Downregulation of Tyrosinase Activity in Human Melanocyte Cell Cultures by Yohimbine

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Treatment of human melanocyte cell cultures with the alpha-2 adrenergic receptor antagonist yohimbine results in a marked down-regulation of tyrosinase activity. A 30% decrease occurs within 12 h of exposure of cells to yohimbine (100 μM), and by 48 h tyrosinase activity in treated melanocytes is less than a fifth that of control cultures. The inhibition is dose dependent and occurs in human melanocytes derived from either black or white skin types, and also in mouse melanoma cells. The yohimbine-induced decrease in tyrosinase activity is reversible, with enzyme levels returning to 90% of control values 48 h after removal of drug. Although tyrosinase activity is markedly suppressed by yohimbine, the compound has no effect on cell proliferation, cellular translation, or DNA synthesis. Treatment of melanocyte cultures with yohimbine blocks the increase in tyrosinase activity by either 3-isobutyl-1-methylxanthine, dibutyryl cAMP, or forskolin. Results of cAMP immunoassays, show that intracellular levels of the cyclic nucleotide are unaffected in cells treated with yohimbine. Tyrosinase inhibition by yohimbine does not involve a decrease in substrate availability since tyrosine uptake studies show that yohimbine has no effect on the amount of tyrosine entering the cell. Incubation of a melanosome-enriched fraction with yohimbine does not cause a lowering of tyrosinase activity, suggesting that an intact cell is required for yohimbine action. In addition, tyrosinase extracts show no reduction in activity when incubated directly with yohimbine, indicating that the drug does not act as a direct inhibitor of the enzyme. Finally, results of western immunoblotting show that yohimbine does not significantly lower the amount of tyrosinase protein in human melanocytes. These findings suggest that yohimbine acts through an as yet unidentified signaling pathway to lower the catalytic activity of pre-existing tyrosinase molecules present in melanocytes. ***Key words: adrenergic receptors/cAMP/forskolin/melanosomes. J Invest Dermatol 114:268–276, 2000***

Many studies have shown that mammalian melanocytes, including human melanocytes, will respond to various hormones and other signaling molecules including α-melanocyte stimulating hormone (Wong and Pawelek, 1973; Iwata *et al*, 1990; Levin *et al*, 1991; Abdel-Malek *et al*, 1995; McLeod *et al*, 1995), prostaglandins (Abdel-Malek *et al*, 1987; Fuller *et al*, 1993), endothelin 1 (Yada *et al*, 1991), and nitric oxide (Romero-Graillet *et al*, 1997) by demonstrating an increase in tyrosinase activity and melanin synthesis. A role for cAMP in mediating the stimulation of tyrosinase activity in mouse and human melanocytes and melanoma has been amply demonstrated from studies with either dibutyryl cAMP (Kreider *et al*, 1973; Hoganson *et al*, 1989; Maeda *et al*, 1992) or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (Halaban *et al*, 1983, 1984). Although the mechanism of cAMP stimulation of tyrosinase in mouse melanoma cells has been shown to involve an increase in tyrosinase gene transcription (Bertolotto *et al*, 1996; Rungta *et al*, 1996), it is not clear, at present, that a similar pathway regulates melanogenesis in human melanocytes. Results of studies to examine tyrosinase expression in different racial skin types have shown that, although tyrosinase activity in melanocytes derived from black skin may be six to eight times higher than in melanocytes from white skinned individuals, tyrosinase mRNA and protein levels are equivalent (Naeyaert *et al*, 1991; Fuller *et al*, 1993; Iozumi *et al*, 1993). Thus, in humans, the level of tyrosinase activity in melanocytes does not simply reflect the abundance of the enzyme or the activity of the tyrosinase gene.

In contrast to the large number of studies that have chronicled the stimulatory effects of a number of hormones on pigmentation in human melanocytes, very few studies have reported on hormones or other signaling molecules that lower tyrosinase activity. The cytokines interleukin-1 and interleukin-6 have been shown to have a slight inhibitory effect on tyrosinase activity (Swope *et al*, 1991), and conflicting reports suggest that retinoic acid may lower tyrosinase activity in human melanocytes (Orlow *et al*, 1990; Talwar *et al*, 1993). Although there have been some reports of endogenous tyrosinase inhibitors in melanoma cells (Kameyama *et al*, 1993), most reports of tyrosinase inhibition in melanocytes have come from studies on compounds that act directly on the enzyme as either competitive or uncompetitive inhibitors (Palumbo *et al*, 1991; Chakraborty *et al*, 1998).

Studies in our laboratory have focused on characterizing the signaling pathways that regulate tyrosinase activity in human

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Abbreviations: MSH, α-melanocyte stimulating hormone; dbcAMP, dibutyryl cyclic 3′,5′-adenosine monophosphate.

melanocytes. Because of the neural crest origin of melanocytes, it seems likely that receptors for some neurotransmitters may be expressed on melanocytes and that these compounds may regulate tyrosinase activity and growth in these cells. Studies on lower vertebrates, including Rana pipiens and Anolis, have shown that pigment dispersion (skin darkening) results from an activation of beta adrenergic receptors and an elevation in cAMP (Goldman and Hadley, 1969a). Conversely, pigment aggregation in melanophores (skin lightening) is mediated through alpha-2 adrenergic receptors. Further, treatment of melanophores with yohimbine prevents the aggregation caused by alpha-2 agonists (Goldman and Hadley, 1969b). In mammalian melanocytes, receptor binding assays have provided evidence for beta adrenergic receptors on human melanoma cells (Steinkraus *et al*, 1990). Moreover, treatment of human melanoma cells with beta adrenergic receptor agonists results in an increase in tyrosinase activity (Karg *et al*, 1989). We have examined the effect of various alpha and beta adrenergic agonists and antagonists on tyrosinase activity in human melanocyte cell cultures. Because the alpha-2 adrenergic receptor antagonist yohimbine acts to block the lowering of cAMP by alpha-2 adrenoceptor agonists, we carried out studies to determine whether yohimbine might increase tyrosinase activity in human melanocytes. Surprisingly, we found that yohimbine and structurally related compounds actually caused a pronounced lowering of tyrosinase activity. We report here on various aspects of this inhibitory effect, and conclude that yohimbine acts through an as yet unidentified signaling pathway to reduce the catalytic activity of pre-existing tyrosinase molecules present in melanosomes.

MATERIALS AND METHODS

Reagents and supplies L-[ring-3,5-^{3}H]tyrosine (46.7 Ci per mmol) was purchased from NEN Life Science Products (Boston, MA). Fetal bovine serum and horse serum (defined equine serum) were purchased from Hy Clone Laboratories (Logan, UT). Nutrient mixture Ham's F-10 was obtained from Mediatech (Washington, DC). Bovine pituitary extract and fibroblast growth factor came from Collaborative Biomedical Products (Becton Dickinson, Bedford, MA). The adrenergic agonists and antagonists Bu224, Efaroxan, Idazoxan, B-HT 933, p-aminoclonidine, and rauwolscine were purchased from Research Biochemicals International (Natick, MA). All other chemicals, including bovine serum albumin, yohimbine, corynanthine, cycloheximide, geneticin, penicillin-G, dihydroxyphenylalanine (L-DOPA), Hank's balanced salt solution, L-tyrosine, IBMX, phenylmethylsulfonyl fluoride (PMSF), streptomycin, Triton X-100, and 12-O-tetradecanoylphorbol 13-acetate (TPA) were purchased from Sigma (St. Louis, MO).

