Opposite Effects of Glucagon and Insulin on Compensation for Spectacle Lenses in Chicks

Xiaoying Zhu and Josh Wallman

PURPOSE. Chick eyes compensate for the defocus imposed by positive or negative spectacle lenses. Glucagon may signal the sign of defocus. Do insulin (or IGF-1) and glucagon act oppositely in controlling eye growth, as they do in metabolic pathways and in control of retinal neurogenesis?

METHODS. Chicks, wearing lenses or diffusers or neither over both eyes, were injected with glucagon, a glucagon antagonist, insulin, or IGF-1 in one eye (saline in the other eye). Alternatively, chicks without lenses received insulin plus glucagon in one eye, and either glucagon or insulin in the fellow eye. Ocular dimensions, refractive errors, and glycosaminoglycan synthesis were measured over 2 to 4 days.

RESULTS. Glucagon attenuated the myopic response to negative lenses or diffusers by slowing ocular elongation and thickening the choroid; in contrast, with positive lenses, it increased ocular elongation to normal levels and reduced choroidal thickening, as did a glucagon antagonist. Insulin prevented the hyperopic response to positive lenses by speeding ocular elongation and thinning the choroid. In eyes without lenses, both insulin and IGF-1 sped, and glucagon slowed, ocular elongation, but glucagon and insulin each increased the rate of thickening of the crystalline lens. When injected together, insulin blocked choroidal thickening by glucagon, at a dose that did not, by itself, thin the choroid.

CONCLUSIONS. Glucagon and insulin (or IGF-1) cause generally opposite modulations of eye growth, with glucagon mostly increasing choroidal thickness and insulin mostly increasing ocular elongation. These effects are mutually inhibitory and depend on the visual input. (Invest Ophthalmol Vis Sci. 2009; 50:24–36) DOI:10.1167/iovs.08-1708

Interest in the control of eye growth has been mobilized in recent years by two factors: the increasing prevalence of myopia among the educated and the evidence from animal research that eyes use defocus to modulate their rate of elongation, resulting in a match between the eye length and the focal length of the optics. In every species studied, postnatal ocular growth is modulated by visual input with the result that the eye grows toward emmetropia—the condition of distant vision. With respect to eye growth, two recent abstracts show that intravitreal injection of insulin or IGF-1 in one eye can have opposite effects depending on the visual input. (Invest Ophthalmol Vis Sci. 2009; 50:24–36) DOI:10.1167/iovs.08-1708

From the Department of Biology, City College, CUNY, New York, New York.

Supported by National Institutes of Health Grants EY-02727 and RR-03060.

Submitted for publication January 8, 2008; revised May 30, July 11, and August 13, 2008; accepted October 30, 2008.

Disclosure: X. Zhu, None; J. Wallman, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Xiaoying Zhu, Department of Biology, The City College of New York, 138th St. and Convent Avenue, New York, NY, 10031; xiaoying@sci.ccny.cuny.edu.

Copyright © Association for Research in Vision and Ophthalmology

24
promotes cell proliferation and differentiation throughout the body and has a high degree of homology with insulin. The receptors for IGF-1 and insulin are also similar, resulting in the cross-reaction of insulin and IGF-1 with the receptors for each other.

In the present study, we ask which of the changes in ocular components that accompany emmetropization or lens compensation are affected by glucagon and its antagonist and by insulin and IGF-1 and whether glucagon and insulin influence each other’s actions. We found that glucagon had generally opposite effects on development toward myopia and hyperopia. As expected, it inhibited development toward myopia primarily by causing choroidal expansion and secondarily by decreasing the rate of ocular elongation; unexpectedly, it partially inhibited development toward hyperopia primarily by increasing the rate of ocular elongation and secondarily by reducing choroidal expansion. Insulin and IGF-1 acted in the opposite direction from glucagon, increasing the rate of ocular elongation and decreasing choroidal thickness in eyes wearing positive lenses, but both insulin and glucagon increased the rate of thickening of the crystalline lens. When glucagon and insulin were combined, a subthreshold dose of insulin prevented a suprathreshold dose of glucagon from thickening the choroid. Some of these results have been presented in preliminary form (Zhu X, et al. IOVS 2001;42:ARVO Abstract 318; Zhu X, et al. IOVS 2007;48:ARVO E-Abstract 5925).

Materials and Methods

Animals

White Leghorn chicks were obtained from either Cornell University (Cornell K-strain; Ithaca, NY) or Truslow Farms (Hyline-W98-strain; Chestertown, MD, only groups 2 and 12; Table 1). The chicks were housed in a heated, sound-attenuated chamber (76 × 61 cm), in a 14:10 light–dark cycle. One- or 2-week-old chicks were used in experiments that lasted 2 to 4 days. Care and use of animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Lenses and Diffusers

We used PMMA plastic lenses (diameter, 12 mm; back optic radius, 7 mm) or glass lenses (not conspicuously curved) of +15, +10, +8, +7, –7, and –15 D, and we used white, plastic diffusers (with a narrow slit in front so that chicks could locate food and water). In all experiments, chicks either wore diffusers or lenses binocularly or had no devices on their eyes. Each lens or diffuser was glued between a rigid plastic ring and a Velcro ring and attached to a mating Velcro ring glued to the feathers around the chicks’ eyes. Lenses were cleaned at least twice daily.

Measurements

Measurements of refractive error and ocular dimensions were conducted with chicks anesthetized with 1.5% isoflurane. Refractive error was measured with a modified Hartinger refractometer. A-scan ultrasonography was used to measure internal ocular dimensions. Ocular length was defined as the sum of anterior chamber depth, lens thickness, vitreous chamber depth, and the thickness of the retina, choroid, and sclera. This measurement, like measurement with calipers, is different from axial length as used clinically—the distance from the anterior surface of the cornea to that of the retina—which is influenced by choroidal thickness, as well as ocular length.

Intravitreal Injection

Intravitreal injections were made daily (unless specified otherwise), immediately after each ultrasound measurement while the chicks were under anesthesia and the lenses or diffusers were removed. The injections were made through the conjunctiva, with an entry site at 12 o’clock approximately 3 mm above the corneal limbus. The needle was pointed toward the posterior pole at approximately 45°, to avoid damaging the lens; eyes that developed cataracts or vitreous infections were not used. We injected either 20 µL through a 26-gauge needle (Hamilton Company, Reno, NV) or 2 to 7.4 µL through a 35-gauge needle (NanoFil syringe; World Precision Instruments, Inc., Sarasota, FL), see Table 1 for details. The smaller needle and volume eliminated the growth-retarding effect that we observed with the larger needle and volume.

