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IN VITRO EFFECTS OF HORMONAL STIMULI UPON TYROSINASE AND PEROXIDASE ACTIVITIES IN MURINE MELANOMAS

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Summary: ACTH and corticosterone altered tyrosinase activity in the B-16, S-91 and Harding-Passey melanomas but did not evoke any changes in peroxidase activity. Tyrosinase activity, but not peroxidase activity, was correlated with the degree of melanin pigmentation present in the melanomas. Thus, tyrosinase is the enzyme of physiological importance in the regulation of melanin pigmentation.

The roles of tyrosinase and peroxidase in melanogenesis have elicited considerable controversy (1-12). Although murine melanomas contain peroxidase activity (4, 5, 7, 12, 13), peroxidase activity has not been shown to be modified by physiological stimuli known to alter melanogenesis. Therefore, the present study compares the changes in tyrosinase and peroxidase activities in response to adrenocorticotropin (ACTH) and corticosterone, both of which have been shown to alter melanogenesis in melanoma (14-18), in order to determine the enzyme of physiological importance in the control of melanin synthesis.

MATERIALS AND METHODS

Tumor sources and preparation. The National Institutes of Health (NIH) B-16, Cloudman S-91, and Harding-Passey (HP) melanomas were maintained in C57Bl/6j, DBA/2j and BALB/c mice, respectively. The tumors were transplanted following the NIH chemotherapy protocol (19). Mice were killed by cervical dislocation, the melanomas were removed and placed in cold (4°C) Krebs-Ringer bicarbonate buffer (pH 7.4) containing 200 mg glucose and 500 mg bovine serum albumin fraction V/dl (KRBGA). The melanomas were trimmed and diced (approximately 2 x 4 mm).

Evaluation of melanoma response. Melanoma dice in KRBGA were treated with ACTH (100 mIU/ml; 150 IU/mg, porcine, chromatographically purified, Schwarz/Mann), corticosterone hemisuccinate (50 ug/ml, Steraloids) and ACTH plus corticosterone (100 mIU/ml plus 50 ug/ml, respectively) in a Dubnoff metabolic shaker (37°C, 95% O_2 + 5% CO_2). All dice were preincubated in KRBGA for 60 min at 37°C. Each die was then transferred to an incubation flask containing 1 ml KRBGA (control) or test agent in this buffer. Three incubates formed a time group. At seven given time intervals (5-240 min) the incubates were terminated by individually quenching the dice in liquid nitrogen.

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The melanoma dice were analyzed for tyrosinase activity by the radiometric assay of Chen and Chavin (20) using 6 mM diethyldithiocarbamate as the control instead of heat inactivation (21). Peroxidase activity was determined by the method of Lundquist and Josefsson (22), using horseradish peroxidase as the standard. This procedure has been validated for use with murine melanoma (8). The protein content of the melanoma dice was quantitated by the Folin-Ciacalteau procedure (23). Tyrosinase and peroxidase activity are expressed as units/ug protein nitrogen.

Where applicable, data are represented as the mean $\pm$ the standard error of the mean. The data obtained were analyzed by the analysis of variance and the group comparison test. Values were considered to be statistically different when $P<0.05$.

RESULTS

Tyrosinase Activity. The control melanoma incubates did not show altered tyrosinase activity during the 4 hr study period in any of the three tumor types. The basal tyrosinase activity in these control tissues was 730 $\pm$ 112, 322 $\pm$ 60 and 805 $\pm$ 74 tyrosinase units/ug protein N in the B-16, S-91 and HP melanoma, respectively. The variation in the control tyrosinase activity correlated with the degree of melanin pigmentation, i.e., the HP melanoma demonstrated the greatest degree of melanization while the S-91 melanoma was lightest in color. ACTH increased B-16 tyrosinase activity at 5 min and HP tyrosinase activity at 15 min but did not alter S-91 tyrosinase activity (Table I). However, the later responses of the B-16 melanoma to ACTH showed depressed tyrosinase activity. Corticosterone depressed B-16 enzymatic activity through most of the incubation period (Table I). In the S-91 and HP melanomas, the alterations in tyrosinase activity evoked by corticosterone were more complex. In the S-91 melanoma, corticosterone stimulated tyrosinase activity during most of the study period, with peak activity at 15 min (Table I). However, at 10 and 30 min, corticosterone depressed S-91 tyrosinase activity. During the other time intervals, S-91 tyrosinase activity was approximately 340% control. Corticosterone initially stimulated HP tyrosinase activity at 5 min. The tyrosinase activity was depressed at 15 min and returned to control levels at 1 and 2 hrs (Table I). Subsequently, HP tyrosinase activity was stimulated at 4 hrs. In the intact mouse, corticosterone release from the adrenal is under ACTH control. Therefore, in vivo,

Table I. In vitro Temporal Tyrosinase Reponse (% Control) of Murine Melanoma to ACTH and Corticosterone Hemisuccinate (B).

