Effect of Testosterone on Imidazole-induced Tyrosinase Expression in B16 Melanoma Cell Culture

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ABSTRACT

To assess the effect of androgens on tyrosinase activity in B16/C3 melanoma cell cultures, proliferating cultures were treated with testosterone (50 nM) or one of several other androgenic analogues and metabolites. None of these compounds influenced basal enzyme activity. Imidazole (10 mM), however, is a potent inducer of tyrosinase in this cell line. Testosterone blocked induction of tyrosinase by imidazole almost completely. This effect was dose dependent, being maximal at 10 nM and half-maximal at ~3 nM, and was rapid, occurring within 15 min. When cultures treated with both imidazole and testosterone were shifted to medium containing only imidazole, enzyme activity approximated that of imidazole alone. These results suggest that testosterone is blocking imidazole induction at a pretranslational level.

INTRODUCTION

Tyrosinase (EC 1.14.18.1.), the rate-limiting enzyme involved in melanogenesis in mammalian cells (1), is influenced by a variety of hormones in both normal and neoplastically transformed tissue. Perhaps the most extensively studied and best characterized regulator of melanin production is melanocyte-stimulating hormone. The response to MSH2 is associated with an increased intracellular cyclic AMP content (2) and is mediated through binding to glycoprotein determinants on the cell surface (3, 4). Disagreement exists concerning the mechanism involved in the stimulation of tyrosinase by MSH. Evidence has been introduced (5) for an activation of preexisting enzyme protein synthesis.

This laboratory (7) reported recently that 3,3',5-L-triiodothyronine, the active thyroid hormone, could inhibit basal and chemically induced tyrosinase activity in B16/C3 murine melanoma cell cultures. The effects were stereospecific and occurred within 2 h of treatment at physiologically relevant concentrations. The effects of 3,3',5-L-triiodothyronine were apparently mediated through pretranslational events independent of mRNA destabilization (7). This cell culture line is thus a useful model for studying hormonal regulation of melanogenesis in vitro.

Sex steroids may be important influences of melanoma behavior in vivo, as suggested by the finding of receptors in melanoma tumors (8) and sex differences in the prognosis and clinical course of these tumors (9). Survival time following metastasis of the primary tumor is significantly greater in females than in males and the secondary tumor-doubling time is slower in female patients (10). These observations prompted us to explore what effect, if any, testosterone and related steroids might have on tyrosinase activity in cultured B16/C3 cells. Here we report that the hormone can block induction of that enzyme by imidazole.

MATERIALS AND METHODS

Chemicals. Testosterone, epitestosterone, and 5a-dihydrotestosterone were purchased from Sigma Chemical Co. (St. Louis, MO), and imidazole was purchased from Fisher Scientific (Pittsburgh, PA). Anastrozolone was obtained from Steraloids (Wilton, NH). All other chemicals used were of the highest purity available.

Cell Culture. The B16/C3 mouse melanoma cell line was a generous gift of Drs. J. Kreider and G. Bartlett (Hershey Medical Center, Hershey, PA). Cultures were incubated at 37°C in 25-cm² Corning flasks (Corning Medical, Medfield, MA) with 5 ml antibiotic-free minimum essential medium supplemented with donor calf serum 10% (vol/vol), as previously described (11). Cells were removed for passage in calcium-free, magnesium-free phosphate-buffered saline containing 0.05% EDTA disodium, pH 7.4. Experimental flasks were seeded with 1.5 x 10⁶ late exponential cells and allowed to attach for 6 h. Fresh medium supplemented with the steroid tested, imidazole, or diluent (95% ethanol) was then added, and cultures were allowed to incubate. Unless indicated otherwise, the final hormone concentrations were 50 nM, and that of imidazole was 10 mM.

Inhibitor Studies. Studies involving inhibitors were conducted as follows. Actively dividing cultures were treated with imidazole for 18 h with or without testosterone. Cultures were then shifted to medium containing the same supplements and cycloheximide (10 μg/ml, a concentration which inhibited >90% of protein synthesis) for 5 h, a period of time which allowed maximal inhibition of the enzyme activity (11). After the cultures had been washed extensively, they were shifted to fresh medium supplemented with only actinomycin D (2 μg/ml, a concentration inhibiting >90% of mRNA synthesis). This time corresponds to time 0 in Figs. 5 and 6.

Tyrosinase Assay. Tyrosinase activity in whole cell sonicates was determined as previously described (12). Briefly, monolayers were rinsed thrice with 0.05% disodium EDTA in calcium-free, magnesium-free phosphate-buffered saline (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-s 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 3,4-dihydroxy-L-phenylalanine in sodium phosphate buffer (26 μmol; pH 6.8), 2.5 μCi L-[^3H]tyrosine (specific activity, 52.5 Ci/mmol; New England Nuclear, Boston, MA), and 0.2 ml cell sonicate (final 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 trichloroacetic acid (10%, v/v). 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 LS-7000 counter (Beckman, Palo Alto, CA), with Aquasol (New England Nuclear) as the scintillant. Protein concentrations were determined by the method of Lowry et al. (13).

