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Effects of Estrogens on Human Melanocytes *In vitro*

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Subjects with elevated serum estrogen concentrations, such as those who are pregnant or ingesting estrogen-containing contraceptive medication, may develop increased skin pigmentation. As little information is available on the mechanism(s) underlying this relationship, the *in vitro* effects of estrogens on melanocytes cultured from normal human skin were examined. Physiological concentrations of 17β-estradiol (10^{-11} to 10^{-9} M) significantly increased the activity of tyrosinase in melanocytes from 15 of 23 subjects. The observed increases ranged from 1.2- to 2.4-fold. Melanin synthesis, which correlated with tyrosinase activity ($r = 0.98$, $P < 0.001$) was increased to a similar extent. Melanin extrusion was also increased by 17β-estradiol (10^{-9} M). The estrogens, estriol (10^{-9} M) and estrone (10^{-9} M) stimulated tyrosinase activity and melanin extrusion to a lesser extent than 17β-estradiol. The analogue 17α-estradiol (10^{-9} M) was shown to have effects on melanocyte tyrosinase activity and melanin extrusion that were equivalent to those of 17β-estradiol. The pure estrogen antagonist ICI 164384 (10^{-6} M) also stimulated tyrosinase activity. Cycloheximide (50 μg/ml) inhibited 17β-estradiol-induced tyrosinase stimulation ($P < 0.001$). These results indicate that several aspects of melanocyte function respond directly to estrogenic stimulation. The equivalent effects of the 17α-analogue and a "pure" anti-estrogen suggest that the 17β-estradiol response may be mediated through a non-classical mechanism which is similar to that described in other tissues of neural crest origin.

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INTRODUCTION

High estrogen levels have been reported to be associated with an increase in skin pigmentation [1]. This effect is particularly evident in pregnancy and in individuals ingesting estrogen-containing oral contraceptives, though many factors have been implicated in the development of melasma including genetic predisposition, elevated progesterone or thyroid hormone concentrations, and solar exposure [2, 3]. We have previously reported that physiological concentrations of 17β-estradiol enhanced tyrosinase activity (E.C.1.14.18.1) of cultured melanocytes from normal human skin [4]. In the current study, the effects of 17β-estradiol and other estrogens on tyrosinase activity were further examined, as well as the effect of 17β-estradiol on melanin synthesis and melanin extrusion, a process whereby formed melanin is secreted into the matrix surrounding the melanocyte [5]. An attempt was made to determine whether the observed effects were mediated through the classical estrogen receptor [6], using the 17α-analogue of 17β-estradiol and two anti-estrogenic compounds.

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EXPERIMENTAL

Materials

All chemicals, including steroid hormones, were obtained from Sigma Chemical Co. (St Louis, MO) except for ICI 164384 which was provided by Dr A. Wakeling (Imperial Chemical Industries, Macclesfield, England). [^{3}H]- and [^{14}C]tyrosine and [^{3}H]17β-estradiol were obtained from Amersham Australia Pty Ltd (Sydney, Australia). Eagles minimum essential medium with Earle's salts (EMEM), Iscove's medium and foetal calf serum (FCS) were obtained from Flow Laboratories Inc. (Sydney, Australia). Plastic culture plates coated with bovine corneal endothelium-derived extracellular matrix (ECM) were obtained from International Biotechnologies Ltd (Jerusalem, Israel), Wood Scientific Ltd. (Sydney, Australia) and Cell Dynamics (Sydney, Australia).

Culture conditions

Human melanocytes were grown from neonatal foreskin samples according to the method of Eisinger and Marko [7] with minor modifications as described previously [8]. The cultures were maintained on uncoated plastic in EMEM with 5% FCS (v/v), pH 7.2, containing phorbol 12-myristate 13-acetate (PMA, 20 nM), cholera toxin (CT, 10 nM), penicillin (60 μg/ml) and streptomycin (100 μg/ml). The cells used in this study had been cultured for 1–4 months.

Experimental conditions

Melanocytes near confluence were detached from the culture flasks with trypsin (0.05%) and ethylenediaminetetraacetic acid (EDTA, 0.02%) in phosphate-buffered saline and seeded at a density of 2×10^4 cells/cm^2 on to ECM-coated 24 well plates unless otherwise stated. Twenty-four to 48 h after seeding, the growth medium was replaced with EMEM containing 5% FCS as the only mitogen. After overnight incubation, this medium was changed to EMEM containing 2% FCS, or Iscove's medium containing bovine serum albumin (0.1% v/v), soybean lecithin (80 μg/ml) and transferrin (30 μg/ml). Melanocytes were then treated with the estrogens or other agents, added 3 times/day for up to 3 days and once on the day of assay, with daily changes of experimental medium.