Measurement of Proteoglycan (GAG) Synthesis

Since the rate of synthesis of glycosaminoglycans (GAGs) in the extracellular matrix of the sclera and choroid has been shown to parallel the changes in the rate of ocular elongation and in choroidal thickness, respectively, we measured these parameters as indicators of the remodeling of the sclera and choroid. For groups 6, 7, 9, and 10 in experiment 1, and all groups in experiments 3 through 5, at the end of each experiment, the newly synthesized choroidal and scleral GAGs were measured as previously described. In brief, 7-mm choroidal and scleral punches were made from the posterior pole and kept in CO2-independent medium (Invitrogen-Gibco, Carlsbad, CA) on ice for 1.5 to 2 hours, until incubation for 3 hours at 37°C in L-15 medium (Millipore Corp., Phillipsburg, NJ) with radioactive Na235SO4 (40 Ci/mL). The tissues were collected and cultured under the same conditions for all these experiments. To assay the newly synthesized GAGs, the tissues were digested overnight at 57°C with protease K (protease type XXVIII; Sigma-Aldrich, St. Louis, MO), and centrifuged at 13,000 rpm for 15 minutes. GAGs were precipitated overnight with cetylpyridinium chloride (Sigma-Aldrich) and unlabeled chondroitin sulfate. The precipitate was captured on filters (GF/F; GE Healthcare-Whatman, Florham Park, NJ), dried overnight and measured in a liquid scintillation counter. Because the cartilaginous layer of the chick sclera incorporates 30 times more sulfate than the fibrous sclera, as shown when the layers are incubated separately, this measurement is dominated by the GAG synthesis of the cartilaginous sclera.

Protocols

Treatment protocols are summarized in Table 1. Pharmacological Agents. For glucagon injections, synthetic glucagon (90% purity; Sigma-Aldrich) was used in groups 1 through 5, glucagon extracted from porcine pancreas (80% purity, Sigma-Aldrich) was used in groups 6 to 10 and 27 to 29. (Glucagon from both sources has the same chemical structure.) A glucagon antagonist (des-His1,-Glu9)-glucagon-amide, purity 97% (Sigma-Aldrich) was used in experiments 2. For insulin injections, insulin isolated from bovine pancreas (95% purity, 27 USP U/mg; Sigma-Aldrich) was used. For IGF-1 injections, IGF-1 (human recombinant, 97% purity; Sigma-Aldrich) was used in experiments 3 and 4. The total dose and the concentration in the vitreous chamber (correcting for percentage purity) of each drug injected are given in Table 1. The volume of the vitreous chamber was estimated to be 400 µL (a 130° sector of a sphere of radius 6.45 mm centered on the optic axis at the level of the limbus, minus the lens volume).

To control for effects of insulin that are not due to its biological activity, some chicks had native insulin injected daily in the experimental eyes and the same concentration and volume of heat-denatured insulin injected in the fellow eyes for 3 days (group 23, Table 1). The insulin was denatured by being maintained at 65°C for 4 hours before injection. Because denaturation usually makes proteins more easily precipitable, we assessed the degree of denaturation by centrifugation at 5000g for 10 minutes and comparing the absorbance of the post-heating supernatant at 280 nm with that of the unheated insulin solution. We found that the absorbance was reduced from 0.92 to 0.11 by denaturation, suggesting that approximately 88% had been denatured.

In experiments 1 to 4, drugs (in 2–20 µL of solution) and PBS were injected daily (unless specified otherwise) into experimental and fellow eyes, respectively. In experiment 5, glucagon plus insulin were
injected in one eye, with the same dose of either glucagon or insulin injected in the other eye. Ocular dimensions were measured with ultrasound daily (except for group 4 in which the birds were not measured on day 2) during each experiment, as was refractive error in groups 1 to 5 and 11 to 17. At the end of the experiment, the synthesis of choroidal and scleral GAGs was measured (except for groups 1 to 5 and 8 in experiment 1, and those in experiment 2). Unless specified otherwise, injections were 20 µL made with a 26-gauge needle.

Experiment 1: Glucagon Injections. Chicks, wearing either diffusers or spectacle lenses over both eyes or with no devices, had glucagon injected into one eye (PBS into the fellow eye) daily, except for group 2, which was injected only once (see Table 1 for details). Three doses of glucagon (0.02, 0.2, and 2 nanomoles) were injected with either a 26-gauge needle or a 35-gauge needle (groups 7 and 10).

Experiment 2: Glucagon Antagonist Injections. Chicks, wearing either positive lenses over both eyes or no lenses, had the glucagon antagonist, des-His²-(Glu)³-glucagon-amide, injected into one eye daily (same volume of PBS into the fellow eye), except for group 11, which was injected only once (see Table 1). To study the effect of the glucagon antagonist on the maintenance of thickening in an already thickened choroid, two groups of birds wore binocular either +10 or +15-D lenses for 2 days before the injection of the glucagon antagonist started (groups 16 and 17).

Experiment 3: Insulin Injections. To study the effect of insulin on normal eye growth or on hyperopia caused by positive lens wear, chicks, either wearing no lenses or binocular +10-D lenses, had insulin injected daily in the experimental eyes with a 26-gauge needle and the same volume of PBS in the fellow eyes for 3 days. The doses of insulin were chosen to ensure maximum binding of insulin to its receptors in chick retina (IC₅₀ = 0.4–0.7 nM). To control for non-biological effects of insulin, in another group, active and heat-denatured insulin of the same concentration and volume were injected into the experimental and fellow eyes, respectively, with a 35-gauge needle (group 23).

Experiment 4: IGF-1 Injections. To test the effect of IGF-1 on normal eye growth, chicks without any devices had IGF-1 injected daily in the experimental eyes and the same volume of PBS in the fellow eyes for 3 days with a 25-gauge needle. We chose doses of IGF-1 so that the vitreous chamber concentration, 36 nM or 301 ng/mL, would be within the physiological range of concentrations in rat plasma and ensure maximum binding to the IGF-1 receptor (half-maximum binding at 3.5 nM in chick retina).

Experiment 5: Interaction of Glucagon and Insulin Effects. To study whether glucagon and insulin oppose each other’s effects or simply act in opposite directions, we took advantage of the fact that 2 nanomoles of glucagon has no effect on ocular elongation, but causes choroidal expansion, whereas 68 picomoles of insulin has no effect on the choroid, but strongly stimulates ocular elongation. Thus we injected glucagon (0.02 or 2 nanomoles) and insulin (68 picomoles or 0.01 U) into one eye, either sequentially (10 minutes apart, group 27), since the order of injection did not make a difference, the data from these birds were pooled) or simultaneously (groups 28–29), and the same amount of either insulin or glucagon alone into the fellow eye on the same schedule.

Statistics

We analyzed our results in four ways, unless otherwise specified: (1) We presented measured values (of ocular parameters) each day in the experimental and fellow eyes in line graphs and compared the interocular difference (the difference of the measured values between paired eyes, X – N) within a group on different days with respect to day 0 using analysis of variance (ANOVA) with Bonferroni post hoc tests. (2) We presented the change in the experimental and fellow eyes over the course of the experiment (ΔX and ΔN) in bar graphs, and used paired Student’s t-tests to compare changes in paired eyes. (3) We analyzed the rate of GAG synthesis by dividing the values from the scintillation counter for the drug-treated eye by those for the fellow eye (X/N) and evaluated the resulting ratio by a one-sample t-test, comparing it to a value of 1. Since the counts were in a range of thousands for chondroïds and tens of thousands for scleras, and the counts from the background were generally within 50, the background counts were not subtracted from each sample. (4) We compared the relative change (change in the experimental eye over the course of the experiment minus the change in the fellow eye, ΔX – ΔN), by ANOVA with Bonferroni post hoc tests to compare different drug doses. All these comparisons are shown in the figures and explained in the captions. In the text, we report only the means and whether the comparisons were significant.

