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Melanocyte stimulating hormone activation of tyrosinase in B16 mouse melanoma cells

Evidence for a differential induction of two distinct isoenzymes

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Tyrosinase induction in murine malignant melanocytes by αMSH is well known, but its molecular basis has not been characterized. Treatment of B16 melanoma cells with theophylline or αMSH mediates a larger induction of tyrosine hydroxylase than of dopa oxidase activity in total cell extracts, and in the melanosomal and microsomal fractions. No evidence for the modulation of a tyrosinase effector was found. SDS-PAGE and specific activity stain demonstrated two forms of tyrosinase, with different degrees of induction by theophylline. These results agree with the recent proposal that two tyrosinases, encoded by different genes, are present in murine melanocytes.

α-Melanocyte stimulating hormone; Tyrosinase; Isoenzyme; Theophylline; Melanogenesis; Melanoma cell

1. INTRODUCTION

It has been known for a long time that αMSH binding to specific receptors in mammalian melanocytes triggers a series of cAMP-mediated events, leading to a large increase in tyrosinase activity [1], the enzyme controlling the rate of melanin synthesis. However, the molecular mechanisms underlying tyrosinase induction are far from clear. It has been suggested that the effect of αMSH is not mediated by a change in the phosphorylation state of tyrosinase or in other post-translational modifications [2,3]. However, there is no agreement as to whether the increase in tyrosinase activity is mainly a function of enzyme abundance [3,4], or reflects both an increased amount of tyrosinase and an activation of previously existing molecules [5], or even the activation of an inactive enzyme pool with little, if any, effect on the rate of enzyme synthesis [6]. As far as the activation of pre-existing tyrosinase molecules is concerned, both the cAMP-mediated removal of an inhibitor [7] or synthesis of an activator [5] have been postulated, although no clear-cut evidence has ever been put forward.

We present evidence suggesting that treatments increasing the cAMP content of B16 mouse melanoma cells in culture trigger the preferential expression of a tyrosinase isoenzyme different from the majority form found in untreated cells. Both forms differ in their electrophoretic mobility in non-reducing SDS-PAGE and in their catalytic properties. The isoenzyme preferentially induced by melanogenic agents displays a larger ratio of tyrosine hydroxylase to dopa oxidase activity, and would therefore mainly affect the first, rate limiting, step of the pathway. This finding conveniently accounts for most of the apparently controversial data in the literature.

Abbreviations: L-dopa, L-3,4-dihydroxyphenylalanine; EDTA, ethylenediaminetetraacetic acid; αMSH, α-melanocyte stimulating hormone; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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2. MATERIALS AND METHODS

2.1. *Cell culture and treatment with melanogenic effectors*

B16-F10 mouse melanoma cells were originally a kind gift of Dr. V. Hearing and have been maintained as described [8]. B16 melanocytes were cultured either on 96-well plates or in 75 cm^2 flasks from Nunc (Denmark). When using 96-well plates, cells were seeded at a density of 5,000 cells per well in 200 μl of medium. After 24 h, serial dilutions of the melanogenic effectors were added in 50 μl of medium, and the plate was further incubated for 48 h. Each concentration was assayed in six independent wells the contents of which were pooled by pairs before measurements so as to assay enzymatic activities in triplicate. To obtain higher amounts of cells for subcellular fractionation experiments, $5 \cdot 10^5$ cells were seeded in 75 cm^2 flasks, incubated for 24 h and then a volume of medium containing the desired concentration of the melanogenic agent was added.

2.2. *Subcellular fractionation*

Cells were harvested by trypsin treatment and washed twice by centrifugation in homogenization buffer (HB, 10 mM phosphate pH 6.8, 0.25 M sucrose, 0.1 mM EDTA, 0.1 mM PMSF). The washed cells were suspended in HB at about 10^7 cells/ml and disrupted in a loose-fitting, manual, glass–teflon homogenizer, at 4°C. A post-nuclear supernatant was obtained by centrifugation at 700 × g, and further

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fractionated by differential centrifugation at 11,000 × g, 30 min, yielding a melanosome-rich pellet. This was followed by centrifugation at 105,000 × g for 60 min to yield the microsomal fraction.

Before activity measurements, the pellets were solubilized in 10 mM phosphate pH 6.8 containing 1% Brij 35 and centrifugation at 105,000 × g, 60 min, using 200 μl of solubilization buffer per 10^7 cells.

2.3. *Activity measurements*

The formation of melanin by cells in culture was directly determined in 96-well plates, as described by Siegrist and Eberle [9]. Tyrosine hydroxylase activity was measured by the method of Pomerantz [10] modified as described elsewhere [11]. Dopa oxidase activity was determined by following spectrophotometrically the formation of dopachrome from L-dopa. One unit of tyrosine hydroxylase activity was defined as the amount of enzyme that hydroxylates 1 μmol L-Tyr/min, whereas one unit of dopa oxidase was defined as the amount of tyrosinase that catalyzes the oxidation of 1 μmol L-dopa/min, as reported previously [11].