Hybridization Studies. Tyrosinase mRNA abundance was quantitated.
using the quick blot hybridization method of Gillespie and Bresser (14) using an [α-32P]dCTP-labeled 20-base pair probe which corresponds to base pairs 224-244 of a cloned B16 tyrosinase sequence similar to that reported previously (15). mRNAs were extracted from cultures using guanidine thiocyanate (14) and equal amounts of total cellular RNA were loaded onto membranes. Hybridization conditions were similar to those suggested (14).

RESULTS

Testosterone, 5α-dihydrotestosterone, and epitestosterone failed to influence tyrosinase activity in proliferating B16/C3 cells in culture (Fig. 1A). Compounds were present for a total of 19 h and none influenced the rate of proliferation. Imidazole (10 mM) induced the activity of that enzyme 2–3-fold (Fig. 1B), consistent with earlier reports (11). We have previously reported (10) that maximal induction of tyrosinase occurs by 19 h in proliferating cultures. When testosterone was added to the culture medium in addition to imidazole, the androgen blocked the increase in activity (Fig. 1B). In contrast, none of the other steroids influenced the imidazole induction. Androstenedione also failed to influence both basal and imidazole-induced activity (data not shown).

The inhibition by testosterone of imidazole induction was dose dependent. As Fig. 2 demonstrates, the threshold of effect occurred at a concentration of 1 nM, was half-maximal at ~3 nM, and was maximal at 10 nM. The addition of up to 10-fold higher concentrations of the hormone failed to inhibit enzyme activity any further.

To determine how rapidly testosterone could affect chemical induction, proliferating cultures which had received imidazole at time 0 were treated with the steroid at 18 or 23 h and assessed for tyrosinase activity. As Fig. 3 demonstrates, there was a rapid decline in enzyme activity regardless of when testosterone was added. Within 3–5 h of testosterone addition, activities fell to levels observed in noninduced cultures. In contrast, plates treated with imidazole alone exhibited increasing activity until 30 h (the end of the study), when levels were almost 4-fold.
above basal levels. This effect was rapidly reversible. Cultures receiving both imidazole and testosterone which were shifted to medium containing only imidazole manifested increasing enzyme activity within 1 h of the shift and levels which were near maximal within 6 h (Fig. 4).

An intact cell is apparently necessary to demonstrate imidazole stimulation of tyrosinase activity and the testosterone blockade thereof. Whole cell sonicates incubated with either imidazole or the steroid and incubated for up to 4 h failed to alter activity (Table 1). In another set of experiments, cells were harvested and sonicated in the presence of sodium molybdate (10 mm), under conditions which stabilize the androgen receptor (16). Results obtained were similar to those contained in Table 1 (data not shown).

Testosterone appears to block imidazole-induced tyrosinase expression at a pretranslational level (Fig. 5). Cultures were incubated with imidazole (10 mm), imidazole plus testosterone (50 nm), or diluent for 18 h. They were then shifted to medium containing cycloheximide (10 µg/ml) and the respective supplements for an additional 5 h (pretreatment). All cultures were then washed and shifted to medium containing actinomycin D (2 µg/ml) alone at the time designated "0" in Fig. 5. As that figure demonstrates, enzyme activity increases 3–4-fold in the 4 h following the shift to actinomycin D in cultures which had previously been treated with imidazole. In contrast, tyrosinase activity in cultures which had received imidazole and testosterone increased less than 2-fold and more closely resembled cultures not pretreated with imidazole. Under these treatment conditions, tyrosinase mRNA accumulated presumably during the pretreatment period, when translation was effectively blocked. Any accumulated message should be available for translational and posttranslational processing during the actinomycin D treatment. These results suggest that imidazole-pretreated cultures accumulated more translatable tyrosinase mRNA than did those which had received imidazole and testosterone or neither compound.

Fig. 6 demonstrates the results of an experiment designed to assess the effect of imidazole and testosterone on preformed, accumulated tyrosinase mRNA. Cultures were treated with imidazole or diluent for 18 h and allowed to proliferate actively. Fresh medium containing cycloheximide with or without imidazole was added for 5 h and then at the time designated 0 in Fig. 6, fresh medium containing actinomycin D and imidazole, testosterone, or diluent was added and cultures were harvested at the times indicated along the abscissa. Those cultures which had received imidazole manifested increasing tyrosinase activity regardless of whether testosterone was present, suggesting that the hormone failed to block the translation or posttranslational processing of preformed mRNA.

The increase in tyrosinase activity following imidazole exposure and the blockade of that induction by testosterone were accompanied by changes in the abundance of cellular RNA which hybridized to a 20-base pair tyrosinase probe. As Fig. 7 clearly demonstrates, imidazole markedly increased the abundance of hybridizable RNA compared to levels in cells receiving testosterone. The hybridization pattern in the testosterone-treated cultures was identical to that in untreated controls (data not shown). When testosterone was added to cultures receiving imidazole, the steroid completely blocked the increase in tyrosinase mRNA abundance.

