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Inhibition of Imidazole-Induced Tyrosinase Activity by Estradiol and Estriol in Cultured B16/C3 Melanoma Cells

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The effect of estrogens on tyrosinase (EC 1.14.18.1) activity was studied in B16/C3 melanoma cultures. Estradiol, estriol, and other related steroids failed to influence tyrosinase activity when added to the medium of proliferating cultures. Imidazole (10 mM), on the other hand, induced the activity of that enzyme 3-fold, as reported previously. Estradiol and estriol blocked imidazole induction, however, unlike the other estrogenic compounds. The blockade occurred within 15 min of hormone addition and was reversible. Dose-response studies revealed that the maximal estradiol effect occurred at 0.75 nM and the half-maximal effect occurred at 0.5 nM. Estriol was more potent, with the maximal blockade occurring at ~ 0.5 nM and half-maximal effect at 0.25 nM. The induction of tyrosinase by imidazole and the blockade of this induction by estradiol and estriol could not be demonstrated in broken cell preparations, suggesting that direct enzyme activation-inactivation was not involved. Studies utilizing inhibitors of protein and RNA synthesis suggest that this effect is mediated at a pre-translational level and is independent of mRNA destabilization.

Melanogenesis, the production of melanin, involves the specialized function of melanocytes in mammals. The oxidative conversion of tyrosine to dopa and of dopa to dopa-quinone is thought to be catalyzed by a single enzyme, tyrosinase (EC 1:14.18.1) (Pawelek, 1976). Control of melanogenesis by the polypeptide hormone, melanocyte stimulating hormone (MSH), has been studied extensively and apparently involves the activation of pre-existing enzyme molecules (Wong and Pawelek, 1975), though some evidence exists for MSH affecting protein synthesis (Fuller and Viskochil, 1979).

Neoplastic transformation of melanocytes occurs in human beings and other mammals, leading to the clinical disease termed melanoma. These malignancies are of great interest because they are associated with significant morbidity and mortality and behave in an unpredictable, capricious manner (Danforth et al., 1982). An intensive search has therefore been undertaken to elucidate the factors which influence the course of the disease. These tumors contain high affinity binding sites for a variety of steroidal hormones which may provide clues about potential roles for these compounds as regulators of tumor growth and behavior (Neifeld and Lippman, 1980).

Melanoma cells can be maintained for prolonged periods in culture, affording an invaluable opportunity to study melanogenesis and melanoma behavior in particular. Established murine cell lines such as B16/C3 have been utilized in studying various aspects of tyrosinase regulation. Recently our laboratories reported that the active thyroid hormone 3,5,3'-L-triiodothyronine (T_3) could inhibit tyrosinase activity in a time- and dose-dependent, stereospecific manner (Kline et al., 1986). In addition, the hormone could block the induction of that enzyme by imidazole. These actions were apparently mediated at a pre-translational level.

The apparent differences in survival between males and females (Rampen and Mulder, 1980), as well as gender-related patterns of metastatic spread (Danforth et al., 1982), have prompted us to examine what influences, if any, several estrogenic compounds have on melanogenesis in the B16/C3 melanoma cells in vitro. In this paper, we report that estradiol (E_2) and estriol (E_3) can block the clinical induction of tyrosinase at a pre-translational level in concentrations which have physiological relevance.

MATERIALS AND METHODS

Chemicals

Estradiol, estriol estrone (E_1), and 17α-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). Imidazole was supplied by Fisher Scientific (Pittsburgh, PA). All chemicals used were of the highest purity available.