Cell cultures Human melanocyte cultures were derived from foreskin as described elsewhere (Iozumi *et al*, 1993). Cell strains were grown in Ham's F-10 nutrient medium containing 10% horse serum, 5% fetal bovine serum, 32 nM TPA, 2 ng basic fibroblast growth factor per ml, 5 μg insulin per ml, 200 units penicillin per ml, and 200 μg streptomycin per ml. The Cloudman S-91 mouse melanoma line (CCL53.1, American Type Culture Collection, Rockville, MD) used in these studies was maintained in Ham's F-10 nutrient medium containing 10% horse serum, penicillin (100 units per ml), and streptomycin (100 μg per ml). Cell counts were made with a Coulter Z-3 cell counter (Beckman Coulter, Ontario, Canada).

***In situ* tyrosinase assays** Tyrosinase activity in melanocyte cell cultures *in situ* was determined as described previously (Fuller *et al*, 1987). Cells were seeded into 60 mm culture dishes at 3×10^5 cells per dish and allowed to attach overnight. The medium was then exchanged for growth medium supplemented, where indicated, with compounds under investigation. Medium was changed daily, and 24 h before the termination of the experiment medium was supplemented with 2 μCi [^{3}H]tyrosine per ml. At the end of experiments, cells were counted, and the radiolabeled medium was assayed for the presence of ^3H_2O using the charcoal absorption method (Pomerantz, 1966). For long-term studies fresh labeled medium was added to the flasks at 24 h intervals and the amount of tritiated water produced during this time was determined.

Measurement of tyrosinase activity in cell homogenates, solubilized tyrosinase, and melanosome-enriched fractions Tyrosinase activity in cell homogenates was determined by sonicating cell pellets in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1 mM PMSF and 1% Triton X-100, and then incubating 50 μl aliquots in 1.0 ml of a reaction mixture containing 0.1 mM tyrosine, 2 μCi [^{3}H]tyrosine per ml, 0.1 mM L-DOPA, and 0.1 mM PMSF in 0.1 M sodium phosphate buffer, pH 6.8. After a 3 h incubation at 37°C, reactions were terminated by the addition of 1 ml of charcoal (10% wt/vol in 0.1N HCl). Samples were centrifuged at $2000 \times g$ for 10 min, and the supernatants were removed and added to scintillation fluid (Scintiverse BD, Fisher Scientific, Pittsburgh, PA) for counting in a Packard 2300TR liquid scintillation counter.

To prepare solubilized tyrosinase, melanocytes (5×10^6 cells) were removed from stock culture flasks and centrifuged at $100 \times g$ for 10 min, and the cell pellet was sonicated in 0.5 ml of 0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and 0.1 mM PMSF. After a 1 h incubation to release tyrosinase from the melanosome membrane, the resulting detergent extract was centrifuged at $40{,}000 \times g$ for 20 min at 4°C. The resulting supernatant contained 95% of all the cellular tyrosinase activity.

Tyrosinase activity in melanosomes was determined by homogenizing melanocytes (10^7 cells) with a Dounce homogenizer in 1.0 ml of Ham's nutrient medium containing 0.1 mM PMSF. Homogenates were centrifuged at $1000 \times g$ for 15 min to pellet nuclei and unbroken cells, and the supernatant was centrifuged at $40{,}000 \times g$ for 20 min at 4°C to obtain a melanosome-enriched pellet. This was resuspended in 0.5 ml of Ham's F-10 nutrient medium containing 0.1 mM PMSF, and used for experiments.

Measurement of DOPA oxidase activity in skin organ cultures Fresh human foreskins were placed in organ culture as previously described (Iwata *et al*, 1990). Cultures were maintained in Iscove's modified Dulbecco's medium containing 10% horse serum, 5% fetal bovine serum, 100 units of penicillin per ml, and 100 μg of streptomycin per ml. To determine the effect of yohimbine on tyrosinase activity, the organ culture medium was supplemented with 100 μM yohimbine. Experimental medium was replaced every 24 h. At the termination of the experiment, skin sections were removed from dishes, placed in Tissue-Tek OCT Compound (Sakura Biochemical, Tokyo, Japan), and quick frozen in liquid nitrogen. Cryostat sections (6 μm thick) were placed on microscope slides (six per slide), fixed in 2% formaldehyde for 4 h at 4°C, and then stained with either hematoxylin and eosin to monitor skin integrity or L-DOPA to visualize tyrosinase activity. DOPA reactions were carried out by incubating skin sections in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.1% L-DOPA for 4 h at 37°C. Following this incubation, sections were passed through water, alcohol, and xylenes and mounted with Permount. Sections were examined at 200× using an Olympus BH-2 microscope equipped with a Polaroid MicroCam camera. Photomicrographs were taken with Polaroid 331 video imaging film. The amount of melanin produced from DOPA by tyrosinase during the 4 h incubation was quantitated with an Alpha Innotech 2000 digital imaging system.

cAMP assays To measure intracellular cAMP levels, an enzyme immunoassay kit (Biotrak, Amersham Life Sciences, Arlington Heights, IL) was used according to the instructions by the manufacturer. Cells were seeded into 25 cm^2 flasks at 10^6 cells per flask and allowed to attach overnight. Compounds to be tested were added for the times indicated in the figure captions, and after this incubation the medium was removed and the cells were washed gently with phosphate-buffered saline. Seven percent perchloric acid was added to the flasks at 4°C for 2 h. The acid supernatants were removed from the flasks and the pH was raised by the addition of 10 N KOH. Tubes were then centrifuged and the supernatants were diluted to 0.05 M acetate buffer (pH 5.8; assay buffer) by the addition of 0.5 M acetate buffer. The amount of cAMP in the supernatants was determined as described in the kit.

Measurement of tyrosine uptake Tyrosine uptake into human melanocytes was determined as described elsewhere (Fuller *et al*, 1987). Briefly, 60 mm culture dishes (Falcon) were seeded with 5×10^5 cells per dish and allowed to attach overnight. Yohimbine (100 μM) was then added to the appropriate dishes for 48 h. At the end of this incubation, dishes were rinsed twice with serum-free nutrient mixture Ham's F-10 and 3 ml of serum-free medium with or without yohimbine and with 4 μCi [^{3}H]tyrosine per ml added for 30 min at 37°C. The labeled medium was then removed, cells were rinsed three times with cold phosphate-buffered saline, and 3 ml of cold 10% trichloroacetic acid was added to each dish. After a 1 h incubation at 4°C to precipitate proteins and recover the tyrosine taken up by the cells, the trichloroacetic acid supernatants were collected and centrifuged at $12{,}000 \times g$ for 10 min, and the radioactivity present in the supernatants was determined by scintillation counting.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of melanocyte extracts Detergent-solubilized melanocyte extracts were subjected to SDS gel electrophoresis as described elsewhere (Fuller *et al*, 1993; Iozumi *et al*, 1993). Briefly, melanocytes (5×10^6 cells) were removed from control or yohimbine-treated culture flasks (75 cm^2 flasks) and pelleted by centrifugation at $100 \times g$ for 10 min, and the pellets were sonicated in 0.05 M sodium phosphate buffer (pH 6.8) containing 0.1 mM PMSF and 1.0% Triton X-100. The resulting homogenate was left at 4°C for 30 min to release tyrosinase from the melanosome membrane and was then centrifuged at 40,000 $\times$ *g* for 20 min at 4°C. The supernatant (50–100 μg protein) was mixed with SDS sample treatment buffer [10% SDS and 10% glycerol in 0.5 M Tris(hydroxymethyl)-aminomethane-HCl (pH 6.8) without beta-mercaptoethanol] at room temperature and electrophoresed on 10% polyacrylamide gels. Beta-mercaptoethanol was omitted to avoid denaturing tyrosinase.