RESULTS

In brief, we found that glucagon and insulin had opposite effects on the modulation of eye growth: Glucagon inhibited growth toward myopia by thickening the choroid and slowing ocular elongation, whereas insulin inhibited compensation for positive lenses by speeding ocular elongation and thinning the choroid. Both insulin and IGF-1 increased the rate of ocular elongation in eyes not wearing lenses. Furthermore, insulin, at a dose too low to affect choroidal thickness by itself, blocked glucagon from thickening the choroid. Changes in ocular length, choroidal thickness, vitreous chamber depth, and refractive error in experimental and fellow eyes over the course of experiments are summarized in Table 1; details of statistical tests are given in figures.

Experiment 1: Treatment with Glucagon

Glucagon increased choroidal thickness and lens thickness, decreased the rate of ocular elongation in negative lens-wearing eyes, and had the opposite effect in positive lens-wearing eyes. Extracted and synthetic glucagon had similar effects.

Diffusers and Negative Lenses. In eyes wearing diffusers, glucagon reversed the normal choroidal thinning, resulting in robust choroidal thickening in diffuser-wearing eyes, even by the first day (Fig. 1A, interocular difference, P < 0.01). Glucagon also inhibited the increased ocular elongation resulting from wearing diffusers, although it acted more slowly, with the effect being significant after 3 days of injections (Fig. 1F). Changes over 4 days in glucagon-injected eyes were half as great as those in fellow eyes (111 µm vs. 267 µm, glucagon versus PBS, P < 0.05, group 1, bar chart in Fig. 1F). The dose used was approximately nine times the EC₅₀ reported by Vessey et al.

In eyes wearing negative lenses, even a single injection of glucagon caused the choroids to expand significantly by 50 µm, whereas the choroids of the PBS-injected fellow eyes also wearing −7-D lenses thinned by 123 µm (bar chart in Fig. 1B, P < 0.01, group 2). It also reduced the increased ocular elongation caused by wearing −7-D lenses for 2 days (glucagon versus PBS, −36 µm vs. 57 µm, P < 0.05, group 2, Fig. 1G). In eyes wearing −15-D lenses, 4 days of daily injections of glucagon caused the rate of ocular elongation to be half that in the fellow eyes (98 µm vs. 182 µm, P > 0.05, group 3, Table 1), but without choroidal thickening. In both of these experiments, the fellow eyes grew less than would have been expected for untreated eyes, presumably because of the daily injections with a 26-gauge needle.

As a result, glucagon caused a hyperopic shift (an average of 3.7 D) in eyes wearing either diffusers or −7-D lenses, relative to the fellow eyes (groups 1 and 2, Table 1).

Furthermore, glucagon increased the rate of lens thickening 2- to 4-fold in eyes wearing diffusers or lenses (Fig. 2A; diffusers: 284 µm vs. 164 µm, P < 0.01; −7-D lenses: 140 µm vs. 77 µm, P < 0.05; −15-D lenses: 233 µm vs. 65 µm, P < 0.001).
### Table 1. Treatment Protocols and Mean Changes over Course of Experiment in Experimental (X) and Fellow (N) Eyes (±SEM)

