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BIOCHEMISTRY OF MELANIN SYNTHESIS

In Vivo Effects of MSH on Tyrosinase and Melanogenesis of Pigmentary System[1]

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Melanogenesis is a process which forms new pigment and is considered to have the function of controlling the overall pigmentation of higher animals, including man. An enhancement of melanogenesis is observed when a prolonged *in vivo* administration of melanocyte-stimulating hormone (MSH) is given to test animals. The mechanism through which MSH induces melanogenesis *in vivo* is not well understood at present. Since tyrosinase is a key enzyme involved in biosynthesis of melanin pigment and is present in appreciable quantities in pigmentary system, it seems likely that it participates in the action of MSH on pigment formation. This communication reports the *in vivo* effect of MSH on tyrosinase activity and melanogenesis of two different pigmentary systems, namely, the benign skin melanocytes of *Rana pipiens* frog and malignant B-16 mouse melanoma. A uniform hypothesis on the mechanism of induction of melanogenesis by this hormone is postulated.

INDUCTION OF MELANOGENESIS IN *RANA PIPIENS* FROGS

Male *Rana pipiens* were used to investigate the *in vivo* effects of MSH on tyrosinase activity and melanogenesis. The detail of the study has been described elsewhere (1). The *in vivo* responses of *Rana pipiens* skin melanocytes to the action of MSH are slow, if the transient darkening of skin coloration is disregarded. After the second week of MSH administration, it became noticeable that the treated frogs remained darker than the control animals when the immediate action of MSH subsided and the animals supposedly returned to their "normal" coloration. It was also noted that the skin debris shed by the treated animals was darker. Both observations are consistent with an elevated rate of synthesis and turnover of melanin in skin melanocytes.

When the isolated skin of these frogs were tested for their ability to darken and lighten in response to external stimuli, two characteristic features were observed,

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the treated group had a lower reflectance reading at all stages of the experiment and a narrower range of variation among individuals as compared to the control animals. The results are shown in Figs. 1 and 2. The capacity of the skin of the treated group to respond to external stimuli remained essentially unchanged, as indicated by the unchanged increase and decrease in reflectance brought about by washing with Ringer solution and by the action of MSH, respectively. These observations may be interpreted as indicating that there was an increase of melanin content of melanosomes but no significant increase in the number of melanosomes

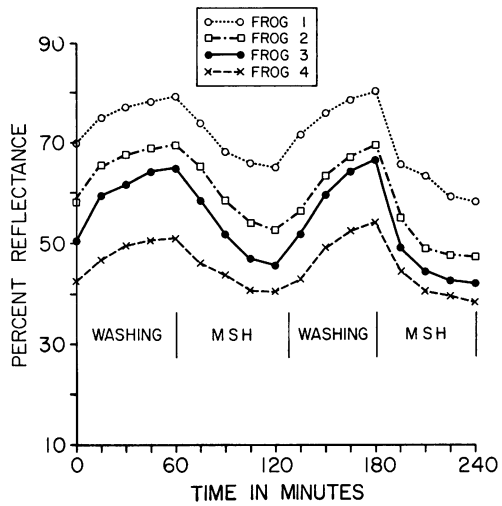

FIG. 1. *In vitro* responses toward washing with Ringer solution and MSH action of the isolated *Rana pipiens* skin from the control group. The washing period consisted of 4 changes of Ringer solution at 15-min intervals; the MSH period was 60 min under the action of 10 units of standard MSH in 20 ml of Ringer solution. Each point on the chart represents the mean value of 4 locations on the body of the frog.

