β-MSH in melanosomes**

Epidermal melanocytes (passages 3–5) were harvested by trypsin exposure. The cells were centrifuged at a low speed and the cell pellet fixed in 0.5% glutaraldehyde (Agar Scientific, Stanstead, Essex, UK), 2% paraformaldehyde (Sigma) in 0.1 M sodium cacodylate buffer (Sigma) containing 0.027 mM CaCl2 (Sigma), buffered to pH 7.4, for 1 h at room temperature. After fixation, cells were washed in PBS for 25 min and rinsed with 0.1 M glycine in PBS for 5 min. The cells were pelleted through low melting point agarose (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to cool down before cutting into 1-mm small pieces. These were fixed in 3% osmium tetroxide (90 min), dehydrated in graded series of ethanol and infiltrated with hydrophilic Unicryl resin (British BioCell International, Cardiff, Wales, UK), finally followed by polymerisation with UV radiation (360 nm) at 4 °C for 72 h.

Using a Reichert-Jung ultramicrotome (Vienna, Austria), ultrathin sections were cut and mounted on 200 mesh carbon-coated nickel grids (Agar Scientific).

Sections processed as above were blocked for 1 h in 10% normal goat serum and 2% BSA in PBS, pH 8.2 (1% normal goat serum in the same blocking buffer was used to dilute the primary and secondary antibodies). The sections were washed twice in PBS containing 2% BSA (pH 8.2) and incubated with polyclonal anti-human β-MSH (Bachem Ltd) primary antibody at a dilution of 1:200 for 18 h at 4 °C. Following incubation, the sections were washed in 2% BSA/PBS (pH 8.2) and incubated in a 1:60 dilution of the secondary goat anti-rabbit antibody conjugated to 10 nm gold particles (British BioCell International) for 1 h at room temperature. Following a final wash in distilled water, the sections were lightly counterstained with 2% Uranyl acetate and Reynold’s lead citrate solution and examined and photographed using a JEM-1200 EX transmission electron microscope (Jeol Tokyo, Japan).

**Western blot analysis**

Normal human epidermal primary keratinocytes and melanocytes were harvested in the presence of a protease inhibitor cocktail (Sigma) at concentrations recommended by the manufacturer. Purified melanosomes from FM55 and FM94 melanoma cells underwent the same procedure. Cell/melanosome lysis was achieved by 6 freeze-thawing cycles followed by centrifugation at 1200 r.p.m. for 5 min. The supernatant was separated in 12% SDS-PAGE and proteins transferred to a PDF membrane (Immobilon, Millipore, Bedford, Beds, UK). The membrane was blocked with 3% BSA in TBS-T buffer (20 mM Tris buffered saline with 0.047% Tween at pH 7.4) and incubated for 2 h with a polyclonal rabbit anti-β-MSH antibody (dilution 1:1000, Bachem Ltd). The blot was washed and incubated for 1 h with an anti-rabbit immunoglobulin G (IgG) peroxidase-conjugated antibody (Fc specific, dilution 1:5000, Sigma). Visualisation of the bands was performed using modified enhanced chemiluminescence (ECL) fixed on a film sheet (X-OMAT, Kodak, USA).

**Sucrose density gradient purification of melanosomes from cultured melanoma cells**

In order to get the necessary quantities for the experiments, melanosomes were purified from human melanoma cells (FM55, FM94) and from murine B16 melanoma cells using the method described by Seiji et al. (1961). Briefly, a cellular homogenate is processed by centrifugation to provide a suspension of the large molecular weight organelles (mitochondria and melanosomes). In the second step, these organelles are separated on a sucrose gradient by ultracentrifugation allowing isolation of purified melanosomes of all maturation stages. Purified melanosomes were lysed via a repeated freeze/thaw cycle to obtain the melanosomal extract. The purity of the final preparation was examined by electron microscopy.

**Protein determination**

Total protein concentrations of melanosomal extracts were determined spectrophotometrically at 280 nm with a Beckman DU-64 UV spectrophotometer as described by Kalb and Bernlohr (1977).