Cell culture

B16/C3 mouse melanoma cell cultures were incubated at 37°C in 25-cm^2 plastic flasks (Corning Medical, Med-

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TABLE 1. Effects of estradiol, estriol, and imidazole on tyrosinase activity in cell sonicates[1]

Compound(s)	37°C Pre-incubation time				
	0 min	30 min	1 h	2 h	3h
No compound	10.37 (± 0.15)	10.09 (± 0.11)	9.48 (± 0.06)	7.87 (± 0.07)	6.27 (± 0.06)
Imidazole (10 mM)	10.37 (± 0.15)	10.07 (± 0.09)	9.27 (± 0.11)	7.94 (± 0.04)	6.41 (± 0.09)
Estradiol (10 nM)	10.37 (± 0.15)	10.22 (± 0.13)	9.31 (± 0.04)	7.79 (± 0.13)	6.34 (± 0.04)
Estriol (10 nM)	10.37 (± 0.15)	10.17 (± 0.13)	9.37 (± 0.09)	7.76 (± 0.10)	6.33 (± 0.11)
Imidazole + Estradiol	10.37 (± 0.15)	10.12 (± 0.08)	9.61 (± 0.10)	7.83 (± 0.03)	6.40 (± 0.07)
Imidazole + Estriol	10.37 (± 0.15)	10.31 (± 0.09)	9.54 (± 0.09)	7.96 (± 0.08)	6.24 (± 0.07)

[1]Sonicates from imidazole-induced cultures were pretreated with the specified compound(s) at 37°C for the times indicated and then assayed for tyrosinase activity. Tyrosinase assays were performed in triplicate and the data given represent the mean ± SEM.

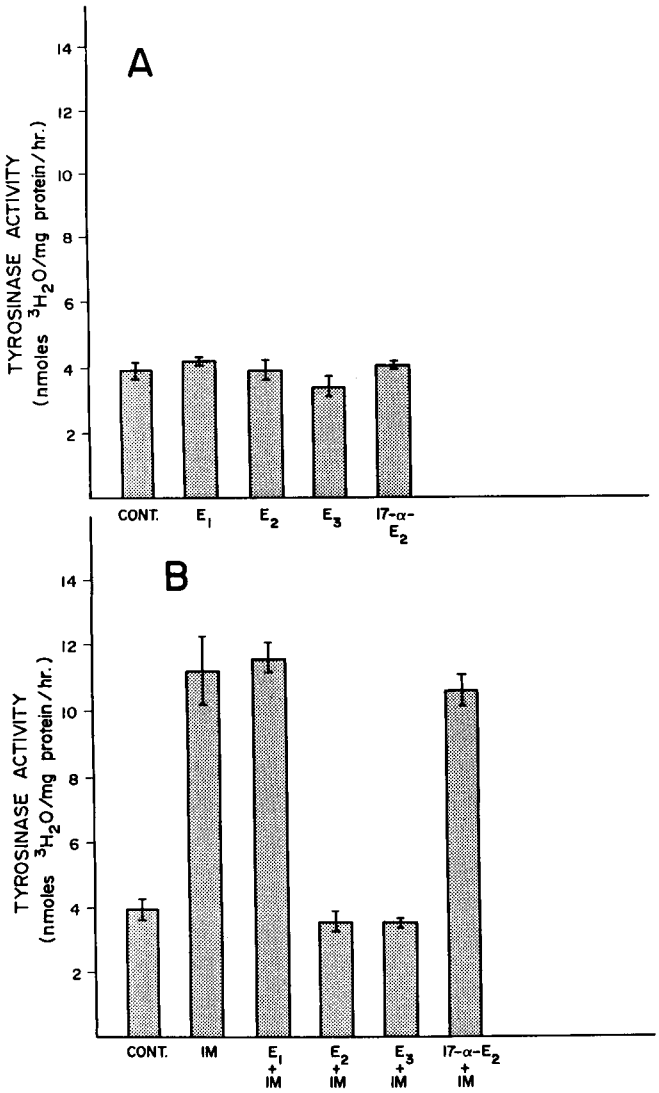

Fig. 1. Effects of estrogenic compounds on tyrosinase activity. Cultures were allowed to proliferate for 19 h in the presence of diluent, or the steroid being tested (10 nM) without (Panel A) or with imidazole (Panel B). Cultures were harvested and analyzed for tyrosinase activity. Each column represents the mean ± range of duplicate plates for one representative experiment.

