

Effects of the Tyrosinase-Dependent Dopaminergic System on Refractive Error Development in Guinea Pigs

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PURPOSE. To determine if myopia in albino guinea pigs is linked to altered ocular dopamine (DA) levels in both the retinal and uveal dopaminergic systems.

METHODS. Retina and retinal pigment epithelium (RPE)/choroid were dissected from eyes of 2-week-old albino myopic (AM) and pigmented hyperopic (PH) guinea pigs. The levels of DA, dihydroxy-phenyl acetic acid (DOPAC), and homovanillic acid (HVA) were determined. Tyrosine hydroxylase (TH) and tyrosinase activities were also measured. PH animals received daily unilateral peribulbar injections of either kojic acid (tyrosinase inhibitor) or vehicle for 2 to 4 weeks. Refractive errors and ocular axial dimensions were measured by eccentric infrared photoretinoscopy and A-scan ultrasonography.

RESULTS. Retinal DA levels were similar between the two strains, but AM eyes had higher levels of DOPAC. RPE/choroid DA and tyrosinase activity in AM eyes were lower than in PH eyes ($P < 0.01$); however, the DA turnover was higher ($P < 0.05$). After 2 weeks of kojic acid treatment, PH eyes developed significant myopia, accompanied by elongated vitreous chambers and axial lengths. Inhibition of tyrosinase activity was linearly correlated with a myopic refraction shift ($R = 0.79$, $P < 0.01$). PH animals that received 62.5 ng/mL kojic acid treatment daily for 2 weeks followed by 625 ng/mL for 2 more weeks became more myopic and had deeper anterior chambers compared to those that received the 62.5 ng/mL dose over this period ($P = 0.04$).

CONCLUSIONS. The uveal tyrosinase-dependent dopaminergic system is involved in the development of guinea pig refraction. Enhancing uveal tyrosinase activity might slow down the development of myopia.

Keywords: tyrosinase-dependent dopaminergic system, refractive development, guinea pigs

Myopia (near-sightedness) is one of the most common disorders of the eye in young people and is currently observed at increasingly younger ages. In general, the earlier myopia starts, the worse it becomes later in life. This in turn results in a higher rate of complications and secondary pathologies, including retinal detachment,¹ subretinal neovascularization,² and glaucoma,³ all of which can result in irreversible vision loss. Myopia that develops after emmetropization is a result of the interaction between genetic and environmental factors. Thus, we were curious as to what, if any, early events differentiate myopia from nonmyopic children after birth.

Diverse animal models have shown that experimentally induced myopia is accompanied by reduced activity of the retinal dopaminergic system (reviewed by Feldkaemper and Schaeffel).⁴ As first reported by Stone et al.,⁵ the reduced activity results in a decrease in the content of retinal dopamine (DA) and is associated with a reduction of tyrosine hydroxylase (TH) activity,⁶ the rate-limiting enzyme in the synthesis of DA. Because DA release is linearly related to light intensity,⁷ bright light partially rescues the drop in DA release in chick form-deprived eyes.⁸ A cross-sectional epidemiological investigation⁹ and a randomized clinical trial¹⁰ also showed that increased outdoor time (mainly bright sunlight) has a protective effect against the incidence of myopia and progression.

The eye has multiple sources of DA. It is catalyzed not only by TH in the retina but also by tyrosinase in the retinal pigment epithelium (RPE) and uvea. In mice, uveal tyrosinase is involved more in DA synthesis than in melanin synthesis during early development after birth.¹¹ The serum level of L-dihydroxyphenylalanine (L-DOPA), the precursor to DA and melanin, is low in albino guinea pigs.¹² Thus we speculated that the level of L-DOPA in the highly vascular choroid of these animals would be lower than that in pigmented guinea pigs. Tyrosinase, which is necessary for the production of L-DOPA from tyrosine in the uveal DA system, is present in the RPE, blood vessel walls,¹³ and the uveal interstitium where there are abundant melanocytes.¹⁴ Additionally, aromatic L-amino acid decarboxylase (L-AADC), which converts L-DOPA to DA, is present in blood and all tissues, including the RPE/choroid.^{10,11} We hypothesized that there is an association between the absence of uveal tyrosinase activity in albino guinea pigs and the development of myopia. This hypothesis is strengthened by the presence of uveal tyrosinase activity in pigmented guinea pigs and the frequent occurrence of hyperopia (71.4%, $+5.14 \pm 2.08$ diopter [D]) in these animals.^{15,16} Because tyrosinase is the key enzyme in the tyrosinase-dependent dopaminergic system, we hypothesized that low or absent uveal tyrosinase activity promotes the development of myopia in albino guinea pigs. Further, we



speculated that the lower levels of retinal and/or uveal DA are responsible for the early onset and high incidence of myopia (70.1%, -6.13 ± 2.70 D) in albino guinea pigs.¹⁵

In this study, we compared the retinal dopaminergic systems of albino myopic (AM) and pigmented hyperopic (PH) guinea pigs. We also attempted to inhibit tyrosinase activity in PH guinea pigs through daily peribulbar injections of kojic acid, a standard inhibitor of tyrosinase,¹⁷ to observe the effect on refractive development.

