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Cyclic Adenosine Monophosphate Activates Retinal Apolipoprotein A1 Expression and Inhibits Myopic Eye Growth

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Purpose. Apolipoprotein A1 (ApoA1) has been shown to inhibit myopia development in chicks, but the underlying biological mechanism remains unknown. Because ApoA1 interacts with cyclic adenosine monophosphate (cAMP) in many cellular systems, we examined whether this interaction is important in myopia development.

Methods. The nonmetabolizable cAMP analogue 8-Bromo-cAMP (8-Br-cAMP) was administered intravitreally to the right eyes of 8-day old chicks for 4 consecutive days. Control eyes received vehicle. Chicks in group 1 received 8-Br-cAMP (0.1 mM or 1 mM) and were fitted with −10 diopter (D) lenses on both eyes, whereas chicks in group 2 (0.1 mM 8-Br-cAMP) wore plano lenses over both eyes. The levels of retinal cAMP and ApoA1 were examined in another two groups of chicks wearing −10 D (group 3) and +10 D lenses (group 4) over their right eyes for 3 days, respectively (plano over left eyes).

Results. The 8-Br-cAMP significantly inhibited development of lens-induced myopia (group 1: 0.1 mM versus vehicle: +1.71 ± 1.22 D versus −8.00 ± 2.19 D; 1 mM versus vehicle: +1.38 ± 1.34 D versus −9.96 ± 1.14 D, mean ± SEM, $P < 0.01$ for both); 1 mM, but not 0.1 mM 8-Br-cAMP increased expression of retinal ApoA1 levels in right eyes ($P < 0.01$). The 8-Br-cAMP had minimal effect on normal eye growth. Both retinal cAMP and ApoA1 levels were significantly increased only in hyperopic eyes (group 4).

Conclusions. The 8-Br-cAMP robustly inhibited development of lens-induced myopia. The increase in retinal ApoA1 observed in cAMP-treated and hyperopic eyes suggested a possible interplay between ApoA1 and cAMP in regulating eye growth.

Keywords: apolipoprotein A1, cyclic adenosine monophosphate, myopia

The prevalence of myopia is high in parts of Asia and has been escalating in the United States (Refs. 1–5; Saw SM, et al. *IOVS* 2011;52:ARVO E-Abstract 2490). Myopia is typically due to an excessive growth of the eyeball,[6] resulting in the optical image being focused in front of the retina and, consequently, blurred vision of distant objects. Although blurred vision can be improved by optical corrections, myopia is more than a minor inconvenience because high myopia is associated with a number of sight-threatening diseases, such as retinal detachment and glaucoma.[7,8] To reduce the risk of vision loss and the socioeconomic burden caused by myopia, an effective treatment to prevent myopia progression is urgently needed. Elucidating the mechanism of myopia would be the first step toward finding a practical solution to myopia.

Myopia can be induced in numerous animal species, including chicks,[9] guinea pigs,[10,11] tree shrews,[12] mice,[13] marmosets,[14] and rhesus monkeys,[15,16] by using either negative-powered lenses (lens-induced myopia) or translucent diffusers (form-deprivation myopia). Signals involved in myopia-associated aberrant eye growth are believed to reside within the eyes, as blockade of eye-to-brain communication by optic nerve transection, or pharmacologic inhibition of ganglion cell action potentials, fails to prevent experimentally induced myopia.[17,18] However, the exact molecular mechanisms regulating eye growth are still poorly understood.

A previous proteomic study examined differentially expressed proteins in the chick retina after lens-induced myopia or hyperopia.[19] Expression of retinal apolipoprotein A1 (ApoA1) was elevated in hyperopic eyes. In addition, ocular growth was inhibited after intravitreal injection of peroxisome proliferator-activated receptor alpha agonists, which increased retinal ApoA1 expression. Therefore, ApoA1 has been proposed as a "STOP" signal in myopia development.[19] Such "STOP" signals are likely evoked when the retina is exposed to myopic defocus produced by wearing a positive lens or when the eye is recovering from experimentally induced myopia after removal of negative lenses.[20]

Apolipoprotein A1 is the major component of high-density lipoprotein that takes part in reverse cholesterol transport from peripheral tissues to the liver for excretion.[21] In reverse cholesterol transport, ApoA1 binds to ATP binding cassette transporter A1 (ABCA1), enabling the transfer of free choles-

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terol and phospholipids out of peripheral cells to high-density lipoproteins.[21] Previous studies have shown that cyclic adenosine monophosphate (cAMP) can stimulate cholesterol efflux in murine macrophages[22] and RAW264 cells.[23] In fibroblasts, cAMP was also shown to induce ABCA1 phosphorylation leading to increased lipid efflux.[24] Because cAMP has been shown to affect ABCA1 phosphorylation and expression,[25] it also may affect the recycling and expression of ApoA1 because ApoA1 recycling is mostly mediated via the ABCA1 transport.[26] To date, the literature studying the effect of cAMP on ApoA1 expression is scarce.

Thus, it is plausible that cAMP interacts with ApoA1 to enhance cholesterol efflux. Currently, it is unclear if cAMP and ApoA1 interact in the signaling pathways controlling either normal or aberrant eye growth. In this study, we explored the relationship between cAMP and ApoA1 levels during normal eye growth and lens-induced myopia development.