The relative *in situ* enzymatic activity of tyrosinase present in each lane of the SDS gel after electrophoresis was visualized by first placing the gel in 0.5 M sodium phosphate buffer (pH 6.5) for 15 min to lower the pH. Gels were then incubated in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.2% L-DOPA. Within 15 min at 37°C, a melanin band became visible on the gel. The amount of DOPA reaction product deposited within the gel during the 15 min incubation correlates with the activity of the enzyme at that position. Upon visualization of the melanin bands, the gel was removed and dried, and the relative amount of melanin formed in each lane was quantitated by determining the integrated density value with an Alpha Innotech Model 2000 digital imaging system.

Western blotting and immunodetection of tyrosinase Tyrosinase abundance in control or yohimbine-treated cells was determined by immunoblotting as previously described (Fuller *et al*, 1993; Iozumi *et al*, 1993). Cell extracts, prepared and electrophoresed as described above, were electrophoretically transferred to Immobilon p membrane (Millipore Corp.) in 25 mM Tris, 192 mM glycine buffer, pH 8.8, containing 20% methanol. The transfer was carried out at 60 V for 2 h under cooling conditions. Following transfer, tyrosinase was quantitated by using a rabbit antityrosinase antibody (Fuller *et al*, 1987, 1993; Iozumi *et al*, 1993) and an alkaline phosphate-based chemiluminescent detection kit (Bio-Rad Laboratories, Hercules, CA). The chemiluminescent signal was detected by exposing the blot to Kodak Biomax MR film followed by development. Exposure times from 5 to 25 s were used for each blot to ensure that the film was not being overexposed. The relative tyrosinase abundance in each sample was quantitated by determining the integrated density value of the chemiluminescence signal with an Alpha Innotech Model 2000 digital imaging system. To determine the linear range of detection of tyrosinase in cell extracts, detergent extracts from human melanocyte cell cultures were prepared as described above, protein amounts ranging from 5 μg to 250 μg were electrophoresed on SDS gels, and tyrosinase was detected by immunoblotting. Tyrosinase was not detectable with 5 μg of total cell extract protein, but the chemiluminescent signal for the enzyme was found to be linear over the range of 20–200 μg cell extract protein. Therefore, sample sizes from 50 to 100 μg of cell protein were used for polyacrylamide gel electrophoresis.

RESULTS

Exposure of either black or white human melanocytes or mouse melanoma cells to yohimbine causes a reversible decrease in tyrosinase activity During studies to assess the effect of various neurotransmitters on melanogenesis in human melanocytes, we discovered that the alpha-2 adrenergic receptor antagonist yohimbine caused a pronounced lowering of tyrosinase activity. The inhibitory effect was evident within 10 h after exposure of cells to yohimbine, and by 48 h tyrosinase activity had been reduced by almost 90% (**Fig 1*a***). Dose–response studies showed that the inhibitory effect was maximal at 100 μM and not detectable at concentrations less than 1 μM (**Fig 1*b***). Since our initial observation of the down-regulation effect of yohimbine was made using melanocytes derived from white (type II-III) skin, studies were carried out to determine if tyrosinase activity in melanocytes derived from type VI skin (black skin) could also be suppressed by yohimbine. We found that yohimbine was equally effective in reducing tyrosinase activity in human melanocyte cultures derived from black skin types even though basal tyrosinase activity in these cells was nine to ten times that measured in white melanocytes (**Fig 2*a***). Yohimbine was also effective in reducing tyrosinase activity in Cloudman S-91 mouse melanoma cells, and did so with approximately the same dose–response profile as seen in human melanocytes. Mouse melanoma cultures showed a 70% decrease in tyrosinase activity when treated with 100 μM yohimbine, a 30% reduction at 50 μM, and no inhibition at concentrations of yohimbine below 25 μM. We found that whereas yohimbine was able to reduce tyrosinase activity in human melanocytes by 80%–90%, the compound had no effect on cell growth, DNA synthesis or protein synthesis (data not shown). Thus, yohimbine is not lowering tyrosinase activity as a result of simply damaging the cell or altering the general metabolic state of melanocytes. Further, as shown in **Fig 3**, the tyrosinase down-regulation effect was completely reversible. Although treatment of melanocytes for 48 h with 100 μM yohimbine produced an almost 90% drop in tyrosinase activity, the activity of the enzyme returned

