Induction of Tyrosinase Gene Transcription in Human Iris Organ Cultures Exposed to Latanoprost

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Objective: Topical administration of latanoprost sometimes induces gradual iris darkening. The present study was undertaken to determine if latanoprost can increase transcription of the gene for tyrosinase, an important enzyme in the biosynthesis of melanin. Results from brown, hazel, and blue irides were compared.

Methods: Iris tissue was isolated from 30 pairs of post-mortem human donor eyes, and 2 iris segments from each eye were incubated in tissue culture medium supplemented with 200nM latanoprost acid or vehicle for 7 days. Tyrosinase messenger RNA (mRNA) was determined using real-time polymerase chain reaction analysis (TaqMan quantitative polymerase chain reaction). Results for tyrosinase mRNA were normalized according to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample.

Results: Tyrosinase mRNA expression was similar in blue and hazel irides, and ranged from 0.7-fold to 12.6-fold greater than GAPDH expression. In contrast, control brown iris culture tyrosinase expression ranged from 6.4-fold to 265-fold greater than GAPDH expression. Induction of tyrosinase mRNA by latanoprost was below threshold in all the blue iris cultures (n=8 pairs), present in 1 of 9 hazel iris cultures, and present in 5 of 13 brown iris cultures. Mean induction in the responding hazel iris cultures was 1.40-fold. Mean induction among the responding brown iris cultures was 2.97-fold.

Conclusions: These observations support the view that iris darkening associated with latanoprost treatment reflects induction of tyrosinase expression. Moreover, they suggest that the probability that latanoprost will increase tyrosinase expression is directly related to the magnitude of tyrosinase expression before treatments are initiated.

Clinical Relevance: The variability of iris darkening with latanoprost may reflect natural variation in the basal transcription of tyrosinase.


Topical treatment with the prostaglandin (PG) analog latanoprost lowers intraocular pressure (IOP) in patients with glaucoma by increasing uveoscleral outflow.1,2 Although this response has few associated systemic side effects, long-term treatment with latanoprost sometimes induces a gradual darkening of iris color.3-6 This local side effect is most commonly seen in hazel-colored irides with patchy brown regions, and it is typically first observed after several months of treatment. Other commonly affected iris types include yellow/brown and blue-gray/brown irides with darker regions near the pupillary margin. Such darkening has not been observed in eyes that are uniformly blue, blue-gray, or dark brown before initiation of latanoprost treatment. Iris darkening also has been observed with another topical PG analog.7 The biological basis for this iris darkening remains unknown.

Several recent studies have investigated the anatomical basis for iris color.8,9 Irides ranging from green to brown generally reveal increasing amounts of pigment-containing melanosomes in melanocytes in the anterior border layer of the iris.10 In contrast, the number and density of melanocytes is relatively constant among irides of different color.9,11 Also constant among irides of different color is the size of the melanosomes containing the pigment.12 Histological evaluation of an iridectomy specimen from a patient with latanoprost-induced iris darkening revealed no evidence of melanocyte mitosis.12 No induction of mitosis was found in iris melanocyte cultures exposed to latanoprost.13 However, iris darkening following topical latanoprost treatment of mon-
key eyes was associated with increased incorporation of a false melanin precursor into iridial melanocytes. This suggests that latanoprost-mediated iris darkening reflects increased melanogenesis. Biosynthesis of the iris melanosome pigment melanin is initiated through the conversion of tyrosine to dihydroxyphenyl alanine by tyrosinase. The finding that mutation of the tyrosinase gene can result in ocular albinism supports a critical role of tyrosinase in the development of eye color. Tyrosinase can also catalyze other reactions associated with mammalian melanogenesis. Although the catalytic activity of tyrosinase can be modified by intracellular modifiers, increases in cellular tyrosinase activity can also reflect increased tyrosinase gene transcription.

