Effects of Positive and Negative Lens Treatment on Retinal and Choroidal Glucagon and Glucagon Receptor mRNA Levels in the Chicken

Christine Buck, Frank Schaeffel, Perikles Simon, and Marita Feldkaemper

PURPOSE. It has been found in the chicken that the amount of retinal glucagon mRNA increases during treatment with positive lenses. Pharmacological studies support the idea that glucagon may act as a stop signal for visually induced eye growth. To gain more insight into the functional role of glucagon, the changes of glucagon and glucagon receptor mRNA concentrations in retina and choroid over time were studied. Furthermore, the abundance of glucagon and the glucagon receptor was studied in different fundal layers (retina, retinal pigment epithelium [RPE], choroid) and the blood.

METHODS. Semiquantitative real-time RT-PCR was used to measure glucagon and glucagon receptor mRNA levels in retina and choroid after positive and negative lens treatment for 2, 6, or 24 hours, by unilateral −7- or +7-D lenses. Contralateral eyes served as the control, and completely untreated animals provided further reference data. Intravitreal colchicine injections (which are known to reduce the number of glucagon cells sharply) were used to verify that the related decline in glucagon mRNA could be measured by real-time RT-PCR.

RESULTS. In the retina, treatment with −7-D lenses induced an initial upregulation of glucagon mRNA in both eyes, followed by a significant downregulation. The treatment with +7-D lenses showed a significant but transient downregulation in the control eye superimposed on a trend toward upregulation in the treated eye. However, the changes in glucagon mRNA expression were not confined to the lens-treated eyes but were also found, although sometimes to a lesser extent, in the non-lens-covered fellow eyes. There was evidence of a transient increase in glucagon receptor mRNA levels in lens-treated eyes after either −7- or +7-D lens wear. In the choroid, no effect of imposed defocus was detected. The injection of colchicine led to the destruction of approximately 75% of the glucagon amacrine cells but the mRNA level of retinal glucagon decreased by only approximately 50%. Glucagon receptor expression was found to be higher in the RPE than the retina and choroid whereas, in the blood, glucagon and glucagon receptor mRNA expression was below detection level.

CONCLUSIONS. The observed bidirectional regulation of glucagon mRNA in correlation with the sign of imposed defocus supports the idea that glucagon may act as a stop-and-go signal for eye growth. This is in line with a previous proposal based on studies of changes of the glucagon peptide content. (Invest Ophthalmol Vis Sci. 2004;45:402–409) DOI:10.1167/iovs.03-0789)

In animal models, it has been shown that development of myopia and hyperopia can be artificially induced by placing the image either behind (negative lenses) or in front of (positive lenses) the retina. During treatment with spectacle lenses, the axial eye growth rates are altered, and this alteration finally results in a close match of eye length and focal length. The retina is able to detect the sign of imposed defocus and neither accommodation nor image processing in the brain is necessary. Little is known about the messengers released by the retina to control axial elongation. A possibly important candidate is glucagon, at least in birds and reptiles. Glucagon has been shown to be a potent stimulator of cAMP production in the chick. Intravitreal quisqualate (QA) injection results in severe retinal degeneration, yet deprivation myopia can still be induced. Among the retinal cells that are almost unaffected by QA are tyrosine-hydroxylase–immunoreactive cells and amacrine cells containing ZENK (also known as Egr-1, NGFI-A) during positive lens treatment and a downregulation during negative lens treatment. The injection of a glucagon agonist inhibited deprivation myopia as well as negative-lens–induced myopia, whereas a glucagon antagonist inhibited the recovery from myopia induced by deprivation.

The glucagon receptor belongs to the family of G-protein-coupled receptors with seven transmembrane domains. Gluca-
gon receptor mRNA is expressed relatively abundantly in the adipose tissue, heart, kidney, liver, ovary, pancreatic islets, spleen, and thymus of the rat. In the adrenal gland, skeletal muscle, small intestine, stomach, and thyroid, the expression levels are lower. In the chick, glucagon receptor mRNA distribution in different tissues has not yet been studied in detail, but a stimulation of adenylate cyclase activity by glucagon in the retinal pigment epithelium (RPE) has been described.

