Tyrosinase Synthesis in Different Skin Types and the Effects of \(\alpha\)-Melanocyte-Stimulating Hormone and Cyclic AMP

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Tyrosinase synthesis and its regulation in human melanocytes was studied by measuring the incorporation of \(^{[35]}S\) methionine into incubated skin biopsies. Tyrosinase was detected in all skin samples with the highest levels in skin type IV and the lowest levels in skin type I. Following psoralen ultraviolet A (PUVA) therapy for several weeks, significant increases in the amounts of tyrosinase were found in skin types III and IV. The presence of \(\alpha\)-melanocyte-stimulating hormone (\(\alpha\)-MSH) (100 \(\mu\)mol/l) or the long-acting analogue [Nle\(^2\), DPh\(^7\)] \(\alpha\)-MSH (1 – 10 \(\mu\)mol/l) in the incubation medium failed to alter tyrosinase levels in the skin biopsies taken from patients both before and after receiving PUVA therapy. Bromo-adenosine 3,5-cyclic monophosphate sodium salt (8-bromo-cAMP) (10 mmol/l), on the other hand, increased the amounts of tyrosinase both before and after PUVA, but these effects were only seen in biopsies of type III and IV skin. These results indicate that MSH fails to stimulate tyrosinase synthesis in human melanocytes. Nevertheless, tyrosinase synthesis and its regulation by cyclic AMP–dependent mechanisms could be important control points in the pigmented response. *J Invest Dermatol* 95:558–561, 1990

Tyrosinase is the rate-limiting enzyme in the melanin pathway and its regulation represents an important control point in the pigmented response. Tyrosinase is regulated by cyclic AMP–dependent mechanisms and factors such as melanocyte-stimulating hormone (MSH) that act via this second messenger system have been shown to stimulate its activity in both normal melanocytes and melanoma cells [1–6]. Although MSH acts to increase the catalytic activity of tyrosinase in melanoma cells [4–6], its main action in normal mouse hair follicular melanocytes is to increase de novo synthesis of the enzyme [2,3]. Whether MSH acts in this way to regulate pigmented responses in man is still not yet clear. Indeed, there is debate as to whether MSH has any pigmentary role in man. Although Lerner and McGuire [7] reported several years ago that \(\alpha\)-MSH increased skin darkening in man, this has never been confirmed. Moreover, we have never found any relationship between skin pigmentation and circulating levels of \(\alpha\)-MSH or other pro-opiomelanocortin (POMC)-derived peptides except when present at extremely high concentrations [8–10]. There have also been several reports that MSH peptides fail to stimulate tyrosinase activity or melanin production in cultured human epidermal melanocytes [11–13].

This lack of effect could reflect the inability of MSH to activate tyrosinase at a post-translational level, but the possibility still remains that MSH peptides act at a transcriptional level in human melanocytes as they do in mouse hair follicular melanocytes. In this study, we have therefore examined the effect of \(\alpha\)-MSH on tyrosinase synthesis in human melanocytes both before and after stimulation of the melanocytes with PUVA. Because of the possibility of facilitatory interactions between MSH and locally produced factors, we have examined the effects of MSH, not in pure melanocyte cultures where such factors may be absent, but in samples of whole skin with the melanocytes in situ. Using a similar approach, we have successfully demonstrated an effect of \(\alpha\)-MSH on tyrosinase synthesis in mouse skin [2,3].

**METHODS**

**Subjects** Sixty-four Caucasian patients were studied. All attended as outpatients for treatment of non-pigmentary disorders, including 41 who received PUVA therapy for either psoriasis or mycosis fungoides. All patients gave their informed consent.