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Dose</th>
<th>Concentration (in Vitro)</th>
<th>Lens or Diffuser (Both Eyes)</th>
<th>Experiment Duration (d)</th>
<th>Δ Ocular Length (µm)</th>
<th>Δ Choroidal Thickness (µm)</th>
<th>Δ Vitreous Chamber Depth (µm)</th>
<th>Δ Refractive Error (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1a: Glucagon (synthetic)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>1 w Diffuser</td>
<td>5</td>
<td>4</td>
<td>111 ± 15*</td>
<td>267 ± 34</td>
<td>175 ± 45**</td>
<td>35 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>2 nmol†</td>
<td>4 µM</td>
<td>1 w −7 D</td>
<td>6</td>
<td>2</td>
<td>−36 ± 53*</td>
<td>57 ± 29</td>
<td>50 ± 35**</td>
<td>−123 ± 24</td>
</tr>
<tr>
<td>3</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>1 w −15 D</td>
<td>7</td>
<td>4</td>
<td>98 ± 42</td>
<td>182 ± 59</td>
<td>−7 ± 54</td>
<td>−14 ± 18</td>
</tr>
<tr>
<td>4</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>2 w +10 D</td>
<td>5</td>
<td>3</td>
<td>131 ± 19*</td>
<td>11 ± 30</td>
<td>74 ± 13</td>
<td>157 ± 70</td>
</tr>
<tr>
<td>5</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>2 w +15 D</td>
<td>4</td>
<td>3</td>
<td>215 ± 57</td>
<td>57 ± 55</td>
<td>141 ± 91</td>
<td>75 ± 78</td>
</tr>
<tr>
<td><strong>Experiment 1b: Glucagon (porcine)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.02 nmol</td>
<td>0.04 µM</td>
<td>1 w None</td>
<td>7</td>
<td>5</td>
<td>60 ± 39*</td>
<td>101 ± 31</td>
<td>−91 ± 24</td>
<td>−71 ± 15</td>
</tr>
<tr>
<td>7</td>
<td>0.02 nmol‡</td>
<td>0.01 µM</td>
<td>1 w None</td>
<td>7</td>
<td>5</td>
<td>217 ± 24</td>
<td>194 ± 17</td>
<td>54 ± 30</td>
<td>5 ± 12</td>
</tr>
<tr>
<td>8</td>
<td>0.2 nmol</td>
<td>0.4 µM</td>
<td>1 w None</td>
<td>5</td>
<td>5</td>
<td>5 ± 24***</td>
<td>120 ± 25</td>
<td>62 ± 42‡</td>
<td>−46 ± 20</td>
</tr>
<tr>
<td>9</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>1 w None</td>
<td>4</td>
<td>3</td>
<td>57 ± 34</td>
<td>86 ± 30</td>
<td>120 ± 54‡</td>
<td>−47 ± 35</td>
</tr>
<tr>
<td>10</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>1 w None</td>
<td>5</td>
<td>3</td>
<td>220 ± 18</td>
<td>165 ± 35</td>
<td>268 ± 63**</td>
<td>39 ± 32</td>
</tr>
<tr>
<td><strong>Experiment 2: Glucagon antagonist</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>26 pmol†</td>
<td>0.7 µM</td>
<td>1 w +7 D</td>
<td>12</td>
<td>2</td>
<td>−32 ± 21**</td>
<td>−91 ± 18</td>
<td>136 ± 16*</td>
<td>92 ± 12</td>
</tr>
<tr>
<td>12</td>
<td>26 pmol</td>
<td>0.7 µM</td>
<td>1 w +15 D</td>
<td>4</td>
<td>4</td>
<td>56 ± 19**</td>
<td>120 ± 54</td>
<td>−76 ± 23</td>
<td>−89 ± 12</td>
</tr>
<tr>
<td>13</td>
<td>26 pmol</td>
<td>0.7 µM</td>
<td>1 w None</td>
<td>6</td>
<td>2</td>
<td>120 ± 61</td>
<td>45 ± 24</td>
<td>31 ± 90</td>
<td>48 ± 18</td>
</tr>
<tr>
<td>14</td>
<td>305 pmol</td>
<td>7.4 µM</td>
<td>2 w +10 D</td>
<td>6</td>
<td>2</td>
<td>119 ± 37**</td>
<td>−57 ± 15</td>
<td>−160 ± 31</td>
<td>88 ± 40</td>
</tr>
<tr>
<td>15</td>
<td>305 pmol</td>
<td>7.4 µM</td>
<td>2 w +15 D</td>
<td>3</td>
<td>4</td>
<td>356 ± 41*</td>
<td>51 ± 28</td>
<td>173 ± 65</td>
<td>208 ± 58</td>
</tr>
<tr>
<td>16</td>
<td>305 pmol‡</td>
<td>7.4 µM</td>
<td>2 w +10 D</td>
<td>5</td>
<td>3</td>
<td>60 ± 40</td>
<td>20 ± 25</td>
<td>64 ± 58</td>
<td>45 ± 31</td>
</tr>
<tr>
<td>17</td>
<td>305 pmol</td>
<td>7.4 µM</td>
<td>2 w +15 D</td>
<td>5</td>
<td>2</td>
<td>140 ± 52</td>
<td>40 ± 50</td>
<td>157 ± 35</td>
<td>83 ± 61</td>
</tr>
<tr>
<td><strong>Experiment 3: Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.001 U‡</td>
<td>0.01 µM</td>
<td>1 w +10 D</td>
<td>4</td>
<td>3</td>
<td>−32 ± 20</td>
<td>−104 ± 13</td>
<td>−54 ± 18*</td>
<td>75 ± 33</td>
</tr>
<tr>
<td>19</td>
<td>0.01 U‡</td>
<td>0.1 µM</td>
<td>1 w +10 D</td>
<td>8</td>
<td>3</td>
<td>582 ± 96*</td>
<td>49 ± 33</td>
<td>−27 ± 36</td>
<td>−63 ± 43</td>
</tr>
<tr>
<td>20</td>
<td>0.1 U‡</td>
<td>1.6 µM</td>
<td>1 w +10 D</td>
<td>9</td>
<td>3</td>
<td>352 ± 94*</td>
<td>104 ± 24</td>
<td>−75 ± 27</td>
<td>45 ± 36</td>
</tr>
<tr>
<td>21</td>
<td>0.001 U</td>
<td>0.01 µM</td>
<td>1 w None</td>
<td>5</td>
<td>3</td>
<td>175 ± 41</td>
<td>93 ± 86</td>
<td>−4 ± 10</td>
<td>−47 ± 21</td>
</tr>
<tr>
<td>22</td>
<td>0.01 U‡</td>
<td>0.1 µM</td>
<td>1 w None</td>
<td>5</td>
<td>3</td>
<td>579 ± 96*</td>
<td>57 ± 16</td>
<td>−15 ± 54</td>
<td>−23 ± 50</td>
</tr>
<tr>
<td>23</td>
<td>0.1 U‡</td>
<td>1.6 µM</td>
<td>1 w None</td>
<td>5</td>
<td>3</td>
<td>661 ± 28***</td>
<td>230 ± 28</td>
<td>21 ± 49</td>
<td>−30 ± 14</td>
</tr>
<tr>
<td><strong>Experiment 4: IGF-1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0.04 pmol‡</td>
<td>0.1 nM</td>
<td>1 w None</td>
<td>8</td>
<td>3</td>
<td>216 ± 20</td>
<td>207 ± 22</td>
<td>−5 ± 19</td>
<td>40 ± 17</td>
</tr>
<tr>
<td>25</td>
<td>0.4 pmol‡</td>
<td>1 nM</td>
<td>1 w None</td>
<td>5</td>
<td>3</td>
<td>195 ± 28</td>
<td>156 ± 18</td>
<td>−2 ± 10</td>
<td>−46 ± 19</td>
</tr>
<tr>
<td>26</td>
<td>15 pmol‡</td>
<td>36 nM</td>
<td>1 w None</td>
<td>8</td>
<td>3</td>
<td>579 ± 54**</td>
<td>209 ± 32</td>
<td>154 ± 44*</td>
<td>50 ± 11</td>
</tr>
<tr>
<td><strong>Experiment 5: X (glucagon [at dose shown] + insulin [0.01 U]) vs. N (insulin or glucagon)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>X: glucagon</td>
<td>0.02 nmol‡</td>
<td>0.04 µM</td>
<td>1 w None</td>
<td>8</td>
<td>3</td>
<td>569 ± 66</td>
<td>495 ± 60</td>
<td>88 ± 41</td>
</tr>
<tr>
<td>28</td>
<td>N: insulin</td>
<td>0.01 U‡</td>
<td>0.1 µM</td>
<td>1 w None</td>
<td>7</td>
<td>3</td>
<td>207 ± 34*</td>
<td>73 ± 22</td>
<td>−11 ± 22</td>
</tr>
<tr>
<td>29</td>
<td>X: glucagon</td>
<td>2 nmol</td>
<td>4 µM</td>
<td>2 w None</td>
<td>5</td>
<td>3</td>
<td>280 ± 46*</td>
<td>481 ± 57</td>
<td>−5 ± 66</td>
</tr>
<tr>
<td>30</td>
<td>N: insulin</td>
<td>0.01 U‡</td>
<td>0.1 µM</td>
<td>1 w None</td>
<td>5</td>
<td>5</td>
<td>579 ± 44</td>
<td>209 ± 32</td>
<td>154 ± 44*</td>
</tr>
</tbody>
</table>

For experiments 1–4, drugs were injected in the experimental (X) eyes, and PBS was injected into the fellow (N) eyes, except for group 25 in experiment 3, in which heat-denatured insulin was injected in the fellow eyes. Concentration in the syringe was 20 times the concentration in the vitreous chamber, except for the following groups: Groups 7 and 24–27, in which the concentration in the syringe was 200 times the concentration in the vitreous chamber, and groups 10 and 23, in which the concentration in the syringe was 54 and 95 times the concentration in the vitreous chamber, respectively. The insulin concentrations in experiment 3, 0.001 U, 0.01 U, and 0.1 U, correspond to 6.8 pmol, 68 pmol, and 680 pmol, respectively.