MATERIALS AND METHODS

Animals

Thirteen albino and 60 pigmented 2-week-old guinea pigs (*Cavia porcellus*, the English short-hair stock) were obtained from the laboratory animal center at Zhejiang University, Hangzhou, China. The animals were reared under a 12-hour light/12-hour dark cycle. After the animals were transferred to Wenzhou Medical University, the lights were turned on at 8:00 AM and off at 8:00 PM. Illuminance at the cage floor was approximately 300 lux, which is reported to be sufficient for animal care but does not cause retinal degeneration in albino animals.¹⁸ Visual exposure was limited to the cage environment, as described previously.¹⁹ Room temperature was kept at 25°C. The animals had free access to standard food and water, and fresh vegetables were provided twice a day. The use of animals for these studies was approved by the Animal Care and Ethics Committee at Wenzhou Medical University, Wenzhou, China. All procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Experimental Protocols

This study had two main goals. Our first goal was to determine if there were differences in the retinal and uveal DA systems between 2-week-old AM and PH guinea pigs. After biometry measurements (described below), the vitreous body, retina, and RPE/choroid were harvested separately. DA and DA metabolites dihydroxy-phenyl acetic acid (DOPAC) and homovanillic acid (HVA) in each tissue were measured by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). Tyrosinase activity in the RPE/choroid was measured by a standard stop assay²⁰ (AM, $n = 3$; PH, $n = 3$), and TH in the retina was assayed by Western blot analysis (AM, $n = 3$; PH, $n = 3$). We found that the retinal TH pathway was not significantly different between the AM and the PH guinea pigs. Therefore, we hypothesized that the tyrosinase-dependent dopaminergic system is another important regulator of the postnatal refractive error development under normal vision conditions, while the TH-dependent retinal dopaminergic system is involved in form-deprivation myopia.

Based upon the hypothesis that DA produced by the uvea regulates postnatal eye growth and refractive error development, at least in part, our second goal was to determine the effect of tyrosinase inhibition on the refractive error development in the eyes of PH guinea pigs. We administered daily peribulbar injections of kojic acid, a tyrosinase inhibitor¹⁷ (K3125-5 g; Sigma-Aldrich Corp., St. Louis, MO, USA) at 62.5 ng/mL ($n = 20$), 625 ng/mL ($n = 9$), or 6250 ng/mL ($n = 9$) in a vehicle of 0.9% saline (100 μ L) for 2 weeks. The same vehicle was also used as the control injection ($n = 8$). For the 62.5 ng/mL group, 11 of the 20 animals received 2 more weeks of injection at the same dose. For 9 of the 20 animals, the dose

was increased to 625 ng/mL for an additional 2 weeks. Vehicle controls were extended accordingly.

The injection procedure has been described previously.²¹ In brief, topical anesthesia was induced in the right eye with one drop of 0.5% proparacaine hydrochloride (Alcon, Puurs, Belgium) every morning at around 10:00 AM. Then 100 μ L vehicle only or vehicle containing the kojic acid was gently injected into the peribulbar space through the lower conjunctival sac of the eyes in control and experimental animals, respectively. Tyrosinase activity in RPE/choroid was measured in some of the drug-treated animals ($n = 12$).

Biometry

Refractive errors (RE) were measured in the vertical pupil meridian using an eccentric infrared photorefractor in alert guinea pigs without cycloplegia, as described previously.¹⁶ The mean of three RE readings was used for statistical analyses.

The axial dimensions of the eyes were measured in alert animals, as described previously,¹⁹ using A-scan ultrasonography (11 MHz, AVISO Echograph Class I-Type Bat; Quantel Medical, Clermont-Ferrand, France) on the same day as the RE were measured. In brief, the cornea was anesthetized topically with one drop of 0.5% proparacaine hydrochloride (Alcon). A stand-off rubber tube was attached to the probe tip, as described by Schaeffel and Howland.²² During the measurement, one hand was used to gently restrict the animal by holding its body, while the other hand placed the modified probe on the center of the cornea until acceptable traces from each interface of the eye components were detected. The recorded parameters included anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD), and axial length (AL) as measured from the anterior surface of the cornea to the anterior surface of the retina. Each eye was measured at least eight times, and the average was used for further analyses. The measurements were made before treatment and after 2 and 4 weeks of treatment.

Vitreous Body, Retina, and RPE/Choroidal Complex Collection, Preparation, and Storage for HPLC

Two-week-old untreated animals were killed by cervical dislocation between 10:00 AM and 11:00 AM. The right eyes were enucleated and put on ice. Eye cups were prepared by coronal section 2 mm behind the limbus. The anterior segment and crystalline lens were removed. Then, under a dissecting microscope, an iris spatula was used to carefully remove the vitreous body and then the retina. The remaining RPE/choroid and sclera were cut in half, and the RPE/choroid was scraped from the sclera. Each tissue was placed into separate 1.5 mL preweighed plastic sealable tubes, which were then reweighed. All procedures were performed within 5 minutes. Immediately after reweighing, 150 μ L homogenizing medium with HPLC-EDC internal standard composed of 0.02 μ M dihydroxybenzylamine (DHBA), 0.1 M perchloric acid, 10 μ M ascorbic acid, and 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA) was added. Each tissue was homogenized manually first and then in an ultrasonic bath. During the whole procedure, the samples were kept in an ice slurry. The homogenates were centrifuged at 4°C for 30 minutes at 18,800g and the supernatants were collected and stored at -80° C until use. All samples were shipped overnight on dry ice from Wenzhou to the Neuron Science Institute, Nanjing Medical University, for analysis by HPLC-ECD (see following section).