A recent investigation showed that tyrosinase activity is elevated in 2 iris melanocyte cell lines following exposure to latanoprost acid, the active form of latanoprost. It is unknown whether this activity reflects alteration in tyrosinase gene transcription. Also, it is unclear how iris darkening is induced by latanoprost in some eyes and not in others. One possibility is that latanoprost-mediated induction of melanogenesis reflects induction of tyrosinase gene transcription, and that this may occur more readily in iris melanocytes that have a higher level of basal tyrosinase gene transcription. To test this hypothesis, we have developed a human iris organ culture system. These cultures were exposed to latanoprost acid or vehicle for 1 week, and the level of tyrosinase gene transcription was measured using real-time

METHODS

Sixty-two eyes from 31 donors aged 40 to 84 years were obtained from the San Diego Eye Bank (San Diego, Calif) within 24 hours postmortem. Mean ± SD age was 67.3 ± 11.9 years. Mean ± SD death-to-enucleation time was 4.4 ± 2.1 hours. After enucleation, the eyes were stored at 4°C for fewer than 24 hours before cultures were generated. Among these eyes, 9 pairs (29.0%) were blue, 9 pairs (29.0%) were hazel, and 13 pairs (41.9%) were brown. One pair of blue eyes was excluded from the present analysis because of a history of glaucoma. Of the remaining, 9 eyes (15%) had pseudophakia. Otherwise, there was no history of eye disease, and no observable signs of abnormal physiology were noted at the time of dissection.

WHOLE IRIS ORGAN CULTURES

Eyes were immersed in 3 changes of Hank’s buffered physiological saline containing 20 µg/mL of gentamicin for 5 to 10 minutes each. Each eye was bisected horizontally 4 mm posterior to the limbus. The anterior segment was placed cornea side down under a dissecting microscope, and the lens was removed by cutting the ciliary zonule. The anterior uveal tract was removed as a unit by carefully peeling it from the sclera. The iris was removed from the uveal tract at the iris root using Castroviejo scissors, and cut into 2 pieces. Pilot experiments showed that half of an intact iris was the smallest portion from which reliable isolation of high-quality RNA could be obtained. These were transferred to 35-mm tissue culture dishes containing 3 µL of MCDB 153 medium (MGM-3, Clonetics Corporation, Walkersville, Md) supplemented as previously described. This medium has been shown to promote the growth and differentiation of human dermal melanocytes in vitro. Three days later, the culture medium was changed to a fresh medium supplemented with 200nM latanoprost acid, the biologically active form of latanoprost, 200nM were observed (B. Sjoquist, PhD, oral communication, May 2000). In view of this observation and the observation that when iris darkening occurs, it is usually after several months of treatment, we chose to evaluate the iris cultures after 7-day exposure to 200nM latanoprost.

MELANOCYTE CULTURES

Human neonatal dermal melanocytes isolated from African American donor foreskins were obtained from Clonetics Corporation, plated in MGM-3 medium, and cultured at 37°C in 5% CO2. Cultures reached 80% confluence within 2 weeks and were passaged using a solution of 0.05% trypsin, 0.53mM ethylenediamide tetraacetic acid, and 0.15 M sodium chloride (GibcoBRL, Grand Island, NY).

TOTAL RNA ISOLATION

Total RNA was isolated using guanidine-isothiocyanate lysis and silica gel–membrane affinity chromatography (RNeasy Mini Kit; Qiagen, Valencia, Calif). Each iris culture was transferred to 350 µL of guanidinium thiocyanate lysis buffer, and homogenized for 60 seconds using a Polytron PT10 homogenizer (Brinkmann, Westbury, NY) fitted with disposable mini generator tips. The homogenate was centrifuged at 16 000g for 3 minutes, and the supernatant was transferred to a fresh microcentrifuge tube. After adding 330 µL of 70% ethanol, the purification was continued according to the manufacturer’s instructions. Total RNA was isolated from fifth-passage dermal melanocyte cultures using the RNeasy Mini Kit according to the manufacturer’s instructions.

REVERSE TRANSCRIPTASE REACTION CONDITIONS

First-strand complementary DNA (cDNA) was synthesized using ribonuclease H–reverse transcriptase purified form Escherichia coli containing the pol gene of Moloney Murine Leukemia virus (Superscript II; GibcoBRL). A 20-µL reaction volume contained 1 to 5 µg of total RNA, 0.5 µg of oligo (deoxythymidine), 50mM tris(hydroxymethyl)aminomethane (pH, 8.3 at room temperature), 75mM potassium chloride, 3mM MgCl2, 0.01M diithiothreitol, 0.5mM dNTPs, and 200 units of reverse transcriptase. The reaction mixture was incubated at 42°C for 30 minutes and terminated by incubation at 70°C for 15 minutes.