2.4. *Other procedures*

Non-reducing SDS-PAGE was performed according to Laemmli [12], but in the absence of β-mercaptoethanol and without any heating in order to preserve enzymatic activities. Before electrophoresis, samples were mixed in a 2:1 (v/v) ratio with sample buffer (0.18 M Tris-HCl, pH 6.8, 15% glycerol, 9% SDS and 0.075% Bromophenol blue) and incubated at room temperature for 30 min. Specific tyrosinase activity stain was performed by incubation of the gels at 37°C for 2 h in 10 mM phosphate buffer pH 6.8 containing 1.5 mM L-dopa supplemented with 0.5 μCi/ml of L-[3-C^{14}]dopa from New England Nuclear. The gels were incubated in Amplify enhancer from Amersham for 30 min, dried, photographed and further analyzed by fluorography using Kodak X-OMAT AR film. Protein was determined as described by Hartree [13].

3. RESULTS AND DISCUSSION

Fig. 1 shows the dose–response curves obtained for unfractionated B16 cells grown in 96-well plates and treated for 48 h with theophylline. The cells responded with a large increase in melanin synthesis and, as shown in Fig. 1, a dramatic and dose-dependent stimulation of the tyrosine hydroxylase activity (approximately 30-fold increase over the control). The dopa oxidase activity displayed a considerably smaller activation, of about 12-fold the control values. A similar situation was found for αMSH and for the more stable and more potent analogue [*N*Leu^4,D-Phe^7]-αMSH (Table I). In principle, this result could be explained by the modulation of a specific effector with different effects on the tyrosine hydroxylase and dopa oxidase activities. Since melanin synthesis is confined to the melanosomes, experiments were performed to investigate the presence of such an effector in the melanosomal fraction of control and theophylline-treated cells. However, as shown in Table II, no evidence pointing to the existence of such an effector was found in experiments where the activity of mixtures of control and treated melanosomal extracts was measured. Moreover, the addition of SDS to reaction mixtures did not have any effect on the activity of either control or treated samples, or their combinations (Table II). It is worth noting that the number of μU in control and treated samples does not reflect the actual induction of tyrosine hydroxylase activity, since the number of cells was lower after theophylline treatment as compared to the controls, and the volume of theophylline-treated extracts in the assay medium was lower than the one of control extracts. In addition, the tyrosine hydroxylation reaction rate of control and treated extracts varied linearly with the amount of extract used in the assays (not shown) as expected for an enzymatic preparation devoid of effectors [14].

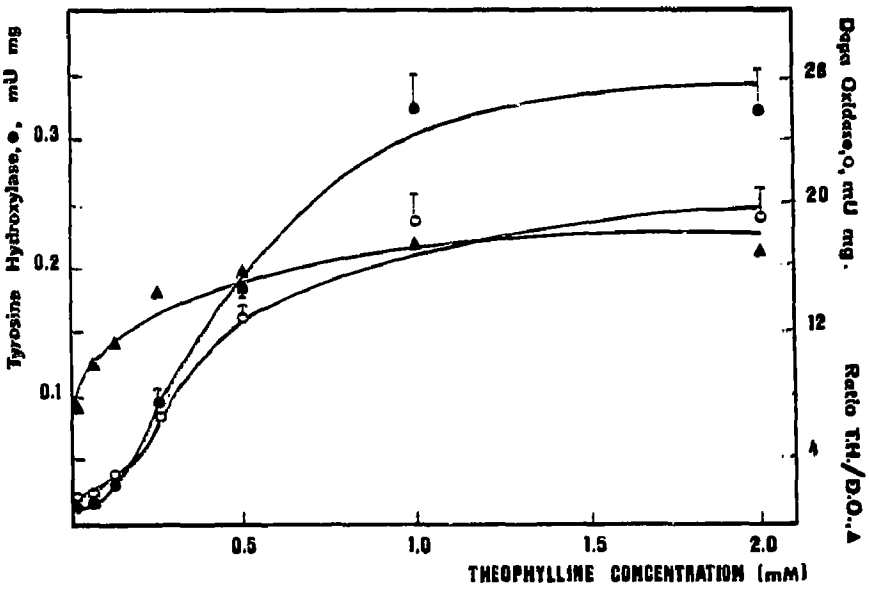

Fig. 1. Dose-response curves of the tyrosinase activities of B16 melanoma cells treated with theophylline. The treatments were carried out as described in Materials and Methods and in Table I. Each point is the mean of three independent experiments. Error bars are shown only when they were larger than the symbol. (●) tyrosine hydroxylase activity, and (○) dopa oxidase activity, expressed in mU/mg protein; (▲) ratio of tyrosine hydroxylase to dopa oxidase activity, expressed in μU/mU.