High-Performance Liquid Chromatography With Electrochemical Detection

DA, DOPAC, and HVA levels in the vitreous bodies, retina, and RPE/choroid tissues were analyzed by HPLC-ECD. Sample supernatants (10 μ L) were injected into the UniMate 3000 auto-sample and pumped by a Thermo UniMate 3000 Pump (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C. The stationary phase in the column was composed of C18 silica (DIONEX AcclaimR RSLC 2.1X100 mm, 2.2 μ m, Thermo Fisher Scientific). DA and the DA metabolites DOPAC and HVA were eluted at a flow rate of 0.2 mL/min in a mobile phase consisting of 90 mM phosphoric acid, 50 μ M EDTA, 1.7 mM sodium 1-octanesulfonate, an ion pairing agent, and 5% acetonitrile. The eluted compounds were detected and quantified using an ESA Coulochem III Electrochemical Detector (Thermo Fisher Scientific), under an applied potential of 350 mV. DA, DOPAC, and HVA peaks were identified by relative retention times, compared to those of extracted standards. The concentration of each eluted compound was determined by comparing peak areas of the samples with those of standards, using the Chromeleon chromatograph workstation (Thermo Fisher Scientific) to collect and analyze the data.

After determining the concentrations of DA and metabolites in each supernatant, the content in each tissue was calculated based on the molecular weight and the initial wet tissue weight. DA and metabolite levels were compared between groups, as well as the ratio of (DOPAC+HVA)/DA as an indicator of DA turnover in the eye.

Enzyme Activity Assays

Posterior eye cups were prepared as described above. After removal of the vitreous body, the retina was gently collected from each collapsed eye cup. For the left eye cup, scissors were used to make three radial incisions of approximately two-thirds of the radius, creating a “petal” shape, and the RPE/choroid complex was scraped from the sclera. The retinal and RPE/choroidal tissues were placed separately into 1.5 mL EP tubes for use in enzyme activity assays.

Western Blotting

Retinas were lysed in 150 μ L RIPA (Beyotime Biotechnology, Shanghai, China) with 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology, Shanghai, China) followed by mechanical and then ultrasonic homogenization. The mixture was centrifuged at 9600g for 1 minute and the supernatant was extracted. Equal volumes of protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (following the EMD Millipore Western Blotting protocol; MilliporeSigma, Darmstadt, Germany). The membranes were then blocked with 5% skim milk powder, incubated with rabbit anti-TH (MilliporeSigma), and rabbit anti-phospho-tyrosine hydroxylase (anti-pSer40; Cell Signaling, Danvers, MA, USA). Secondary antibodies with anti-mouse IgG were applied and developed using enhanced chemiluminescence detection reagents.

Tyrosinase DOPA Oxidase Activity Assay

In the EP tubes with RPE/choroidal tissue, several porcelain beads were added, followed by 200 μ L homogenization buffer composed of 0.1% Triton X-100 and 0.001% PMSF. The mixtures were vibrated at 30 Hz for 5 minutes using a ball miller (Ball Mill, MM400; Retsch GmbH, Hann, Germany), then centrifuged for 10 minutes at 4°C at 18,800g. The supernatants

were extracted, and the proteins were quantified using the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA).

Tyrosinase DOPA activity was measured in a stopped spectrophotometric assay²⁰ that is specifically sensitive to detect pmol level of product produced by tyrosinase in mammalian cells expected to express the enzyme. In brief, the reaction solution was freshly prepared using 250 μ L assay buffer (100 mM potassium phosphate [pH 7.1], with 4% [vol/vol] N,N'-dimethylformamide, 0.1% [vol/vol] Triton X-100), 100 μ L 5 mM L-DOPA (D9628-5G, Sigma-Aldrich Corp.), and 120 μ L 25 mM 3-methyl-2-benzothiazonone hydrazine (MBTH, 129739-5G; Sigma-Aldrich Corp.). Equal volumes of freshly prepared enzyme protein solution and different concentrations of kojic acid stock (62.5, 625, or 6250 ng/mL) were mixed, respectively. The mixture was incubated in a water bath at 37°C for 1 hour. The enzyme/kojic acid mixture or freshly prepared enzyme protein solution without kojic acid (30 μ L) was added to the reaction solution (500 μ L final volume) and incubated at 37°C for 30 minutes. The oxidation of L-DOPA by tyrosinase produces dopaquinone that reacts with the MBTH to form a pink product with a sharp absorption peak at 505 nm. The reaction was terminated with the addition of 500 μ L 1 M perchloric acid, which also stabilized the pink product. The mixture was microcentrifuged and the supernatants were transferred to transparent 24-well plates (Becton Dickinson Labware, Bedford, MA, USA). The absorbance was measured at 505 nm with an MD SpectraMax M5 (Molecular Devices, LLC., Sunnyvale, CA, USA). As recommended in the assay protocol,²⁰ the data were represented as 1000 times the absorbance at 505 nm/mg protein.

Statistics

The means \pm standard errors of all data were determined. Unpaired 2-tailed Student's *t*-tests were used to compare levels of DA, DA metabolites, and tyrosinase activity between the AM and PH guinea pigs. One-way ANOVA was performed to evaluate the effects of kojic acid on tyrosinase activity in vitro and on RE development in vivo. Inhibition of tyrosinase activity by kojic acid was calculated as a ratio ([treated eye – fellow eye] / fellow eye). Linear regression assessed the correlation between the enzyme inhibition ratio of tyrosinase in the RPE/choroid and the RE shift after 2 weeks of kojic acid treatment.

To determine the effect of extended kojic acid treatment, the interocular differences (treated eyes – fellow eyes) of RE, ACD, LT, VCD, and AL at different age points were determined and analyzed by repeated measures ANOVA and 1-way ANOVA.