* P < 0.05, ** P < 0.01, *** P < 0.001.
† Injected once at the beginning of the experiment.
‡ Injections made with a 35-gauge needle.
‡‡ Chicks were positive lenses for 2 days prior to the injections.
# Heat-denatured insulin injected into the fellow eyes.
Glucagon increased choroidal thickness (A, B, D, J) and decreased the rate of ocular elongation (F, G, I, J) in eyes wearing diffusers, −7-D lenses, or no devices. The opposite effect was found in eyes wearing positive lenses (C, H, J). Glucagon also increased the rate of choroidal GAG synthesis (E) in eyes without lenses or diffusers. (J) In eyes wearing positive lenses, glucagon (2 nanomoles) increased the rate of ocular elongation in nearly all birds and reduced choroidal thickening in half of them. (Seven birds, five 1-week old and two 2-week old, are from pilot experiments and are not shown in Table 1.) Bar charts show the mean changes and SEMs over the course of the experiments shown in the line graphs, in which solid and dashed lines show the time course of the experimental and fellow eyes, respectively. For choroidal GAG synthesis, the ratios of the scintillation counts in paired eyes (glucagon/PBS) of individual chicks (circles) are superimposed on the mean and SEM of each group. ANOVA with Bonferroni post hoc tests were used to test the statistical significance of interocular difference (X − N) among different days within a group with respect to day 0 (asterisks on line graphs), and of relative changes (ΔX − ΔN) among the different doses (asterisks over horizontal lines in D and I). For ocular length and choroidal thickness, asterisks over bars indicate paired, one-tailed Student’s t-tests comparing changes in experimental and fellow eyes, except for (C) and (H), in which paired, two-tailed Student’s t-tests were used. For GAG synthesis, one-sample t-tests were used to compare the ratio of the counts from paired eyes (glucagon/PBS, or X/N) to a value of 1. *P < 0.05, **P < 0.01, ***P < 0.001.
Glucagon

**A. With lens or diffuser (2 nmol)**

![Graph](image)

- **diffuser**
- **-7 D lens**
- **-15 D lens**
- **+10 D lens**
- **+15 D lens**

\[ \Delta \text{Lens thickness (mm)} \]

- **n = 5**
- **n = 6**
- **n = 7**
- **n = 5**
- **n = 5**

**B. No lens or diffuser**

![Graph](image)

- **0.02**
- **0.2**
- **2**

- **doses (nmol)**

- **: glucagon (26-gauge needle)**
- **: glucagon (35-gauge needle)**
- **: PBS**

**Figure 2.** The effect of glucagon on lens thickness. (A) Glucagon increased lens thickness in eyes wearing diffusers or negative lenses. (B) Glucagon increased lens thickness in a dose-dependent fashion in eyes not wearing lenses. Asterisks over horizontal lines show comparisons of relative changes (ΔX/ΔN) that were significant by ANOVA with Bonferroni post hoc test. Asterisks over bars show significant difference in changes in experimental and fellow eyes from paired, two-tailed Student’s t-tests, as in Figure 1.

**Positive Lenses.** In eyes wearing positive lenses, however, glucagon (2 nanomoles) acted in the opposite direction: First, ocular elongation was less inhibited in the glucagon-injected eyes than in the fellow eyes in nearly all birds (increase in ocular length, for +10-D lenses: 131 μm vs. 11 μm, for +15-D lenses: 215 μm vs. 57 μm, glucagon versus PBS, P < 0.05, one-sample sign test, data pooled, groups 4 and 5 in Table 1 and bar charts in Figs. 1H, 1J). Second, in eyes wearing positive lenses, the choroids were not further thickened by glucagon, whereas they were thickened by glucagon in all other groups (for relative change, glucagon with positive lenses versus glucagon without lenses, P < 0.001, by either an unpaired, two-tailed Student’s t-test or a Mann-Whitney U test, bar chart in Figs. 1C, 1J). Notice in Figure 1J that, in contrast to the glucagon-treated eyes wearing negative lenses or diffusers, all of which had thicker choroids and slower elongation than the fellow eyes did, the glucagon-treated eyes wearing positive lenses nearly all had increased elongation. As a consequence, glucagon reduced the compensatory hyperopia in eyes wearing positive lenses (for birds wearing +10-D lenses, glucagon versus PBS, reduced by 2.9 D vs. 6.9 D, P > 0.05, group 4; for birds wearing +15-D lenses, 3.5 D vs. 6.3 D, P > 0.05, group 5).

Glucagon also increased lens thickness in eyes wearing positive lenses (changes in lens thickness for birds wearing +10-D lenses, glucagon versus PBS, 139 μm vs. 48 μm, P < 0.001, group 4; for +15-D lens-wear, 100 μm vs. 14 μm, P > 0.05, group 5, Fig. 2A).

**No Lenses or Diffusers.** In eyes without lenses or diffusers, glucagon thickened choroids in a dose-dependent fashion (Fig. 1D, groups 6–10). Because daily injections of PBS with a 26-gauge needle, but not a 35-gauge needle, thinned the choroid (26- vs. 35-gauge needles, ~58 μm vs. 19 μm, pooled data from fellow eyes, P < 0.001), we repeated the highest and lowest doses (0.02 and 2 nanomoles) with the finer needle: in both cases, the differences in the effects of these two doses of glucagon were significant (P < 0.01 with the 35-gauge needle, P < 0.05 with the 26-gauge needle). Glucagon also reduced the rate of ocular elongation, at least at the 0.2-nanomole dose (Fig. 11, groups 6 through 10).

Furthermore, the rate of choroidal GAG synthesis was increased by 30% after 3 days of the lowest dose of glucagon (P < 0.05, Fig. 1E) and approximately doubled at the highest dose (P < 0.05 with the 26-gauge needle, P < 0.01 with the 35-gauge needle).

**Experiment 2: Treatment with the Glucagon Antagonist**

The glucagon antagonist reduced development toward hyperopia by reducing the inhibitory effect on ocular elongation of wearing positive lenses.

**Ocular Length.** The glucagon antagonist reduced and, at the higher dose, eliminated the inhibition of ocular elongation caused by wearing positive lenses (Fig. 3A, the dotted line shows the daily growth of 70 μm in normal eyes).

**Choroidal Thickness.** The glucagon antagonist generally had no effect on choroidal thickness, except that it blocked further thickening of the choroid in eyes that had worn either +10 or +15-D lenses for 2 days before the injections began (choroid in these eyes was already thick before the injection; mean 365 μm for group 16, and 344 μm for group 17, P > 0.05, Fig. 3B).

**Refractive Error.** Consistent with its effect on choroidal thickness, the glucagon antagonist significantly reduced in six of seven birds development toward hyperopia in chicks injected during the 4 days of wearing positive lenses (mean difference, ~3.8 D, P < 0.05, paired t-test, groups 12 and 15) but not in chicks in which the injections started after 2 of the 4 days of positive lens wear (mean difference ~2.3 D, P > 0.05, paired t-test, groups 16 and 17).

**Experiment 3: Treatment with Insulin.** In eyes not wearing lenses, insulin had an effect opposite to that of glucagon, in that it increased the rate of ocular elongation, and in eyes wearing +10-D lenses, it increased ocular elongation and scleral GAG synthesis and decreased choroidal thickness.

**Ocular Length and Scleral GAG Synthesis.** When combined with positive lens wear, which normally stops the eye from elongating, insulin, at the two higher doses, caused the eye to elongate at a rate considerably greater than in normal eyes (0.01 U, 382 μm vs. 49 μm, P < 0.001; 0.1 U, 352 μm vs. ~104 μm, P < 0.001; if the absolute change in the experimental eyes alone was used, the middle dose had significantly greater effect than the lowest dose, P < 0.05, Fig. 4A).

In keeping with the stimulatory effect on ocular elongation, insulin also increased scleral GAG synthesis by approximately 100% in eyes wearing positive lenses (Fig. 4I).