field, MA) with 5 ml antibiotic-free Minimum Essential Medium supplemented with 10% calf serum (vol/vol), as previously described (Montefiori and Kline, 1981). Cells were removed for passage in calcium-free, magnesium-free phosphate buffered saline containing 0.05% EDTA disodium, pH 7.4 (0.05% EDTA disodium in CMF-PBS). Experimental flasks were seeded with 1.5×10^5 late exponential cells and allowed to attach for 6 h. Fresh medium supplemented with a steriod, imidazole, or diluent (95% ethanol) was then added, and cultures were allowed to incubate for the various times indicated in the figure legends. Unless indicated otherwise, the final hormone concentrations were 10 nM, and that of imidazole was 10 mM.

Tyrosinase assay

Tyrosinase activity in whole cell sonicates was determined as previously described (Pomerantz, 1968). Briefly, monolayers were rinsed thoroughly with 0.05% EDTA disodium in CMF-PBS (5 ml), suspended in 1.5 ml sodium phosphate buffer (80 mM; pH 6.8), and frozen at −20°C until assayed. Samples were thawed and disrupted at 4°C with two 15 sec sonication bursts with a microtip probe (sonicator model W-220F, Heat Systems, Plainview, NY). The assay mixture contained 0.4 μmol L-tyrosine, 0.04 μmol L-dopa in sodium phosphate buffer (26 μmol; pH 6.8), 2.5 μCi L-[^{3}H]tyrosine (SA, 52.5 Ci/mm, New England Nuclear, Boston, MA), and 0.2 ml cell sonicate (final reaction volume, 0.4 ml). Reactions were carried out at 37°C for 1 h in a shaking water bath and terminated with the addition of 0.5 ml ice-cold trichloracetic acid (10% , vol/vol). Unreacted L-tyrosine was extracted by adding 0.7 ml activated charcoal suspension (100 mg/ml; Norit-A, Fisher) and centrifuging. An aliquot of supernatant was subjected to liquid scintillation spectroscopy in a Beckman LS7000 counter (Beckman, Palo Alto, CA), with Aquasol (New England Nuclear) as the scintillant. Protein concentrations were determinated by the method of Lowry et al. (1951).

RESULTS

Effects of estrogens and related steroids on tyrosinase activity and cellular proliferation

When proliferating B16 melanoma cell cultures were incubated for 19 h in medium containing E_1, E_2, E_3, or 17α-estradiol (all 10 nM), no significant alteration in tyrosinase activity occurred compared to untreated con-