RESULTS

Comparison of Retinal Dopaminergic System Activity in AM and PH Guinea Pigs

Under normal visual and light conditions, the refraction of 2-week-old AM guinea pigs ($n=7$) was -8.25 ± 1.40 D, while for PH guinea pigs it was $+6.50 \pm 0.93$ D ($P < 0.001$). Based on Western blot analysis, there were no differences between AM and PH animals in the retinal protein levels of either TH or the phosphorylated isoform Ser40 (Fig. 1A). In AM guinea pigs, the retinal DA and HVA levels, 0.43 ± 0.17 and 0.035 ± 0.002 μ g/g, respectively, were not significantly different from those in PH guinea pigs, 0.31 ± 0.11 and 0.033 ± 0.002 μ g/g, respectively ($P > 0.05$ each, unpaired *t*-test, Fig. 1B). However, there was statistically more DOPAC in AM retinas, 0.22 ± 0.01 , than in PH retinas, 0.18 ± 0.01 μ g/g retina ($P=0.02$, unpaired *t*-test, Fig. 1B). Nevertheless, the metabolite conversion ratio

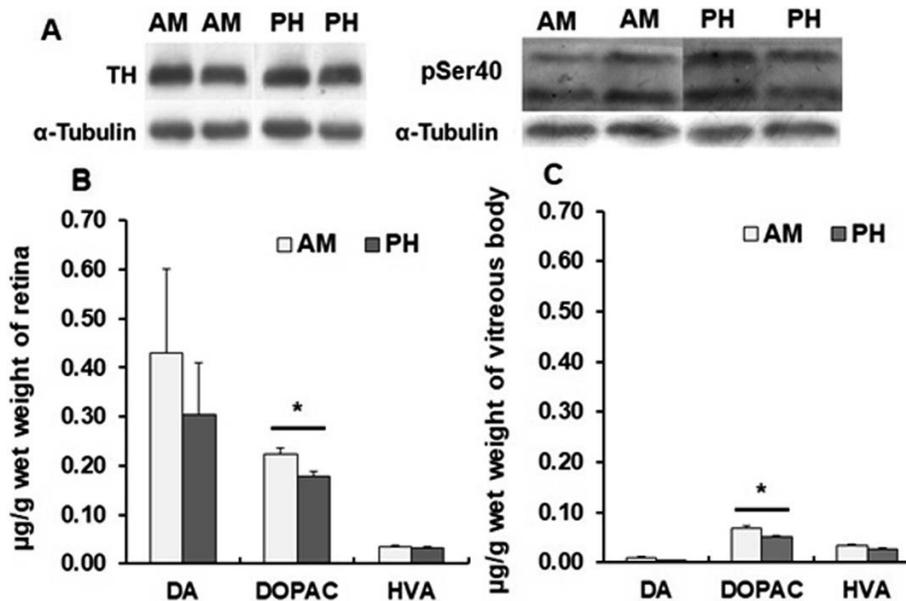


FIGURE 1. Comparison of retinal dopaminergic systems in 2-week-old AM and PH guinea pigs. (A) Protein levels of retinal tyrosine hydroxylase (TH) and the phosphorylated isoform (pSer40) were quantified by Western blots. α -Tubulin was the loading control. Each lane corresponds to one retina. (B) Retinal DOPAC, DA, and HVA ($\mu\text{g/g}$ wet weight). (C) Vitreous body DOPAC, DA, and HVA ($\mu\text{g/g}$ vitreous body). Light gray bars: albino guinea pigs ($n = 7$); dark gray bars: pigmented guinea pigs ($n = 8$). * $P < 0.05$.

for AM guinea pigs, measured as the ratio of (DOPAC+HVA)/DA, was 2.77 ± 0.79 , which was not statistically different from the PH guinea pig retinas, 1.60 ± 0.46 ($P = 0.25$, unpaired t -test).

It is accepted that DA and DA metabolites in the vitreous body accurately reflect the dopaminergic activity in the retina.²³ In the current study, we also assayed DA and DA metabolites in the vitreous body. In AM guinea pigs, the vitreous body DA and HVA levels, $0.009 \pm 0.002 \mu\text{g/g}$ and $0.034 \pm 0.003 \mu\text{g/g}$, respectively, were not significantly different from those in PH guinea pigs, $0.0050 \pm 0.0003 \mu\text{g/g}$ and $0.028 \pm 0.002 \mu\text{g/g}$, respectively ($P > 0.05$ each, Fig. 1C). Consistent with the retina results, the DOPAC level in vitreous body in the AM animals, $0.068 \pm 0.005 \mu\text{g/g}$, was significantly greater than that in the PH animals, $0.052 \pm 0.003 \mu\text{g/g}$ ($P = 0.02$, Fig. 1C). The metabolic conversion ratio for AM guinea pigs, measured as described above, 14.07 ± 1.68 , was not significantly different from that of PH guinea pigs, 16.35 ± 1.22 .