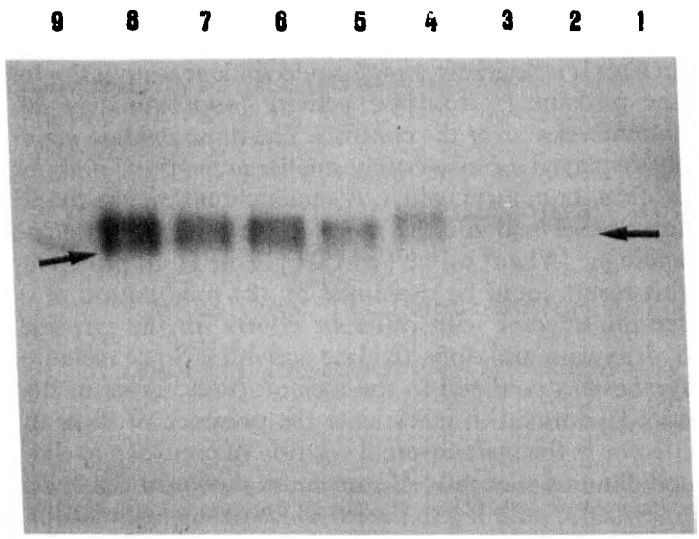

Fig. 2. Activity stain of SDS-PAGE non-reducing gels of the microsomal and melanosomal fractions of control and theophylline-treated cells. Control and cells treated with theophylline (1 mM) for different times were submitted to subcellular fractionation and their melanosomal and microsomal fractions solubilized. A volume of the solubilized extracts corresponding to $3 \cdot 10^5$ cells was electrophoresed and the gel stained for activity with radioactive L-dopa and analyzed by fluorography. The time of exposure was 5 days. Lanes 1 to 4, microsomal extracts of control cells (1) and cells treated for 16, 24 and 48 h (2 to 4); lanes 5 to 8, melanosomal extracts control cells (5) and cells treated for 16, 24 and 48 h (6 to 8); lane 9, reductively methylated BSA.

Another possible explanation for the relative change in the ratio of tyrosine hydroxylation to dopa oxidation was the cAMP-mediated induction of a different isoenzyme with distinct kinetic parameters for both activities. Such a possibility will be consistent with the proven necessity of continued transcription and translation for the apparent activation process [5] and with the recently reported existence of more than one tyrosinase in mammalian melanocytes, the b and c proteins [15,16]. To check this possibility, two approaches were followed. First, the activation of both tyrosinase activities was analyzed in the microsomes (containing newly synthesized enzyme 'en route' to the melanosomes) and the melanosomal fraction. As shown in Table III, the newly synthesized microsomal tyrosinase already displayed a ratio of tyrosine hydroxylation to dopa oxidation higher in theophylline treated cells than for control cells. More conclusively, a sensitive activity stain of SDS-PAGE gels based on the fluorographic detection of radioactive melanin (to be published) showed a different pattern of activity bands in control and theophylline-treated cells (Fig. 2). In control cells, an activity band of lower electrophoretic mobility was the predominant form in the melanosomal and microsomal fractions. However, upon theophylline treatment, a form of slightly higher electrophoretic mobility (shown with arrows in Fig. 2) became increasingly evident, accounting for approximately 50% of the total stain in the melanosomal fraction after 48 h. This higher-mobility form was, however, the minority activity band in control cells, especially in the microsomal fraction. Therefore, SDS-PAGE showed a different degree of induction for two active forms of tyrosinase.

It has recently been shown that two different genes mapping to the brown (b) and albino (c) loci in mice might encode for active tyrosinase molecules [16]. These proteins display extensive sequence similarity and should therefore share antigenic determinants. In the light of the results herein presented, it can be proposed

Table I

Effect of different melanogenic agents on the dopa oxidase and tyrosine hydroxylase activities of B16 mouse melanoma cells

Treatment	Tyrosine hydroxylase (μU/mg protein)	Dopa oxidase (mU/mg protein)	Ratio (μU/mU)
Control	11.4 ± 2.6	1.6 ± 0.3	7.1
Theophylline	327.1 ± 25.9 (28.7)	20.2 ± 2.3 (12.5)	16.2
αMSH	65.0 ± 3.7 (5.7)	4.8 ± 0.3 (3.0)	13.5
*N*Leu⁴,DPhe⁷-αMSH	117.2 ± 11.3 (10.3)	6.1 ± 0.5 (3.8)	19.2

All treatments were carried out for 48 h with cells grown in 96-well culture plates. Theophylline was 1 mM, whereas αMSH and its analogue were 10^{-7} M. Culture media were removed and the cells were solubilized by addition of 1% Brij 35 in 10 mM phosphate buffer, pH 6.8, using 100 μl of buffer/well. After 1 h incubation at room temperature, the solubilized samples were centrifuged at 105,000 × g, for 1 h, and measurements were carried out on the supernatant fractions. The stimulation achieved is shown in brackets. The results are the mean ± S.E.M. for two independent experiments, each one carried out in triplicate.