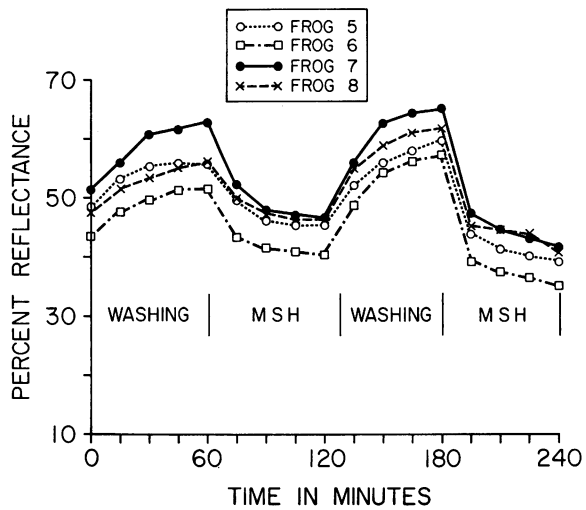

FIG. 2. *In vitro* Responses toward washing with Ringer solution and MSH action of the isolated *Rana pipiens* skin from the treated group, received daily injection of MSH for 21 days. The same conditions as those indicated in Fig. 1 were employed.

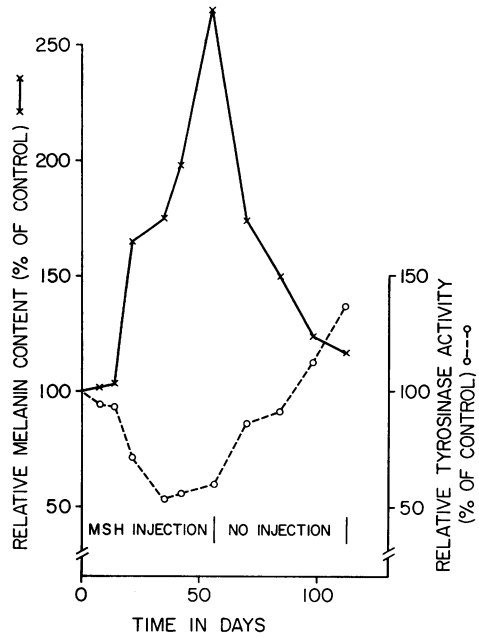

FIG. 3. Effect of long-term MSH treatment on the melanin content and tyrosinase activity in the skin of *Rana pipiens* frogs.

or melanocytes. Moreover, these melanized melanosomes behaved normally toward external stimuli after being in a dispersed state for long periods of time.

Tyrosinase of *Rana pipiens* skin is a soluble cytoplasmic protyrosinase which requires activation by a proteolytic enzyme. Trypsin, chymotrypsin, and a fungal protease (*Streptomycetes griseus*) were found equally effective, indicating that the activation of the protyrosinase did not involve the cleavage of a specific type of peptide bonds. A spectrophotometric procedure measuring the formation of dopachrome from L-tyrosine was used to measure the tyrosinase activity of the skin extract. Since trypsin was included in the assay mixture, therefore, the procedure measured the total soluble tyrosinase activity regardless of the state of activation of the enzyme in the specimens. The melanin content of skin specimens was measured spectrophotometrically by solubilizing the melanin in dilute alkali after removal of proteins by peptic digestion. Figure 3 shows the melanin content and tyrosinase activity of skin specimens obtained from frogs given daily injection of MSH for 8 weeks, followed by a period of 8 weeks during which no injection was given. The tyrosinase activity of the skin decreased while the rate of melanin synthesis was elevated. An inverse relationship between the tyrosinase activity and melanin content was observed. This finding supports the hypothesis that the administration of MSH may release a bound, intracellular proteolytic enzyme which activates the inactive protyrosinase. The tyrosinase molecule, once activated, attaches itself to the protein matrix of a subcellular particle and initiates melanin synthesis, leading to the MSH-induced melanogenesis. The conversion of the soluble protyrosinase to active tyrosinase bound to subcellular particles causes a decrease of the soluble protyrosinase content of the skin as measured by the dopachrome procedure under the conditions used.

INDUCTION OF MELANOGENESIS IN B-16 MOUSE MELANOMA

Female C57BL/6J mice of the same age (6–9 weeks old) were used in this study (2). The pigmentary system of B-16 mouse melanoma was found to be more responsive toward the action of MSH than amphibian skin melanocytes. Striking changes were observed in relatively short periods of time. On account of the short life span of the animal carrying B-16 melanoma, experiments in this study were not carried beyond 30 days after the implantation of the tumor. Mouse melanoma tyrosinase is tightly bound to melanosomes and is not readily solubilized by common laboratory aqueous buffers. Due to its insoluble nature, it was not possible to measure the enzyme activity by the spectrophotometric procedure. For this reason, we used a modification of the hydroxylation procedure (3) which is amenable to the measurement of particulate tyrosinase.