Methods

The care and use of the animals in these experiments were in accordance with the ARVO resolution on the Use of Animals in Research and also in compliance with university guidelines set forth by the Animal Subjects Research Ethnic Sub-committee of The Hong Kong Polytechnic University. White Leghorn chicks (*Gallus gallus domesticus*) were bred from Specific Pathogen-Free eggs (Jinan Spafas Poultry Co., Jinan, China). The chicks were reared under a 12/12-hour light/dark diurnal cycle. Food and water were provided ad libitum.

Experiment 1: Effect of Intravitreal Injection of 8-Bromo-cAMP on Refraction, Ocular Component Dimensions, and Retinal ApoA1 Expression

Overview. Two groups of chicks (group 1 and group 2) were given daily intravitreal injections of the cAMP analogue 8-Bromo-cAMP (8-Br-cAMP) into their right eyes and of vehicle into their left eyes for 4 days, beginning when they were 8 days old. Chicks in group 1 were fitted with −10 diopter (D) lenses over both eyes for the duration of the treatment period, whereas chicks in group 2 were fitted with plano lenses. Lenses were fitted using a Velcro ring.[17] The chicks in group 1 received 8-Br-cAMP injections of either 0.1 mM (group 1A, $n = 6$) or 1 mM (group 1B, $n = 6$). The chicks in group 2 ($n = 8$) all received injections of 0.1 mM 8-Br-cAMP. Ocular component dimensions and ocular refraction were measured before the first injection (baseline) and at the end of the 4-day treatment period.

The expression level of retinal ApoA1 protein was measured in groups 1A and 1B at the end of the treatment period using Western blotting.

Methodologic Details. The 8-Br-cAMP was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and dissolved in PBS. Intravitreal injections were administered through the conjunctiva approximately 3 mm above the superior corneal limbus in a volume of 10 µL using a 30-gauge needle attached to a Hamilton syringe. Injection at or near blood vessels on the conjunctiva was avoided and the needle was pointed toward the vitreous chamber to avoid damage to the crystalline lens. Injections were administered while chicks were anesthetized with 2% isoflurane (Minrad, Inc., Bethlehem, PA, USA) in oxygen. All injections were given between 11 AM and 1 PM. The ocular components were examined using a high-frequency A-scan ultrasound system (Panametrics, Waltham, MA, USA) with a 30-MHz transducer sampled at a rate of 100 MHz. Refractive errors were measured by streak retinoscopy. The spherical equivalent refractive error was calculated as the sum of spherical power and half of the cylindrical power. Assuming the vitreous chamber volume for 8-day-old chicks is 150 to 200 µL[27,28] and there is no loss of drug during delivery, the concentration of 8-Br-cAMP in the vitreous was calculated to be 5 µM and 50 µM for the 0.1 mM and 1 mM injections, respectively. For ease of interpretation, we refer to the drug dose concentrations rather than the expected eye tissue concentrations.

Sample Preparation for Western Blotting. After measuring refractive error and ocular component dimensions, chicks in groups 1A and 1B were euthanized by overexposure to carbon dioxide. Their eyes were enucleated and the retinas were isolated by carefully peeling them off from the posterior pole. Visible RPE was carefully removed using forceps. The retinas were immediately frozen in liquid nitrogen and kept at −80°C until further use. Each frozen retinal sample was mixed with 300 µL lysis buffer and homogenized in a liquid nitrogen–cooled Teflon freezer mill (Mikrodismembrator Braun Biotech, Melsungen, Germany) for 6 minutes at 16100*g*.[29] Lysis buffer contained 7 M urea, 2 M thiourea, 40 mM Tris, 0.2% (wt/vol) Biolytes, 1% (wt/vol) dithiothreitol (DTT), 2% (wt/vol) CHAPS, 1% (wt/vol) ASB14 (Calbiochem, San Diego, CA, USA), and 1 tablet of Complete, Mini protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) in 10 mL buffer. The sample was resuspended in lysis buffer and incubated at room temperature for 20 minutes, and then centrifuged at 16,100*g* for 20 minutes at 4°C. The supernatant was collected and its protein concentration was measured using a 2D Quant Kit (GE Healthcare Life Science, Buckinghamshire, England).

Western Blotting. Aliquots of retinal protein (50 µg) were mixed with loading buffer (0.3 M Tris, 10% SDS, 50% vol/vol glycerol, 3.6 M beta-mercaptoethanol, and 0.5% bromophenol blue), heated at 95°C for 5 minutes, and separated on a 10% SDS-PAGE gel. Following electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad, San Diego, CA, USA) at 4°C.