The purpose of this study was to gain more information about the possible role of glucagon in the regulation of eye growth. Therefore, the temporal sequence of glucagon and glucagon receptor mRNA expression in the retina and the choroid was studied after imposed defocus. Furthermore, glucagon and glucagon receptor transcript levels in different fundal layers were compared. The influence of colchicine injections (which are known to reduce the number of glucagon cells sharply) on glucagon expression was studied. Semiquantitative real-time RT-PCR was chosen as a convenient and reliable method to analyze mRNA expression changes with respect to a reference gene.

Methods

Treatment of Animals

Lens Treatment. Male White Leghorn chickens were raised under a 12-hour light–dark cycle. Six groups of animals aged between 12 and 17 days were unilaterally treated with positive and negative lenses (+7 D and −7 D) for different periods (2, 6, and 24 hours), while the contralateral eyes remained uncovered. The lenses were attached to Velcro rings that had been glued to the feathers around the eyes of anesthetized animals 1 day before the experiments. In addition, one group of totally untreated chickens served as an independent control. Each group consisted of five or six animals. The experimental treatment was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was approved by the institutional review board.

Colchicine Injections. Injections were made into the vitreous chamber on the day of hatching while chicks were under diethylether anesthesia. One eye was injected with 20 μL of water (control), and the other eye was injected with 20 μL of 25 μM (0.2 μg) colchicine (Sigma-Aldrich, Diesenhofen, Germany). This group consisted of nine animals. Colchicine has been reported to destroy most of the retinal ganglion cells and the glucagon amacrine cells and should therefore reduce the amount of glucagon mRNA drastically. With this experiment, we intended to verify the sensitivity of the real-time RT-PCR method. Refractive states were measured by automated infrared photorefractometry, and axial length was measured by A-scan ultrasound.

Blood Analysis

Because the variability of the glucagon receptor mRNA content in the choroid was very high, residual blood was considered to be a source of variable expression. Blood samples were taken from three totally untreated animals killed by decapitation at age 13 and the concentrations of control and target gene mRNA expression were measured.

Tissue Preparation

All animals were killed in the early afternoon by an overdose of diethylether. The eyes were enucleated and vertically cut with a razor blade. The anterior part containing the lens was discarded, and the posterior part (eyecup) was placed in a Petri dish containing Ringer’s solution chilled on ice to retard further metabolic changes. The different layers were carefully separated under visual control of a dissecting microscope. Only retinas or pieces of retinas that could be isolated without RPE were collected. For RPE cell collection, the remaining eyecups were radially incised several times (approximately 3 mm in depth), put on a cover slide, and flattened by filter paper (type HA, 0.45 μm; Millipore, Eschborn, Germany). Most of the RPE cells adhered to the filter paper, and the remaining eyecup was put back into the Petri dish. RPE residues on the choroid were removed, although contamination of the choroid by a few RPE cells cannot be excluded. All tissues were snap frozen in liquid nitrogen and stored at −70°C until RNA extraction. The blood samples were obtained by decapitation and collection of the blood in a sterile Petri dish already containing heparin. After centrifugation at 2000 rpm for 5 minutes, 50 μL of the cellular phase was used for subsequent RNA isolation. As erythrocytes of birds are nucleated, enough RNA could be extracted for quantitative analyses.

RNA Isolation and cDNA Synthesis

Total RNA from retina, choroid, and RPE was isolated with a kit (RNasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer’s instructions. The tissues were homogenized for 1 minute at a range of speed that increased in four steps from 11,000 to 20,000 rpm (Dixx 900 Homogenizer; Heidelberg, Kelheim, Germany). The isolation of total RNA from the blood was performed with extraction reagent TRIzol (Invitrogen, Karlsruhe, Germany), according to the manufacturer’s instructions, because the application of the extraction kit for isolation of blood RNA was not successful. One reason for the failure may be the different composition of avian blood (red blood cells contain nuclei). Blood homogenization was achieved with a sterile syringe with a needle of 0.9-mm diameter and by shearing the blood-extraction reagent (TRIzol; Invitrogen) mixture several times. All RNA samples were treated with RNase-free DNase I (Roche, Mannheim, Germany). The quality of RNA obtained from each tissue was tested by gel electrophoresis (1 μg) RNA from each retina, choroid, and blood sample and 0.25 μg RNA of each RPE sample (because of the relatively low RNA yield of <1 μg) were reverse transcribed (SuperScript II RNase H–reverse transcriptase; Invitrogen) using 0.5 μg oligo(dT)18 primer (Roche), according to the manufacturer’s instructions. To assure stable conditions for all samples during transcription, the reverse transcriptase was added to the mix containing DTT, 5× first-strand buffer, and RNase inhibitor (RNasin; Promega, Madison, WI) instead of being added to each sample separately.