Melanoma Type	Time (min)	ACTH (100 mIU/ml)	B (50 ug/ml)	ACTH + B (100 mIU/ml + 50 ug/ml)
B-16	5	135 ± 17[a]*	44 ± 2*	90 ± 30
	10	71 ± 7	56 ± 6	107 ± 40
	15	122 ± 78	79 ± 17	295 ± 21*
	30	33 ± 7	46 ± 10	55 ± 10*
	60	41 ± 12*	38 ± 8*	37 ± 8*
	120	71 ± 15	45 ± 9*	58 ± 19
	240	45 ± 12*	28 ± 17*	25 ± 6*
S-91	5	85 ± 23	301 ± 81**	44 ± 17*
	10	114 ± 48	68 ± 12*	45 ± 2*
	15	98 ± 31	699 ± 83**	442 ± 88**
	30	95 ± 39	55 ± 19*	173 ± 67
	60	73 ± 13	333 ± 80**	210 ± 75*
	120	182 ± 58	348 ± 85**	374 ± 75**
	240	89 ± 52	343 ± 78**	734 ± 90**
Harding-Passey	5	134 ± 53	173 ± 35*	100 ± 14
	10	104 ± 14	42 ± 35	72 ± 27
	15	149 ± 30*	9 ± 3*	132 ± 27
	30	140 ± 30	66 ± 23	141 ± 28*
	60	78 ± 27	105 ± 34	111 ± 35
	120	85 ± 5	108 ± 17	32 ± 15*
	240	102 ± 21	155 ± 24*	181 ± 10

[a]Mean per cent control values ± SEM of three samples.
*P < 0.05
**P < 0.01

murine melanomas are exposed simultaneously to both ACTH and corticosterone. In the present study, combined hormone treatment produced an early peak in tyrosinase activity in all three tumors. In the B-16 and S-91 melanomas, this peak in tyrosinase activity was observed at 15 min, while in the HP melanoma the peak in tyrosinase activity was delayed until 30 min (Table I). Subsequent to this peak in tyrosinase activity, enzyme activity was decreased in all three tumors. The S-91 and HP melanomas later showed another increase in tyrosinase activity.

Peroxidase activity. Peroxidase activity occurred in each tumor examined. The enzymatic activities of the control incubates were unchanged during the study period. The B-16, S-91 and HP melanomas contained

204 ± 33, 77 ± 19 and 2.9 ± 1.6 pU peroxidase/ug protein N, respectively. Unlike tyrosinase activity, peroxidase activity was not correlated with the degree of melanoma pigmentation. Hormonal treatment did not evoke statistically significant alterations in peroxidase activity when compared to the controls in any of the three melanoma types at any time interval. Upon comparison of hormone induced alterations in tyrosinase activity versus peroxidase activity (Fig 1a-c), it is clear that alterations in tyrosinase activity are not accompanied by alterations in peroxidase activity.

DISCUSSION

Although hormonal control of melanin pigmentation in melanoma (16-18, 24, 25) requires elucidation, in the present report we deal only with the question of the biologically important enzyme involved in melanin synthesis. The involvement of peroxidase in melanin synthesis within melanocytes was suggested from histochemical studies on hemopoietic tissue (for review see 26) which indicated that L-3,4-dihydroxyphenylalanine may be oxidized to melanin by peroxidase. Histochemical differentiation of integumental tyrosinase and peroxidase activities (1) led to the proposal that the oxidation of tyrosine to melanin in melanocytes was mediated by peroxidase not tyrosinase. When these histochemical procedures (1) were applied to homogenates, tyrosinase mediated melanin formation could be demonstrated in mammalian, avian and teleostean eyes (2, 3, 9, 22), mammalian and teleostean skin (2, 9, 28) as well as murine melanoma (7-9).

The presence of peroxidase in murine melanoma homogenates has been reported (13) and is presently confirmed. However, peroxidase activity has not been demonstrated in melanosomes (6, 8, 9), the site of melanin formation. Nevertheless, it has been claimed that murine melanoma tyrosinase was incapable of oxidizing tyrosine to melanin (6, 28). On the other hand, tyrosine has been demonstrated to be oxidized to melanin by purified murine hairbulb tyrosinase (4, 29), purified murine melanoma tyrosinase (4, 7, 12) and purified human melanoma tyrosinase (30, 31). Thus, studies utilizing