**PUVA Therapy** PUVA therapy was given 3 times weekly and was continued for 6–12 weeks. The dose of UVA used was determined by an individual’s skin type as follows: Skin type I, initial dose 1.5 J/cm\(^2\); type II, initial dose 2.5 J/cm\(^2\); type III, initial dose 3.5 J/cm\(^2\); type IV, initial dose 4.5 J/cm\(^2\). All doses were increased weekly by 0.5 J/cm\(^2\).
Figure 1. Immunoprecipitation of [³⁵S]-methionine-labeled tyrosinase with tyrosinase antibodies. Skin biopsies were incubated for 16 h and immunoprecipitation carried out as in Materials and Methods. The autoradiographs of immunoprecipitated tyrosinase are shown (with the position of molecular weight markers) in skin types I, II, III, and IV.

Skin Samples Skin biopsies (4-mm diameter) were taken under local anaesthesia from the upper arm region. The biopsies were trimmed of fat and incubated as described below.

Drugs and Hormones Synthetic α-MSH was kindly provided by Ciba Geigy Ltd. and [Nle⁴,D-Phe⁷]α-MSH by Professor Mac Hadley, University of Arizona, USA. Bromo-adenosine 3',5'-cyclic monophosphate sodium salt (8-bromo-cAMP) was obtained from Sigma Ltd., Poole, Dorset, UK.

Metabolic Labeling of Skin Biopsies with [³⁵S] Methionine Single-skin biopsies were incubated in 1 ml low-methionine (67 μmol/l) Dulbecco's modified Eagle's medium, containing 60 μCi/ml [³⁵S]-methionine (5 Ci/mmol, Amersham International plc), for up to 16 h. At least two biopsies were taken from each patient, one of which served as the control incubation. The other was incubated in the presence of either α-MSH (100 μmol/l), [Nle⁴,D-Phe⁷]α-MSH (1–10 μmol/l) or 8-bromo-cAMP (1–10 mmol/l). After incubation, the biopsies were harvested immediately or at intervals during a subsequent chase with non-radioactive methionine (3.9 mmol/l).

Isolation and Quantification of Immune Complexes The following steps were carried out at 4–6°C. Biopsies were washed 3 times in 0.5 ml of phosphate-buffered saline containing methionine (2 mg/ml) and then lysed for 30 min in 120 μl of buffer containing 50 mmol NaCl/l (BDH, Poole, Dorset), 0.05% (v/v) Nonident P-40 (NP-40, BDH), 0.5% (v/v) sodium deoxycholate (BDH), and 0.1 mmol phenylmethylsulphonyl fluoride/l (Sigma Ltd.). The lysate was then centrifuged at 16,000 × g for 5 min and the cleared supernatant divided into aliquots, two of which were used for estimation of protein. The remaining four were incubated for 30 min with either control rabbit immunoglobulin (Sigma Ltd.) or excess rabbit immunoglobulin G (IgG) isolated from a polyclonal tyrosinase antiserum that has been well characterized [4]. They were then incubated for a further 30 min after the addition of 64 μl protein A-Sepharose slurry (20 mg/ml, Sigma Ltd.). The bound immune complexes were sedimented at 16,000 × g for 2 min, washed 3 times with 0.5 ml phosphate buffer containing 0.5% NP-40, and then released by incubating the pellets at 100°C for 5 min in 20 μl sample buffer containing 20% (v/v) glycerol, 4% w/v sodium dodecylsulphate (SDS), 10% v/v mercaptoethanol, 0.125 mol Tris- HCl/l (pH 6.8), and 0.002% (w/v) bromophenol blue. The protein A-Sepharose was then removed by centrifugation at 16,000 × g for 5 min. Samples of the supernatant were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by fluorography and trichloroacetic acid precipitation and scintillation counting. Fluorography was carried out using the method of Bonner and Laskey [14] with preflashed Kodak X-omat film at -70°C [15]. Exposures of up to 14 d were used and densitometry carried out using an LKB scanning densitometer.