Tyrosinase activity

Tyrosinase activity was measured by a modification of the method of Pomerantz [9], as described previously [8]. This method measures the amount of [^{3}H]H_2O released from [^{3}H]tyrosine as a result of the tyrosine hydroxylase activity of the tyrosinase enzyme. All values were corrected for non-enzymic hydroxylation by subtracting the amount of [^{3}H]H_2O formed by cell extracts containing 1 mM phenylthiourea (PTU), a specific inhibitor of the tyrosinase enzyme [10]. Cell numbers from replicate wells were counted in a Coulter Counter or haemocytometer.

Melanin synthesis

[^{14}C]Tyrosine incorporation into TCA-precipitable melanin by live, whole cells was measured by a modification of the method of Chen and Chavin [11], as described previously [8]. The counts obtained in PTU-inhibited controls were subtracted from all values obtained. Under these conditions, melanin formation was linear with cell number, with substrate concentration up to 8.0 μCi/ml, and with time up to 6 h.

Melanin extrusion

The extrusion of [^{14}C]melanin into the medium was measured by a slight modification of the melanin synthesis method. After 8 h in the presence or absence of estrogens, [^{14}C]tyrosine (2 μCi/ml) and dihydroxyphenylalanine (DOPA, 10 μg/ml) were added to the wells, which were incubated for a further 16 h. The medium was then removed and the cells washed and lysed with Triton X-100 (0.05% v/v) in phosphate buffered saline. Melanin in the medium or cell extracts was precipitated with trichloroacetic acid as described previously [8]. Correction for non-specific precipitation of counts was made by subtracting the counts obtained from cells incubated in the presence of PTU.

Estrogen receptor assay

The estrogen receptor immunocytochemical assay (ER-ICA) kit was obtained from Abbott Labs (North Chicago, II). Melanocytes and frozen sections of a human breast cancer specimen were fixed and incubated with anti-estrogen receptor monoclonal antibody according to the method described by Bilous *et al.* [12]. In an attempt to induce the estrogen receptor, melanocytes were also preincubated with 17β-estradiol (10^{-10} to 10^{-8} M) for 24 h before staining with antibody. Uptake binding studies of [^{3}H]17β-estradiol were carried out in whole cells as described previously for another steroid, 1,25-dihydroxyvitamin D_3 [4].

Statistical analysis

Statistical comparisons of the data were made using Student's t-test for unpaired observations and by Analysis of Variance where appropriate. Where results were expressed as ratios such as tyrosinase activity/number of cells, the standard deviations of the ratios were calculated using the method of Colquhoun [13] before application of the t-test.

RESULTS

Incubation with 17β-estradiol significantly increased tyrosinase activity in melanocyte strains from 15 of 23 donors. In melanocytes from those subjects responsive to estradiol, the maximal increases ranged from 1.2- to 2.4-fold with a mean increase of 1.6 ± 0.1 (SEM)-fold. The dose–response relationship for 3 donors is shown in Fig. 1. When tyrosinase activity was unaltered by 17β-estradiol treatment, there was also no change in the cell numbers (data not shown). In contrast, significant increases in tyrosinase activity were accompanied by decreases in cell numbers. There was, however, no correlation between the magnitude of the increase in tyrosinase activity and the magnitude of the corresponding decrease in cell numbers ($n = 12$, $r = 0.255$, $P > 0.3$). The stimulatory effect of 17β-estradiol was only seen when melanocytes were plated on ECM and not when they were maintained on uncoated plastic.

Tyrosinase activity and melanin synthesis were positively correlated under basal conditions [8], and when tyrosinase activity and melanin synthesis were compared before and after treatment with various concentrations of 17β-estradiol, there was a strong positive correlation between these parameters ($r = 0.98$,