**Determination of β-MSH by radioimmunoassay**

Melanosomal extracts were prepared as described above. β-MSH concentrations were determined in duplicate using a commercial radioimmunoassay kit for the peptide (Peninsula Laboratories, Inc. Division of Bachem, Torrance, CA, USA) following the manufacturer’s protocol. This antibody does not cross-react with α-MSH or β-lipotrophin according to the manufacturer. The results were based on a β-MSH standard curve, which was determined at the same time and together with the samples.

**Pterin analysis of melanosomal extracts by HPLC**

Pterin analysis was based on the established HPLC method described by Ziegler and Hülter (1992). Briefly, purified...
melanosomes from human melanoma cells were obtained as described and were immediately re-suspended in 0.05 M Tris–HCl (pH 7.5) and snap frozen in liquid nitrogen. Extracts were made by grinding frozen preparations in a pre-cooled micro-pestle and mortar followed by centrifugation at 700 g for 5 min. In order to determine the pterin content in melanosomes, melanosomal extracts were oxidised under acidic conditions, followed by separation of the pterins with DOWEX (BioRAD, Hemel Hempstead, Herts, UK).

HPLC analysis was conducted on a Dynamax SD-300 instrument with a 25 cm C-18 reverse-phase column (Sphereclone 5 µM ODS1, Phenomenex, Macclesfield, Cheshire, UK) maintained at 22 ºC, coupled to a Shimadzu RF-535 fluorescence detector (λ EX 350 nm, λ EM 450 nm) interfaced with a computer and Dynamax PC version 1.6 software. For pterin analysis a filtered acidic mobile phase was used (1% acetonitrile, 2% methanol and 1 × 10⁻³ M H₃PO₄ in HPLC grade water (Fisher Chemicals, Loughborough, Leics, UK)). The flow rate was maintained at 1.0 ml/min and 100 µl melanosomal extract were injected per analysis in the presence or absence of 1 µM 7-biopterin standard. Experiments were carried out in duplicates.

[^3]H/6BH₄ uptake in purified melanosomes

In order to follow possible uptake of cytosolic 6BH₄ into melanosomes, preparations of intact melanosomes from B16 melanoma cells were resuspended in PBS containing 0.5 M dithiothreitol (DTT), pH 7.4. For each assay 700 µg melanosomal protein were mixed with 10 µl 0.75 mM unlabelled (cold) 6BH₄ (Schricks Laboratories, Jona, Switzerland) and 15 µl (33 KBq) [^3]H/6BH₄ (20 Ci/mmol, 2.9 µmol/l) in a final reaction volume of 75 µl PBS/DTT buffer. Reactions were mixed and incubated at 37 ºC over time (5–15–25–35 min). Immediately following incubation, the melanosomes were centrifuged at 1200 r.p.m. for 5 min at 4 ºC followed by a gentle wash of the pellet in 500 µl PBS/DTT buffer, and centrifugation. This step was repeated 3 times. In order to ensure that the washing procedure cleaned all free radiolabel, 50 µl of the last supernatant was counted in 4 ml scintillation cocktail (Ready-Safe Liquid Scintillation cocktail, Beckman, Fullerton, CA, USA) in the ^3H channel in a Wallac1209 Rackbeta liquid scintillation counter. The uptake of the ^3H-labelled 6BH₄ into the melanosomes was determined in the pellet after the fourth wash at each time point. Before counting, the pellet was re-suspended in 100 µl PBS/DTT buffer. ^3H-labelled 6BH₄ was a kind gift from E Werner, University of Innsbruck, Austria.

Inhibition of mushroom tyrosinase by 7BH₄

Mushroom tyrosinase was obtained from Sigma and 7BH₄ was from Schircks Laboratories. Tyrosinase activities were measured by following the formation of dopachrome at 475 nm. All reactions were performed in 0.1 M KH₂PO₄ buffer at pH 7.4 and reaction rates were determined in the linear phase by measuring AOD₄₇₅/2 min. The inhibition of tyrosinase by 7BH₄ was determined over a concentration range of 0–0.45 × 10⁻³ M to confirm the previously established Kᵢ value (Wood et al. 1995). V vs S (V, velocity; S, substrate concentration) results were compared with the control values without the addition of inhibitor in the presence of different concentrations of the substrate L-tyrosine (0–3 × 10⁻³ M). Lineweaver-Burk plots were obtained from the V vs S data to ascertain the inhibitor mechanism.