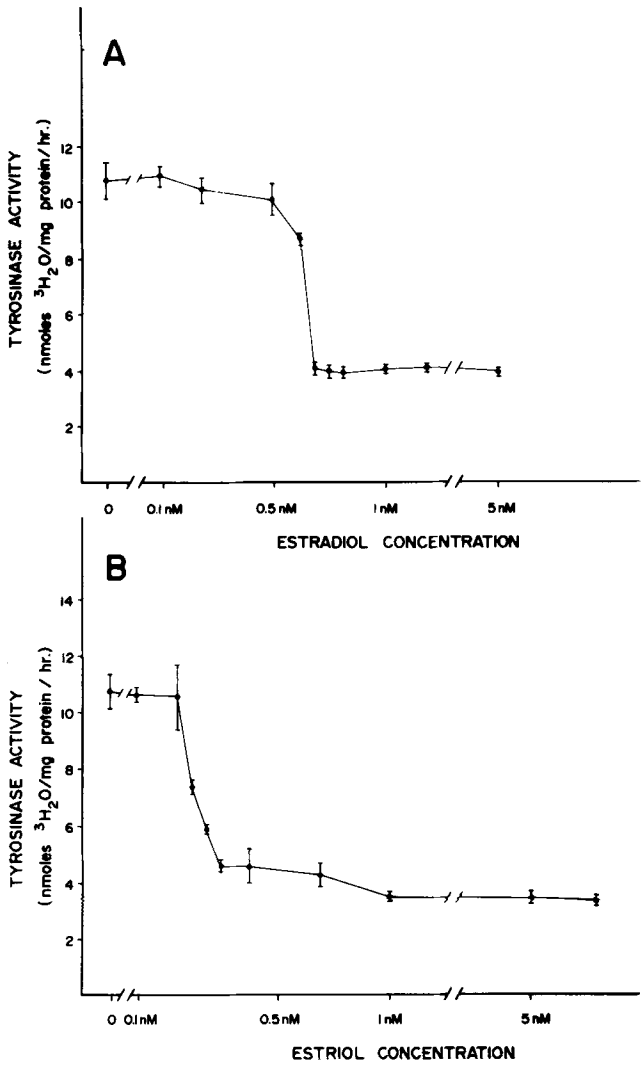

Fig. 2. Dose-response curves for estradiol (Panel A) and estriol (Panel B). Cultures were allowed to proliferate for 19 h in the presence of imidazole (10 mM) and the concentrations of hormones indicated along each abscissa. Each datum point represents the mean ± range of activities for duplicate cultures.

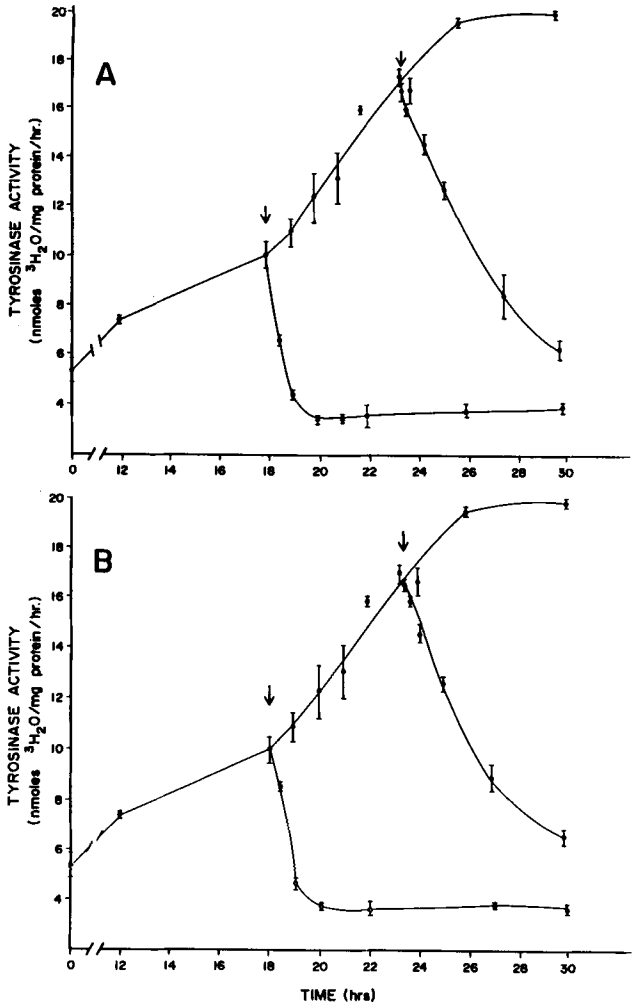

Fig. 3. Time course of the effect of estradiol (Panel A) or estriol (Panel B) on imidazole-induced tyrosinase activity. Cultures were allowed to proliferate in the presence of imidazole (10 mM). At either 18 or 23 h, some cultures were shifted (arrows) to medium containing either steroid (5 nM) in addition to imidazole, while some plates were continued in the presence of imidazole alone. Cultures were harvested at the times indicated along the abscissa and analyzed for tyrosinase activity. Each datum point represents the mean ± range of activities from duplicate culture plates.

trol cultures (Fig. 1A). The rate of cellular proliferation was unaffected under these culture conditions (data not shown). In the presence of imidazole (10 mM), tyrosinase activity increased 2.7 fold (Fig. 1B). Estradiol and estriol blocked this induction of the enzyme to levels observed in control cultures. In contrast, the other steroids failed to influence imidazole stimulation.