Histology

To determine the extent to which the glucagon amacrine cells diminished after colchicine injection, frozen sections of colchicine- and water-injected eyes were made and stained for glucagon. The primary antibody solution contained a monoclonal mouse antibody (1:100) specific for the N-terminus of glucagon (Gordon Ohning, University of California Los Angeles, Los Angeles, CA). The secondary antibody solution contained 1:500 Oregon green–conjugated goat anti-mouse IgG. The method used has been described elsewhere.

Semiquantitative Real-Time RT-PCR

The sequences of the 18S rRNA (AF173612) and the preproglucagon (Y07539) genes were obtained from the European Molecular Biology Laboratory nucleotide sequence database (EMBL; http://www.embl-heidelberg.de/; Heidelberg, Germany). The nucleotide sequence amplified by the glucagon primers contained glucagon and a part of the intervening peptide (nucleotides +248 through +379 of the preproglucagon sequence). Primers for β-actin were complementary to sense nucleotides +2190 through +2207 and antisense nucleotides +2861 through +2840 of the β-actin sequence (National Center for Biotechnology Information [NCBI] no. X00182; www.ncbi.nlm.nih.gov/ provided in the public domain by the National Institutes of Health, Bethesda, MD) spanning an intron. The chicken glucagon receptor gene was sequenced by an author (Feldkaemper M, unpublished data, 2000)
but part of the glucagon receptor sequence is now also available in the NCBI nucleotide database (BU249554). Primer design was performed on computer with a commercial program (Prime; GENIUSnet HUSAR; KYE Systems, Heidelberg, Germany). The primer sequences used for specific amplification of the target and control genes are listed in Table 1. Primer synthesis was also performed on computer with a commercial service (Interactiva; Thermo BioSciences GmbH, Ulm, Germany), and the specificity of the PCR reaction was verified by gel electrophoresis and melting-curve analysis. In addition, PCR products amplified by the glaucagon and glucagon receptor primers were verified by automated sequencing. Primer concentrations for semiquantitative real-time RT-PCR ranged from 0.3 to 1 µM depending on tissues and genes.

The PCRs were performed in a thermocycler (iCycler iQTM Multi-Color Real Time PCR Detection System; Bio-Rad, Hercules, CA) using a fluorescence detection kit (Quantitect SYBR Green PCR kit; Qiagen). Primer annealing was performed at 55°C for 30 seconds and elongation at 72°C for 60 seconds. Fluorescence was measured, with every cycle at 72°C. The volume of a single reaction added up to 15 µL. The template amounts varied between the tissues, depending on the target mRNA abundance and the efficiency of the PCR reaction. A template amount corresponding to 4 ng RNA per well was used for retina, RPE, and blood samples, whereas a template amount corresponding to 4 ng RNA was used for choroidal samples. The higher template concentration was chosen because of the relatively low target mRNA expression.

Statistics and Data Analysis

Based on the threshold cycles ($C_T$) of the PCR products, statistical data analysis was conducted. The $C_T$ is defined as the PCR cycle at which the fluorescence intensity of a transcript crosses a threshold line, which is placed in the exponential amplification phase. The $C_T$ provides information about the amount of starting material. Standard curves for the amplification efficiencies of the four primer sets were generated by a dilution series, using template amounts ranging from 4 to 0.125 ng per well. The efficiencies ($E$) for each primer pair were calculated according to the following equation: $E = 10^{(-1/\text{slope})}$, giving a value between 1 and 2, whereby 1 corresponds to 0% efficiency and 2 to 100%. The slope ($S$) was determined by plotting the mean $C_T$ of each of the six cDNA dilution samples against the logarithm of the sample concentration.

Figure 1 shows an example of slope calculation in retina samples. Slopes and corresponding efficiencies for all tissues and primers are shown in Table 2.