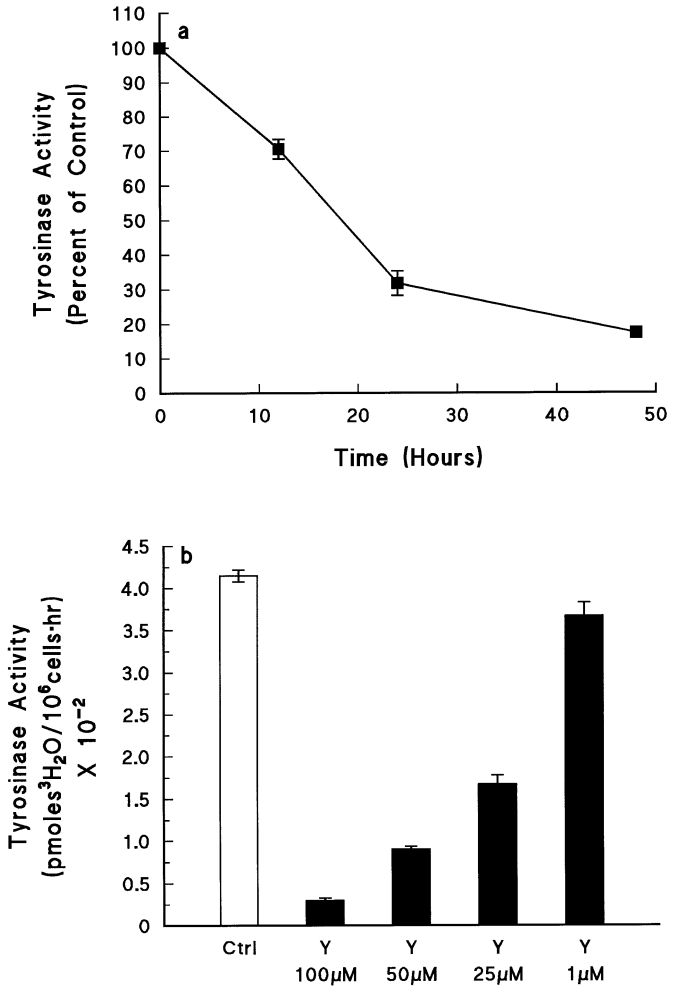

Figure 1. Yohimbine causes a dose-dependent decrease in tyrosinase activity in human melanocytes. (*a*) Melanocytes were seeded into 60 mm culture dishes at 3×10^5 cells per dish and were treated, where indicated, with 100 μM yohimbine for 12, 24, and 48 h, in medium containing 2 μCi [^{3}H]tyrosine per ml. At the times indicated, cells were removed and counted, and the medium was removed and assayed for tyrosinase activity by determining the amount of ^3H_2O produced, as described in *Materials and Methods*. Values are the averages of six determinations ± SD. (*b*) Melanocytes were seeded into 60 mm culture dishes at 3×10^5 cells per dish and were treated with the indicated amounts of yohimbine (Y) for 48 h. For the time period 24–48 h, the medium was supplemented with 2 μCi [^{3}H]tyrosine per ml. At the end of the experiment cells were removed and counted and the medium was assayed for ^3H_2O. Values are the averages of six determinations ± SD. The experiments in (*a*) and (*b*) were repeated at least three times with similar results.

to control levels 48 h after removal of the drug from the culture medium.

To determine if the inhibitory effect of yohimbine could be demonstrated in a more *in vivo* model, we assessed the antagonist's effect on melanocytes in a human skin organ culture system (Iwata *et al*, 1990). Skin cultures can be maintained in a viable state in this system for many days and the effect of hormones on tyrosinase activity can be determined by measuring the conversion of L-DOPA into melanin in sectioned material. Melanocytes with active tyrosinase make and deposit melanin, thereby becoming visible in sections of skin, whereas those melanocytes in which tyrosinase is absent or inactive cannot convert DOPA to melanin and remain undetectable. As shown in **Fig 4**, pronounced basal DOPA oxidase activity of tyrosinase was detected in human skin organ cultures derived from black skin. When these skin type VI cultures were treated with yohimbine for 48 h, the amount of DOPA reaction product formed was markedly decreased (approximately fivefold) indicating that yohimbine was inhibiting tyrosinase activity.

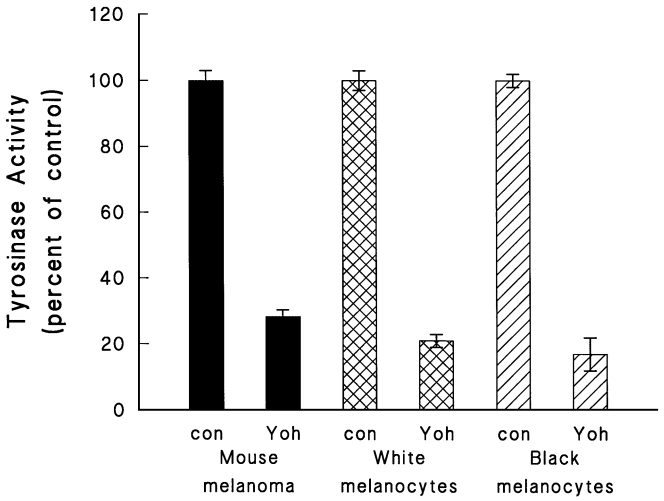

Figure 2. Yohimbine lowers tyrosinase activity in human melanocytes derived from either black or white skin types and in mouse melanoma cells. Human melanocyte cell strains, derived from foreskins of newborns with black (type VI) or white (type II-III) skin types or mouse melanoma cells (Cloudman S-91 mouse melanoma) were seeded into 60 mm culture dishes and yohimbine (100 μM; YOH) was added to the appropriate flasks for 48 h. For the last 24 h of treatment, the medium was replaced with fresh medium containing 2 μCi [^{3}H]tyrosine oer ml and, where indicated, yohimbine. At the end of this time the medium was assayed for the amount of tritiated water produced during the 24 h period. Values are the averages of six determinations ± SD and are expressed as a percentage of controls. The actual tyrosinase values (pmol per 10^6 cells per h) are as follows: Cloudman melanoma cells, control, 32 ± 2.5; yohimbine, 9 ± 1.5; white melanocytes, control, 90.7 ± 7.2; yohimbine, 19.3 ± 3.5; black melanocytes, control, 824 ± 22; yohimbine, 139 ± 20. Experiments were repeated at least three times with similar results.

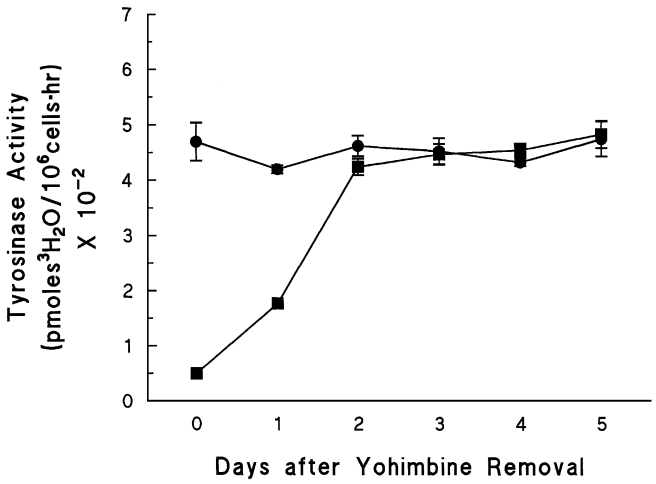

Figure 3. The inhibition of tyrosinase in human melanocytes by yohimbine is completely reversible. Human melanocytes were seeded into 60 mm culture dishes and either left untreated or treated with yohimbine (100 μM) for 48 h to lower tyrosinase activity. During the last 24 h of incubation, medium in some dishes was supplemented with 2 μCi [^{3}H]tyrosine per ml to determine the level of inhibition. Yohimbine was removed from the remaining dishes and tyrosinase activity was determined in control (●) and yohimbine-pretreated (■) cultures every 24 h for 5 d. Values are the averages of six determinations ± SD. This experiment was repeated three times with similar results.