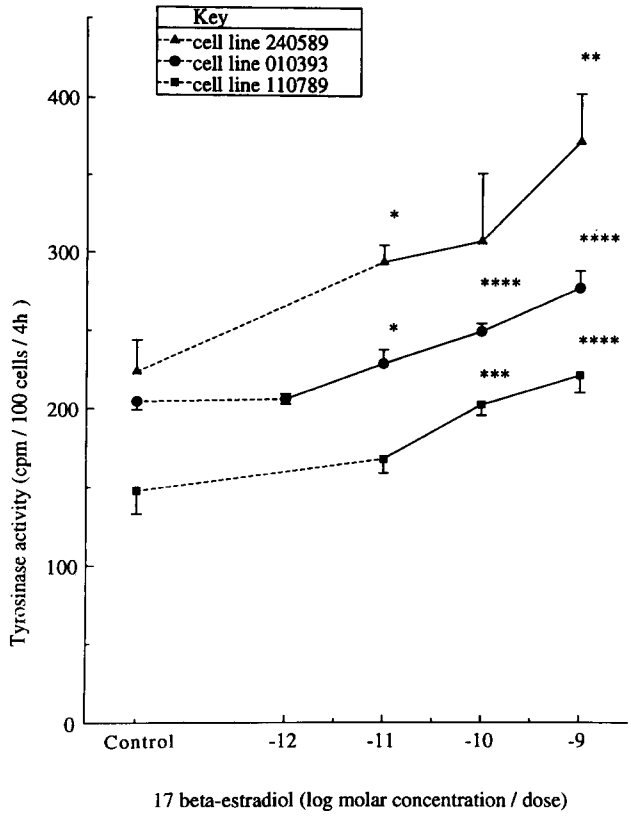

Fig. 1. Linear dose–response relationship between 17β-estradiol and melanocyte tyrosinase activity. Data are the mean ± SEM (bars) of triplicate determinations. Significantly different from control values, *$P < 0.05$; **$P < 0.025$; ***$P < 0.01$; ****$P < 0.0025$.

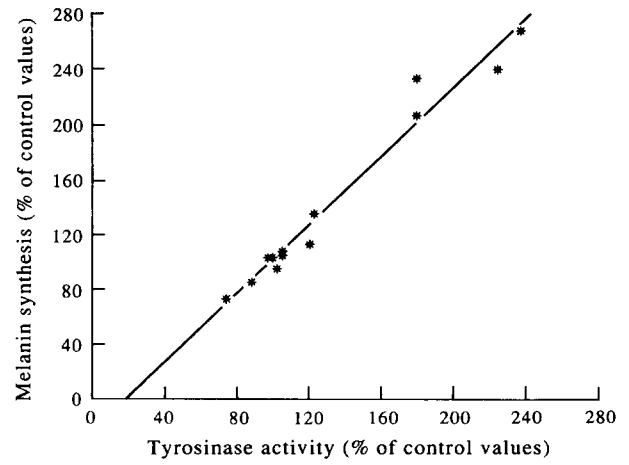

Fig. 2. Linear regression analysis of tyrosinase activity versus melanin synthesis after incubation with 17β-estradiol (10^{-12} to 10^{-9} M). The responses of melanocyte strains from 3 different donors to 17β-estradiol were calculated as percentages of control values. Data are the mean of triplicate determinations for each parameter.

Table 1. Effects of estrogens (10^{-9} M) on melanocyte tyrosinase activity cell numbers and melanin extrusion (mean ± SEM)

Estrogen	Tyrosinase activity (cpm/100 cells/4 h)	Cells/well (in duplicate)	Melanin extruded (cpm/100 cells/16 h)
Control	548 ± 21	29774	6.4 ± 2.0
β-estradiol	1015 ± 60***	16350	22.0 ± 0.1***
Estrone	615 ± 29*	26700	8.7 ± 1.1
Estriol	733 ± 58**	24720	3.8 ± 2.4

Significantly different from control values; *$P < 0.02$; **$P < 0.01$; ***$P < 0.001$.

$P < 0.001$; Fig. 2). In addition to increased melanogenesis, melanin extrusion into the medium was also enhanced by 17β-estradiol. The relative effects of 17β-estradiol, estrone and estradiol on tyrosinase activity, cell numbers and melanin extrusion are shown in Table 1. Preliminary results indicate that the catechol estrogen 2-hydroxyestradiol at 10^{-9} M increased tyrosinase activity and melanin synthesis by 1.13 ± 0.04 (SEM)-fold ($P < 0.05$) and 1.25 ± 0.04 (SEM)-fold ($P < 0.05$), respectively without affecting cell numbers.

The presence of cycloheximide, in a dose shown to inhibit [^{3}H] leucine incorporation [14], abolished the stimulatory effect of 17β-estradiol on tyrosinase activity [Figure 3(A)]. Cycloheximide markedly increased melanin extrusion into the medium in otherwise untreated cells from 754 ± 23 to 3192 ± 540 cpm/10^4 cells/16 h ($P < 0.001$). No radiolabelled melanin was detected in either control or estradiol-treated melanocytes in the presence of cycloheximide.