Reactivation of the tyrosinase/7BH₄ inhibitor complex by β-MSH

Following the dopachrome formation at 475 nm/min, the standard tyrosinase reaction was allowed to reach linear kinetics (at 4.5 min) before 7BH₄ was added to completely inhibit the reaction. The chosen concentration of 7BH₄ was 65 µM based on the Kᵢ value. At 6.5 min 10 µM β-MSH was added to re-activate the enzyme. After the β-MSH bound 7BH₄, the enzyme was again inhibited by the remaining 7BH₄ in the reaction mixture. Reactivation was achieved upon addition of a further 10 µM β-MSH.

Reduction of dopachrome to L-dopa in the presence of 7BH₄

The formation of dopachrome from L-tyrosine, by tyrosinase, at a pH of 7.4 was followed in the presence and absence of 7BH₄ (0–134 × 10⁻⁶ M). The observed reduction of dopachrome to L-dopa was subsequently examined via HPLC analysis as previously described, with the fluorescence detector at λ EX 280 nm λ EM 320 nm. The mobile phase was methanol:heptanesulphonic acid buffer (2:5:32 (v/v)) and the flow rate was maintained at 1.0 ml/min. L-Dopa standards were prepared as 1 mM stock solutions and L-tyrosine standards were prepared as 10 mM stock solutions, pH 9.2. The optimum concentration for dopachrome reduction was 134 µM 7BH₄. Samples were taken every 2 min from the standard tyrosinase reaction mixture in the presence and absence of 7BH₄. Samples were immediately diluted 1:10 with heptanesulphonic acid buffer and kept on ice. Twenty microlitres of the diluted sample were injected per analysis, with or without appropriate addition of standards. Internal controls included the analysis of the reaction mixture in the absence of 7BH₄ and tyrosinase. In order to compare the reduction of dopachrome between 6BH₄ and 7BH₄, reactions were followed upon addition of 0–134 µM 6BH₄. Experiments were carried out in duplicate.

Detection of L-dopa in melanosomes by HPLC

Purified melanosomes were immediately resuspended in 100 µl heptanesulphonic acid buffer pH 4.0 (6 × 10⁻³ M...
sodium heptanesulphonate (Fluka Chemicals, Gillingham, Kent, UK) and 7·5 \( \times 10^{-2} \) M sodium dihydrogen orthophosphate dihydrate (Fisher Chemicals) and snap frozen in liquid nitrogen according to the method of Said et al. (1990). Extracts were prepared in a micro-pestle and mortar as previously described and the supernatant de-proteinised by the addition of 5% (v/v) 2 N perchloric acid. Protein precipitates were removed by centrifugation and the supernatant was used for HPLC analysis. All samples were analysed within 30 min of extraction. HPLC was conducted as described above. The mobile phase was methanol:heptanesulphonic acid buffer (2:5:32 (v/v)) and the flow rate was maintained at 1·0 ml/min.

\( \beta \)-Dopa standards were prepared as 1 mM stock solutions and \( \beta \)-tyrosine standards were prepared as 10 mM stock solutions pH 9·2. Melanosomal extract (100 µl) was injected per analysis with or without addition of standard. Analysis was carried out in duplicate of two different melanosomal preparations from FM55 and FM94 cells.

**Results**

\( \beta \)-MSH is present in epidermal melanocytes and melanosomes

Immunofluorescence demonstrated the presence of \( \beta \)-MSH throughout the human epidermis (Fig. 1A).