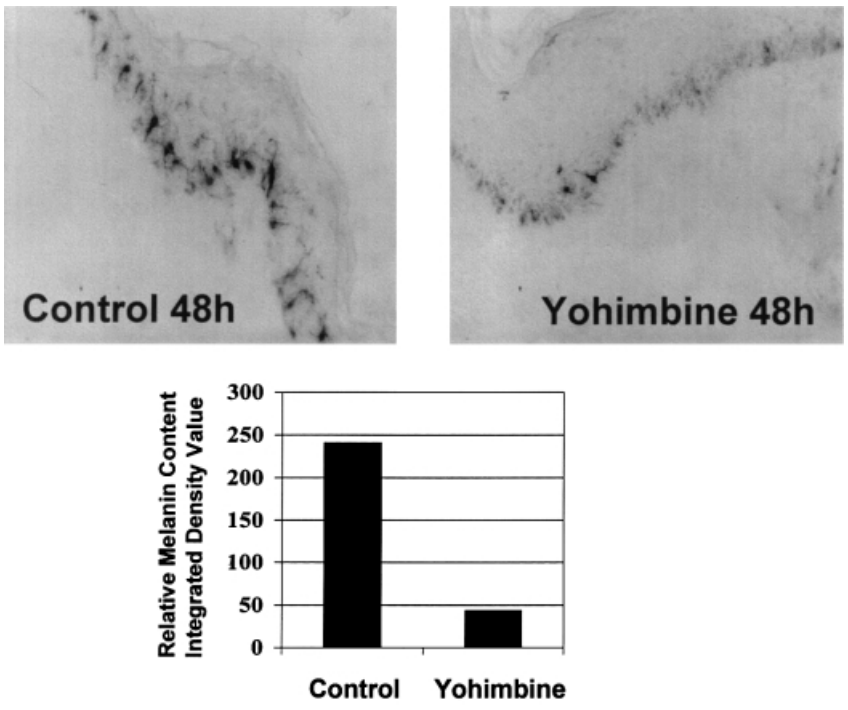

Figure 4. Yohimbine lowers tyrosinase activity in human skin organ cultures. Human foreskin organ cultures were established as described elsewhere (Iwata *et al*, 1990). Cultures either were left untreated or were treated with yohimbine (100 μM) for 48 h, and then embedded, sectioned, and incubated in L-DOPA to visualize tyrosinase activity as described in *Materials and Methods*. Photomicrographs were taken of random areas from each of the six sections mounted on each slide. Each photograph was then analyzed with an Alpha Innotech digital imaging system to determine the relative amount (density) of DOPA reaction product produced in the skin section. The relative amount of reaction product is expressed by the imaging system as an integrated density value (IDV) where IDV = Σ(each pixel value - background). A representative photomicrograph of control and yohimbine-treated organ cultures is shown. The bar graph shows the integrated density values of the DOPA reaction product in these two sections. This organ culture study was repeated at least three times with similar results.

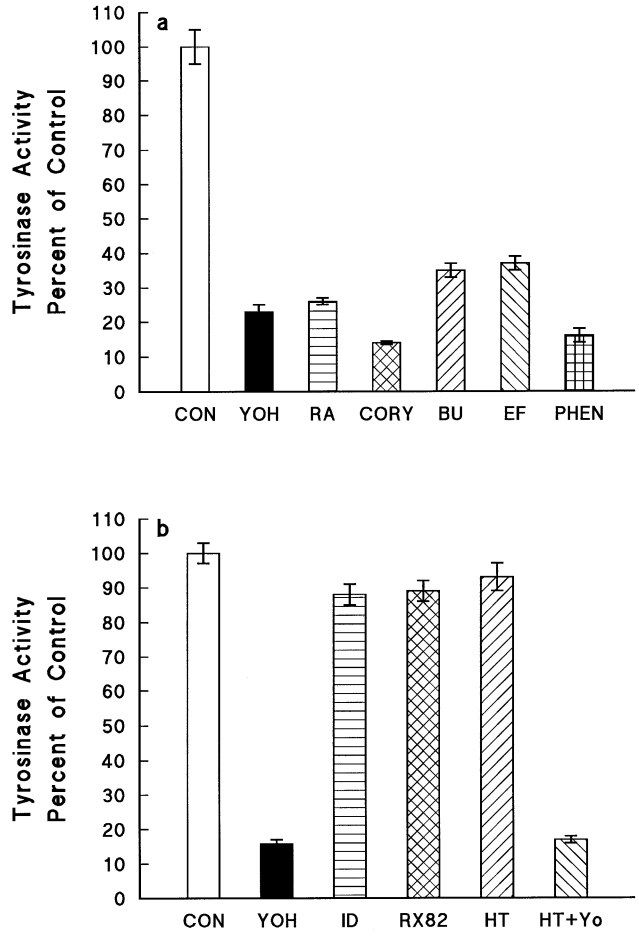

Figure 5. Yohimbine isomers and alpha-2 adrenergic receptor antagonists lower tyrosinase activity in human melanocytes, whereas alpha-2 adrenergic receptor agonists have no effect on enzyme activity. Human melanocytes were treated with the indicated compounds for 48 h. During the last 24 h, cells were incubated in medium containing [^{3}H]tyrosine, and at the end of this time, tyrosinase activities were determined as described in *Materials and Methods*. (*a*) YOH, yohimbine. Yohimbine stereoisomers: RA, rauwolscine (100 μM); CORY, corynanthine (100 μM). Alpha-2 receptor antagonists: BU, BU224 (100 μM); EF, efaroxan (100 μM); PHEN, phentolamine mesylate (100 μM, general alpha receptor antagonist, and not specific for alpha-2 adrenergic receptors). (*b*) Alpha-2 receptor agonists: ID, idazoxan (1 μM); RX82, Rx821002 (100 μM). Alpha-2 adrenergic receptor agonist, HT, B-HT933 (100 μM). HT + Yo, B-HT933 + yohimbine. Values are the averages of six determinations ± SD. Experiments were repeated at least three times with similar results.

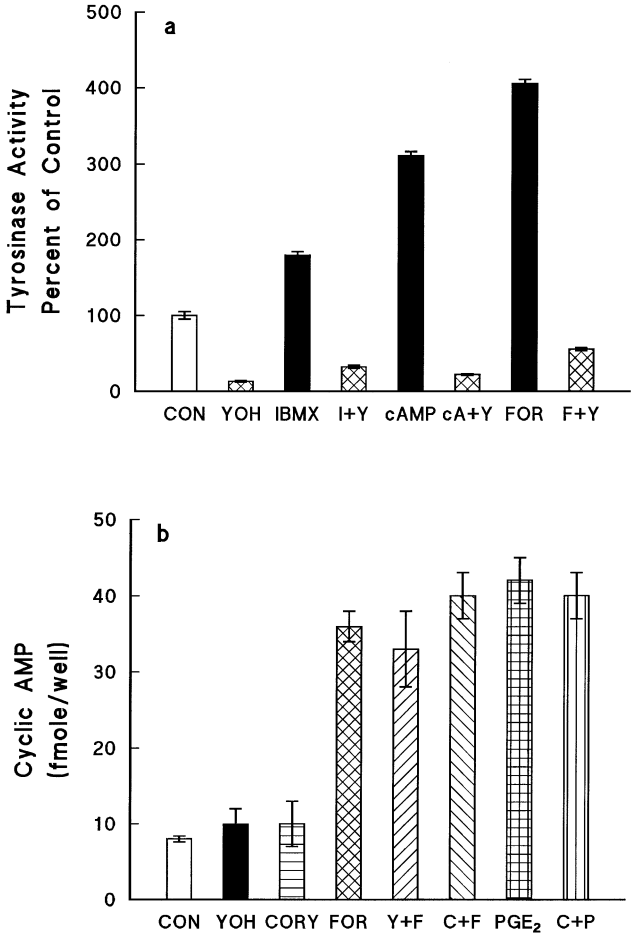

Figure 6. Yohimbine blocks the cAMP-mediated increase in tyrosinase activity but does not alter cAMP levels. (*a*) Human melanocytes were seeded into 60 mm dishes and were treated with the indicated compounds for 48 h. During the last 24 h, cells were incubated in medium containing [^{3}H]tyrosine, and at the end of this time the medium was assayed for the presence of ^3H_2O as described in *Materials and Methods*. YOH, yohimbine (100 μM); IBMX, 3-isobutyl-1-methylxanthine (100 μM); cAMP, dibutyryl cAMP (100 μM); FOR, forskolin (10 μM); I + Y, IBMX + yohimbine; cA + Y, dibutyryl cAMP + yohimbine; F + Y, forskolin + yohimbine. Values are averages of six determinations ± SD. Experiments were repeated at least three times with similar results. (*b*) Melanocytes were seeded into 60 mm culture dishes and were treated with the indicated compounds for 40 min. At the end of this incubation cells were processed for cAMP measurements as described in *Materials and Methods*. CON, control; YOH, yohimbine (100 μM); CORY, corynanthine (100 μM); FOR, forskolin (10 μM); Y + F, yohimbine + forskolin; C + F, corynanthine + forskolin; PGE_2, prostaglandin E_2; C + P, corynanthine + PGE_2. Values are the averages of triplicates ± SD. Cyclic experiments were repeated twice with similar results.