To control and adjust for the variability of the PCR amplification, triplets of each tested sample were analyzed for all genes under investigation, and the coefficient of variation (CV) was calculated by dividing the standard deviation of each triplet by the mean $C_T$. Table 3 shows the mean CV ± SD of all triplet replicates. The mean $C_T$ of each triplet was used for final statistical data analysis.

The mean normalized expression (MNE)$^{25}$ was used to compare relative expression levels among different groups (both lens-treated groups and the colchicine-treated group) and was calculated as follows:

$$\text{MNE} = \frac{(E_{\text{reference}})^{C_{T\text{reference}}}}{(E_{\text{target}})^{C_{T\text{target}}}}$$

where $E$ is the efficiency of the PCR reaction (see Table 2), 18S rRNA and beta actin are the references, and glucagon and the glucagon receptor are the targets. For statistical comparison of the different treatment groups, one-way ANOVAs were applied and a significant ANOVA result was followed by a Tukey-Kramer test as a post hoc analysis. Within one ANOVA, either the lens-treated eyes (2, 6, and 24 hours) or the uncovered, contralateral eyes (NL) were compared with the totally untreated control group (CG). In addition, paired $t$-tests were performed to compare the MNE of the lens-treated eyes with that of the contralateral, uncovered eyes. The Bonferroni correction was applied to results obtained by paired $t$-tests.

Considering the variability in MNEs, the estimated power to detect a 50% change in expression is 0.8 for glucagon and 0.68 for the glucagon receptor in the retina. In the choroid, the power is 0.36 for glucagon and 0.08 for the glucagon receptor.

Influence of Lens Treatment on Reference Gene Expression

To assure the independence of the reference genes under treatment conditions, the reference gene $C_T$ of the lens-treated and the contralateral, uncovered eyes were compared with the $C_T$ of the totally untreated eyes of the control group. In the retina, neither 18S rRNA nor beta actin mRNA expression levels were influenced by lens treatment. Taking into account the results of the CV calculation, expression rates in the retina were all normalized to beta actin. However, similar results were obtained by normalization to 18S rRNA. In the choroid, beta actin mRNA expression was significantly influenced by the lens treatment. The expression of 18S rRNA was decreased in positive lens-treated eyes after 2 hours relative to the untreated control group. However, no further mRNA expression changes in the different treatment groups could be found. Therefore, results for the choroid were normalized to 18S rRNA. For comparison of target gene expression in different fundal layers of the eye, expression levels were normalized to the same reference gene.

Correlations of the Mean Normalized Expression in Retina and Choroid

To check whether the RNA expression correlated in the pairs of eyes of untreated control animals, the MNEs for glucagon and the glucagon receptor of each eye pair were plotted against each other and the
correlation coefficient $R$ was determined. No correlation could be found for the MNE of glucagon (based on $\beta$-actin and 18S rRNA) and glucagon receptor (based on $\beta$-actin) in the retina, but the MNE of glucagon receptor mRNA based on 18S rRNA showed a significant correlation ($R = 0.996, P < 0.05$). In the choroid, no correlation between the MNEs of both target genes was found. The data obtained from the 12 eyes of the six totally untreated control animals were therefore pooled.

**RESULTS**

**Effect of Defocus Imposed by $-7$- and $+7$-D Lenses on Concentrations of Glucagon and Glucagon Receptor mRNA in the Retina**

The treatment with negative lenses (Fig. 2A) resulted in an initial increase of the glucagon mRNA concentration in the lens-treated eyes ($+27.5\%$ after 2 hours). This was followed by a decrease of $-25.3\%$ and $-58.8\%$ after 6 and 24 hours, respectively, relative to the totally untreated control group (CG). All these changes were significant. The contralateral, uncovered eyes (NL) showed changes that were in close correlation to those in the lens-treated eyes. After 2 hours, the glucagon mRNA concentration was upregulated by $+11.4\%$, whereas after 6 and 24 hours mRNA concentration was down-regulated by $-19.2\%$ and $-38.1\%$, respectively. However, these changes achieved significance, relative to the untreated control group, only after 24 hours. Only within the group treated for 24 hours was a significant difference in the glucagon mRNA concentration between treated eyes and their contralateral, uncovered eyes determined by a paired $t$-test ($P < 0.05$).