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PRIMERS AND PROBE DESIGN

Primers and probe for tyrosinase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are presented in the Table. They were chosen for tyrosinase using the computer program Primer Express (Applied Biosystems, Foster City, Calif). The primers contained minimal internal structure (eg, hairpins and primer dimer formation as predicted from sequence) and had melting temperatures within 10°C of each other. The probe that was selected had a melting temperature that was approximately 5°C to 10°C higher than the matching primer pair. The PCR amplicon was 70 base pairs (bp). This allowed rapid nucleotide extension and minimized interactions. The primers and probe sequences for GAPDH are well established in many different systems for specificity and were obtained from Applied Biosystems.

REAL-TIME PCR REACTION CONDITIONS

Real-time PCR was performed in triplicate for each sample and each probe according to the method of Heid et al26 using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The reaction mixture contained 5.5mM MgCl₂, 200mM dNTPs, AmpErase Uracil-N-glycosylase (Applied Biosystems), AmpliTaq Gold DNA polymerase, buffer (Universal PCR Master Mix, Applied Biosystems), 200nM probe, (Perkin-Elmer Branchburg, NJ) of each primer (300nM), and 5.0 µL of sample cDNA. The PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. During the extension phase of each PCR cycle (60°C), fluorescence signal strength within each tube is assessed with a short laser light flash every 8 seconds, and the average reading of the last 3 assessments within a particular cycle is recorded. Taq polymerase–mediated extension under conditions used in PCRs can proceed in excess of 60 nucleotides per second.33 Hence, when the concentration of PCR product in the tube is relatively low, there is ample time to achieve complete replication of amplicons that are less than 100 bp and obtain a reliable reading of the fluorescence signal. The concentration of product and the fluorescent signal increases exponentially with each PCR cycle until a point at which the PCR reaction is inhibited by the PCR products, and the growth of the fluorescent signal approaches a plateau. Threshold for the determination of critical cycle number (Cₚ) was established at 7% of maximal increase in fluorescence and was determined for each experimental run.

An interpolation algorithm was used to calculate Cₚ to within 1/1000 of a cycle.

SPECIFICITY

To verify specificity of the reaction, 15 µL of the reaction mixture containing PCR products from amplified reaction plates was separated by electrophoresis in 4% agarose gel (1% agarose, DNA Grade, High Melting; Fisher Scientific, Pittsburgh, Pa; 3% NuSize 3:1 agarose; FMC Bioproducts, Rockland, Me; and TBE Buffer; Novex, San Diego, Calif). Standards included a 100-bp ladder and a 25-bp ladder (Promega, Madison, Wis). The gel was developed using ethidium bromide and photographed on a light box with 360 nm of excitation (Transilluminator 4000; Stratagene, La Jolla, Calif).

In addition to analysis of the PCR product by electrophoresis, the identity of the PCR product was also analyzed by sequencing. Following real-time PCR, the amplified DNA was purified from the other reaction mixture components by binding to a silica-gel membrane column in a high-salt buffer and then elution in a low-salt buffer (QIAquick; Qiagen, Valencia, Calif). The purified DNA was replicated by PCR using fluorescent dye terminator deoxy-nucleotides, AmpliTaq polymerase FS (BigDye Terminator, Version 2.0, Applied Biosystems), and the forward primer from the real-time PCR.34-36 The reaction products were analyzed by gel electrophoresis within an automated DNA sequencer (ABI PRISM 377, Applied Biosystems). During the electrophoresis, the fluorescent fragments were identified as they passed through a laser beam detector positioned over the gel. Electropherograms from the sequencer were then analyzed using EditView 1.0.1 (Applied Biosystems).

STANDARD CURVES

Standard curves were generated for each real-time PCR experiment using cDNA from neonatal dermal melanocytes. Concentration of RNA was estimated by measuring optical density at 260 nm and comparing this value against purified calf liver RNA standards (Sigma, St Louis, Mo). Dermal melanocyte cDNA aliquots were stored at −80°C. A serial dilution series was generated extending from 50 ng to 0.78 ng per assay, and each dilution was assayed by real-time PCR in triplicate for tyrosinase and for GAPDH.
STANDARD CURVES

To calibrate experimental measurements, standard curves for both tyrosinase and GAPDH were generated using serial dilution of RNA from cultured human neonatal dermal melanocytes obtained from African American donors. As shown in Figure 2, the relationship between $C_T$ and the log of concentration was linear for both tyrosinase and GAPDH throughout the range of 0.78 ng to 50 ng of starting cDNA ($R^2=0.99$ and 0.97, respectively). The difference between the $C_T$ values for tyrosinase ($C_{Tt}$) and those for GAPDH ($C_{TG}$) was constant throughout the dilution series (mean±SD $C_{Tt}=2.58±0.35$). This difference can be used to calculate the ratio of tyrosinase mRNA to GAPDH mRNA as follows:

\[
\text{Fold Increase} = 2^{- (C_{Tt} - C_{TG})}
\]