Comparison of the RPE/Choroidal Dopaminergic System Activity in AM and PH Guinea Pigs

We assessed DA levels and tyrosinase activity in the RPE/choroid complexes of the two guinea pig strains. The DA level was significantly lower in the RPE/choroid complex of AM guinea pigs, $0.010 \pm 0.002 \mu\text{g/g}$ tissue, compared to PH guinea pigs, $0.07 \pm 0.02 \mu\text{g/g}$ tissue ($P < 0.01$, Fig. 2A). In contrast, the concentration of the DA metabolites, DOPAC and HVA, was significantly higher in the AM RPE/choroid complex than in the PH guinea pigs (DOPAC: AM 0.15 ± 0.01 vs. PH $0.12 \pm 0.01 \mu\text{g/g}$ RPE/choroid tissue, $P < 0.05$; HVA: AM 0.030 ± 0.002 vs. PH $0.020 \pm 0.002 \mu\text{g/g}$ RPE/choroid tissue, $P < 0.01$). This difference indicated greater DA turnover in AM than in PH RPE/choroid tissue (22.01 ± 3.13 vs. 4.44 ± 1.39 , $P < 0.001$). Because the retinal DA levels in both strains were similar, the possibility was small that an inadvertent transfer of DA and metabolites originating from the retina to the RPE/choroid

complex during tissue separation had a significant effect on the assay.

There was no significant difference in the tyrosinase content of the AM and PH RPE/choroid complexes as determined in Western blots (Fig. 2B). The AM RPE/choroid complex tyrosinase-dependent DOPA oxidase activity, 0.20 ± 0.05 , was higher than in the retinal controls from both AM and PH guinea pigs, -0.14 ± 0.05 and -0.08 ± 0.05 , respectively (1-way ANOVA, $P < 0.001$). However, it was lower than the PH RPE/choroid complex DOPA oxidase activity (0.20 ± 0.05 vs. 2.06 ± 0.24 , $P < 0.001$, Fig. 2C).

Effects of Kojic Acid on Tyrosinase Activity and on Refractive Development in PH Guinea Pigs

In an in vitro assay, we validated the inhibitory effect of kojic acid on tyrosinase activity. The activity decreased in a concentration-dependent manner, reaching 50% of the control activity when incubated with 6250 ng/mL ($P < 0.0001$, 1-way ANOVA, Fig. 3A). Based upon these levels of inhibition, we selected doses of 62.5, 625, and 6250 ng/mL for daily peribulbar injection.

Interocular differences at the end of the treatment period, normalized to pretreatment values, were calculated to evaluate treatment-induced changes in RE. The kojic acid-treated groups consistently showed significant myopic shifts in RE compared with the control group, which showed a hyperopic shift (1-way ANOVA, $P = 0.001$): 62.5 ng/mL, $-1.43 \pm 0.25 \text{ D}$; 625 ng/mL, $-2.01 \pm 0.33 \text{ D}$; and 6250 ng/mL, $-1.64 \pm 0.44 \text{ D}$ (Fig. 3B). Each treated group was significantly different from the control vehicle-injected group $+0.34 \pm 0.17 \text{ D}$ ($P = 0.009$, $P = 0.001$, $P = 0.021$, post hoc multiple comparisons). However, there were no statistically significant differences in the biometric parameters among the drug-treated groups. None of the kojic acid doses induced statistically significant change in ACD, LT, VCD, or AL after 2 weeks of treatment (Fig. 3C). However, there was an apparent tendency for deepening of the anterior chamber, vitreous chamber, and elongation of the AL in eyes treated with 625 and 6250 ng/mL kojic acid (Fig. 3C).

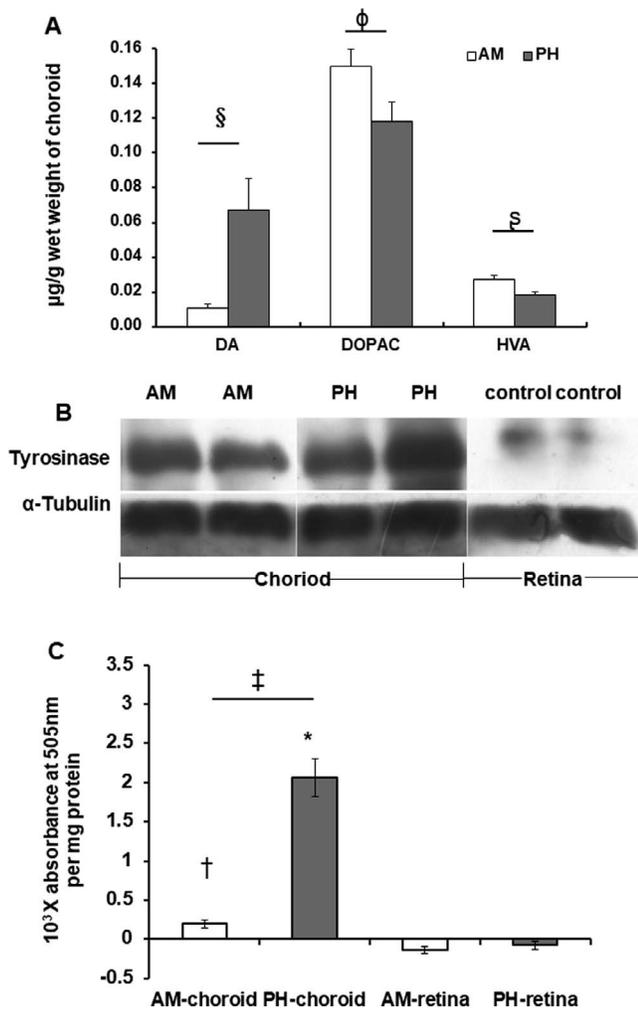


FIGURE 2. Comparison of the dopaminergic system in the RPE/choroid complex of AM and PH guinea pigs. (A) Levels of DA, DOPAC, and HVA in the RPE/choroid complex of AM (light gray bar) and PH (dark gray bars) guinea pigs. $\S P < 0.05$; $\phi P < 0.04$; $\S P < 0.01$. (B) Western blots of tyrosinase in the RPE/choroid complex and retina. Control, tyrosinase negative control using PH retina. (C) Tyrosinase activity, measured by DOPA oxidation of the stopped MBTH assay, in AM and PH guinea pigs. Retinas from AM and PH guinea pigs served as negative controls. *Comparison of PH-RPE/choroid with the negative control, $P < 0.001$; †comparison of AM-RPE/choroid with the negative control, $P < 0.01$; ‡comparison between AM-RPE/choroid and PH-RPE/choroid, $P < 0.001$; independent t -test.