After the treatment with positive lenses (Fig. 2B), a steady increase of the glucagon mRNA concentration was observed over time in the lens-wearing eyes, although this increase ($+38.4\%$ after 24 hours relative to the control group) was not statistically significant and also not as pronounced as the decrease of the mRNA concentration after negative lens treatment. Again, the contralateral, uncovered eyes were co-regulated and the significant difference of the glucagon mRNA concentration (as determined by a paired $t$-test, $P < 0.001$) between the treated and the contralateral, uncovered eyes after 2 hours of positive lens treatment is due to the down-regulation of glucagon mRNA in the contralateral, uncovered eyes.

The glucagon receptor mRNA level was significantly up-regulated ($+41.7\%$ compared with the control group and by $+33.1\%$ in comparison with the contralateral, uncovered eyes) after 2 hours of treatment with negative lenses (Fig. 2C). Glucagon receptor mRNA concentration did not change each control level after 6 and 24 hours of negative lens wearing. The results obtained by treatment with positive lenses (Fig. 2D) strongly resembled those obtained by negative lens treatment. After 2 hours, a clear difference of glucagon receptor mRNA expression between the lens-treated and their contralateral, uncovered eyes was seen, although significance was not achieved due to large standard errors. After 6 and 24 hours, the glucagon receptor mRNA level was not different from the control group level.

**Changes in Glucagon and Glucagon Receptor mRNA Expression in the Choroid after Imposed Defocus**

Lens treatment neither influenced glucagon nor glucagon receptor mRNA expression in the choroid (Fig. 3). Although glucagon mRNA expression remained relatively stable within all treatment groups, the concentration of the glucagon receptor mRNA varied considerably, as can be seen from the large standard errors of the mean in Figure 3.

**Altered Glucagon and Glucagon Receptor mRNA Expression after Colchicine Treatment**

Immunohistochemical analysis revealed that after 14 days, the number of glucagon amacrine cells was sharply reduced ($-75\%$) in the colchicine-injected eyes (C) relative to the contralateral, water-injected eyes (W). The anterior chamber depth was significantly reduced ($P < 0.05$; $0.96 \pm 0.06$ mm [C] vs. $1.10 \pm 0.03$ mm [W]) in the colchicine-injected eyes and they became $5.7$ D more hyperopic than the water-injected fellow eyes ($8.3 \pm 0.33$ D [C] vs. $2.6 \pm 0.2$ D [W]). Vitreous chamber depth did not change significantly. Most of the ganglion cells were removed in the retinas of colchicine-injected eyes. The MNE of glucagon mRNA was significantly reduced ($50.2\%$ compared with the control group and with the contralateral, water-injected eyes ($42.8\%$; Fig. 4A, colchicine-ver-

<table>
<thead>
<tr>
<th>Table 2. Slopes and Corresponding Efficiencies of Tissue-Specific PCR Amplifications Determined by Dilution Series</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes</strong></td>
</tr>
<tr>
<td><strong>Slope</strong></td>
</tr>
<tr>
<td>18S rRNA</td>
</tr>
<tr>
<td>$\beta$-Actin</td>
</tr>
<tr>
<td>Glucagon</td>
</tr>
<tr>
<td>Glucagon receptor</td>
</tr>
</tbody>
</table>

E, efficiency; NM, non-measurable.

<table>
<thead>
<tr>
<th>Table 3. Coefficients of Variation for Investigated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genes</strong></td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
</tr>
<tr>
<td>$\beta$-Actin</td>
</tr>
<tr>
<td>Glucagon</td>
</tr>
<tr>
<td>Glucagon receptor</td>
</tr>
</tbody>
</table>

NM = non-measurable.
sus water-injected group: \( P < 0.01 \), paired Student’s \( t \)-test). The concentration of glucagon receptor mRNA (Fig. 4B) was found to be significantly increased (47%) in the colchicine-injected eyes compared with the control group and with the controllateral, water-injected eyes (59.5%; colchicine–versus water-injected group: \( P < 0.001 \), paired Student’s \( t \)-test).