This formula is based on consideration that the amount of PCR product and fluorescence signal doubles with each cycle. Hence, the ratio of tyrosinase mRNA to GAPDH mRNA in the present example was $6.14±1.41:1$. Both tyrosinase and GAPDH standards were generated for each iris culture analyzed and used to calculate the amount of each transcript.

TYROSINASE EXPRESSION IN IRIS CULTURES

To measure tyrosinase expression in the control iris cultures, the mean $C_T$ from tyrosinase expression in cDNA from each culture was compared with the mean $C_T$ from GAPDH determinations from the same cDNA samples. The amount of tyrosinase mRNA among the control samples ranged from the same to more than 200-fold greater than GAPDH mRNA (Figure 3). Among blue and hazel iris control cultures, the range of tyrosinase expression was similar, and it extended from 0.7-fold to 12.6-fold greater than GAPDH expression (mean±SD expression for blue iris cultures, $5.38±4.16$; for hazel iris cultures, $4.23±2.29$; $P=.41$ by unpaired $t$ test). In contrast, brown iris control culture tyrosinase expression ranged from 6.4-fold to 265-fold greater than GAPDH expression. This was significantly higher than the mean of the blue and hazel iris tyrosinase expression (mean±SD expression of brown irides, $57.1±76.5$-fold; of blue and hazel irides, 4.87±3.27-fold; $P=.009$ by unpaired $t$ test). Nevertheless, there was substantial overlap among the blue, hazel, and brown iris cultures (Figure 4). Hence, control cultures expressing from 6-fold to 12-fold more tyrosinase mRNA than GAPDH mRNA included blue, hazel, and brown irides.

INDUCTION OF TYROSINASE EXPRESSION BY LATANOPROST ACID

The amount of tyrosinase mRNA in iris cultures treated with latanoprost acid was compared with the amount of tyrosinase mRNA in vehicle-treated control cultures. In each case, the results were normalized according to GAPDH expression, and then each of the 2 measurements of the treated cultures was compared against the mean of the measurements from the corresponding control cultures and reported as fold induction. When each
range was expressed as a percent of the corresponding induction, the mean±SD of all the ranges obtained was 19.0%±13.5%. Thus, for any culture set from a particular donor, if the lowest measurement among the treated cultures was greater than 1.19-fold of the mean expression of tyrosinase mRNA in the corresponding control cultures, this was considered a positive induction of tyrosinase expression. Smaller changes were considered to be unconfirmed.

Tyrosinase mRNA induction among treated blue iris cultures compared with corresponding controls ranged from 0.79-fold to 1.63-fold, with a mean±SD induction of 1.23±0.33-fold (Figure 4A). However, in each case, the range of induction from each donor set came to within 19% of 1.0 (0.81-fold to 1.19-fold). Hence, induction was not confirmed among any of the blue iris cultures. Tyrosinase induction among the 9 hazel iris cultures ranged from 0.89-fold to 1.47-fold, with a mean±SD value of 1.17±0.22-fold (Figure 4B). In 1 culture set from an 85-year-old donor, the induction was 1.40 ± 0.05-fold. As the lowest induction was greater than 1.19-fold, this indicates induction of tyrosinase mRNA with latanoprost. Among the 13 brown iris cultures, tyrosinase induction ranged from 0.82-fold to 5.79-fold (Figure 4C). In 8 of these cultures, the lowest induction was less than 1.19-fold, suggesting no induction of tyrosinase (mean±SD, 1.00±0.23). In contrast, the lowest induction was greater than 1.19-fold in 5 brown iris cultures, suggesting latanoprost-mediated induction of tyrosinase mRNA. Mean induction values among the responding brown iris culture sets ranged from 1.96-fold to 5.79-fold (overall mean±SD, 2.97±1.60-fold; P<.01 compared with mean of nonresponding brown iris cultures). Comparison of the responding vs nonresponding donors did not suggest correlations between responders and age, race, sex, or cause of death. Also, death-to-enucleation times did not seem to be critical, as both responders and nonresponders were observed with death-to-enucleation times of up to 6.7 hours.