After blocking the PVDF membrane with 5% nonfat dry milk (Carnation nonfat dry milk; Nestle, Vevey, Switzerland) in 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl containing 0.05% Tween-20 (TBST), it was probed with the anti-chick ApoA1 antibody (1:1000 in 0.3% nonfat dry milk in TBST) for 1.5 hours at room temperature. A rabbit polyclonal antibody for chick ApoA1 was kindly provided by Patrizia Tarugi, PhD, Department of Biomedical Sciences, University of Modena and Reggio Emilia, Italy. After six washes in TBST (10 minutes each), the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000 in 0.3% nonfat dry milk in TBST; Zymed Laboratories, San Francisco, CA, USA) for another 1.5 hours at room temperature. The membrane was washed again as above, and labeling was visualized using Pierce SuperSignal West Pico Chemiluminescent substrate (ThermoFisher Scientific, Waltham, MA, USA). An anti-tubulin antibody (1:2000 in 0.3% nonfat dry milk in TBST, Santa Cruz Biotechnology, Dallas, TX, USA) was used as loading control, as retinal tubulin expression is unaffected by wearing −10 D lenses for 3 days.[30]

Experiment 2: Effect of Lens Wear on Retinal Levels of cAMP and ApoA1

Overview. In this experiment, the endogenous level of retinal cAMP was examined in chicks wearing −10 D or +10 D lenses. Two further groups of chicks were used (groups 3 and 4). The chicks in group 3 ($n = 8$) had a −10 D lens fitted over their right eye and a plano lens left over their left eye. The chicks in group 4 ($n = 6$) had a +10 D lens fitted over their right

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Table. The Effect of 0.1 mM and 1 mM 8-Br-cAMP on Ocular Parameters and Refractive Errors After Four Daily Intravitreal Injections (Mean ± SEM)

Group	Sample Size	Lens	Intravitreal Injection	Axial Length, mm	Refractive Errors, D	Anterior Chamber Depth, mm	Lens Thickness, mm	Vitreous Chamber Depth, mm	Retinal Thickness, mm	Choroidal Thickness, mm	Scleral Thickness, mm
1A	6	R: −10 D	0.1 mM 8-Br-cAMP	8.441 ± 0.066	+1.71 ± 1.22‡	1.250 ± 0.045	2.167 ± 0.016	5.025 ± 0.088*	0.244 ± 0.015	0.143 ± 0.019*	0.097 ± 0.003
		L: −10 D	Vehicle	8.601 ± 0.046	−8.00 ± 2.19	1.080 ± 0.045	2.171 ± 0.030	5.350 ± 0.078	0.218 ± 0.008	0.090 ± 0.006	0.089 ± 0.004
1B	6	R: −10 D	1 mM 8-Br-cAMP	8.312 ± 0.097*	+1.38 ± 1.34‡	1.259 ± 0.039	2.075 ± 0.029	4.978 ± 0.051†	0.233 ± 0.002†	0.177 ± 0.026	0.085 ± 0.006
		L: −10 D	Vehicle	8.654 ± 0.053	−9.96 ± 1.14	1.271 ± 0.038	2.064 ± 0.037	5.319 ± 0.050	0.208 ± 0.004	0.108 ± 0.008	0.087 ± 0.005
2	8	R: Plano	0.1 mM 8-Br-cAMP	8.432 ± 0.072	+3.66 ± 0.56*	1.293 ± 0.033	2.180 ± 0.028	4.959 ± 0.052	0.252 ± 0.006	0.186 ± 0.013*	0.106 ± 0.005
		L: Plano	Vehicle	8.480 ± 0.063	+1.25 ± 0.35	1.267 ± 0.034	2.190 ± 0.024	5.023 ± 0.037	0.234 ± 0.009	0.156 ± 0.006	0.103 ± 0.005

The 8-Br-cAMP was injected into the right eye (R) and vehicle was injected into the left eye (L) as control. *Asterisks* indicate 2-tailed paired *t*-tests comparing data from 8-Br-cAMP– and vehicle-treated eyes.

* $P < 0.05$.

† $P < 0.01$.

‡ $P < 0.001$.

eye and a plano lens left over their left eye. In both groups, lenses wear applied to 2-day-old chicks and worn for 3 days. Refractive errors and ocular component dimensions were measured before and after lens treatment. Retinas were collected after the 3-day period to determine endogenous cAMP and ApoA1 levels.

Methodologic Details.

Tissue Preparation. Retinas were harvested as described in experiment 2. The frozen retinas were homogenized in 5% trichloroacetic acid (TCA) using a Polytron-type homogenizer. The homogenized samples were then centrifuged at 1600*g* for 10 minutes at 4°C. The supernatant was collected and purified using water-saturated ether, and the pellet was kept and measured for its protein content. The top layer of the water-saturated ether mixture was carefully removed and discarded. The extract containing cAMP was then heated at 70°C for 5 minutes to remove the residual ether. The pellet (protein extract) was washed with ice-cold acetone and then ground in lysis buffer directly in the microcentrifuge tube using a sterilized Kontes pellet pestle (Fisher Scientific, Waltham, MA, USA). Grinding continued until no visible pellet was left. The protein concentration of the resuspended pellet sample was measured using a 2D Quant Kit (GE Healthcare Life Science). The ApoA1 content was then measured by Western blotting as described in experiment 1.

Measurement of Retinal cAMP Concentration. The cAMP concentration in the TCA-ether extracts was measured using a commercial immunoassay kit (cAMP EIA Kit; Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. A standard curve consisting of different concentrations of cAMP was prepared by reconstituting the cAMP standard provided in the kit. The cAMP levels were expressed as pmol per mg protein.