Dose response of estradiol and estriol inhibition on tyrosinase activity

The dose response for estradiol and estriol blockade of imidazole-induction of tyrosinase was determined by incubating imidazole-treated cultures with increasing concentrations of these compounds for 19 h. Decreased tyrosinase activity was observed in cultures receiving estradiol in a concentration as low as 0.25 nM. Half-maximal inhibition occurred at 0.5 nM and a maximal response was achieved at 0.75 nM (Fig. 2A). The lowest concentration of estriol which inhibited imidazole-induction was 0.13 nM. Half maximal inhibition occurred at 0.25 nM and maximal at 0.5 nM (Fig. 2B).

Effects of estradiol, estriol, and imidazole on preformed tyrosinase enzyme

To determine whether the effects of the estrogens and imidazole were a result of activation or inactivation of preformed tyrosinase, broken cell preparations were treated with the compounds or diluent for up to four hours and then assayed for tyrosinase activity. Enzyme activities (Table 1) indicate that no effects occurred in these preparations. These results suggest that the alterations of tyrosinase activity did not result from direct interactions with the enzyme.

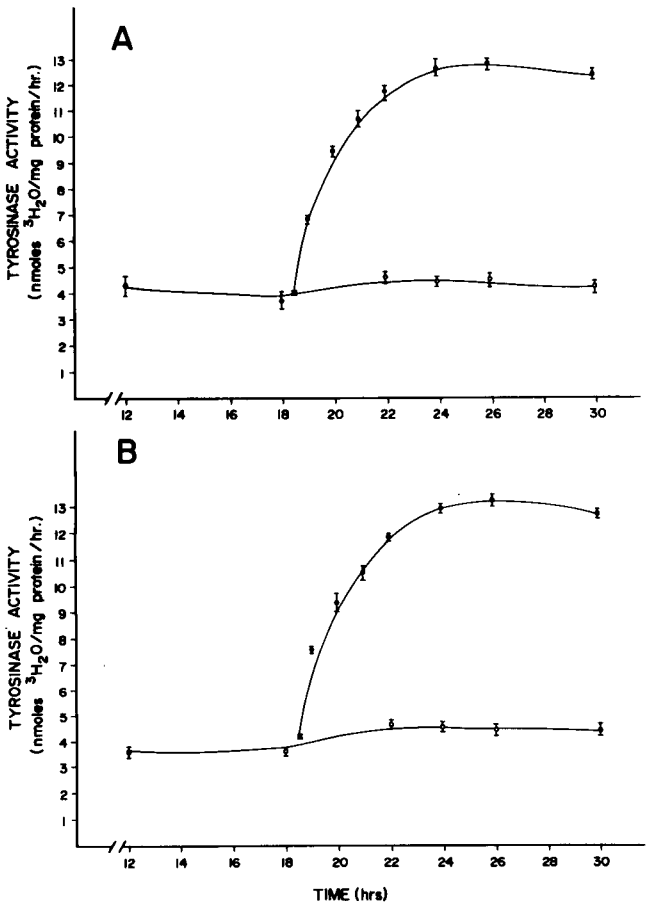

Fig. 4. Reversibility of the blockade of imidazole induction of tyrosinase by estradiol (Panel A) and estriol (Panel B). Cultures were allowed to proliferate for 18 hours in the presence of imidazole (10 mM) and either estradiol (5 nM) or estriol (5 nM). After 18 h, some cultures were shifted to medium containing imidazole alone (●), while others were continued in the presence of the steroids as well (○). Cultures were harvested at the times indicated along the abscissa and analyzed for tyrosinase activity. Each datum point represents the mean ± range of activities from duplicate culture plates from one representative experiment.