**RNA Expression Patterns in Different Fundal Layers**

To compare the relative expression level of target gene mRNA in different tissues of the eye, the MNE based on \( \beta \)-actin was determined in untreated animals (Fig. 5). The glucagon mRNA level was highest in the retina and was rather low in the choroid and RPE. Glucagon receptor mRNA concentration was found to be higher in the RPE than in both the choroid and the retina. As can be seen by the error bars, variations in glucagon receptor mRNA concentration that occur in the retinas of negative-lens–treated eyes is not understood, but a similar effect has been observed in earlier studies on the expression of ZENK mRNA.26 Finally, after 6 hours of monococular lens treatment, visual treatments that are known to enhance axial eye growth (negative lenses) or decrease axial eye growth (positive lenses) in the treated eyes were associated with opposite changes of the mRNA levels that occurred both in the treated and contralateral control eyes.

**DISCUSSION**

**mRNA Expression Changes in the Retina after Imposed Defocus**

Visual conditions that induce hyperopia or myopia resulted in a trend toward upregulation and significant downregulation, respectively, of the glucagon mRNA concentration. The increasing glucagon mRNA expression in response to positive lens treatment complies with results from a previous study14 in which an upregulation of glucagon mRNA concentration after 6 hours of positive lens treatment was shown by Northern blot analysis. It is also in line with the idea that glucagon may function as a stop signal for eye growth. Downregulation of glucagon mRNA during negative lens treatment could result in axial eye elongation because the inhibitory effect of glucagon decreases. The observed changes in mRNA expression soon after the beginning of lens treatment (detectable after only 2 hours) persist for 24 hours. The initial increase of glucagon mRNA concentration that occurs in the retinas of negative-lens–treated eyes is not understood, but a similar effect has been observed in earlier studies on the expression of ZENK mRNA.26 Finally, after 6 hours of monococular lens treatment, visual treatments that are known to enhance axial eye growth (negative lenses) or decrease axial eye growth (positive lenses) in the treated eyes were associated with opposite changes of the mRNA levels that occurred both in the treated and contralateral control eyes.

Recently, it has been shown that brief periods of myopic defocus imposed by positive lenses prevent myopia that is induced by wearing of negative lenses all day.27,28 Measuring glucagon mRNA content in the retina, we found a significant difference between the positive-lens-treated eye and the control group resulted in F ratio = 3.7324; Prob > \( F \) 0.0254; significances determined by post hoc analysis were: CG versus –7 D/2 hours* and –7 D/2 hours versus –7 D/6 hours*. For clarity, significance levels of glucagon mRNA expression changes are not denoted in the figure but are presented in Table 4.

**Table 4. ANOVA Results of Glucagon mRNA Expression Changes after Lens Treatment in the Retina**

<table>
<thead>
<tr>
<th>Control group (CG)/contralateral, uncovered eyes (NL)</th>
<th>Treatment</th>
<th>F Ratio</th>
<th>Prob &gt; ( F )</th>
<th>Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group (CG)/lens treated eyes</td>
<td>Negative lens</td>
<td>4.7028</td>
<td>0.0094</td>
<td>CG vs. NL/24 h*; NL/2 h vs. NL/24 h*</td>
</tr>
<tr>
<td></td>
<td>Positive lens</td>
<td>9.5177</td>
<td>0.0002</td>
<td>CG vs. NL/6 h*; NL/2 h vs. NL/6 h**; NL/24 h**</td>
</tr>
<tr>
<td>Control group (CG)/lens treated eyes</td>
<td>Negative lens</td>
<td>17.0045</td>
<td>&lt; 0.0001</td>
<td>CG vs. –7/24 h***; –7/2 h vs. –7/6 h***; –7/24 h***</td>
</tr>
<tr>
<td></td>
<td>Positive lens</td>
<td>2.3240</td>
<td>0.0983</td>
<td></td>
</tr>
</tbody>
</table>

CG, untreated control group; NL, contralateral, uncovered eyes.

\( ^* P < 0.05; ^{**} P < 0.01; ^{***} P < 0.001. \)
tralateral eye as early as 2 hours after lens wear began, whereas temporal changes in glucagon mRNA expression in the negative lens-treated eyes differed from the fellow control eye only after 24 hours. These results complement each other, both suggesting that defocus imposed by positive lenses is a more powerful and faster stimulus for emmetropization.