Data Analysis

In all experiments, changes in ocular parameters and refractive errors between treated and control eyes were compared using the paired *t*-test. Densitometric readings of Western blots were analyzed by paired *t*-test to compare retinal ApoA1 expression between treated and control eyes. Normalized cAMP levels were calculated relative to the control eyes and analyzed by paired *t*-test in each group. $P < 0.05$ was regarded as statistically significant. All values are presented as mean ± SEM.

Results

Experiment 1: Effect of Intravitreal 8-Br-cAMP on Refraction and Ocular Component Dimensions

Refractive errors of eyes with either 0.1 mM (group 1A) or 1 mM 8-Br-cAMP (group 1B) were significantly different from the contralateral control eyes (paired *t*-test, $P < 0.001$ for both groups, Table). Strikingly, in all cases, the refractive error of 8-Br-cAMP–treated eyes remained hyperopic (group 1A: +1.71 ± 1.22 D; group 1B: +1.38 ± 1.34 D) despite the nearly complete compensation for the contralateral eyes receiving vehicle injections (−8.00 ± 2.19 D and −9.96 ± 1.14 D in groups 1A and 1B, respectively).

Vitreous chamber depth (VCD) of the vehicle-injected eyes increased in response to negative lens wear, statistically significantly different from the 8-Br-cAMP–injected eyes (paired *t*-test treated versus control eyes, $P < 0.01$ for both group 1A and 1B, Fig. 1A).

The choroidal thickness of 8-Br-cAMP–injected eyes was numerically increased versus vehicle-injected eyes but the

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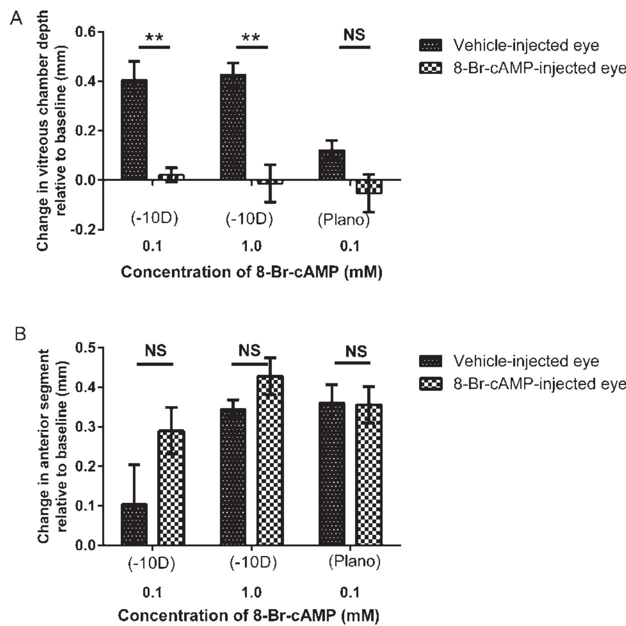

Figure 1. Change in VCD (**A**) and anterior segment (**B**) in eyes wearing plano or −10 D lenses binocularly and receiving intravitreal injections of vehicle, or 0.1 mM or 1 mM 8-Br-cAMP. *Asterisks* indicate statistically significant difference versus vehicle-treated eye (mean ± SEM, $n = 6$ for −10 D and $n = 8$ for plano; paired *t*-test, $^{**}P < 0.01$).

difference was statistically significant only in group 1A receiving 0.1 mM 8-Br-cAMP (paired *t*-test, $P < 0.05$ for group 1A, $P = 0.054$ for group 1B, Table). Furthermore, the retinas in 8-Br-cAMP–treated eyes were thicker than in vehicle-injected eyes (paired *t*-test, $P < 0.01$ for group 1B, Table). No significant differences were observed in the anterior chamber depth and lens thickness after injection of 8-Br-cAMP (Fig. 1B).

The effect of 8-Br-cAMP on normal ocular growth was also investigated (group 2; 0.1 mM and both eyes wearing plano lenses). Eyes receiving a daily injection of 0.1 mM 8-Br-cAMP were slightly, but significantly, more hyperopic than the contralateral vehicle-injected eyes (+3.66 ± 0.56 D versus +1.25 ± 0.35D, respectively; paired *t*-test, $P < 0.05$). Choroidal thickness was found to be significantly increased in cAMP-injected chicks (paired *t*-test, $P < 0.05$; Table). No change in anterior segment parameters, including anterior chamber depth and lens thickness, was observed with injection of 0.1 mM 8-Br-cAMP into normal eyes (paired *t*-test, $P > 0.05$; Fig. 1B).

Effect of Intravitreal 8-Br-cAMP on Retinal ApoA1 Levels

After intravitreal injections and −10 D lens wear, retinas from chicks in groups 1A and 1B were collected and analyzed for ApoA1 protein levels by Western blotting. As shown in Figures 2A and 2B, the ApoA1 level was more than 2-fold higher in eyes that had received 1 mM 8-Br-cAMP compared with vehicle-treated eyes (paired *t*-test, $P < 0.05$). However, at a dose of 0.1 mM, 8-Br-cAMP did not change the retinal ApoA1 level significantly.