Table II

Tyrosine hydroxylase activity of mixtures of control and theophylline treated melanosomal fractions

Sample	μU		% Activity respect theoretical sum
Exp. 1			
Control	9.05	27.85	
Theophylline	18.8		
Control + theophylline	24.8		89
Control + theophylline + 0.5% SDS	26.1		94
Exp. 2			
Control	5.7	15.6	
Theophylline	9.9		
Control + theophylline	18.4		118
Control + theophylline + 0.5% SDS	17.5		112

Control and theophylline-treated (1 mM, 48 h) B16 cells were grown to semiconfluence, harvested, fractionated and the melanosomal fractions purified as described in Materials and Methods. The samples were mixed if necessary and incubated at room temperature for 30 min, prior to the addition of substrates for tyrosine hydroxylase activity measurements.

Table III

Activation of the dopa oxidase and tyrosine hydroxylase activities of tyrosinase in the microsomal and melanosomal fractions of B16 mouse melanoma cells treated with theophylline

	Control		Theophylline	
	Microsomes	Melanosomes	Microsomes	Melanosomes
Tyrosine hydroxylase (μU/10^6 cells)	1.1 ± 0.15	6.0 ± 1.0	16.7 ± 0.8	60.5 ± 5.5
Dopa oxidase (mU/10^6 cells)	0.12 ± 0.02	0.68 ± 0.11	0.75 ± 0.05	2.7 ± 0.3
Ratio (μU/mU)	9.2	8.8	22.3	22.4

Control and theophylline treated (1 mM, 48 h) melanoma cells were grown to semiconfluence in 75-cm^2 flasks, harvested, counted and fractionated. Microsomes and melanosomes were purified as described in Materials and Methods. The results are the mean ± S.E.M. for five independent experiments carried out in duplicate.

that the activation of tyrosinase mediated by αMSH might affect to different extents the biosynthesis of both isoenzymes. At least in B16 cells, the resting cells appear to contain predominantly a form of lower tyrosine hydroxylase and higher dopa oxidase specific activity, especially in the microsomal fraction, consistent with a basal situation where the rate of the melanization pathway is kept low. Upon αMSH stimulation, there is a larger increase in the activity of a protein with higher tyrosine hydroxylase specific activity, thus resulting in a larger stimulation of the rate-limiting step of the pathway. Such a situation would simulate both an apparent activation of pre-existing tyrosinase as well as new synthesis of enzyme if the specific activity of tyrosinase is determined by measuring the amount of enzyme by means of immunologic techniques with a polyclonal antibody, which can be predicted to recognize both forms of the enzyme [5]. Conversely, if the amount of enzyme is determined with a monoclonal antibody directed against the form present in higher amounts under basal conditions, little increase in the amount of enzyme will be observed, and the large increase in tyrosine hydroxylase activity due to the accumulation of a different isoenzyme not recognized by the antibody could be reasonably assigned to the activation of a preexisting pool of the enzyme [6]. Finally, if the specific activity is measured under conditions where the initial situation is such that both enzyme forms are already present in comparable amounts, for example after a previous αMSH stimulation, or if the antibody employed to determine tyrosinase amounts is mainly directed against the higher specific activity protein, the preferent stimulation of this form might lead to an interpretation of the results in terms of an αMSH induction without any activation of the enzyme [3,4]. In any case, a variety of experiments performed with specific antibodies have shown that αMSH treatment of melanocytes results in little differences in the patterns of synthesis of both tyrosinases, which were increased no more than about two-fold by the hormone [16]. Therefore, the induction of tyrosinase activity cannot be accounted for merely on the basis of increases in the synthesis of the isoenzymes. Other mechanisms such as hormone-mediated alterations in post-translational processing or routing to the melanosome might also contribute to the observed differences in isoenzyme distribution. An interesting possibility which is currently under study in our laboratory would be a differential stabilization of the isoenzymes, whose half-life 'in vivo' is relatively short as result of active degradation [6].

In summary, the results here presented clearly point out the existence of two different forms of tyrosinase, as proposed by Hearing and co-workers [15,16]. These isoenzymes would have distinct catalytic properties, and different responses to αMSH. These findings provide a reasonable explanation for the conflicting data on the stimulation by αMSH of tyrosinase in mammalian melanocytes.

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