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Figure 1 (A) \( \beta \)-MSH is expressed throughout the human epidermis. Co-localisation with NKI/beteb shows the presence of \( \beta \)-MSH in melanocytes along the stratum basale (i) DAPI, (ii) \( \beta \)-MSH (iii) NKI/beteb and (iv) \( \beta \)-MSH merged (magnification \( \times 400 \)). (B) \( \beta \)-MSH expression within melanocytes derived from normal human skin phototype III (Fitzpatrick classification). (i) DAPI, (ii) \( \beta \)-MSH, (iii) \( \beta \)-MSH merged with NKI/beteb (magnification \( \times 400 \)), (iv) insert of (iii) showing perinuclear co-localisation in a granular pattern. (C) Immunogold labelling demonstrates the presence of \( \beta \)-MSH in all maturation stages. \( \beta \)-MSH-bound gold particles (arrows) are present in (A) early stage, lightly pigmented, (B) moderately pigmented and (C) heavily pigmented melanosomes in epidermal melanocytes of skin phototype IV (Fitzpatrick classification) (magnification \( \times 100000 \)). (D) Western blot analysis confirms the presence of \( \beta \)-MSH in epidermal melanocytes and keratinocytes as well as in melanosomes. KC, keratinocytes; MC, melanocytes; FM55 and FM94, melanosomal preparation of human melanoma cells. \( \beta \)-MSH was used as a positive control.
Co-localisation of this peptide with the pre-melanosomal marker gp100 (NKI/beteb) indicated that β-MSH is present in the melanocyte (Fig. 1A,B). This was also demonstrated in cultured epidermal melanocytes (Fig. 1B). However, only some melanocytes yielded co-localisation, supporting the presence of cellular sub-populations even in situ. The granular pattern suggested expression within the melanosomes. In order to test this assumption, human epidermal melanocytes were examined with immunogold labelling and electron microscopy. The results showed the presence of β-MSH in melanosomes of all maturation stages (Fig. 1C). Western blot analysis confirmed the presence of β-MSH in keratinocytes and melanocytes as well as in melanosomes (Fig. 1D). The result also showed antibody specificity for β-MSH because the single band corresponded to the molecular weight of β-MSH. There was no cross-reactivity observed with α-MSH and β-lipotrophin. Moreover, β-MSH concentrations were determined in melanosomal extracts by radioimmunoassay following the manufacturer’s protocol. The results yielded close to 10 pg β-MSH/mg melanosomal protein.

7-Biopterin is present in melanosomes

Besides being a cofactor for the aromatic amino acid hydroxylases and the nitric oxide synthases, it was shown earlier that 6BH₄ and 7BH₄ could function as regulators of tyrosinase to control melanogenesis by allosteric inhibition (Wood et al. 1995). Since melanocytes have cytosolic PAH and TH activities while only TH and tyrosinase activities are present in the melanosomes, the presence of 7BH₄ in these organelles was very likely. To test this assumption, melanosomal extracts from two different melanoma cell lines were subjected to acidic oxidation prior to analysis via HPLC using the standardised methods from Ziegler and Hültner (1992). Figure 2 shows the quantitative separation of a 100 µl aliquot containing 1·35 mg protein of oxidised melanosomal extract. Two suspect peaks were detected demonstrating the characteristic double peak of 6- and 7-biopterin. To confirm the identity of the compound a second aliquot was analysed upon addition of 5·8 pmoles 7-biopterin. The result confirmed that the suspected peak at 8·2 min was indeed 7-biopterin. The amount was 4·2 pmol of this pterin/1·35 mg melanosomal protein. There was no 6-biopterin detectable after spiking with this pteridine standard. The nature of the second peak needs to be assigned.

Transport of [³H]6BH₄ into melanosomes

Based on the above result, we decided to follow the possible melanosomal uptake of cytosolic 6BH₄ since this is the essential precursor for the non-enzymatic formation of 7BH₄. For this purpose we used [³H]-labelled 6BH₄ and cold 6BH₄ (10⁻⁴ M) and studied the uptake over time in melanosomal preparations. The results of these experiments proved a time-dependent uptake into these organelles. The total uptake in 35 min was 52 × 10⁻⁶ M 6BH₄ (Fig. 3). This slow uptake rate suggests facilitated diffusion rather than active transport of 6BH₄ in melanosomes.
Mixed allosteric inhibition of tyrosinase by 7BH₄

It has been well documented that under experimental in vitro conditions 6BH₄ acts as an allosteric uncompetitive inhibitor of tyrosinase (Wood et al. 1995). Using Lineweaver-Burk plot analysis, we confirmed classical uncompetitive enzyme kinetics in the case of 6BH₄ (Wood et al. 2004); meanwhile the 7-isomer acts via mixed inhibition kinetics (Fig. 4). This result suggests at least two alternative mechanisms for inhibition of tyrosinase by 7BH₄, i.e. competitive and uncompetitive modes of action.