The only statistically significant effect concerning the glucagon receptor content in the retina was an upregulation of the mRNA expression after 2 hours of negative lens treatment. A similar result was obtained with positive lenses but significance was not achieved. Because the pattern of glucagon receptor mRNA expression was similar in both negative and positive lens-treated eyes, the upregulation after 2 hours could be an unspecific response to blurring retinal images. With regard to the glucagon receptor mRNA, no co-regulation was observed in the contralateral, uncovered eyes.

The expression of glucagon in the choroid was not altered by imposed defocus and remained stable over time. The source of glucagon in this tissue is still unknown. The blood level of target gene mRNA was too low to be measured and it cannot therefore account for the glucagon mRNA that was detected in the choroid. There could be glucagon-producing cells that are not responsive to imposed defocus. Although the glucagon mRNA level did not change significantly over time, the glucagon receptor mRNA concentration varied by several orders of magnitude. Between both eyes of the same animal, the glucagon receptor mRNA concentrations differed by up to seven cycles in the PCR, which corresponds to a 128-fold difference. These variations occurred randomly with little correlation to the treatments, although the variability appeared to reach a

Glucagon and Glucagon Receptor mRNA Expression in the Choroid

The expression of glucagon in the choroid was not altered by imposed defocus and remained stable over time. The source of glucagon in this tissue is still unknown. The blood level of target gene mRNA was too low to be measured and it cannot therefore account for the glucagon mRNA that was detected in the choroid. There could be glucagon-producing cells that are not responsive to imposed defocus. Although the glucagon mRNA level did not change significantly over time, the glucagon receptor mRNA concentration varied by several orders of magnitude. Between both eyes of the same animal, the glucagon receptor mRNA concentrations differed by up to seven cycles in the PCR, which corresponds to a 128-fold difference. These variations occurred randomly with little correlation to the treatments, although the variability appeared to reach a

FIGURE 3. MNEs over time for glucagon (A, B) and glucagon receptor (C, D) mRNA in the choroid. Error bars, SEM. Six animals were tested in each treatment group. Description of the key is provided in Figure 2.

FIGURE 4. MNEs for glucagon (A) and glucagon receptor (B) mRNA expression in the retina 14 days after intravitreal water or colchicine injection. Error bars, SEM. Six animals were tested in the treatment group. Control, totally untreated animals (12 eyes of six animals); colchicine, colchicine-injected eyes; water, contralateral water-injected eyes. Horizontal brackets: significant differences between colchicine-injected, contralateral water-injected eyes, and totally untreated eyes of the control group were determined by ANOVA (glucagon: F ratio = 13.3055; Prob > F 0.0002. Glucagon receptor: F ratio = 8.5035; Prob > F 0.0025). Vertical brackets: significant differences in glucagon and glucagon receptor mRNA expression between the colchicine-injected and the contralateral water-injected eyes were detected by paired t-tests: *P < 0.05; **P < 0.01; ***P < 0.001.
maximum after 2 hours of lens treatment. It is unlikely that variations in the blood content of the tissue account for the variations in glucagon receptor mRNA levels, given that the mRNA was not detectable in blood alone. Local variations in glucagon receptor mRNA content in the tissue, which was randomly sampled depending on the preparation, seem also unlikely. Contamination by RPE cells could certainly account for minor variations in glucagon receptor mRNA content, but the range observed in this study cannot be explained by small amounts of residual RPE cells. In summary, the source of this variability remains obscure at present, and therefore no conclusions as to whether the lens treatment influences glucagon receptor expression in the choroid can be drawn.

Colchicine Treatment as a Measure of Sensitivity

Even though it has been shown that semiquantitative real-time RT-PCR is a powerful technique for measuring relative mRNA levels, another test was performed to verify its performance in our protocols. We chose colchicine, a drug with a known effect on the glucagon amacrine cells, the only cells in the avascular chick retina that are known to produce glucagon. In colchicine-injected eyes, glucagon mRNA concentration was downregulated by approximately 50%, whereas the histologic analysis of the colchicine-treated retinas revealed the disappearance of approximately 75% of the glucagon amacrine cells.