Studies were carried out to investigate the time course of induction of melanogenesis by MSH. The mice received a single injection of 2 mg of α-MSH at the beginning of the experiment and, thereafter, received no further injection. A significant increase of tyrosinase activity and melanogenesis of the tumor was observed. There were, however, marked differences in the rate and extent of enzyme induction between experiments. The time in which tyrosinase activity and melanogenesis reached the maximum value also varied widely. Figure 4 represents the results of a typical series of experiments. The initial increase of the enzyme activity appeared between 1 and 2 days after the injection of α-MSH and reached a maxi-

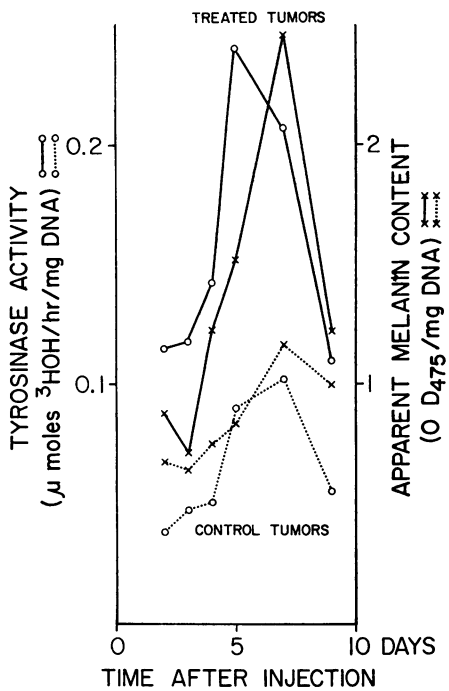

FIG. 4. Time course of induction of tyrosinase and melanogenesis in B-16 melanoma produced by one injection of 2 mg of α-MSH to mice bearing this tumor. (O——O) Tyrosinase activity of α-MSH-treated tumor; (O - - - - - O) tyrosinase activity of control tumor; (X——X) melanin content of α-MSH treated tumor; (X - - - X) melanin content of control tumor.

TABLE 1

EFFECTS OF α-MSH ON TYROSINASE ACTIVITY AND MELANIN CONTENT OF B-16 MELANOMA

Expt no.	Dose (mg)	No. per group	First injection[a]	No. of injections[b]	Tyrosinase activity (μ moles ^{3}HOH/hr/mg DNA)			Melanin content (OD_{475}/mg DNA)		
					BSA	α-MSH	Increase	BSA	α-MSH	Increase
1	2	6	Day 1	12	0.195	1.720	1.525			
				19	0.168	1.730	1.562			
2	2	6	Day 1	12	0.133	1.800	1.667	0.77	2.90	2.13
				19	0.152	2.190	2.038	1.25	4.81	3.56
3	0.5	4	Day 8	2	0.472	0.526	0.054	0.42	0.85	0.43
				4	0.416	0.745	0.329	0.60	1.75	1.15
				6	0.548	0.942	0.494	0.89	2.13	1.24
				9	0.627	1.849	1.222	1.85	4.19	2.34
				12	0.608	1.968	1.360	1.44	3.76	2.33
				21	0.461	2.060	1.559	2.50	5.14	2.64

[a] Day 1 was defined as 24 hr after tumor implantation.
[b] The animals were sacrificed 24 hr after the last injection.