RESULTS

Bio synthesis and Degradation of Tyrosinase Incorporation of [³⁵S]-methionine into total protein was found at all times studied. The immune-complexes that were isolated with tyrosinase antibodies normally contained less than 2% of the radioactivity incorporated into total protein. Following SDS-PAGE and fluorography, two main bands with molecular weights ranging between 69,000 and 90,000 were found (Fig 1). A polypeptide with a molecular weight of approximately 70,000 was evident after 4 h of labeling with [³⁵S]-methionine and an additional species with a molecular weight of approximately 80,000 appeared at later times. These two immunoprecipitated bands had a precursor-product relationship, as previously described by ourselves [2,3] and others [16], and possessed tyrosinase catalytic activity determined by dopa staining [17]. The half life of total tyrosinase as determined from the pulse chase experiments was found to be 10 h.

Effect of MSH Peptides and 8-bromo-cAMP The results are shown in Table 1. Alpha-MSH had no effect on the incorporation of [³⁵S]-methionine into tyrosinase immune complexes isolated from skin biopsies that were incubated for up to 16 h. The long-acting and potent analogue [Nle⁴,D-Phe⁷]α-MSH was also without effect. However, 8-bromo-cAMP (10 mmol/l) caused a threefold increase in the incorporation of [³⁵S]-methionine into tyrosinase immune complexes.

Effect of PUVA In this and subsequent experiments, skin type was taken into account. The incorporation of [³⁵S]-methionine into

### Table 1. Effect of α-MSH, [Nle⁴,D-Phe⁷]α-MSH and 8-bromo-cAMP on Tyrosinase Synthesis in Biopsies of Human Skin Incubated for 16 h

<table>
<thead>
<tr>
<th>Condition</th>
<th>% [³⁵S]-Methionine Incorporation</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>2.10 ± 0.72</td>
</tr>
<tr>
<td>α-MSH</td>
<td></td>
</tr>
<tr>
<td>100 μmol/l</td>
<td>1.97 ± 0.83</td>
</tr>
<tr>
<td>[Nle⁴,D-Phe⁷]α-MSH</td>
<td></td>
</tr>
<tr>
<td>1 μmol/l</td>
<td>3.01 ± 0.80</td>
</tr>
<tr>
<td>10 μmol/l</td>
<td>2.42 ± 0.59</td>
</tr>
<tr>
<td>8-bromo-cAMP</td>
<td></td>
</tr>
<tr>
<td>1 mmol/l</td>
<td>2.78 ± 0.24</td>
</tr>
<tr>
<td>10 mmol/l</td>
<td>7.96 ± 0.82</td>
</tr>
</tbody>
</table>

*Tyrosinase synthesis is expressed as dpm of [³⁵S]-labeled tyrosinase/dpm of labeled total protein X 100. Results given as means ± SEM (with numbers of subjects in parentheses). Student t test was used to test for significance of differences.

p < 0.001.
tyrosinase immunocomplexes was lowest in skin type I and increased progressively in skin types II, III, and IV (Fig 1). PUVA increased the incorporation of [35S]-methionine into tyrosinase in skin type III and IV, but not in skin types I (Fig 2). There was a small increase following PUVA in skin type II, but this was not significant (Fig 2).

**Effect of α-MSH and 8-bromo-cAMP Following PUVA**

Alpha-MSH (10 μmol/l) had no effect on [35S]-methionine incorporation in any of the skin types following PUVA (Fig 3). [Nle4,D-Phe7]-α-MSH was also without effects (results not shown). 8-bromo-cAMP (10 mmol/l) had no effect on [35S]-methionine incorporation in skin types I and II but increased incorporation in skin type III. There was also an increase in skin type IV, but this was just short of statistical significance (Fig 4).