Dopachrome is reduced to L-dopa in the presence of 7BH₄

To study the above finding in more detail, we decided to explore a possible competitive mechanism. Since it was shown recently that 6BH₄ reduces dopachrome back to L-dopa, although at relatively high concentrations (Jung et al. 2004), here we show that only 10⁻⁶ M 7BH₄ can reduce dopachrome to L-dopa in a dose-dependent manner (Fig. 5). HPLC analysis confirmed the formation of L-dopa during the standard tyrosinase reaction in the presence of 134 µM 7BH₄ which was the established optimum concentration for the reduction (Fig. 6).
However, the natural co-factor 6BH₄ was unable to have the same effect at this concentration. HPLC analysis of melanosomal extracts demonstrated that β-Dopa is indeed present in human melanosomes. β-Dopa elutes under these conditions with a peak at 4·6 min, together with an α-tyrosine peak at 5·4 min (Fig. 7). This result proves a competitive mechanism involving the reduction of dopachrome back to β-Dopa thus explaining the observed mixed kinetics shown in Fig. 4.

Reactivation of the tyrosinase 7BH₄ inhibitor complex by β-MSH
As shown earlier, both α- and β-MSH can reactivate the 6BH₄/tyrosinase inhibitor complex by binding to the pterin (Marles et al. 2002). Figure 8 demonstrates that β-MSH reactivates the 7BH₄ inhibited tyrosinase in a dose-dependent manner. Earlier, it was shown that α-MSH has no effect on this inhibitor complex (Schallreuter et al. 1999).

Discussion
This study demonstrates that melanosomes in human melanocytes have the capacity to produce β-MSH (Peters et al. 2000, Kauser et al. 2005) besides the production of α-MSH, ACTH and β-endorphin. Moreover, 7BH₄, the non-enzymatic isomer of the important co-factor 6BH₄ is present in these organelles. We provide evidence that these 7BH₄ concentrations are sufficient to regulate tyrosinase by two alternative mechanisms. We have shown uncompetitive inhibition of tyrosinase by 7BH₄ and competitive inhibition of the enzyme by this pteridine leading to reduction of dopachrome to β-Dopa (Figs 4 and 5). The tyrosinase inhibitor complex can be reactivated in the presence of β-MSH but not by α-MSH. Therefore, it can be concluded that both mechanisms depend on the concentration of 7BH₄ and β-MSH in the melanosome.

Since 7BH₄ is a much more potent inhibitor of tyrosinase compared with 6BH₄, these results suggest, for the first time, a physiological function for this isomer. However, it must be noted that the presence of 7BH₄ in melanosomes also implicates 6BH₄ in this organelle, since the 7-isomer is only produced by the aromatic amino acid hydroxylases from 6BH₄ via 4a-OH-carbinolamine in the absence of PCD (Curtius et al. 1990a, Davis et al. 1991, Adler et al. 1992). It is noteworthy that the presence of tyrosine hydroxylase isoform I (TH) has been demonstrated in melanosomes while PAH is absent in this organelle (Marles et al. 2003). Whether the third hydroxylase, i.e.

Figure 7 HPLC analysis identifies the presence of L-dopa in melanosomes. (A) HPLC of L-tyrosine and L-dopa standards (50 pmoles). (B) Separation of a 120 μl aliquot (13·5 mg protein) of melanosomal extract. Suspected L-dopa and L-tyrosine eluted at 4·6 and 5·4 min respectively. (C) Separation of a 120 μl aliquot (13·5 mg protein) of melanosomal extract spiked with 60 pmol L-dopa confirming the identity of the peak at 4·6 min.