Incubation of melanocytes with the pure estrogen antagonist ICI 164384 (10^{-6} M) also increased tyrosinase activity [Fig. 3(B)]. When both 17β-estradiol and ICI 164384 were preincubated with the cells, tyrosinase activity was no higher than with either agent alone. In each of three experiments, the stimulation of tyrosinase by 17β-estradiol was mimicked by 17α-estradiol (Table 2). In an experiment where treatment with 10^{-9} M 17β-estradiol increased melanin extrusion 3.4 ± 0.01 (SEM)-fold ($P < 0.001$), treatment with 10^{-9} M 17α-estradiol also increased melanin extrusion 2.0 ± 0.3 (SEM)-fold ($P < 0.001$). Cell numbers were similar after both treatments. Nuclear magnetic resonance studies indicated that the spectra of the 17β- and 17α-estradiol compounds were consistent with the expected stereochemical configurations (data not shown).

No estrogen receptors were found using the Abbott monoclonal antibody estrogen receptor kit, which showed clearly positive results with breast cancer cells. Since it has been proposed that apparent estrogen binding to pigment cells may be an artefact due to [^{3}H]H_2O release from tritiated estradiol by tyrosinase [29], specific binding of [^{3}H]17β-estradiol was tested in the presence of the tyrosinase inhibitor PTU. Specific binding was not demonstrated under these conditions.

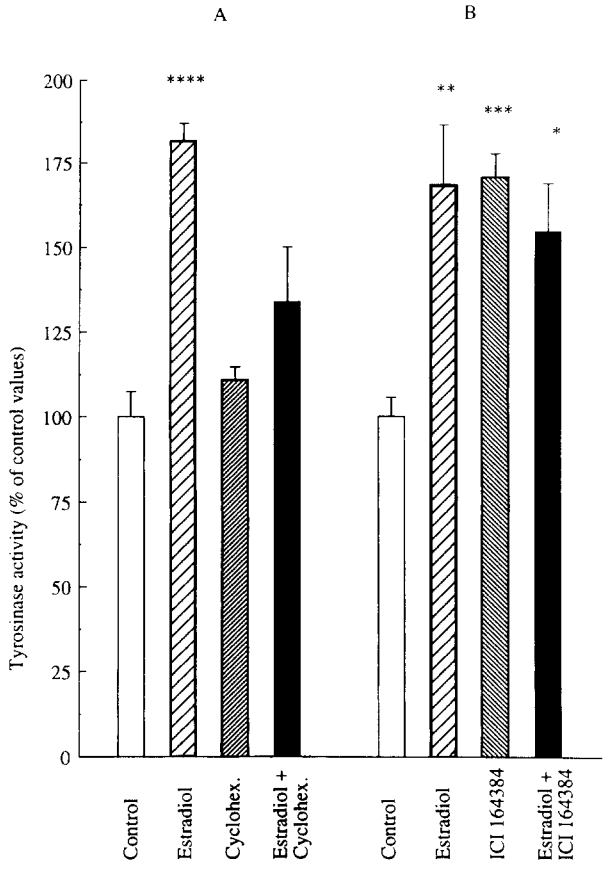

Fig. 3. (A) Tyrosinase activity in melanocytes after treatment with 17β-estradiol (10^{-9} M) in the presence or absence of cycloheximide (50 μg/ml) (B) Tyrosinase activity in melanocytes after treatment with 17β-estradiol (10^{-9} M) in the presence or absence of ICI 164384 (10^{-6} M). Data are the mean ± SEM (bar) of triplicate determinations. Significantly different from control values, *$P < 0.025$; **$P < 0.01$; ***$P < 0.0025$; ****$P < 0.001$.

DISCUSSION

Skin pigmentation *in vivo* is determined by genetic, environmental, local and endocrine factors which influence both melanin synthesis within each melanocyte and the distribution of melanin throughout the epidermis [15]. In a previous report, we demonstrated that 17β-estradiol increased melanocyte tyrosinase activity *in vitro* [4]. These studies have now been extended to show that responsiveness to 17β-estradiol is donor-dependent and that melanin synthesis and extrusion is also increased by the hormone.

Table 2. Comparison between the effects of 17β-estradiol (10^{-9} M) and 17α-estradiol (10^{-9} M) on melanocyte tyrosinase activity in cpm/100 cells/4 h (mean ± SEM)

	Control	β-Estradiol	α-Estradiol
Experiment 1	548 ± 21	1015 ± 60**	988 ± 78**
Experiment 2	671 ± 12	965 ± 68*	900 ± 57*
Experiment 3	1079 ± 42	1473 ± 28**	1750 ± 47**

Significantly different from control values; *$P < 0.005$; **$P < 0.001$.