TABLE 2

SUBCELLULAR DISTRIBUTION OF TYROSINASE ACTIVITY OF B-16 MELANOMA

	Tyrosinase activity (μmoles ^{3}HOH/hr/mg tumor DNA equivalent)[a]											
	Injections											
	2		4		6		9		12		21	
	BSA	α-MSH	BSA	α-MSH	BSA	α-MSH	BSA	α-MSH	BSA	α-MSH	BSA	α-MSH
Whole	0.472	0.526	0.416	0.745	0.548	0.942	0.627	1.849	0.608	1.968	0.461	2.06
Fraction 1	0.070	0.067	0.095	0.125	0.050	0.071	0.086	0.180	0.091	0.260	0.051	0.257
Fraction 2	0.281	0.312	0.226	0.450	0.261	0.462	0.338	0.976	0.343	1.109	0.243	1.102
Fraction 3	0.099	0.123	0.060	0.128	0.166	0.300	0.141	0.529	0.105	0.415	0.108	0.503
Fraction 4	0.023	0.024	0.031	0.042	0.071	0.103	0.062	0.164	0.068	0.184	0.059	0.192

[a] The tyrosinase activity of all subcellular fractions were normalized to 100% recovery of the fractionation. The average recovery was 90% with a range of 77–110%.

mum value on the 5th day. Thereafter, the enzyme activity dropped sharply to approximately twice the control level on the 9th day when the experiment was terminated. The time course of melanogenesis of the tumor followed that of the enzyme with a lag period of 2 days. It is of interest to note that the melanin content of the tumor fell rapidly to nearly the control value after the maximum melanogenesis was reached, indicating a high turnover rate of melanin in the tumor pigmentary system.

When the daily injection of α-MSH was maintained over a period of time, more extensive induction of melanogenesis and enzyme activity occurred as shown in Table 1. The results suggest that the induction of melanogenesis was proportional to the dosage as well as to the length of administration of α-MSH. The subcellular distribution of tyrosinase activity was investigated by fractionating the tumor into subcellular fractions according to the procedure of Seiji *et al.* (4). The results are shown in Table 2. The tyrosinase activity of all subcellular fractions was increased manifold by the administration of α-MSH and more than 50% of the total increaase of tyrosinase activity was found in Fraction 2, which contained the major portion of mature melanosomes. Moreover, there was a considerable amount of enzyme activity in Fraction 4, the soluble fraction of the tumor, indicating that there are 2 types of tyrosinase activities: one is attached to subcellular particles and the other is a soluble enzyme. These observations are compatible with the hypothesis on the biosynthesis of melanosomes postulated by Seiji *et al.* (4). Figure 5 shows

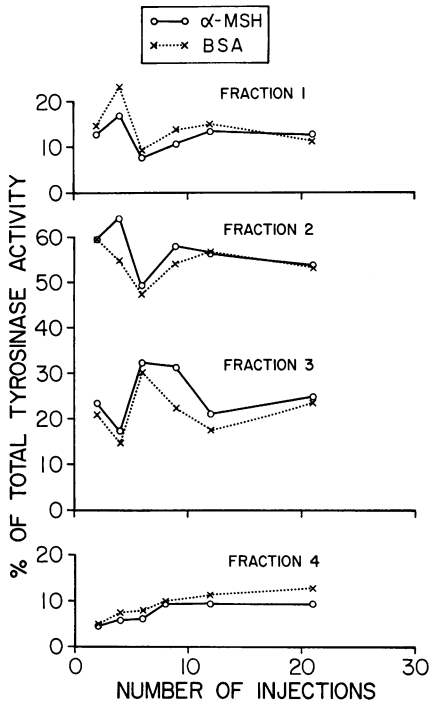

FIG. 5. Percentage distribution of tyrosinase activity in subcellular fractions from α-MSH-treated and control B-16 melanoma after 2, 4, 6, 9, 12, and 21 injections. The dosage used was 0.5 mg of α-MSH or BSA per animal. (O——O) α-MSH-treated tumor; (X - - - - X) control tumor.

the percent of the total tyrosinase activity of the tumor contributed by each subcellular fraction at various stages of the experiment. The most striking feature of these diagrams is that there was no remarkable difference between the treated and the control animals. In other words, the subcellular distribution pattern of tyrosinase activity was not significantly altered by the hormone treatment, although the tyrosinase activity of each of these subcellular fractions was increased manifold.