Figure 8 Reactivation of 7BH₄ inhibited tyrosinase by β-MSH. Reaction mixture contained 0·1 M phosphate buffer (pH 7·4), 1·0 mM tyrosine and 30 μg tyrosinase. Upon the addition of 7BH₄ at 4·5 min, tyrosinase was completely inhibited. Reactivation was achieved upon sequential addition of β-MSH (10 μM) at 6·5 min and at 10·5 min.
tryptophan hydroxylase, is present remains to be shown. HPLC analysis of the oxidised melanosomal extract detected only 7-bioppterin. However, in this study we present evidence that ¹H-labelled 6BH₄ transport occurs into melanosomes (Fig. 3). Given the above circumstances, it is tempting to conclude that the 7-isomer originates from the TH reaction after uptake of cytosolic 6BH₄ into melanosomes. In this context it is important to recognise that TH does require 6BH₄ while 7BH₄ fails to act as a co-factor or inhibitor for this reaction (Davis et al. 1992). Our results demonstrated that 7BH₄ is a potent inhibitor of tyrosinase with mixed inhibition kinetics (Fig. 4) and that this inhibitor complex is under specific regulation by β-MSH (Fig. 8). Importantly, the presence of 7BH₄ in the 10⁻⁶ M range in the melanosomes is also a potential source for l-dopa formation from dopachrome (Fig. 5). Since l-dopa is required as an electron donor for the activation of the two Cu²⁺ centres in tyrosinase (Prota et al. 1992) and considering that the pH optimum for tyrosinase is 7-4, the above mechanism could provide an additional source for l-dopa formation in melanosomes to foster dopachrome oxidase activity of tyrosinase (Wood et al. 1995).

Previous work on the upregulation of tyrosinase activities and melanin synthesis in cultured melanocytes by β-MSH indicated that this POMC peptide was implicated in the regulation of melanogenesis (McLane & Pawelek 1988, Chakraborty et al. 1991, Pawelek et al. 1992). However, the mechanism of action remained unknown. The presence of β-MSH in human melanosomes is not surprising since the entire POMC machinery has previously been demonstrated in these organelles (Peters et al. 2000, Kauser et al. 2003, 2005).

The results of the study presented herein could call into question the regulation of tyrosinase by 6BH₄/α- and β-MSH as proposed earlier by our group, despite the fact that α-MSH and its function as an activator of the 6BH₄/tyrosinase inhibitor complex was well documented under the experimental conditions used in those studies (Wood et al. 1995, Schallreuter et al. 1998, 1999, Moore et al. 1999, Marles 2002). Since we showed uptake of cytosolic ³¹H]6BH₄ into melanosomes by facilitated diffusion, but only found its product 7BH₄ in this organelle, the question remains as to whether 6BH₄ can indeed regulate tyrosinase in the melanosomes.

In summary, based on our results we propose a novel receptor-independent function for the melanocortin β-MSH in the regulation of tyrosinase. In support of this mechanism it has been shown that β-MSH is a very weak agonist for the MC1-R with a Kᵢₐ 10-fold higher than α-MSH (Suzuki et al. 1996, Wikberg 1999). Moreover, due to concentration-dependent mixed inhibition kinetics by 7BH₄ we propose that this pteridine can exercise two physiological functions in the regulation of tyrosinase. This hypothesis is supported by the concentrations of β-MSH, l-dopa and 7BH₄ found in melanosomes.

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References


Davis MD, Ribeiro P, Tipper J & Kaufman S 1992 ‘7-Tetrahydrobiopterin,’ a naturally occurring analogue of tetrahydrobiopterin, is a cofactor for and a potential inhibitor of the aromatic amino acid hydroxylases. PNAS 89 10109–10113.


Moore J, Wood JM & Schallreuter KU 1999 Evidence for specific complex formation between alpha-melanocyte stimulating hormone and 6(R)-t-erythro-5,6,7,8-tetrahydrobiopterin using near infrared Fourier transform Raman spectroscopy. Biochemistry 38 15317–15324.


Suzuki I, Cone RD, Im S, Nordlund J & Abdel-Malek ZA 1996 Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. Endocrinology 137 1627–1633.


Tsatmali M, Yukitake J & Thody AJ 1999 ACTH1–17 is a more potent agonist at the human MC1 receptor than alpha-MSH. Cellular and Molecular Biology 45 1029–1034.


Wood JM, Chavan B, Hafeez I & Schallreuter KU 2004 Regulation of tyrosinase by tetrahydropteridines and H2O2. Biochemical and Biophysical Research Communications 325 1412–1417.

Ziegler I & Hülner I 1992 Tetrahydro-6-biopterin is associated with tetrahydro-7-biopterin in primary murine mast cells. FEBS Letters 307 147–150.

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