Time course of estradiol and estriol effects on imidazole-induced tyrosinase activity

Induction of tyrosinase by imidazole (10 mM) achieved a 1.5-fold increase at 12 h and a 2- to 2.5-fold increase by the first cell doubling (18 h). During the next 6 h, a 4-fold increase above the non-induced controls was observed (Fig. 3). This level of tyrosinase activity was maintained for the duration of the experiment (30 h), when a confluent state was reached. When estradiol or estriol was added to cultures which had been treated with imidazole for 18 h, inhibition of tyrosinase became apparent within 30 min (Fig. 3). This inhibition reached a maximum within approximately 2 h after the hormone addition. Shifting cultures treated with both imidazole and either steroid to medium supplemented with imidazole alone resulted in a rapid reversal of the inhibition of tyrosinase induction (Fig. 4). Within 6 hours of hormone depletion, enzyme activity approximated that observed in cultures never receiving either steroid.

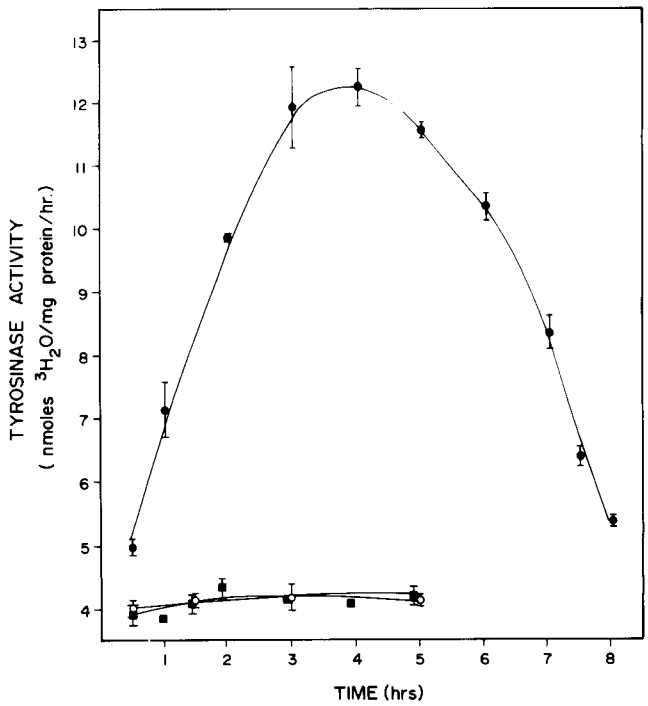

Fig. 5. Protein and RNA inhibitor effects on the imidazole induction of tyrosinase and the blockade of that induction by estradiol. Cultures were allowed to proliferate for 18 h in medium supplemented with imidazole (10 nM) (●), imidazole plus estradiol (10 nM) (■) or diluent (control, ○). Cultures were then shifted to medium containing the respective supplements plus cycloheximide (10 μg/ml) for 5 h (pretreatment). Plates were then washed extensively and incubated in medium to which only actinomycin D (2 μg/ml) was added at time "O" and were harvested at the times indicated along the abscissa and assayed for tyrosinase activity. Each datum point represents the mean ± range of duplicate culture plates.

Protein and RNA inhibitor effects on imidazole-induced tyrosinase activity

Estriol and estradiol appear to inhibit imidazole-induced tyrosinase activity at a pre-translational level (Fig. 5), independent of translational or post-translational modification of the enzyme (Fig. 6; Table 1). Exponentially proliferating cultures were treated with imidazole (10 mM) for 18 h, shifted to medium containing imidazole and cycloheximide (10 μg/ml) with or without estradiol (1 nM) and incubated for an additional 5 h (pretreatment). At this time, each culture was then shifted to medium containing only actinomycin-D (2 μg/ml), the zero time point in Figure 5. These inhibitor concentrations blocked > 90% of protein and RNA synthesis, respectively. Tyrosinase activity manifested during the actinomycin D treatment period could result from residual enzyme from prior to the pretreatment with cycloheximide or enzyme translated de novo from mRNA which had accumulated during cycloheximide exposure.