Experiment 2: Effect of Lens Wear on Retinal Levels of cAMP and ApoA1

The right eyes of young chicks (aged 2 days old at the start of the lens-wearing period) fitted monocularly with either a −10 D or +10 D lens for 3 days developed a significant amount of myopia (group 3, $n = 8$; −4.07 ± 0.72 D) or hyperopia (group 4, $n = 6$; +11.38 ± 0.44 D), respectively. The left, plano-wearing, control eyes, however, were slightly hyperopic after 3 days.

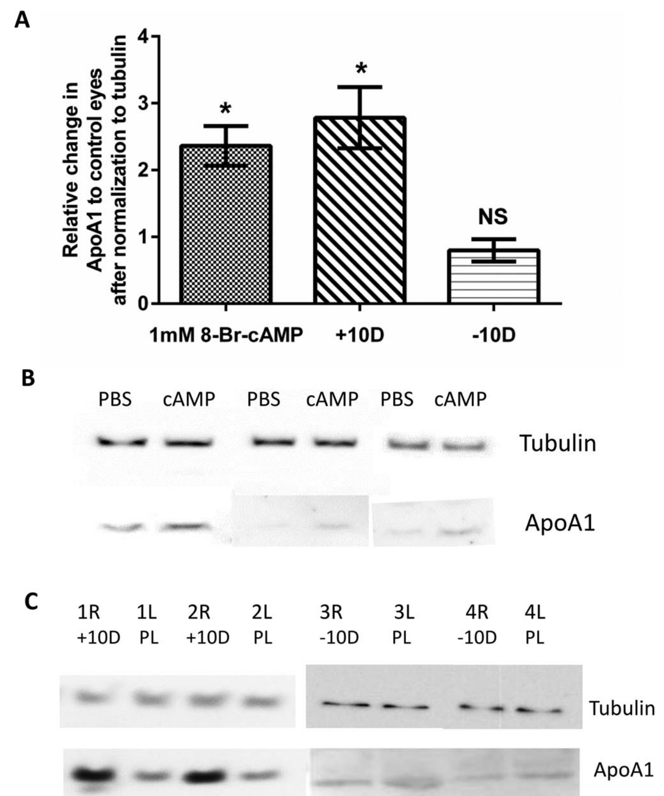

Figure 2. Effect of 1 mM 8-Br-cAMP and lens wear on retinal ApoA1 level. (**A**) Level of retinal ApoA1 in eyes receiving 1 mM 8-Br-cAMP or wearing a +10 D or −10 D lens. Data shown represent expression relative to that of control eyes and normalized against tubulin expression (paired *t*-test comparing data from right and left eyes; mean ± SEM, $^{*}P < 0.05$). (**B**) Representative Western blots from retinas of three pairs of eyes. Right eye (cAMP) received 10 µL 1 mM 8-Br-cAMP daily, and the left eye (PBS) received 10 µL PBS. All eyes wore −10 D lenses. (**C**) Representative Western blots from retinas of four pairs of eyes. A significant upregulation of retinal ApoA1 expression was found in eyes that wore a +10 D lens for 3 days (mean ± SEM, $n = 6$ for +10 D and $n = 8$ for −10 D, paired *t*-test, $^{*}P < 0.05$).

The VCD in eyes wearing a −10 D lens (group 3, right eyes) increased significantly after 3 days (paired *t*-test, $P < 0.01$; Fig. 3). Conversely, the eyes wearing a +10 D lens (group 4, right eyes) had shorter VCD than their contralateral control eyes (Fig. 3A).

Normalized against its concentration in the contralateral control eye, the retinal cAMP level in eyes wearing a −10 D lens was not altered significantly (group 3; normalized cAMP level = 1.089 ± 0.084; absolute retinal cAMP level in myopic eye = 1.374 ± 0.074 pmol/mg; Fig. 3B). By contrast, the normalized retinal cAMP level in +10 D lens-wearing eyes was increased by approximately 30% compared with contralateral control eyes (group 4; normalized cAMP level = 1.308 ± 0.129; absolute retinal cAMP level in hyperopic eye = 2.529 ± 0.396 pmol/mg of protein). Notably, the absolute retinal cAMP concentration in the contralateral eyes of chicks in group 4 were higher than in the control eyes of group 3 (group 3 versus group 4; 1.279 ± 0.062 vs. 1.923 ± 0.239 pmol/mg of protein, respectively), which may indicate the presence of cross-talk between the

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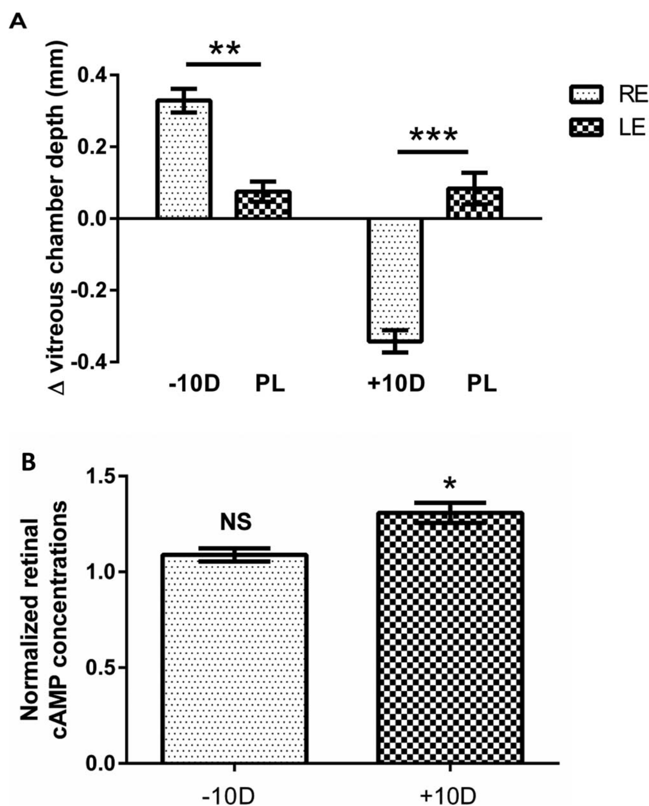