Yohimbine-related compounds and some other alpha-2 adrenergic receptor antagonists inhibit tyrosinase Since yohimbine is known to be an alpha-2 adrenergic receptor antagonist, we looked at the effect of other such antagonists on the activity of tyrosinase in human melanocyte cultures. Two yohimbine stereoisomers, corynanthine and rauwolschine, were found to be as effective as yohimbine in reducing tyrosinase activity. In addition, the synthetic alpha-2 adrenoceptor antagonists BU224 and efaroxan, and the nonselective alpha adrenergic receptor antagonist phentolamine, also inhibited tyrosinase activity (**Fig 5*a***). Whereas these data suggest that the inhibitory effect of yohimbine may be mediated through an alpha-2 adrenergic receptor event, additional alpha-2 adrenergic receptor antagonists, including Idazoxan and RX821002, were found to be ineffective in down-regulating tyrosinase activity (**Fig 5*b***). Further, treatment of melanocytes with the alpha-2 adrenergic receptor agonists B-HT933 and p-aminoclonidine did not block the inhibitory effect of yohimbine (**Fig 5*b***).

Yohimbine blocks cAMP action in human melanocytes Tyrosinase activity is increased in human melanocytes treated with hormones, such as α-melanocyte stimulating hormone (MSH), and prostaglandins, and to compounds that raise cAMP levels, including forskolin, methylxanthines, and dibutyryl cyclic 3′,5′-adenosine monophosphate (dbcAMP). We therefore looked at the effect of yohimbine on tyrosinase activity in cells stimulated with IBMX, dbcAMP, or forskolin. As shown in **Fig 6(*a*)** yohimbine was effective in reducing tyrosinase activity below control levels, even in cells in which tyrosinase activity had been stimulated fourfold to sixfold. Since an increase in intracellular cAMP in human melanocytes leads to an increase in tyrosinase activity, we investigated the possibility that yohimbine might down-regulate

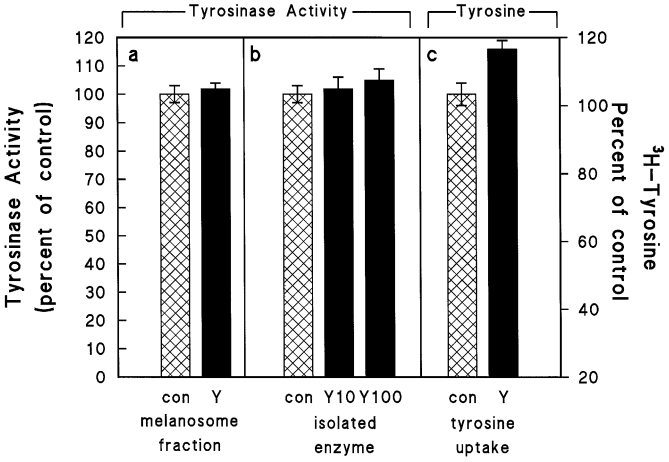

Figure 7. Yohimbine has no direct inhibitory effect on melanosomal-bound tyrosinase, or on isolated tyrosinase, and has no effect on tyrosine uptake into melanocytes. (*a*) Stock cultures of melanocytes from four 75 cm^2 flasks were used to prepare a melanosome-enriched fraction as described in *Materials and Methods*. Melanosomes were incubated with or without yohimbine (100 μM) in medium containing [^{3}H]tyrosine (2 μCi per ml) for 18 h, and the amount of tritiated water produced during this time was determined. CON, control; Y, yohimbine. Values are the averages of six assays ± SD. (*b*) Tyrosinase was released from the melanosome membrane by detergent treatment as described in *Materials and Methods*. The sonicates were centrifuged at 40,000 × g and aliquots of the supernatant containing soluble tyrosinase were incubated without or with either 10 μM (Y10) or 100 μM (Y100) yohimbine for 3 h at 37°C in phosphate buffer (pH 6.8) containing 0.1 mM L-DOPA, 0.1 mM tyrosine, and 2 μCi [^{3}H]tyrosine per ml. The amount of tritiated water produced during this time was determined as described in *Materials and Methods*. Values are averages of six assays ± SD. (*c*) Human melanocytes were treated with yohimbine (100 μM) for 48 h. After this incubation, the medium was removed and replaced with serum-free medium containing 4 μCi [^{3}H]tyrosine per ml for 30 min. At the end of this incubation the amount of tyrosine taken up by the cells was determined as described in *Materials and Methods*. Values are the averages of four determinations ± SD. Experiments were repeated at least three times with similar results.

tyrosinase activity by lowering cAMP levels. When cAMP levels were determined, we found that neither yohimbine nor its stereoisomer corynanthine had any effect on basal cAMP levels in human melanocytes. In addition, these compounds did not block the stimulation of cAMP by forskolin or PGE-2 (**Fig 6*b***). Thus, the reduction in tyrosinase activity in human melanocytes by yohimbine apparently does not proceed through a cAMP-dependent mechanism.

Yohimbine does not lower tyrosinase activity by directly interacting with melanosomes or isolated tyrosinase, or by lowering tyrosine entry into melanocytes Although it seems most likely that yohimbine is exerting its inhibitory effects by binding a surface receptor to activate some as yet unidentified signaling pathway, it is also possible that yohimbine may enter the cell and interact directly with the melanosome to cause a lowering of melanosome-bound tyrosinase activity. Alternatively, the compound may bind directly to tyrosinase and act as either a competitive or an uncompetitive inhibitor. To determine if yohimbine causes an inhibition of tyrosinase activity by directly interacting with the melanosome, a melanosome-enriched granule fraction was incubated with 100 μM yohimbine for up to 18 h and the effect of this incubation on tyrosinase activity was determined. As shown in **Fig 7(*a*)** tyrosinase activity in melanosomes was unchanged by the incubation with yohimbine. To determine whether yohimbine might act directly on tyrosinase to inhibit the catalytic activity of the enzyme, a tyrosinase extract was prepared from melanocytes homogenized in a detergent buffer to release tyrosinase from the melanosome membrane and tyrosinase activity in the extract was determined in the presence of either 10 μM or 100 μM yohimbine. No inhibitory effect of yohimbine was observed (**Fig 7*b***). These findings suggest that tyrosinase inhibition in human melanocytes by yohimbine requires an intact cell, and that the drug has no direct effect on either melanosomes or tyrosinase.