Tyrosinase activity increased within 1 h of the shift to actinomycin D in cultures pretreated with imidazole. Cultures pretreated with estradiol plus imidazole were indistinguishable from control cultures (Fig. 5). Within 3 h of the shift to actinomycin D, enzyme activity had increased in the imidazole treated culture plates 3-fold

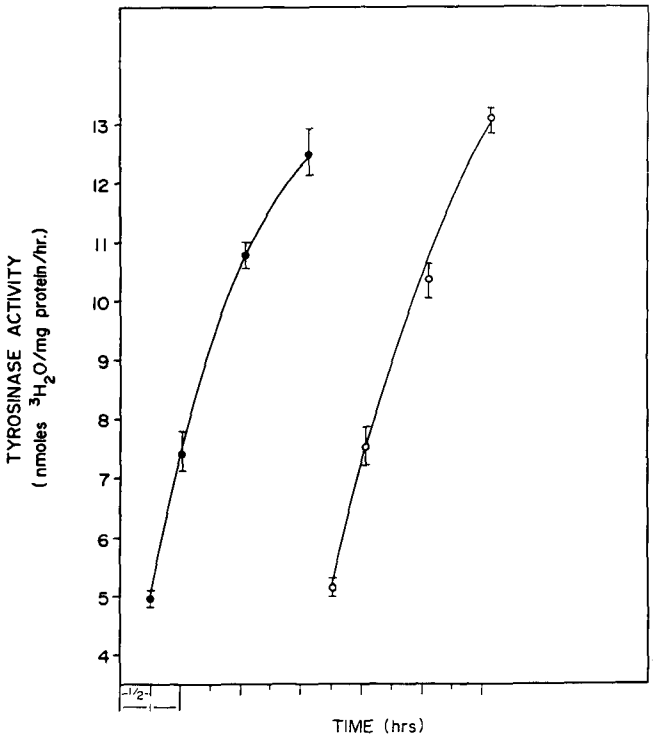

Fig. 6. Effects of estradiol on the translation of pre-formed tyrosinase mRNA from imidazole induced B16/C3 cultures. Cultures were allowed to proliferate in the presence of imidazole (10 mM) for 18 h. They were then shifted to medium supplemented with imidazole and cycloheximide (10 μg/ml) for 5 h (pretreatment). Cultures were then washed extensively and shifted to medium containing actinomycin D without (●) or with (○) estradiol (10 nM) and harvested and analysed for tyrosinase activity. Each datum point represents the mean ± range of activities from duplicate culture plates.

above controls while estradiol completely blocked this increase. In another experiment, a set of cultures was pretreated with imidazole plus actinomycin D and then shifted to medium containing only actinomycin D as in these studies. Like the active estrogenic steroids, actinomycin D pretreatment blocked the rise in tyrosinse activity during the 5 h following the medium shift. Estriol (1 mM) had an identical effect (data not shown).

Imidazole treated cultures were shifted to medium containing that compound and cycloheximide for 5 h. Cultures were then washed and shifted to medium supplemented with actinomycin D plus diluent or estradiol and tyrosinase activity was assessed. As Figure 6 demonstrates, there was a parallel increase in activity in both treatment groups. Since the expression of activity under these conditions presumably reflects the translation of mRNA which had accumulated during the cycloheximide blockade, it appears that estradiol failed to affect the translational or posttranslational processing of tyrosinase, consistent with the results reported with T_3 (Kline et al., 1986). It suggests further that the turnover of preformed tyrosinase mRNA is not being altered by either steroid.