To confirm that tyrosinase activity in the RPE/choroid complex was related to the changes in refraction, the activity in drug-treated eyes ($n = 12$) was tested in vitro. The enzyme activity inhibition ratio was calculated as the ratio of ([treated eye – fellow eye]/fellow eye). There was a significant linear correlation between the enzyme inhibition ratios and the interocular differences in refraction: $y = 2.88x - 0.58$ ($R = 0.79$, $P = 0.002$, Fig. 4), where y is the interocular difference in refraction after 2 weeks of drug treatment and x is the enzyme inhibition ratio. Based on the correlation, inhibition of approximately 20% of the enzyme activity was associated with 1 D of interocular difference. Three of the kojic acid-treated eyes showed slight increases in tyrosinase activity (Fig. 4). For two of these eyes (Fig. 4, upper right quadrant), the development of a hyperopic interocular difference in refraction was not inhibited.

TABLE. Interocular Differences Before and After Kojic Acid Treatment

Interocular Differences (Treated Eyes – Fellow Eyes)			
Kojic Acid	2 wk	4 wk	6 wk
Refractive error, D			
CL	-0.36 ± 0.13	-1.95 ± 0.45	-1.11 ± 0.45
ED	-0.28 ± 0.04	-1.89 ± 0.46	$-3.97 \pm 0.39^*$
Anterior chamber depth, μm			
CL	3 ± 12	-5 ± 10	-9 ± 3
ED	-3 ± 6	-4 ± 6	$12 \pm 10^\dagger$
Crystalline lens thickness, μm			
CL	7 ± 13	9 ± 8	14 ± 6
ED	0 ± 6	7 ± 7	6 ± 13
Vitreous chamber depth, μm			
CL	8 ± 7	35 ± 14	16 ± 16
ED	14 ± 5	24 ± 17	33 ± 12
Axial length, μm			
CL	17 ± 7	35 ± 15	21 ± 17
ED	11 ± 7	27 ± 16	51 ± 11

Kojic acid dosages: CL, continuously low dosage of 62.5 ng/mL; ED, enhanced dosage varying from 62.5 to 625 ng/mL.

* Significant difference between CL and ED groups at 6 weeks of age, $P < 0.001$, 1-way ANOVA.

† Significant difference between CL and ED groups at 6 weeks of age, $P = 0.04$, 1-way ANOVA.

In the low dosage only (LD) group, the interocular difference at 4 weeks of age (after 2 weeks of the initial treatment), -1.95 ± 0.45 D, shifted to -1.11 ± 0.45 D at 6 weeks of age (after the 2 additional weeks of treatment) (Table). In the enhanced dosage (ED) group, the interocular differences increased from -1.89 ± 0.46 D at 4 weeks of age to -3.97 ± 0.39 D at 6 weeks of age. Overall, the interocular differences between the two groups were significant over the 4 weeks of treatment ($F[1,18] = 4.9$, $P = 0.04$, repeated measures ANOVA). At the end of treatment period, the interocular difference for the LD group, -1.11 ± 0.45 D, was significantly less than for the ED group, -3.97 ± 0.39 D, ($P < 0.01$, independent t -test). Interocular differences in ACD increased over time for the ED group, resulting in significant differences between the LD and ED groups by the end of the treatment period (LD versus ED: -9 ± 3 vs. 12 ± 10 μm , $P = 0.04$, independent t -test).

Although none of the other interocular differences of biometric parameters were statistically significant over the 4 weeks of drug treatment (repeated measures ANOVA), the changes were consistent with and may have contributed to the myopic interocular differences in the ED group (detailed in Table). Specifically, the interocular differences of AL and VCD tended to decrease in the LD group but increased in the ED group over time. In contrast, the interocular difference of LT increased in the LD group but decreased in the ED group over time.

DISCUSSION

Our findings suggest that tyrosinase is involved in eye growth and RE development. Tyrosinase activity in the RPE/choroid complex of developmentally myopic albino guinea pigs was significantly lower than in the developmentally hyperopic pigmented guinea pigs. Furthermore, when tyrosinase activity in the PH eyes was partially inhibited, interocular difference in RE showed myopic shifts that were linearly correlated with the degree of enzyme inhibition. Extending kojic acid treatment with a higher dose resulted in greater myopic interocular differences accompanied by a deepening of the AC. The latter