The possibility that yohimbine might be lowering tyrosinase activity simply by slowing or blocking the uptake of tyrosine into human melanocytes was investigated by measuring the rate of [^{3}H]tyrosine uptake into untreated cells and in cultures treated with yohimbine for 48 h. As shown in **Fig 7(*c*)**, the amount of tyrosine taken up into a melanocyte was, if anything, slightly increased in cells treated with yohimbine. Thus, it is unlikely that the inhibitory effect of yohimbine on tyrosinase activity can be attributed to a decrease in tyrosine uptake into the cell. Whether or not the drug may cause changes in the movement of tyrosine across the melanosome membrane remains to be determined.

We next investigated the possibility that yohimbine might reduce the amount of tyrosinase present in human melanocytes. Effects of hormones and other signaling molecules on tyrosinase abundance and gene activity in mouse melanoma cells are well known (Halaban *et al*, 1984; Fuller *et al*, 1987; Hoganson *et al*, 1989; Bertolotto *et al*, 1996; Rungta *et al*, 1996; Price *et al*, 1998). In order to investigate the effect of yohimbine on tyrosinase abundance, detergent extracts of control and yohimbine-treated cells were electrophoresed on SDS gels, and tyrosinase abundance was quantitated by western immunoblotting. As part of this procedure we routinely measured the activity of tyrosinase in homogenates prepared from control and yohimbine-treated cells prior to loading samples on polyacrylamide gels. When this was done we found, surprisingly, that, although *in situ* tyrosinase assays of cell cultures treated with yohimbine for 48 h showed an 80%–90% loss in enzyme activity, when the activity of tyrosinase in detergent solubilized cell homogenates from these same cells was determined, the yohimbine inhibition effect had been completely lost (**Fig 8*a***). The activity of tyrosinase released from the melanosomes of yohimbine-treated cells was as high as that in control homogenates. Further, when detergent solubilized melanocyte extracts were electrophoresed on SDS polyacrylamide gels and tyrosinase activity was visualized by incubating the gels in L-DOPA buffer, the amounts of melanin formed from DOPA in lanes with control or yohimbine extracts were the same, indicating that the catalytic activities of tyrosinase in extracts from control and yohimbine-treated cells were equivalent (**Fig 8*b***). Given these findings, it seemed unlikely that yohimbine was acting on human melanocytes to lower the amount of tyrosinase protein in the cells. To confirm this, the relative amount of tyrosinase protein in control melanocytes and in cells treated for 48 h with yohimbine was determined by western immunoblotting. As shown in **Fig 8(*c*)**, we found no significant difference in tyrosinase abundance between control and yohimbine-treated cells, even though the *in situ* activity of the enzyme from yohimbine-treated cells was a fifth of that measured in control cultures.

DISCUSSION

The finding that yohimbine produces an almost 90% inhibition of tyrosinase activity in human melanocytes is, to our knowledge, the first observation of such a pronounced, and reversible, inhibitory effect of any hormone or chemical messenger on tyrosinase in human pigment cells. It is interesting that the yohimbine effect occurs without any alteration in cell proliferation, DNA synthesis, protein synthesis, or tyrosine uptake. Further, unlike a number of tyrosinase inhibitors, such as hydroquinones, yohimbine does not act simply as a competitive or uncompetitive inhibitor of the enzyme. The compound is equally effective in lowering tyrosinase from either black or white melanocytes, and does so in a completely reversible manner. Although the final target site for yohimbine action must be melanosome-bound tyrosinase, the drug does not appear to act directly on either melanosomes or tyrosinase but, rather, requires an intact cell to exert its inhibitory effect.

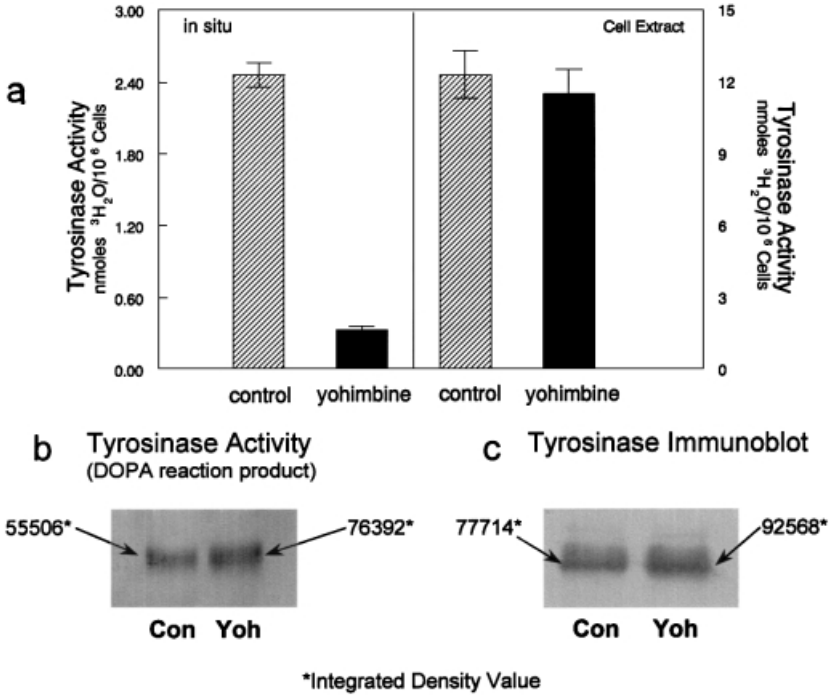

Figure 8. Yohimbine does not reduce the amount of tyrosinase protein present in human melanocytes. Human melanocytes were seeded into either 75 cm^2 culture flasks (for western blots and immunodetection) or into 60 mm culture dishes (for *in situ* tyrosinase assays) and then either left untreated or treated with yohimbine (100 μM) for 48 h. (*a*) [*in situ*] During the last 24 h of treatment with yohimbine, cells were incubated in medium containing [^{3}H]tyrosine, and at the end of this time the amount of ^3H_2O produced was determined. (*a*) [cell extract] Melanocytes, either treated with yohimbine for 48 h or left untreated, were removed from flasks, and a detergent solubilized tyrosinase extract was prepared and assayed for tyrosinase activity as described in *Materials and Methods*. (*b*) Detergent extracts from control and yohimbine-treated cells were electrophoresed on SDS polyacrylamide gels. After electrophoresis, the gels were incubated in L-DOPA to visualize tyrosinase. The resulting melanin bands were quantitated with an Alpha Innotech Model 2000 digital imaging system, and the density of the bands was expressed as integrated density value. (*c*) Cell extracts from control and yohimbine-treated cells were electrophoresed on SDS polyacrylamide gels and the proteins was electroblotted onto PVDF membrane, as described in *Materials and Methods*. The relative amount of tyrosinase in control and yohimbine samples on the blots was then determined by immunodetection and chemiluminescence, followed by quantitation of the autoradiograms with an Alpha Innotech digital imaging system as described in *Materials and Methods*. The relative abundance of tyrosinase is expressed by the imaging system in pixels as an integrated density value. These values are shown next to the autoradiogram bands of tyrosinase. The experiments were repeated at least four times with similar results.

Finally, yohimbine does not appear to lower tyrosinase gene activity since the abundance of the enzyme is the same in control and yohimbine-treated cells, even though enzyme activity in yohimbine-treated cells is a fifth of the activity in control cultures.