There did, however, seem to be a relationship between tyrosinase mRNA induction in latanoprost-treated cultures and tyrosinase mRNA expression in control cultures. As shown in Figure 5, no induction of tyrosinase expression was observed in cultures in which tyrosinase mRNA expression in the controls was less than 8-fold more than GAPDH expression. In contrast, latanoprost treatment increased tyrosinase expression in 6 of the 13 cultures in which control

![Figure 2](image-url)  
*Figure 2. Standard curves plotting time (CT) as a function of starting complementary DNA (cDNA) from dermal melanocytes for both tyrosinase (A) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (B). In both cases, the relationships were linear. Error bars indicate standard deviation of triplicate determinations.*

![Figure 3](image-url)  
*Figure 3. Mean tyrosinase messenger RNA (mRNA) in control iris cultures expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and grouped by eye color. Note that the range of blue and hazel control culture tyrosinase was quite similar. In contrast, the range of brown iris culture tyrosinase was an order of magnitude larger than either the blue or hazel iris culture tyrosinase. Error bars indicate the range of results from the duplicate-treated cultures from each pair of eyes.*
These results show that incubation of iris tissue with 200 nM latanoprost for 7 days increased expression of tyrosinase in cultures from 6 (20%) of 30 donors examined. Five of the 6 responding culture sets were brown irides, while the remaining set was hazel. In contrast, increased tyrosinase expression was not observed in any of the blue iris cultures and in 8 (89%) of 9 of hazel iris cultures. These results support the view that increased expression of tyrosinase occurs in some eyes after topical latanoprost treatment, and that this may contribute to the darkening of eye color that is occasionally observed. Moreover, induction of tyrosinase by latanoprost was only observed in those culture sets in which control expression of tyrosinase mRNA was 8-fold or more greater than GAPDH mRNA. These results suggest that variability in the initial expression of tyrosinase among patients receiving latanoprost treatment may contribute to the variability in the induction of gradual iris darkening.

The choice of GAPDH to serve as a reference for mRNA loading in the real-time PCR measurements is supported by experiments that directly compared the amount of cardiac myocyte mRNA loaded with real-time PCR measurements. It was observed that the ratio of GAPDH mRNA to total mRNA was the same in cells exposed to control medium or to 1µM dinoprost (PGF2α), a treatment known to induce myocyte hypertrophy. At the same time, transcription of genes for c-fos and atrial natriuretic factor in these cells were increased 35-fold and 800-fold, respectively. However, a doubling of total mRNA per cell in the treated cultures was noted. If an increase in total mRNA per cell occurred in the present cultures, it would have underestimated tyrosinase transcription induction by latanoprost acid. Unlike cardiac myocytes, no change was noted in the appearance or cell number of cultured human iridal melanocytes exposed to 10µM PGF2α or 10µM latanoprost acid, a concentration 50-fold greater than used in the present study. Hence, the present increase in tyrosinase mRNA measurements relative to GAPDH mRNA in the treated cultures is likely either to directly reflect or to underestimate the increase in tyrosinase mRNA copies per cell.

The differential responsiveness of the cultures in the present study seems to reflect intrinsic differences among the various donors. Evaluation of the age of the donors showed that both responsive and unresponsive cultures were obtained among cultures from donors ranging from the fourth to the eighth decades of life. Furthermore, neither cause of death, sex, nor race appeared to be related to responsiveness. Finally, both responders and nonresponders were observed among the cultures from donor eyes in which death-to-preservation time spanned from 3.5 to 6.7 hours, suggesting that this is not the determining factor for responsiveness in the present study. However, no induction was observed among cultures in which basal tyrosinase transcription was less than 8-fold more than GAPDH expression but was present in 6 of the 16 cultures in which basal tyrosinase transcription exceeded 8-fold more than GAPDH expression. Substantial evidence indicates that modulation of tyrosinase gene transcription involves the interactions of several regulatory influences and that the contributions of these influ-
ences can be either additive or synergistic.\textsuperscript{23,24,38} Hence, it is likely that the induction of tyrosinase transcription by 200nM latanoprost acid reflects the addition of FP-receptor-activated signals to other signals regulating the tyrosinase promoter. Variable response to these signals may reflect differences in the basal expression of these signals as well as differences in the tyrosinase promoter.