In eyes not wearing positive lenses, insulin caused a two- to six-fold increased rate of elongation (0.001 U, 173 μm vs. 93
Anterior Chamber Depth. Insulin increased anterior chamber depth significantly in eyes both with and without lenses (Fig. 5A). In eyes not wearing lenses, this stimulation was clearly dose-dependent, in that at 0.1 U, insulin increased anterior chamber depth significantly more than 0.001 U ($P < 0.05$ for relative change; if the absolute change in the experimental eye alone was used, the highest dose of insulin also increased anterior chamber depth significantly more than at 0.01 U, $P < 0.001$). At the two higher doses, the anterior chambers of the eyes injected with insulin were deeper than the fellow eyes (for 0.01 U: 52 μm vs. −53 μm, $P > 0.05$; for 0.1 U: 273 μm vs. 39 μm, $P < 0.001$). At the highest dose, insulin caused the anterior chamber to deepen by 23%, whereas the ocular length increased by only 8%. In eyes wearing positive lenses, the anterior chambers were 8% to 10% deeper at the two higher doses (0.01 U: 100 μm vs. 11 μm; 0.1 U: 126 μm vs. −3 μm; $P < 0.05$ for both concentrations, Fig. 5A), whereas the eyes were only 4% longer. The responses were not dose dependent. This increase in anterior chamber depth is much too small to account for the increased ocular length caused by insulin (Figs. 4A, 5A).

Lenses Thickness. Insulin also increased lens thickness, at least in eyes wearing positive lenses (changes in lens thickness of eyes wearing positive lenses, for 0.01 U: 188 μm vs. 57 μm, $P < 0.001$; for 0.1 U: 96 μm vs. 32 μm, $P < 0.05$; changes in eyes without lenses, 0.01 U: 214 μm vs. 125 μm, $P > 0.05$; Fig. 5B). This effect seemed to be at its maximum at the intermediate dose tested, significantly more than at either 0.001 U for eyes wearing positive lenses ($P < 0.05$) or 0.1 U for eyes without lenses ($P < 0.01$).

Experiment 4: Treatment with IGF-1

Similar to insulin, IGF-1 increased the rate of ocular elongation and the anterior chamber depth. Unlike the choroids in insulin-injected eyes, those in the IGF-1-injected eyes thickened.

Ocular Length. At the highest dose tested (15 picomoles), IGF-1 caused a threefold increase in the rate of ocular elongation (IGF-1 vs. PBS, 579 μm vs. 209 μm, $P < 0.001$, group 26; Fig. 6A), significantly more than at the two lower doses (0.04 and 0.4 picomoles, $P < 0.001$).

Choroidal Thickness. In contrast to choroids in eyes injected with insulin, those in eyes injected with the highest dose of IGF-1 thickened significantly more than those in the PBS-injected eyes (154 μm vs. 30 μm, $P < 0.05$; Fig. 6B), significantly more than at the lowest dose (0.04 picomoles, $P < 0.01$).

Anterior Chamber Depth. At the highest dose, IGF-1 increased anterior chamber depth (15 picomoles, IGF-1 vs. PBS: 219 μm vs. 81 μm, $P < 0.05$, Fig. 6C), significantly more than at the two lower doses (14.7 picomoles vs. 0.04 or 0.4 picomoles, $P < 0.01$). The highest dose of IGF-1 caused the anterior chamber depth to deepen by 11%, whereas the ocular length increased by only 6%. Again, the increase in anterior chamber depth is much too small to account for the increase in ocular length caused by IGF-1 (Figs. 6A, 6C).

Experiment 5: The Interaction between Glucagon and Insulin

We were interested in whether glucagon and insulin act independently. Thus, we injected glucagon (at a dose that did not reduce the rate of ocular elongation; Fig. 11) together with insulin (at a dose that increased the rate of ocular elongation; Figs. 4A, 4H) into one eye (considered the experimental eye, or X) and the same dose of either glucagon or insulin alone into the fellow eye (N, groups 27–29, Table 1). Thus, we had three groups of birds with the combined injections, with the fellow eye receiving either glucagon or insulin. If their combined...
effects were the result of their separate effects, it would argue that glucagon and insulin act independently; if one drug, at a subthreshold level, inhibited the effect of the other one, it would argue that it acts through the pathway by which the effect of the other one is mediated.

Although insulin by itself had no effect on choroidal thickness in eyes not wearing lenses, it completely inhibited the strong effect of glucagon. Conversely, the same dose of glucagon, which by itself had no effect on ocular length, reduced the strong stimulatory effect of insulin.

**Choroidal Thickness and GAG Synthesis.** The most striking result of injecting glucagon and insulin into the same eyes was that, although insulin (at 0.01 U, 68 picomoles) alone did not reduce choroidal thickness, when injected with glucagon, it completely blocked the choroidal thickening effect of glucagon at 2 nanomoles (combination versus glucagon: 280 μm vs. 481 μm, P < 0.05, group 29; Fig. 7E). The combination of the lower dose of glucagon (0.02 nanomoles) and insulin caused a small increase in the rate of choroidal elongation compared with that in the insulin-injected eyes (combination versus insulin alone: 569 μm vs. 495 μm, P < 0.05; group 27).

As mentioned, insulin (at 0.01 U, 68 picomoles) caused a doubling of scleral GAG synthesis (Fig. 4I), whereas glucagon had no effect. The combination of glucagon (2 nanomoles) and insulin reduced the stimulatory effect of insulin on the rate of scleral GAG synthesis in 4 of 5 birds (mean reduction overall: combination/insulin, 25%, P < 0.05; right bar in Fig. 7F, group 29), but did not eliminate it, since eyes injected with both had a higher rate of scleral GAG synthesis in four of five birds compared with that in the fellow eyes injected with glucagon alone (mean increase, combination/glucagon, 45%, P < 0.05; right bar in Fig. 7F, group 28).

**DISCUSSION**

**Do Glucagon and Insulin Mimic the Effects of Positive and Negative Lenses?**

A prominent feature of the control of eye growth by vision is that the control is bidirectional, in that wearing positive spectacle lenses has effects opposite to those of wearing negative lenses, relative to normal eyes. In light of the facts that the visual modulation of eye growth persists in eyes detached from
and that retinal neurons have been identified that show opposite responses to positive and negative lenses, it is tempting to suppose that the retina exports chemical signals that direct the choroid and sclera to change in compensatory directions. One can further suppose that, because the levels of glucagon mRNA increase with positive lenses and exogenous glucagon inhibits the response to negative lenses, perhaps increased glucagon is the signal to halt ocular elongation and to thicken the choroid, while either decreased glucagon or an increase in another molecule does the opposite. Our results from treating eyes with glucagon, a glucagon antagonist, insulin, or IGF-1 partially support this simple scenario. We found, as others have, that glucagon inhibits ocular elongation and thickens the choroid, whereas insulin and IGF-1 tend to do the opposite (see summary in Table 2). However, when examined in detail, our results are not supportive of the simple notion of a STOP and a GO signal that regulates compensation for spectacle lenses.