Significant stimulation of tyrosinase activity by 17β-estradiol was seen in 65% of donors. Melanocyte strains from 8 out of 23 donors showed no significant alteration in tyrosinase activity in response to the hormone. Despite the reproducibility of the stimulatory effect of 17β-estradiol in most donors, both the basal tyrosinase activity and the degree of stimulation varied noticeably in melanocytes from different donors, as would be expected from *in vivo* studies. Estrogen responsiveness could not be predicted by basal tyrosinase activity or plating/proliferative capacity as assessed by cell numbers in vehicle-treated wells. A response to hormones was only observed in melanocytes plated on ECM in the absence of PMA and CT. The ECM is understood to resemble the basal lamina of skin where melanocytes normally reside, and it maintains melanocytes in a relatively quiescent but highly differentiated state in the absence of mitogens which might otherwise interfere with hormone signal processing [8]. The authors are also aware that, whilst most studies on melanocytes have depended on cells from neonatal skin, some caution must be exercised in extrapolating any of the results to the adult situation.

The strong correlation between tyrosinase activity and melanin synthesis in cells treated with 17β-estradiol is not surprising since melanin content in mammalian pigment cells is quantitatively dependent on tyrosinase activity [4, 16, 17]. The subsequent transfer of melanin from melanocytes to keratinocytes is believed to occur by two processes. The first process involves the contact of a melanocytic dendrite with the cell membrane of a keratinocyte, which then pinches off the dendritic tip containing melanosomes and encloses it within a phagocytic vacuole prior to melanin dispersion [15]. The second process involves the extracellular secretion of melanosomes from melanocytic dendrites, and their subsequent engulfment by the neighbouring keratinocytes [5]. Our studies with cycloheximide, which inhibited the increase in tyrosinase induced by 17β-estradiol and caused almost complete melanin extrusion under basal conditions, is consistent with the suggestion that melanin extrusion may be tonically inhibited by a protein with a relatively short half-life. That 17β-estradiol directly enhances melanogenesis and melanin extrusion in melanocytes *in vitro* provides some evidence to support a role for estrogens in modulating skin pigmentation and in the development of melasma.

The biological effects of estrogens in classical target tissues, such as the uterus and breast, are believed to be mediated by the binding of estrogen to an intracellular steroid receptor protein and the subsequent alteration of gene expression [6]. The affinities of estrone and estradiol for the estrogen receptor in uterine tissues are approx. 20 and 5%, respectively of that of 17β-estradiol [6]. The reduced activity of estrone and estriol

on the tyrosinase activity of melanocytes, together with the abolition of the estrogenic effects in the presence of cycloheximide, are consistent with a classical estrogen receptor-mediated mechanism, but are also consistent with other mechanisms. However, despite the reports that ICI 164384 is a purer anti-estrogen than tamoxifen and has little agonist activity [18–20], a stimulatory effect of this agent was observed in our melanocyte system.

More strikingly, the concept of a classic receptor-mediated mechanism is inconsistent with our observation that the effects of 17α-estradiol were very similar to those of 17β-estradiol. Similar effects of 17α and 17β-estradiol have previously been reported in the estrogen receptor negative MDA-MB-330 human breast cancer cell line [21]. While it is possible that 17α-estradiol can be isomerized to 17β-estradiol *in vitro* [22], other experiments indicate that in some tissues of neural crest origin the phenolic 17β-estradiol can be converted by 2- and 4-hydroxylase enzymes to compounds which resemble catecholamines [23, 24]. These catechol estrogens exhibit reduced affinity for the estrogen receptor [25] but, by virtue of their catechol structure, have biological activity in several *in vitro* systems [26, 27]. Moreover, 17α-estradiol can be converted to catechol estrogens in a similar manner to 17β-estradiol [28], and is equipotent to 17β-estradiol in inhibiting tyrosine hydroxylase and catechol-*o*-methyltransferase activity in rabbit and rat brain *in vitro* [25, 28]. The relatively modest effects of 2-hydroxyestradiol in this preliminary study may be due to the likely rapid metabolism of this metabolite when it is provided as single doses rather than being synthesized over a period of time from its substrate [29].

Evidence for the presence of catecholamine biosynthetic pathways within melanoma cells has been presented by McEwan and Parsons [30]. Furthermore, it has been shown that tyrosinase can utilize estradiol as a substrate and hydroxylate it to a catechol-like compound [31]. This may be possible because the A ring of estradiol has structural resemblance to the phenolic group of tyrosine and thus may substitute for tyrosine. The preliminary observation in our laboratory of melanocyte tyrosinase stimulation by the catechol estrogen 2-hydroxyestradiol suggests the possible regulation of tyrosinase by catechol-like compounds. However, the precise mechanism by which estrogens may affect melanocyte function through catechol intermediates is as yet unknown.

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