**DISCUSSION**

Although previous studies have shown that tyrosinase synthesis occurs in human melanocytes in culture [11], this is the first demonstration of tyrosinase synthesis in melanocytes in situ in human skin. Our results show that by using a metabolic labeling procedure it is possible to measure tyrosinase synthesis in single biopsies of human skin. The level of tyrosinase synthesis was not the same in the different skin types and was at its lowest in skin type I and its highest in skin type IV. The results demonstrate that newly synthesized tyrosinase in human skin has a molecular weight of around 70,000 and that this is then converted to a form with a higher molecular weight of approximately 80,000. These results are consistent with the precursor-product relationship that has been reported for tyrosinase in human melanocytes [11] and also in mouse-hair follicular melanocytes [2,3]. Pulse-chase experiments revealed that the half-life of labeled tyrosinase was around 10 h. This is higher than the 4 h we have previously reported for tyrosinase in mouse-hair follicular melanocytes, but it falls within the range of 3−14 h found in cultured human melanocytes, as reported by Halaban et al [11].

MSH is generally considered to be a pigmentation hormone [10], but in the present study, in contrast to our findings in mouse-hair follicular melanocytes [2,3], we found no increases in the amounts of tyrosinase with α-MSH. The long-acting and potent analogue [Nle4,D-Phe7]α-MSH, which is resistant to enzymatic degradation [18] and has been shown to penetrate human skin [19], was also without effect. Therefore, it seems unlikely that α-MSH is able to increase the synthesis of tyrosinase in human melanocytes as it is able to do in mouse-hair follicular melanocytes [2,3]. It is also unlikely that α-MSH acts at a post-translational level to activate tyrosinase as it does in mouse melanoma cells [4,6], in view of reports that the peptide fails to stimulate tyrosinase activity and melanogenesis in cultured human melanocytes [12,13]. Whether it can affect skin pigmentation through some other action, i.e., the transfer of melanin, remains unknown.

On the other hand, 8-bromo-cAMP increased the amounts of tyrosinase in human skin and this is consistent with our findings in mice [2,3]. These findings confirm that in human melanocytes, as in those of other species, tyrosinase synthesis is regulated through cyclic AMP-dependent mechanisms [2,3]. They also indicate that the lack of response to MSH by the human melanocytes cannot be explained by a defect in the cyclic AMP system; one possible explanation is that human melanocytes fail to express MSH receptors. However, Halaban and co-workers have reported binding of [125I]β-MSH to human melanocytes, although the level was considerably lower than that found for mouse melanoma cells, which respond well to MSH [11]. It has also been reported that MSH increases cyclic AMP production in cultured human melanocytes [13], but the increases were small and may be insufficient to activate tyrosinase synthesis. There are reports that in mouse melanoma cells MSH receptors are expressed discontinuously and are only functional during the G2 phase of the cell cycle [20]. The same may be true in human melanocytes. Because the majority of melanocytes in human skin are probably in the resting G0 phase, MSH receptor expression could be extremely low. It has also been reported that UVB may increase MSH receptor activity, thus increasing responsiveness to MSH [21]. We have been unable to confirm this in mice [22,23]; in the present study PUVA, which is known to activate melanocytes, failed to bring about a response to α-MSH. However, the possibility that UVB is able to alter MSH receptor expression in human melanocytes cannot be excluded.

PUVA alone did, however, increase the amounts of tyrosinase and further increases were found when 8-bromo-cAMP was present in the incubation medium. These effects were only seen in patients with skin types III and IV and it would seem that PUVA greatly emphasizes the differences in the amounts of tyrosinase seen in the different skin types. It is recognized that the patients with skin types III and IV received slightly higher doses of PUVA than those with skin types I and II, but this is unlikely to account for the large differences in tyrosinase synthesis. The higher levels of tyrosinase in skin types III and IV could be due to increased numbers of active melanocytes or, alternatively, they could represent differences in tyrosinase transcription within the melanocytes of the various skin types. We are now studying these possibilities using a tyrosinase cDNA probe and in situ hybridization.

**REFERENCES**


15. Laskey RA, Mills AD: Quantification film detection of \( ^{3} \)H and \( ^{14} \)C in polyacrylamide gels by fluorography. Eur J Biochem 56:335–341, 1975