An important consideration is whether the latanoprost-mediated induction of tyrosinase mRNA observed in the present study reflects changes within the iridial melanocytes, which would be important for eye color, or the iris pigment epithelium (IPE). Previous studies have observed that adult rodent IPE contains tyrosinase activity and premelanosomes suggesting ongoing melanogenesis.\textsuperscript{39-41} However, analysis of monkey eyes following injection of the labeled false melanin precursor, methimazole, revealed uptake into iris melanocytes, but not into IPE cells.\textsuperscript{14} Moreover, in monkey eyes where iris darkening was observed following topical latanoprost treatment, there was increased uptake of methimazole in the iris stromal melanocytes compared with untreated contralateral eyes, but no uptake within IPE cells.\textsuperscript{19} This indicates that melanogenesis does not occur in the IPE of normal or latanoprost-treated monkey eyes. In view of these observations, it is likely that the increased tyrosinase mRNA observed in a portion of human iris cultures treated with latanoprost acid reflects changes within the iris stromal melanocytes and not the IPE.

Previous studies evaluating melanogenesis in cultured human iridial melanocytes exposed to latanoprost acid reported increased tyrosinase and melanogenesis in 1 study,\textsuperscript{25} and no change in melanogenesis in the other study.\textsuperscript{13} One possible explanation for this difference is that the melanocyte cell lines tested in the first study (where increased melanogenesis was observed) were from donor eyes with brown-containing irides (one with brown irides and the other with brown-blue irides). In contrast, the cell line evaluated in the study in which no induction of melanogenesis was observed was from a single donor with green irides. In view of the results of the present study, it is possible that these different responses reflect intrinsic differences in the responsiveness of the melanocytes from these different donors. This would be consistent with the variable induction of iris color change observed among patients receiving topical latanoprost treatment.\textsuperscript{8} Indeed, the present study showed that induction of tyrosinase mRNA was substantially more prevalent among the brown iris cultures than the hazel iris cultures.

Several features regarding the variation of tyrosinase mRNA expression in the control iris cultures are similar to the variation of melanosomes in iris melanocytes in situ. First, the observation of greater tyrosinase expression in brown irides than in blue or hazel irides is similar to the observation of greater melanosomes per melanocyte in dark brown irides than in blue irides.\textsuperscript{8} This suggests that a relationship exists between tyrosinase expression and melanogenesis. Supporting this view, a direct relationship was observed between increased tyrosinase expression and increased melanogenesis within ultraviolet light-irradiated dermal melanocytes.\textsuperscript{42} Nevertheless, there was substantial variability in mean melanosomes per melanocyte in irides of the same color classification.\textsuperscript{8} Moreover, like the case with tyrosinase expression, there was substantial overlap in the ranges of mean melanosomes per melanocyte among the various color categories so that it is not possible to predict iris color based on this parameter.\textsuperscript{8} This is similar to the substantial overlap in tyrosinase expression among the different color irides seen in the present study. Also, it has been observed that there is a capacity for the modulation of tyrosinase catalytic activity in human dermal melanocytes.\textsuperscript{21,22} Thus, it is possible that under certain circumstances, the relationship between increased tyrosinase gene transcription and iris darkening may not be direct.

In addition to differential modulation of tyrosinase transcription, several other factors may contribute to the variability of iris darkening observed with topical PG analog therapies. First, several other enzymes are critically involved in the biosynthesis of melanin including 5,6-dihydroxyindole carboxylic acid oxidase (tyrosinase related protein-1) and dopachrome tautomerase (tyrosinase related protein-1).\textsuperscript{43} Differential expression of each of these enzymes can alter the formation of melamin.\textsuperscript{42,44} Moreover, many other proteins are involved in the assembly and regulation of melanosome function.\textsuperscript{45} Variations in each of these are potential targets for modulating iridial melanocyte responsiveness to latanoprost. Recent experiments suggest that sympathetic tone mediated by the sympathetic innervation of the iris also can modulate latanoprost-mediated iris color change.\textsuperscript{45,46} Hence, these additional factors also may contribute to iris darkening that sometimes occurs with topical PG analog treatment.

In conclusion, there is a relationship between tyrosinase mRNA expression and iris color that is similar to the relationship between melanogenesis and eye color. Moreover, the amount of basal tyrosinase gene transcription may positively influence whether exposure to latanoprost will increase tyrosinase. These observations support an important role for tyrosinase gene transcription in latanoprost-mediated iris darkening.

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