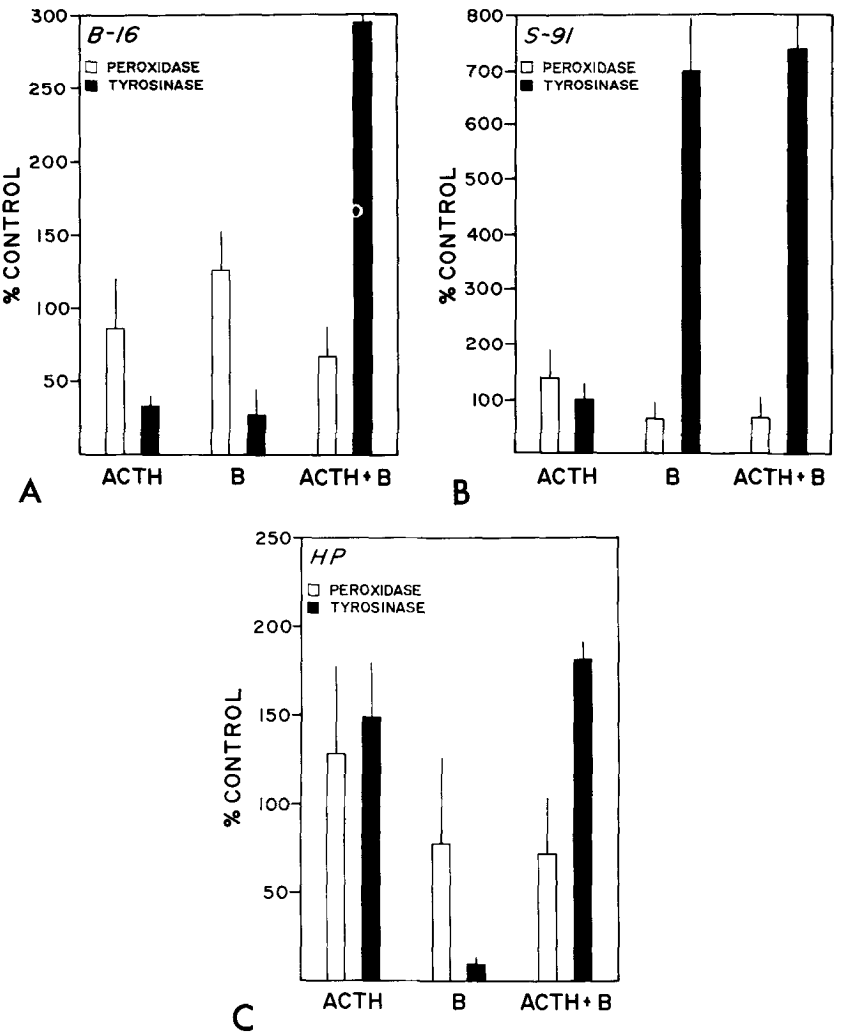

Figure 1. Comparison of the (a) B-16, (b) S-91 and (c) HP melanoma tyrosinase activity and peroxidase activity responses to ACTH and corticosterone. The maximal in vitro change in tyrosinase activity in response to ACTH (100 mIU/ml) and corticosterone hemisuccinate (50 ug/ml, B) alone and in combination, with the corresponding change in peroxidase activity are shown. Each bar represents the mean per cent control values ± SEM of three samples.

purified tyrosinase from a variety of sources unequivocally demonstrate that tyrosinase, in fact, can oxidize tyrosine to melanin.

The validity of the ^{14}C-tyrosine-tyrosinase assay (20), as well as the ability of the assay to differentiate between tyrosinase and peroxidase mediated melanin formation has been questioned (Discussion after 7, 13, 32). If this

tyrosinase assay measures total melanogenesis and if peroxidase is truly the key enzyme involved in melanogenesis, then the changes observed with the tyrosinase assay should be accompanied by appropriate and similar changes in peroxidase activity. Although ACTH and corticosterone treatment significantly alter tyrosinase activity in all three tumors of the present study, peroxidase activity remains unaltered by any hormone treatment in any of these same melanomas. Further, peroxidase activity is not correlated with the degree of pigmentation of the melanomas used in the present study, whereas, tyrosinase activity shows such a positive correlation. Correlation of tyrosinase activity with the degree of melanin pigmentation has been shown in every class of vertebrate studied (for reviews see 33, 34) as well as in human melanomas (30). Such a correlation has not been demonstrated for peroxidase. In fact, an inverse relationship between melanin pigmentation and peroxidase activity was found in murine melanoma using histochemical techniques (35).

Although peroxidase is present in murine melanoma, this enzyme does not respond to those normally occurring physiological stimuli (ACTH, corticosterone) capable of increasing melanin synthesis. Further, the activity of this enzyme does not correlate with the degree of melanin pigmentation. Therefore, peroxidase appears to be of little significance in the physiological control of melanin synthesis. Thus, the results of the present study reveal that tyrosinase is the key enzyme involved in the regulation of melanin formation.

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