We found that glucagon countered the changes associated with myopia from wearing negative lenses or diffusers by slowing ocular elongation and thickening the choroid; Vessey et al. had similar results, and Feldkaemper and Schaeffel found that a glucagon agonist slowed ocular elongation. We, like others, found that glucagon injected into the eye reduced the rate of ocular elongation and caused the choroid to thicken, thereby opposing the changes that make eyes myopic as a result of form deprivation or wearing negative lenses. Conversely, in eyes wearing positive lenses, we, like others, found that an antagonist to glucagon partially restored ocular elongation to normal levels. These results are consistent with an inhibitory action of glucagon on ocular growth. However, we did not find a thinning of the choroid by the antagonist, although the hyperopia caused by compensation for the positive lenses was reduced by the antagonist, as Feldkaemper and Schaeffel also found.

More provocatively, we found that glucagon reversed its action in eyes wearing positive lenses: instead of inhibiting the rate of ocular elongation, glucagon restored the normal rate, despite the positive lenses, and reduced the choroidal expansion that positive lenses normally produce. (The effect on ocular elongation was also found by Feldkaemper and Schaeffel using a glucagon agonist.) We have no persuasive argument for why this should be the case. Perhaps endogenous glucagon levels are high enough when positive lenses are worn that either the glucagon receptors reverse their action when fully occupied or the number of receptors is downregulated or another lower affinity receptor is involved. Furthermore, at the
dose (2 nanomoles) that strongly inhibited ocular elongation resulting from wearing diffusers or negative lenses, glucagon had little if any effect on eyes not wearing lenses or diffusers, evidence also consistent with conjecture that when endogenous glucagon (perhaps at higher levels in normal than in eyes wearing negative lenses or diffusers) is added to the exogenous glucagon, something different happens. Whatever the mechanism of action, the opposite action of glucagon in eyes wearing lenses of opposite signs suggests the possibility that the action of glucagon, rather than being a consistent “stop” signal, is modulated by the presence and sign of defocus.

When chicks wear lenses for only a fraction of the day, we find that positive lenses have a greater effect on the rate of ocular elongation than on choroidal thickness, whereas the opposite is true of negative lenses. Contrary to what would be expected if glucagon were acting much like a positive lens, we find that glucagon acts more on the thickness of the choroid in eyes wearing negative lenses or diffusers, whereas the glucagon antagonist acts more by inhibiting ocular elongation, with no effect on choroidal thickness (unless the eyes had been already wearing positive lenses when the injections started). This conclusion must be viewed with caution, because the pharmacology of the glucagon antagonist in the avian eye is poorly understood.

The situation is similar with respect to insulin. In birds not wearing lenses or diffusers, insulin as much as triples the rate of ocular elongation, as one would expect by a GO signal, antagonizing the effect of glucagon. (Feldkaemper et al. also found a smaller increase in axial length, probably attributable to increased depth of anterior chamber.) However, insulin increased ocular elongation without thinning the choroid, a situation that could not be achieved by lens wear. Only when eyes wear positive lenses, which on their own would cause choroidal thickening, does insulin thin the choroid, as well as accelerate ocular elongation. (Feldkaemper et al. found a much larger acceleration of ocular elongation with positive lenses.)

With IGF-1, the deviation from expectations was even greater, in that the drug increased ocular elongation, together with...
with thickening rather than thinning the choroid. Unfortunately, little is known about the similarity of avian physiological responses to IGF-I and insulin.

Finally, with either insulin or IGF-I, the depth of the anterior chamber increases greatly, and with either insulin or glucagon, the lens thickens, both changes that are not seen with short periods of lens wear. Feldkaemper et al.\textsuperscript{37} report similar findings for insulin, and Vessey et al.\textsuperscript{29} for glucagon. The finding that glucagon and insulin both increase lens thickness also argues that the peptidergic control of lens growth may differ from that controlling ocular elongation and choroidal thickness.

All in all, although the general direction of effects of glucagon is like that of positive lenses and the general direction of effect of insulin and IGF-I is like that of negative lenses, there are too many differences in their actions to give one confidence that the various visually modulated components of eye growth are mediated by two simple signals.

**Role of Glucagon**

These findings complicate a simple view of the role of glucagon. In particular, the finding that both glucagon and its antagonist restore normal ocular elongation in the eyes wearing positive lenses is puzzling. When we examined the responses of individual animals (Fig. 1I), we found that in the animals wearing positive lenses, the glucagon-treated eye was longer than the fellow eye (which also wore a positive lens) and had a thinned choroid. Considering the individual animals, all the glucagon-treated eyes had a greater rate of ocular elongation, and only half showed the usual association of decreased choroidal thickness with increased ocular elongation. A possible explanation for this dissociation is that glucagon inhibits ocular elongation only within a range of concentrations. When high doses of exogenous glucagon are added to high levels of endogenous glucagon resulting from the positive lens wear, it may be out of the inhibitory range, and the action of glucagon is therefore reversed, perhaps because desensitization of the glucagon receptors may remove a tonic inhibition of insulin action by glucagon. Our results in animals not wearing lenses hint in this direction to the extent that high levels of glucagon did not inhibit ocular elongation (Fig. 1I).

Fischer et al.\textsuperscript{34} have recently shown that the effect of glucagon is exerted much more on the equatorial diameter than on the ocular length. They suggest that the glucagon secreted by two populations of neurons near the retinal margin (bullwhip and mini-bullwhip cells) acts on the adjacent RPE, choroid, and sclera, all of which express glucagon receptor mRNA, thereby inhibiting growth. These findings suggest that there may be a way for the glucagon secreted by the retina to act on the choroid and sclera, despite the barrier of the RPE. The findings that killing the bullwhip and mini-bullwhip neurons exerts an effect mostly on the equatorial diameter of the eye and that giving glucagon can reverse this effect suggest that the normal action of glucagon may be at the equator and that glucagon has privileged access to the choroid and sclera there. If the growth at the equator of the eye were isotropic, one would expect a great inhibition of equatorial growth and a small inhibition of axial growth, the latter being what we found. It may be the case that the effects we saw on choroidal thickness come about from glucagon entering the choroid at the equator of the eye and being circulated within the choroid to the posterior pole, where our measurements took place. On the other hand, this conjecture goes against our findings that myopia, localized to part of the retina, results in local expansion of the choroid.\textsuperscript{35} Fischer et al.\textsuperscript{34} also show by in situ hybridization a higher level of the glucagon receptor in the fibrous sclera than in the cartilaginous sclera. In conditions that stimulate ocular elongation, such as form-deprivation or negative lenses, the fibrous sclera grows less (as shown by decreased thickness\textsuperscript{36,37} and decreased GAG synthesis\textsuperscript{23}). The physiological state of the fibrous sclera has been shown to modulate the cartilaginous sclera.\textsuperscript{25} One is tempted to conjecture that, if a principal action of glucagon is on the sclera, it may stimulate the growth of the fibrous sclera and perhaps, according to the hypothesis of Kusakari et al.\textsuperscript{30}, inhibit transdifferentiation of fibroblasts into chondrocytes, thereby decreasing ocular elongation. The signaling pathways controlling eye growth in mammals are almost certainly different from those in chicks insofar as glucagonergic amacrine cells have not been shown to exist in the mammalian retina.\textsuperscript{59}