FIGURE 3. Lens-induced change in VCD (A) and retinal cAMP concentrations after 3 days of +10 D and −10 D lens wear. (A) The right eyes of chicks were fitted with a −10 D (group 3, $n = 8$) or +10 D (group 4, $n = 6$) lens, respectively. Left eyes in both groups were fitted with a plano lens. The VCD (mean ± SEM) increased significantly after 3 days of −10 D lens wear, and it decreased significantly after 3 days of +10 D lens wear (paired *t*-test, $^{**}P < 0.01$, $^{***}P < 0.001$). (B) The retinal cAMP concentration was normalized against that of plano lens-wearing eyes. Retinal cAMP levels were significantly increased by approximately 30% in the hyperopic eyes (mean ± SEM, $n = 6$ for +10 D and $n = 8$ for −10 D, paired *t*-test, $^{*}P < 0.05$).

eyes. Although there was a greater variability in retinal cAMP concentrations in both the +10 D lens and plano-wearing eyes of chicks in group 4, a significant increase in cAMP was still found in chicks wearing +10 lens ($P < 0.05$).

Interestingly, the level of ApoA1 in the retina of +10 D lens-wearing eyes was increased by more than 2-fold when compared with fellow, plano-lens-wearing eyes (paired *t*-test, $P < 0.05$; Figs. 2A, 2C). The retinal ApoA1 level in −10 D lens-wearing eyes was slightly, but not significantly, lower than in fellow control eyes (Figs. 2A, 2C).

DISCUSSION

In the present study, consecutive intravitreal injection of cAMP analogue (8-Br-cAMP) for 4 days could effectively prevent myopic eye growth in chicks wearing −10 D lenses. It strongly inhibited the elongation of the vitreous chamber and thickened the choroid of eyes that underwent lens-induced myopic growth. Despite eyes wearing −10 D lenses for 4 days, slight hyperopia was maintained by 8-Br-cAMP treatment, indicating the potency of the treatment. In contrast to its strong inhibitory effect on myopic growth, cAMP had less effect on normal development. Although slight hyperopia and choroidal thickening were found, there were no significant changes in VCD and axial length (AXL) after injecting cAMP in eyes wearing plano lenses. The effect of cAMP seems to be rather specific in retarding myopic eye growth because only changes in VCD and AXL were found in eyes with −10 D lenses but not with plano lenses.

In a recent study with myopic guinea pigs, cAMP was injected to the subconjunctival space of normal and form-deprived eyes and its effect on eye growth was studied.[31] Injection of cAMP had no effect on form-deprived myopic eyes, but induced myopia in normal guinea pig eyes. The exact reason for this discrepancy is unknown but it could be related to the mode of administration of cAMP because cAMP was injected subconjunctivally rather than intravitreally. The sites of action with subconjunctival injections were likely those external tissues, such as the sclera. However, intravitreal injection would target mainly intraocular sites such as the retina. It is plausible that cAMP could act differently on different ocular tissues in terms of eye growth.

It remains unknown by what mechanism cAMP prevents myopia development induced by negative lenses. ApoA1 is a candidate downstream mediator of cAMP effects. In the current study, intravitreal injection of high-dose 8-Br-cAMP (1 mM) significantly increased retinal ApoA1 expression. The result is consistent with the notion that the inhibitory effect of cAMP may be mediated through upregulation of ApoA1. Because myopic eye growth was prevented after ocular injection with both low- and high-dose 8-Br-cAMP (Fig. 1A), it was expected that retinal ApoA1 level would be quantitatively correlated with the concentrations of injected cAMP. However, only the high-dose 8-Br-cAMP (1 mM) led to a detectable increase in retinal ApoA1 expression. Although the level of ApoA1 expression required for inhibiting myopic growth is unknown, low-dose 8-Br-cAMP may have triggered too small an increase in ApoA1 expression to be detectable by Western blotting,[32] but still potent enough to abolish myopic eye growth. This speculation will need to be further investigated. The fact that 8-Br-cAMP can directly modulate ApoA1 expression and halt animal myopia suggests that cAMP may be another "STOP" signal in the biochemical reactions that regulate eye growth.