DISCUSSION

This study demonstrates that estradiol and estriol can block imidazole-induced tyrosinase expression in B16 melanoma cell cultures. This inhibition was time- and dose-dependent and was rapidly reversible. Neither steroid could influence the non-induced activity of this enzyme nor was there any effect in broken cell preparations. Studies involving inhibitors of protein and RNA synthesis suggest that the blockade of imidazole induction occurs at a pre-translational level and is independent of mRNA destabilization.

Imidazole can induce tyrosinase in B16/C3 cells in vitro without affecting intracellular cAMP content (Montefiori and Kline, 1981) and is one of a series of low molecular weight compounds which can alter gene transcription at a specific promoter site in procaryotic cells and is a metabolite gene regulator (Kline et al., 1979, 1980a,b, 1984). While the imidazole effect in B16 melanogenesis appears pre-translational on the basis of inhibitor studies (Montefiori and Kline, 1981), whether transcription of the tyrosinase structural gene is itself being regulated is not yet known.

Steroidal control of melanogenesis has been observed previously. Estrogen and progesterone cause the dispersion of melanin granules in frog skin and increased melanin pigmentation in human skin in vivo (Parker, 1981). Glucocorticoids on the other hand stimulated tyrosinase activity in the Cloudman S-91 melanoma cell line in vitro, an action which was independent of changes in cellular cAMP content (Abromowitz and Chavin, 1978a). The same authors demonstrated an inhibition of the enzyme by corticosterone in B16 cells (Abromowitz and Chavin, 1978b), suggesting differential regulation in heterologous tumor lines. We have been unable to document any effects of glucocorticoids or progesterone on melanogenesis in B16/C3 cultures (unpublished observation). These differing results may reflect clonal drift or subtle differences in culture conditions. These cells do appear to contain high affinity cytosolic glucocorticoid binding sites which conform to the physicochemical expectations for putative receptors (Bhakoo et al., 1981).

The mechanism by which estradiol and estriol attenuate the response to imidazole is not known. Imidazole may be inducing the transcription of the tyrosinase gene and these steroids may be directly blocking this induction. Alternatively, the estrogens could influence the synthesis of an inhibitor protein. This later possibility seems unlikely since cytosol from estrogen treated cultures failed to alter tyrosinase activity in imidazole-induced cell sonicates (unpublished observation). Preliminary studies quantitating hybridizable tyrosinase mRNA using a cDNA probe suggest that imidazole increases the abundance of that mRNA and that estradiol blocks this effect (Kline et al., in preparation).

The role of estrogen receptors in the mediation of these effects on melanogenesis has not been established. Estrogen receptors have been demonstrated in human melanoma (Fisher, 1976; Rampen and Mulder, 1980), but their functional importance has not. The B16 cell line exhibits a differential growth rate in male and female mice (Proctor et al., 1976) and the number of metastases is affected by estrogen treatment (Proctor et al., 1981).

Unlike estradiol and estriol, estrone failed to influence the imidazole induction of tyrosinase. It is possible that estrone is incapable of binding to the cytosolic estrogen receptor or that the resulting hormone receptor complex fails to translocate into the cell nucleus, as has been

demonstrated previously in rat liver (Dickson et al., 1980). In any event, the differential potency of estrogenic compounds in regard to effects in a number of tissues is not without precedent (Jordan et al., 1985).

Tyrosinase activity in cultured B16 melanoma cells is apparently regulated by several hormones, including T_3 (Kline et al., 1986), testosterone (submitted for publication) and certain estrogenic steroids. All of these hormones can block imidazole induction of the enzyme at a pre-translational level without altering the half-life of translatable mRNA. In addition, T_3, but not the other classes of hormone, can inhibit noninduced tyrosinase activity. Whether these effects represent direct control of the structural tyrosinase gene or whether they are mediated through the regulation of intermediate protein(s) is currently being examined. It would appear that the estrogens comprise an important component of the hormonal milieu which conditions the response of these cells to exogenous chemicals. Whether or not there is any relationship between these observations and the clinical behavior of this tumor is yet to be determined.

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