Although this study has elucidated many of the basic features of the yohimbine inhibition of tyrosinase activity in human melanocytes, the results raise a number of questions about yohimbine action. For example, we do not know what receptor yohimbine is binding to, what signaling pathway is generated, and what downstream effector(s) regulates tyrosinase activity at the level of the melanosome. Since yohimbine is classified as an alpha-2 adrenoceptor antagonist, the most obvious target site for yohimbine action on human melanocytes would presumably be an alpha-2 adrenergic receptor. Such receptors have been shown to be present on frog melanophores (Eberle and Girard, 1985), but no evidence has been presented to suggest their presence on either mouse or human melanocytes. The lack of a consensus effect of alpha-2 receptor antagonists on tyrosinase, the inability of alpha-2 agonists to block yohimbine action, and the relatively high concentrations of yohimbine needed to elicit the inhibition suggest that the down-regulation of tyrosinase activity in human melanocytes is probably not being mediated through an effect at an alpha-2 adrenergic receptor. Further, since alpha-2 adrenergic receptors, when activated by an agonist, cause a lowering of cAMP (Lomasney *et al*, 1995), it seems unlikely that yohimbine is acting on this type of receptor to elicit its inhibitory effect in melanocytes. If yohimbine were binding this receptor, one might expect the drug to block the lowering of cAMP and, as a result, actually cause an increase in tyrosinase activity.

A possible melanocyte receptor target for yohimbine could be an alpha-1 adrenergic receptor. Interestingly, as shown in this study, the yohimbine stereoisomer corynanthine, which is actually classified as an alpha-1 receptor antagonist, causes the same inhibitory effect as yohimbine. Since the activation of alpha-1 adrenergic receptors results in the stimulation of phospholipase C (Lomasney *et al*, 1995), if yohimbine is acting as an antagonist to this receptor, the lowering of tyrosinase activity must be somehow linked to downstream effectors produced by phospholipase C. Phospholipase C causes the hydrolysis of polyphosphoinositides, resulting in the production of diacylglycerol and inositol triphosphate (Rhee and Bae, 1997). Increased amounts of inositol triphosphate lead to the release of intracellular calcium, whereas the production of diacylglycerol results in the activation of kinase C. If yohimbine is acting as an antagonist to alpha-1 receptors on human melanocytes (which have not yet been shown to exist) the drug may be lowering the activity of phospholipase C, thereby causing a decrease in intracellular calcium and kinase C activity. If basal tyrosinase activity in both black and white melanocytes is dependent upon some threshold level of intracellular calcium and kinase C activity, then if this level falls because of a yohimbine-mediated down-regulation of phospholipase C activity, tyrosinase activity would be expected to decrease. A role for kinase C in regulating tyrosinase activity in both mouse and human melanocytes has been suggested by Gilchrest and coworkers (Park *et al*, 1993) who reported an increase in tyrosinase in melanocytes transfected with a kinase C expression plasmid. This group has also shown that melanin production in human melanocytes can be stimulated by diacylglycerol, a kinase C activator (Gordon and

Gilchrest, 1989). Although these studies implicate a role for kinase C in controlling human pigmentation, racial differences in basal tyrosinase activities and melanin levels in human melanocytes derived from black and white skin types seem to be unaffected by kinase C activation. Tyrosinase activities in human melanocytes derived from black skin are typically seven to ten times higher than in white melanocytes even though both cell strains are grown for months in medium containing the kinase C activator, TPA. Since kinase C might be expected to be activated to the same extent in all melanocytes grown in TPA-containing medium, if kinase C activity was the primary regulator of tyrosinase activity one might expect all melanocytes cultured in medium containing TPA to have approximately the same tyrosinase activity, regardless of racial origin. This is not the case. In contrast to a possible role of kinase C in up-regulating human pigmentation, studies in the mouse melanocyte model have shown that kinase C activation leads to a down-regulation of tyrosinase gene expression (Fuller *et al*, 1990; Oka *et al*, 1993; Bertolotto *et al*, 1998), and that lowering kinase C activity increases pigmentation (Englaro *et al*, 1998). Thus, whereas it is intriguing to speculate that yohimbine may somehow interfere with a phospholipase C/kinase C pathway, further work is needed to ascertain the role of kinase C in the regulation of pigmentation in human melanocytes, and to determine whether or not an alpha-1 adrenergic receptor is involved in yohimbine action.

Our finding that yohimbine does not alter the abundance of tyrosinase present in human melanocytes suggests that the drug is somehow reducing the catalytic activity of pre-existing tyrosinase. Since more than 90% of tyrosinase is bound to the melanosome membrane, any model to explain the action of yohimbine must include the melanosome as the final target site. We know from our isolated melanosome experiments that yohimbine does not appear to have any direct effect on melanosome-bound tyrosinase activity, nor does it alter the catalytic activity of isolated enzyme. The data from tyrosine uptake experiments argue against a role for yohimbine in altering tyrosine transport across the plasma membrane of human melanocytes. Our studies did not examine the effect of yohimbine on tyrosine uptake into melanosomes. Certainly, if yohimbine blocked tyrosine entry into the melanosome, tyrosinase activity would be inhibited. Studies are in progress to determine whether tyrosine transport is inhibited in yohimbine-treated cells

When one considers that the inhibitory response of human melanocytes to yohimbine is fairly slow (30% reduction in 12 h), if the final target site for yohimbine action is the melanosome, it seems likely that marked changes in the melanosome environment must be taking place over this time to alter tyrosinase activity. Such changes could include the appearance or disappearance of melanosome proteins that regulate tyrosinase activity or changes in tyrosinase itself. Given the absence of an effect of yohimbine on tyrosinase abundance, if the melanosome-bound enzyme is structurally altered by yohimbine action, these changes would probably take the form of post-translational modifications such as phosphorylation, glycosylation, or acylation. It seems unlikely that yohimbine is promoting any post-translational modification of tyrosinase, since SDS gel electrophoresis patterns of tyrosinase show no difference in the apparent molecular weight of the enzyme from control and yohimbine-treated cells. Further, when the enzyme in yohimbine-treated cells is solubilized away from the melanosome membrane, the activity returns to control levels, further suggesting that yohimbine has not caused some covalent modification of tyrosinase. As the decrease in tyrosinase activity in yohimbine-treated cells becomes detectable only after prolonged exposure to the drug, it seems likely that yohimbine may exert its effect through some alteration of gene expression. As the most likely final target site for the yohimbine signaling pathway seems to be the melanosome, the most likely gene candidates for yohimbine action are melanosome proteins, either those regulating tyrosine transport into the organelle, or those capable of interacting directly with tyrosinase to alter the enzyme's catalytic activity. With regard to the second possibility, tyrosinase has been shown to complex to other melanosome proteins and the suggestion has been made that this interaction may be required for tyrosinase function (Orlow *et al*, 1994). Two-dimensional gel electrophoresis studies are now in progress to determine whether yohimbine treatment leads to changes in the composition of melanosomal proteins.

This study has shown that yohimbine is an effective reversible inhibitor of tyrosinase activity in human melanocytes, and that the compound causes neither stimulation nor inhibition in cell growth. Its potential use as a safe and effective skin lightening product for hyperpigmentation disorders is now under clinical investigation.

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