Moreover, elevated expression levels of ApoA1 and cAMP were found in the hyperopic retina, further substantiating a role as "STOP" signal for cAMP and ApoA1 in the regulation of eye growth. However, there was no decrease in the retinal cAMP concentration in myopic eyes. In chicks, eye size is growing rapidly at postnatal day 3. Hence, we expected to be able to capture changes in cAMP levels at this time point. However, because the temporal change in cAMP levels is not known, it is possible that changes in retinal cAMP levels may occur before day 3. In addition, the temporal and dose-response characteristics of defocus-induced biochemical changes may be different between hyperopic and myopic eyes. A more detailed profiling of retinal cAMP levels over time in lens-induced myopic eyes will be required to verify if cAMP levels change transiently during myopic eye growth. It is also plausible that, physiologically, cAMP may contribute mainly to the retardation of eye growth (as in hyperopia) rather than to accelerated eye growth (as in myopia). Therefore, retinal cAMP expression was not reduced in the myopic eye (being maintained at a "housekeeping" level) but was significantly increased in the hyperopic eye. Although cAMP and ApoA1 could effectively inhibit myopia development, exactly how they interact with other candidate compounds in orchestrating eye growth is yet to be revealed. Published literature suggests potential interactions with a number of neurotransmitters known to be important in regulating eye growth.

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One of the possible candidate regulators for retinal cAMP is glucagon. Intravitreal injection of glucagon was shown to slow myopia development in chicks.[33] Glucagon can activate adenylate cyclase and stimulate cAMP production,[34] and it can also increase ApoA1 mRNA expression in cultured rat hepatocytes.[35] Therefore, cAMP and ApoA1 could be the downstream effectors of glucagon in regulating eye growth.

Furthermore, dopamine receptors, members of the G-protein coupled receptor family, have the potential to influence retinal cAMP levels. Stimulation of D1-like receptors (D1 and D5) activates adenylate cyclase and enhances cAMP production. On the other hand, activation of D2-like receptors (D2, D3, and D4) produces the opposite effect by inhibiting adenylate cyclase.[36] Previous studies have shown that D2-specific dopamine agonists inhibited ocular elongation in response to negative lenses and diffusers.[37] In addition, administration of D2-specific dopamine agonists was expected to result in decreased cAMP levels. These results do not directly support our current findings (i.e., that increasing cAMP level inhibits development of myopia); however, D2 agonists can have additional downstream effects, and it is unclear if the biological effects of D2 agonists on eye growth are solely or primarily mediated via decreased levels of cAMP. Whether D1-like receptors are involved in myopia development is unknown. A study by Nickla et al.[38] initially suggested both D1- and D2-like receptors are important in inhibiting myopia because D1-specific dopamine agonists could slow ocular elongation in lens-induced myopia, but not as effectively as D2-specific agonists. However, a recent study from the same laboratory found no contribution of D1 receptors to both form-deprived and lens-induced myopia.[39] Therefore, exactly how dopamine interacts with cAMP in regulating ocular growth remains unclear.

To conclude, the current study provided the first direct evidence that cAMP plays a key role in retardation of eye growth. Its inhibitory effect on myopia development was strong and specific. The 8-Br-cAMP also activated the expression of another "STOP" signal ApoA1. Because cAMP is a ubiquitous second messenger in many signaling pathways, its role in regulating eye growth is likely multiple and complex. Investigating differential protein expression in cAMP-treated retinas may help understand the signaling pathways associated with inhibiting myopia development in chicks. Based on the potency of cAMP in inhibiting myopia development, elucidating its exact role(s) and related biochemical pathways may open up novel drug targets for future therapeutic treatment of myopia.