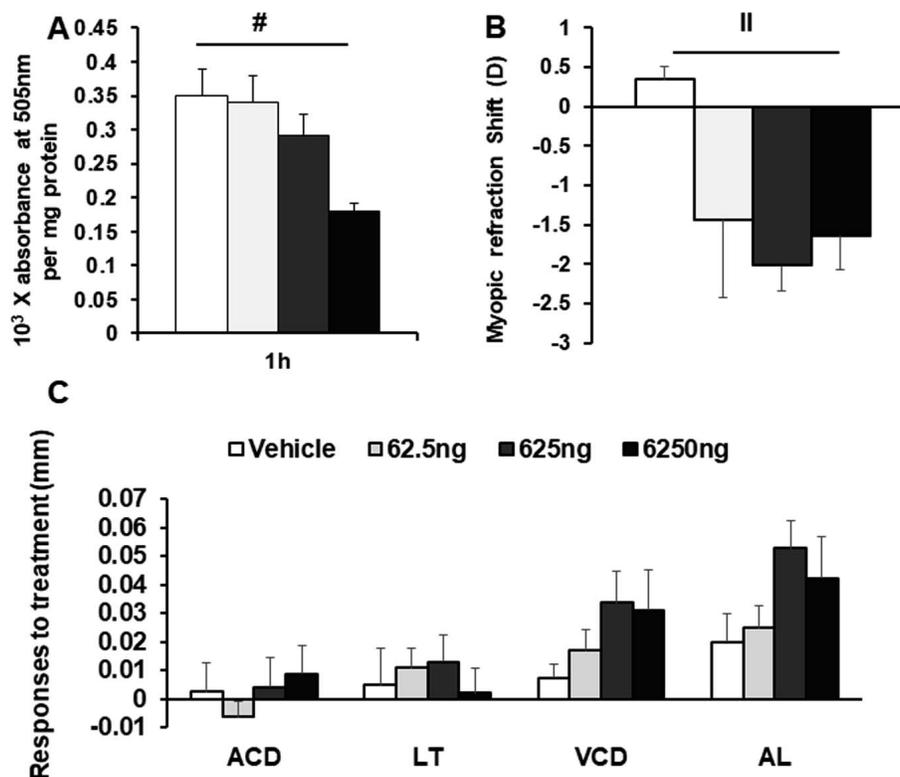


FIGURE 3. Effects of the tyrosinase inhibitor, kojic acid, on tyrosinase DOPA oxidase activity in the RPE/choroid complex and on refractive error development in PH guinea pigs. (A) In extracts prepared from RPE/choroid complexes of PH guinea pigs, kojic acid inhibited tyrosinase DOPA oxidase activity in a concentration-dependent manner ($\#P < 0.001$ 1-way ANOVA), but only the highest concentration significantly inhibited enzyme activity compared to the blank control ($P < 0.01$). (B) Two weeks of daily drug administration induced a significant myopic refraction shift ($\|P < 0.01$, 1-way ANOVA). (C) Changes in the normalized eye parameters showed apparent dose-dependent patterns for the ACD, VCD, and AL.

result suggests the involvement of tyrosinase-derived DA in anterior segment development.

Involvement of the RPE/Choroidal Tyrosinase-Dependent Dopaminergic System in RE Development

Guinea pigs, as precocial animals, are born with well-developed retinas.²⁴ However, pigmented and albino animals undergo different RE development after birth. At birth, the pigmented guinea pigs used in our current and past studies typically have hyperopic RE that decrease over time, but remain hyperopic ($+8.26 \pm 4.51$ D at birth, $+6.50 \pm 0.93$ D at 2 weeks, $n = 24$; retinoscopy) (Jiang L, Wei Z, unpublished data, 2014). In contrast, while the albino guinea pigs used in our current and past studies also have hyperopic RE at birth, their RE rapidly progress toward myopia within first 2 weeks of age ($+4.49 \pm 4.48$ D at birth, -8.25 ± 1.40 D at 2 weeks, $n = 8$; retinoscopy) (Jiang L, Wei Z, unpublished data, 2014).

A question of interest is why the AM guinea pigs develop myopia so rapidly while their pigmented counterparts remain hyperopic. There were no differences of retinal TH enzyme activity and DA level between the AM and PH animals. Thus, spontaneous myopia in albino guinea pigs is different from experimental form-deprivation myopia that is linked to lower levels of DA and a reduction of TH activity in the retina.^{4,5} Bergen et al.²⁵ reported that retina-specific TH knockout (rTHKO) pigmented mice maintained the same degree of refraction as wild-type littermates up through 4 weeks of age, and after that a small amount of DA was still detected in their retinas at 10 weeks of age.²⁵ The residual DA may have

originated from the RPE/choroid (tyrosinase-dependent DA), and could have contributed to the maintenance of postnatal RE development in the rTHKO pigmented mice. Moreover, in chickens, the application of 6-hydroxy DA (a neurotoxin to DA amacrine cells) suppressed deprivation myopia.²⁶ One possible explanation for this paradoxical result is that the long-term reduction in retinal DA caused an upregulation in retinal DA

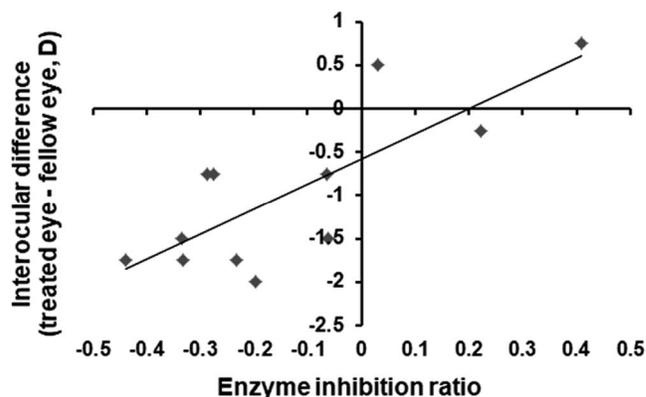


FIGURE 4. Correlation of interocular differences in refractive error with inhibition ratios of tyrosinase activity. The enzyme inhibition ratio was calculated as the ([treated eye - fellow eye]/fellow eye) in RPE/choroid complex after 2 weeks of daily peribulbar injection of kojic acid (62.5–625 ng) in PH guinea pigs. The correlation between the myopic interocular difference in refraction and the enzyme inhibition ratio was described by the equation $y = 2.88x - 0.58$ ($R = 0.79$, $P = 0.002$), where y was the relative shift in myopia and x was the inhibition ratio of the enzyme.