**Possible Receptors Mediating Responses to Injected Insulin**

Although our findings of opposite actions of insulin and glucagon are consistent with the opposite actions of insulin and glucagon in other biological contexts, it is an open question whether the insulin we injected is acting on insulin receptors rather than IGF receptors, one of which is activated by insulin, although less effectively than by IGF-I. In our results, the effective dose of IGF-I was somewhat lower than that of insulin, especially on the ocular length, but the difference is not great enough to allow one to discriminate the receptor involved. Although the fact that insulin acts in the opposite direction from glucagon in promoting the proliferation of retinal progenitor cells\textsuperscript{6,9} originally provoked this study, we do not regard this as indicating that insulin is the active molecule with respect to control of eye growth, especially because it has been shown that IGF-I is secreted by photoreceptors and acts to increase the number of rod progenitor cells in fish.\textsuperscript{40}

Although receptors for insulin and IGF-I have been found in chick retina\textsuperscript{26} and sclera,\textsuperscript{41} and proinsulin has been found in chick embryo retina,\textsuperscript{42} it is not clear whether insulin synthesis occurs in the retina of post-hatching chicks. Several lines of evidence favor IGF-I’s playing a role in the control of eye growth. First, the level of mRNA expression for the IGF-I receptor in the RPE has been shown to be modulated in opposite directions by having chicks wear negative versus positive lenses (Zhang Z, et al. \textit{IOVS} 2007;48:ARVO E-Abstract 4417). Second, the sclera of chicks contains a layer of cartilage, and IGF-I profoundly affects the growth of cartilage, in general. In particular, IGF-I has been shown to increase proteoglycan synthesis in chick sclera (Luebke A, et al. \textit{IOVS} 2003;44:ARVO E-Abstract 414). Third, retinoic acid, the synthesis of which is changed in opposite directions by positive and negative lenses in both the retina and choroid, has been shown to affect proteoglycan synthesis in the chick sclera.\textsuperscript{43} In other systems, the activities of chondrocytes are modulated in opposite directions by retinoic acid and IGF-I.\textsuperscript{44} One mechanism by which retinoic acid might modulate scleral IGF-I levels is by changing the level of IGF- binding proteins.\textsuperscript{45,46}

**Relationship of Glucagon and Insulin Effects**

In eyes wearing neither lenses nor diffusers, insulin increases the rate of ocular elongation, with no effect on choroidal thickness, whereas glucagon thickens the choroid with little effect on the rate of ocular elongation. One can ask, then, whether these actions are the normal functions of these two molecules—that is, acting separately with different effects. To investigate this question, we treated eyes with combinations of the two drugs. We found that glucagon, at a dose that had no effect on ocular elongation, greatly reduced the stimulation of ocular elongation by insulin, and, conversely, insulin, having no effect on choroidal thickness, completely eliminated the choroidal thickening caused by glucagon. We interpret these
results as showing that the actions of glucagon and insulin are not independent, but must act together on some tissue to determine both the effects on the rate of ocular elongation and choroidal thickness. The most likely site of interaction is the retina, not only because both molecules act on retinal neurons, but more directly, because Feldkampfer et al. have shown that insulin injections block the increase in glucagon mRNA expression caused by wearing positive lenses and that they also block the increase in the fraction of glucagonergic amacrine cells that express the immediate early gene ZENK (egr-1). However, the interaction could as well occur at the level of the RPE, for which there is evidence of receptors for glucagon, insulin and IGF-1 (Zhang Z, et al. IOVS 2007;48: ARVO E-Abstract 4417), and the level of glucagon receptor mRNA is higher in the RPE than in the retina. We have preliminary evidence that insulin has a greater effect on the choroidal thickness of in vitro eye cups lacking the retina if the RPE is present (Sheng CK, et al. IOVS 2008;49:ARVO E-Abstract 1734). Furthermore, expression of mRNA for glucagon receptors has been found in the choroid and sclera, and so these are also possible sites of interaction.

Relation of Choroidal and Ocular Length Changes
Both in normal chicks and in those wearing lenses, an increased or decreased rate of elongation of the eye is associated with thinning or thickening of the choroid, respectively. Are changes in choroidal thickness and ocular elongation independent, or are they linked? On the side of independence, having chicks wear lenses briefly twice daily can affect one component without affecting the other, and having chicks wear very weak diffusers over positive lenses can block the effect of the positive lenses on the choroidal thickening but not on the ocular length inhibition. On the side of the two components being linked, a variety of manipulations that cause inhibition of ocular elongation all cause choroidal thickening, although in some cases the thickening lasts for only a few hours, suggesting that our findings of ocular inhibition without the choroidal thickening mentioned above may have been because the choroidal thickness was not measured at the right time.

Our present results, taken together, tend to support the linkage of choroidal thickening to inhibition of ocular elongation, at least in birds wearing lenses (Table 2). Specifically, we found that treatment with glucagon caused the choroid to thicken in parallel with slowed ocular elongation when the birds wore negative lenses or diffusers. When birds wearing positive lenses were treated with the glucagon antagonist, both the inhibition of ocular elongation and the choroidal thickening were blocked, although the choroid was affected only during the second 2 days of treatment. As for insulin, when it was administered to eyes wearing positive lenses, it both thinned the choroids and accelerated the rate of ocular elongation, the normal linkage. However, as discussed earlier, when glucagon, insulin, or IGF-1 was administered without the lenses, the coupling of choroidal thickness with the rate of ocular elongation was lost. We interpret these findings as indicating that coupling of choroidal thickness and ocular elongation depends on the eye’s being in a state of compensating for defocus and that, under these conditions, the coupling tends to be maintained despite the effect of the drugs.

Possible Mechanisms of Action of Glucagon and Insulin
As mentioned at the start of the Discussion, the simplest view of how glucagon and insulin might control emmetropization would be that insulin stimulates the eye to elongate and the choroid to thin, thus acting like a negative lens, whereas glucagon does the reverse, slowing the elongation and causing the choroid to thicken, thus acting like a positive lens. We conclude that the situation is considerably more complex.

We conjecture that, whether glucagon and insulin act at the retina, RPE, or choroid, their end-effect is to change the physiological state of the choroid, which, in turn, modulates both choroidal thickness and scleral growth, the latter being manifested as a change in the rate of ocular elongation. The evidence in support of the choroid’s modulating the sclera comes from in vitro studies in which choroids from eyes with accelerated or decelerated rates of elongation were cocultured with pieces of sclera from normal eyes. In this situation, the scleras increased or decreased their rate of proteoglycan synthesis, respectively, implying that the physiological state of the choroid determines the rate of growth of the sclera and, therefore, the rate of elongation of the eye, a finding recently replicated. Furthermore, the choroid synthesizes and releases retinoic acid, the amount of which increases in eyes wearing positive lenses and decreased in eyes wearing negative lenses; the retinoic acid produced by choroids of eyes wearing positive lenses inhibits proteoglycan synthesis in scleral explants.

In our view, in addition to the accepted functions of the choroid as a source of oxygen and nutrients for the outer retina, as a provider of lymphatic drainage for the eye, and as an effector to move the retina forward and back toward the focal plane, the choroid also acts as a secretory tissue modulating the growth of the sclera and hence of the eye in response to signals from the retina, two of which are likely to be glucagon and insulin or IGF-1.

References


29. Nickla DL. Transient increases in choroidal thickness are consistently associated with brief daily visual stimuli that inhibit ocular growth in chicks. Exp Eye Res. 2007;84:951–959.