**DISCUSSION**

These results demonstrate that testosterone can block imidazole induction of tyrosinase rapidly at concentrations which have physiological relevance. This inhibitory effect apparently involves de novo protein synthesis.

There exists a priori evidence to suspect steroid hormones as significant regulators of melanoma behavior and metabolism. High affinity binding for androgens, estrogens, progesterone, and glucocorticoids has been demonstrated in human melanoma (8, 17–19), yet there is generally a lack of clinical response to endocrine therapy (8). Thus, the binding activities may represent an association with inactive receptors or with unrelated proteins.

We found that testosterone but none of the other related steroids influences the induction of tyrosinase activity in B16 cells in vitro. The lack of response to 5α-dihydrotestosterone suggests that a 5α reductive pathway is not involved in the mediation of this effect, consistent with androgen action in normal dermal tissue not derived from the genital anlage (20). Whether any alternative biotransformation of testosterone (3α reduction or aromatization to estradiol, as examples) occurs in

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**Table 1 Effects of testosterone and imidazole on tyrosinase activity in cell sonicates**

<table>
<thead>
<tr>
<th>Compound</th>
<th>0 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent</td>
<td>10.87 ± 0.23</td>
<td>10.89 ± 0.17</td>
<td>9.48 ± 0.16</td>
<td>7.87 ± 0.17</td>
<td>6.27 ± 0.12</td>
<td>5.02 ± 0.07</td>
</tr>
<tr>
<td>Imidazole</td>
<td>10.87 ± 0.23</td>
<td>10.43 ± 0.12</td>
<td>9.69 ± 0.14</td>
<td>7.66 ± 0.24</td>
<td>6.40 ± 0.05</td>
<td>5.11 ± 0.23</td>
</tr>
<tr>
<td>Testosterone</td>
<td>10.87 ± 0.23</td>
<td>10.53 ± 0.14</td>
<td>9.52 ± 0.23</td>
<td>7.39 ± 0.10</td>
<td>6.18 ± 0.16</td>
<td>5.21 ± 0.19</td>
</tr>
</tbody>
</table>

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Fig. 5. Protein and RNA synthesis inhibitor effects on imidazole and testosterone regulation of tyrosinase activity. Proliferating cultures were incubated for 18 h with imidazole (10 μM; △), testosterone (50 μM; ○), and imidazole plus testosterone (10 μM; ●) or diluent (●). They were then shifted to medium with these respective supplements as well as cycloheximide (10 μg/ml) for 5 h. All plates were then shifted to medium to which only actinomycin D (2 μg/ml) was added (time 0) and harvested at the times indicated along the abscissa. These concentrations of inhibitors blocked >90% of protein and RNA syntheses, respectively. Points, means ± range (bars) of tyrosinase activity in duplicate cultures.

Fig. 6. Effect of testosterone on the translation of preformed tyrosinase mRNA. Cultures were treated for 18 h with imidazole (10 μM; ○, △) or diluent (▲) and then shifted to medium containing these respective supplements and cycloheximide (10 μg/ml) as well as cycloheximide (10 μg/ml). At time 0, cultures were shifted to medium containing actinomycin D plus either imidazole (●), testosterone (A), or diluent (▲) and were harvested at the times indicated along the abscissa. These concentrations of inhibitors blocked >90% of protein and RNA syntheses, respectively. Points, means ± range (bars) of tyrosinase activity in duplicate cultures.

these cells and precedes the blockade of tyrosinase induction is not known. Our recent finding (21) that estradiol can also block the induction by imidazole raises the possibility that these effects might be mediated through the estrogen receptor.

Imidazole induction of tyrosinase in B16/C3 cells in vitro is independent of increased intracellular cyclic AMP content (11), and unlike the action of MSH on these cells (22) it involves de novo synthesis of enzyme. This compound can alter gene transcription at specific promoter sites in prokaryotic cells as a "metabolite gene regulator" (23–25). From the data presented here and those recently reported (7, 21), it would appear that 3,3',5-triiodothyronine, estradiol, and testosterone can influence significantly the chemical induction of tyrosinase. While the effects of all three classes of hormone appear to be pretranslational and independent of mRNA destabilization, whether transcription is affected is uncertain. Preliminary hybridization studies with the 20-base pair deoxyoligonucleotide probe confirm that imidazole increases the cellular content of mRNA for tyrosinase and that both 3,3',5-triiodothyronine and estradiol block this induction, similar to the effect of testosterone reported here.

Chemical-induced melanogenesis in B16/C3 cells in vitro is thus apparently under multihormonal control, consistent with a variety of other cellular events including α2c-globulin gene expression (26, 27), growth hormone synthesis (28), and hyaluronate synthesis (29). This culture model may be ideal for studying the nature of hormone-hormone interactions at the level of gene expression and for examining the mechanisms involved in hormonal conditioning of the cellular response to chemicals, as has been described in the liver (30, 31).

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REFERENCES


4 J. T. Warren, Jr. T. J. Smith, and E. L. Kline, manuscript in preparation.
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