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References

1. Matsumura H, Hirai H. Prevalence of myopia and refractive changes in students from 3 to 17 years of age. *Surv Ophthalmol*. 1999;44:S109–S115.
2. Lin LL, Shih YF, Hsiao CK, Chen CJ. Prevalence of myopia in Taiwanese schoolchildren: 1983 to 2000. *Ann Acad Med Singapore*. 2004;33:27–33.
3. Vitale S, Sperduto RD, Ferris FL III. Increased prevalence of myopia in the United States between 1971–1972 and 1999–2004. *Arch Ophthalmol*. 2009;127:1632–1639.
4. Morgan IG, Ohno-Matsui K, Saw SM. Myopia. *Lancet*. 2012;379:1739–1748.
5. Jones D, Luensmann D. The prevalence and impact of high myopia. *Eye Contact Lens*. 2012;38:188–196.
6. Zadnik K. The Glenn A. Fry Award Lecture (1995). Myopia development in childhood. *Optom Vis Sci*. 1997;74:603–608.
7. Gilmartin B. Myopia: precedents for research in the twenty-first century. *Clin Experiment Ophthalmol*. 2004;32:305–324.
8. Wong TY, Klein BE, Klein R, Knudtson M, Lee KE. Refractive errors, intraocular pressure, and glaucoma in a white population. *Ophthalmology.* 2003;110:211–217.
9. Wildsoet CF. Active emmetropization—evidence for its existence and ramifications for clinical practice. *Ophthalmic Physiol Opt*. 1997;17:279–290.
10. Howlett MH, McFadden SA. Form-deprivation myopia in the guinea pig (*Cavia porcellus*). *Vision Res*. 2006;46:267–283.
11. Howlett MH, McFadden SA. Spectacle lens compensation in the pigmented guinea pig. *Vision Res*. 2009;49:219–227.
12. Norton TT. Experimental myopia in tree shrews. *Ciba Found Symp*. 1990;155:178–194.
13. Schaeffel F, Burkhardt E, Howland HC, Williams RW. Measurement of refractive state and deprivation myopia in two strains of mice. *Optom Vis Sci*. 2004;81:99–110.
14. Troilo D, Judge SJ. Ocular development and visual deprivation myopia in the common marmoset (*Callithrix jacchus*). *Vision Res*. 1993;33:1311–1324.
15. Norton TT, Siegwart JT Jr. Animal models of emmetropization: matching axial length to the focal plane. *J Am Optom Assoc*. 1995;66:405–414.
16. Hung LF, Crawford ML, Smith EL. Spectacle lenses alter eye growth and the refractive status of young monkeys. *Nat Med*. 1995;1:761–765.
17. Wildsoet C, Wallman J. Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Res*. 1995;35:1175–1194.
18. McBrien NA, Moghaddam HO, Cottriall CL, Leech EM, Cornell LM. The effects of blockade of retinal cell action potentials on ocular growth, emmetropization and form deprivation myopia in young chicks. *Vision Res*. 1995;35:1141–1152.
19. Bertrand E, Fritsch C, Diether S, et al. Identification of apolipoprotein A-I as a "STOP" signal for myopia. *Mol Cell Proteomics*. 2006;5:2158–2166.
20. Morgan IG. The biological basis of myopic refractive error. *Clin Exp Optom*. 2003;86:276–288.
21. Wang N, Silver DL, Costet P, Tall AR. Specific binding of ApoA-I, enhanced cholesterol efflux, and altered plasma membrane morphology in cells expressing ABC1. *J Biol Chem*. 2000;275:33053–33058.
22. Bernard DW, Rodriguez A, Rothblat GH, Glick JM. cAMP stimulates cholesteryl ester clearance to high density lipoproteins in J7774 macrophages. *J Biol Chem*. 1991;266:710–716.
23. Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem*. 2000;275:34508–34511.
24. Haidar B, Denis M, Krimbou L, Marcil M, Genest J Jr. cAMP induces ABCA1 phosphorylation activity and promotes cholesterol efflux from fibroblasts. *J Lipid Res*. 2002;43:2087–2094.

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25. Witzlack T, Wenzeck T, Thiery J, Orth M. cAMP-induced expression of ABCA1 is associated with MAP-kinase-pathway activation. *Biochem Biophys Res Commun*. 2007;363:89–94.
26. Krimbou L, Marcil M, Genest J. New insights into the biogenesis of human high-density lipoproteins. *Curr Opin Lipidol*. 2006;17:258–267.
27. Schmid KL, Wildsoet CF. Inhibitory effects of apomorphine and atropine and their combination on myopia in chicks. *Optom Vis Sci*. 2004;81:137–147.
28. Vessey KA, Lencses KA, Rushforth DA, Hruby VJ, Stell WK. Glucagon receptor agonists and antagonists affect the growth of the chick eye: a role for glucagonergic regulation of emmetropization? *Invest Ophthalmol Vis Sci*. 2005;46:3922–3931.
29. Lam TC, Li KK, Lo SC, Guggenheim JA, To CH. A chick retinal proteome database and differential retinal protein expressions during early ocular development. *J Proteome Res*. 2006;5:771–784.
30. Chun RKM, Li KK, Lam TC, Lui Q, To CH. Proteome analysis of myopic retina—characterization of the role of apolipoprotein A1. HUPO 9th Annual World Congress; September 19–24, 2010; Sydney, Australia.
31. Tao Y, Pan M, Liu S, et al. cAMP level modulates scleral collagen remodeling, a critical step in the development of myopia. *PLoS One*. 2013;8:e71441.
32. Scholl B, Liu HY, Long BR, et al. Single particle quantum dot imaging achieves ultrasensitive detection capabilities for Western immunoblot analysis. *ACS Nano*. 2009;3:1318–1328.
33. Zhu X, Wallman J. Opposite effects of glucagon and insulin on compensation for spectacle lenses in chicks. *Invest Ophthalmol Vis Sci*. 2009;50:24–36.
34. Exton JH. Mechanisms of hormonal regulation of hepatic glucose metabolism. *Diabetes Metab Rev*. 1987;3:163–183.
35. Hall GP, Redgrave TG, Yeoh GC. The ontogeny of apolipoprotein expression in rat liver. mRNA levels in developing liver and cultured fetal rat hepatocytes. *Eur J Biochem*. 1995;228:332–336.
36. Witkovsky P. Dopamine and retinal function. *Doc Ophthalmol*. 2004;108:17–40.
37. McCarthy CS, Megaw P, Devadas M, Morgan IG. Dopaminergic agents affect the ability of brief periods of normal vision to prevent form-deprivation myopia. *Exp Eye Res*. 2007;84:100–107.
38. Nickla DL, Totonelly K, Dhillon B. Dopaminergic agonists that result in ocular growth inhibition also elicit transient increases in choroidal thickness in chicks. *Exp Eye Res*. 2010;91:715–720.
39. Nickla DL, Totonelly K. Dopamine antagonists and brief vision distinguish lens-induced- and form-deprivation-induced myopia. *Exp Eye Res*. 2011;93:782–785.

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