The results of time-course studies strongly suggested that the action of α-MSH on the melanogenesis of mouse melanoma was mediated through an increase of tyrosinase activity of the tumor. The question arises as to whether the increase of tyrosinase activity came about by an increased rate of synthesis of the enzyme or by other means. If an increased synthesis did take place, it would be expected that a soluble tyrosinase was synthesized, and these tyrosinase molecules would orient themselves with structural proteins to form premelanosomes and melanosomes. Thus, we should find a remarkable increase of tyrosinase activity in the soluble fraction of the tumor at the onset of induction of the enzyme and a subsequent gradual shift of the increase to the "small" and "large" granule fractions. We observed a universal increase and an unaltered distribution pattern of tyrosinase activity among all subcellular fractions of the tumors, at early as well as late stages of the experiment, ruling out the possibility that an increased synthesis of the enzyme took place. Since the existence of a tyrosinase inhibitor, which may act as a regulator of the melanogenesis of the tumor, has been confirmed by several investigators (5–7), it is tempting to speculate that the administration of α-MSH

may release an "activator" which counteracts the tyrosinase inhibitor to activate the tyrosinase activity of the tumor.

Based on these considerations, a working hypothesis may be formulated as follows: In both pigmentary systems, the action of MSH is mediated through an increase of tyrosinase activity. In amphibian-skin pigmentary system, the administration of MSH may cause a slight physiochemical change in the intracellular environments leading to the release of a bound proteolytic enzyme, which in turn activates the soluble protyrosinase. The tyrosinase, once activated, attaches itself to the protein matrix of a melanosome and initiates melanin synthesis. This scheme is consistent with the observation that, during MSH-induced melanogenesis in *Rana pipiens,* a decrease in the content of soluble protyrosinase of the skin occurred. On the other hand, the tyrosinase of mouse melanoma exists in a partially inhibited state under the influence of an intracellular tyrosinase inhibitor, and the administration of MSH may trigger the release of an "activator" which counteracts the inhibitor, leading to the activation of inhibited tyrosinase. This is consistent with the observation that there was a universal increase but an unaltered distribution pattern of tyrosinase activity among all subcellular fractions of the tumor during the entire period of administration of the hormone. There is ample evidence that intracellular cyclic AMP is a mediator of the transient action of MSH, involving the dispersion of melanosomes to produce skin darkening in fish and amphibians. It was also observed that MSH activated *in vitro* the adenylcylase system of melanoma (8). Whether this enzyme system also plays a role in the *in vivo* MSH-induced melanogenesis is not known at present.

REFERENCES

1. Lee, T. H., and Lee, M. S., Studies on MSH-induced melanogenesis: effect of long-term administration of MSH on the melanin content and tyrosinase activity. *Endocrinology* **88,** 155 (1971).
2. Lee, T. H., Lee, M. S., and Lu, M. Y., Effects of α-MSH on melanogenesis and tyrosinase of B-16 melanoma, *Endocrinology* **91,** 1180 (1972).
3. Pomerantz, S. Y., The tyrosine hydroxylase activity of mammalian tyrosinase. *J. Biol. Chem.* **241,** 161 (1966).
4. Seiji, M., Shimao, K., Birbeck, M. S. C., and Fitzpatrick, T. B., Subcellular localization of melanin biosynthesis. *Ann. N.Y. Acad. Sci.* **100,** 497 (1963).
5. Hirsch, H. M., Inhibition of melanogenesis by tissues and the control of intracellular autooxidants. *In* "Pigment Cell Biology " (Gordon, M., Ed.), p. 327. Academic Press, New York, 1959.
6. Chian, L. T. Y., and Wilgram, G. F., Tyrosinase inhibition. Its role in suntanning and in albinism. *Science* **155,** 198 (1967).
7. Satoh, G. J. Z., and Mishima, Y., Tyrosinase inhibitor in Fortner's amelanotic and melanotic malignant melanoma. *J. Invest. Dermatol.* **48,** 301 (1967).
8. Bitensky, M. W., and Demopoulos, H. B., Activation of melanoma adenylcyclase by MSH. *J. Invest. Dermatol.* **54,** 83 (1970) (Abs.).