receptors (Mangel S, et al. *IOVS* 1996;37:ARVO Abstract S139), with which other nonretinal sources of DA interacted to normalize retinal DA activity. One such source could be the DA secreted by the RPE/choroid, for which, at least in chickens, the tyrosinase-dependent DA system was unaffected by deprivation.²⁷

Our results showed that there was still residual tyrosinase activity in the AM RPE/choroid complex when the animals were 2 weeks of age, but as expected, it was dramatically lower than in PH RPE/choroid complex. Thus, the AM eyes were hyperopic shortly after birth and then developed myopia rapidly later. Accordingly, the DA level was significantly lower in the AM RPE/choroid complex than in the PH complex. But the DA metabolites DOPAC and HVA were much higher in the AM RPE/choroid complex than in the PH complex. We are uncertain whether this increase in DA turnover is a compensation for low DA levels or whether it leads to DA neurotoxic effects, as proposed as an explanation for similar findings in Parkinson's disease.²⁸ In the specific AM animal model in the current study, the low DA level accompanied by high DA turnover occurred concurrently with the rapid progression of myopia development.

Tyrosinase is the key enzyme in the uveal tyrosinase-dependent dopaminergic system. The myopic refraction shift in the PH guinea pigs after uveal tyrosinase activity inhibition by kojic acid supported the proposed role of the uveal DA system in modulating eye growth. The linear correlation between uveal tyrosinase activity and induced myopia, expressed as interocular differences, provides additional support for this idea. Two additional weeks of kojic acid at the higher dosage induced larger myopic changes, accompanied by increases in ACDs. It is not clear why the ACD changed after extended kojic acid application at the higher dose of 625 ng/mL. A possible explanation is that the high dosage of kojic acid diffused into the anterior chamber to inhibit local tyrosinase activity, thereby depleting L-DOPA levels, which, in turn, altered the development of the anterior chamber.²⁹

A recent report showed that systemic administration of L-DOPA to AM guinea pigs reduces both spontaneous myopia and form-deprived myopia.³⁰ Guinea pigs have no retinal blood vessels,³¹ and the retinal DA levels in AM and PH guinea pigs were similar. Therefore, we speculate that systemic injection of L-DOPA increased DA levels in the RPE/choroid, thereby inhibiting myopic changes by way of the proposed supplemental uveal dopaminergic pathway.

Relevance of Tyrosinase Activity on RE Development in Humans

Our in vitro results showed that kojic acid inhibited tyrosinase activity in a dose-dependent manner. Furthermore, in vivo, inhibition of tyrosinase induced myopic changes in PH guinea pigs that increased with the magnitude of inhibition. It may be relevant that the incidence of myopia (≤ -1 D spherical equivalent) in non-Hispanic whites (35.2%) is more common than in non-Hispanic blacks (28.6%) and Mexican Americans (25.1%).³² The differences are even bigger in cases of severe myopia (< -5 D): non-Hispanic whites, 7%; non-Hispanic blacks, 4.7%; and Mexican Americans, 3.6%. The tyrosinase activity in the skin of black neonates is significantly greater than in white neonates.³³ If similar activity levels are present in the uvea, it may partially explain the disparity in incidences of myopia and high myopia among the different racial/ethnic groups.

Many reports now suggest that outdoor activity can retard the development of myopia. Norton and Siegwart³⁴ reviewed reports that high-intensity indoor light can partially inhibit form-deprivation myopia in chickens,³⁵ macaques,³⁶ and tree

shrews. It can also slow compensation to the defocus imposed by negative lenses in chickens³⁷ and tree shrews. While indoor high-intensity light did not alter the degree of negative lens-induced myopia in macaques,³⁸ 3 hours of outdoor light exposure partially inhibited lens-induced myopia in rhesus monkeys.³⁹ In guinea pigs, high-intensity light partially inhibited the induction of myopia by negative lenses⁴⁰; however, blue light (470 ± 5 nm) almost completely inhibited lens-induced myopia.⁴¹

It is worth mentioning that catecholaminergic balance can be modified by environmental and behavioral factors (such as stress, social interactions, and diet), and can lead to obesity⁴² and hypertension.⁴³ The increased incidence of myopia in young Asians shares many similar contributing factors such as intense academic focus and high incidence of stressors. Therefore, understanding how environmental and behavioral factors influence the homeostatic regulation that maintains the balance of retinal and uveal DA systems, and how these systems mutually modulate refractive development, could provide insight for the treatment and prevention of myopia.

CONCLUSIONS

Under normal vision conditions, RE development in PH guinea pigs undergoes a myopic refraction shift after tyrosinase inhibition by daily peribulbar injection of kojic acid. Furthermore, a higher dosage of kojic acid increased ACD and contributed to a larger shift in myopic refraction. Based upon the great difference between AM and PH guinea pigs in their postnatal RE development yet similar retinal DA levels, we speculate that a supplemental uveal DA system may also be involved in the development of RE.

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