The effect of colchicine on glucagon mRNA levels was clear and confirmed that the technique provided a reliable detection method for mRNA expression changes. Furthermore, it provided information on the range of changes that could be expected. A possible explanation for the still relatively high glucagon mRNA levels after the destruction of three quarters of the cells may be a compensatory increase in transcription in the remaining glucagon amacrine cells. Another explanation may be that some glucagon amacrine cells are still viable and transcriptionally active, although they are histologically undetectable. Glucagon and glucagon receptor expression seem to be negatively coupled, because the downregulation of glucagon mRNA content after colchicine injection resulted in an upregulation of glucagon receptor mRNA. The result is in good accordance with a previous study, which showed that glucagon dose dependently decreases glucagon receptor mRNA expression in hepatocytes. This “downregulation” is thought to be essential for physiological adaptation induced by endogenous ligands, as well as in the clinical effects of exogenously administered drugs that are used chronically or repeatedly.

Comparison of RNA Expression in Different Tissues

We found that glucagon receptor mRNA expression is fairly high in the RPE, which constitutes the blood-brain barrier in the eye and has an important function in controlling the passage of nutrients from the blood to the retina. It is not known whether the glucagon receptors preferably face the choroid or the retina, and it is similarly unclear whether retinal glucagon or blood-borne glucagon is the primary ligand for the glucagon receptors in the RPE. In the choroid, only low mRNA levels of glucagon and glucagon receptor were measured, whereas retinal levels were much higher. It may therefore be that retinal glucagon exerts its effect on eye growth through binding at glucagon receptors on the retinal side of the RPE. It has been shown that glucagon peptide content in the retina was decreased after negative lens treatment for 24 hours, which may have influenced the glucagon receptor saturation in the RPE. Finally, it remains to be shown that the glucagon receptors in the RPE can trigger changes in scleral growth.

Coregulation in Contralateral Eyes

Glucagon mRNA expression in the retina was coregulated in the contralateral, uncovered eyes with normal vision. In contrast, glucagon receptor mRNA expression was independently regulated in both eyes. A coregulation of ZENK protein, and other proteins has been found in prior studies. Given that the changes in eye growth were confined to the treated eyes, this effect is surprising. The mechanism through which the two eyes may communicate and the way in which changes in axial length can be confined to the treated eye, even though the fellow eye shows similar alterations in biochemical pathways, are both yet unknown. It could occur through humoral factors, systemically through the blood flow, or by a direct neuronal linkage between both eyes. Moreover, it could be that local conditions in the control eye stimulate pathways that override the input from the treated eye. Our results emphasize that both eyes of the chicken cannot be considered independent and that it is crucial to have comparisons available to an independent control group of untreated animals.

Glucagon Amacrine Cells and Regulation of Eye Growth

Initially, the finding of Fischer et al. that amacrine cells, which are immunoreactive for both glucagon and the immediate early gene product ZENK, display an upregulation of ZENK during positive lens treatment and a downregulation during negative lens treatment suggested that these cells are involved in control of visual eye growth. We and others have therefore started to study whether glucagon is really involved. The above-described bidirectional regulation of glucagon mRNA in correlation with the positive and negative lens treatment supports the idea that glucagon may act as a stop-and-go signal for eye growth. However, one has also to keep in mind that most
of the changes in gene expression were not confined to the treated eye. Until the question of coregulation in the contralateral eye is resolved, the significance of the changes in gene expression remains unclear. A large number of transcription factors, including Pax6 and cAMP response element-binding protein (CREB) bind to the promoter region of the human glucagon gene, but an Egfr (Zenk in chicks)-binding site could not be demonstrated in the human promoter sequence. Until now, the chicken glucagon promoter has not been characterized, and it remains unclear whether an increase in the transcription factor ZENK may induce an increase in glucagon production directly. Although previous studies, especially of Stell et al.17 and of our group, have suggested a role of glucagon itself, this does not exclude that glucagon-immunoreactive cells use other proglucagon-derived peptides for this task (for example, glucagon-like peptide 1) and/or nonpeptide neurotransmitters. For example, 50% of the glucagon ZENK-immunoreactive cells are GABAergic. Very recently, the role of γ-aminobutyric acid (GABA) during eye growth regulation was studied.18 The authors concluded that GABA receptors mediate eye growth and refractive development. The ZENK-immunoreactive glucagon cells are a small, homogenous population. Until now, it has been shown that they do not express cellular retinoic acid binding protein, choline acetyltransferase, somatostatin, tyrosine hydroxylase, vasoactive intestinal polypeptide or parvalbumin. A detailed investigation of the neurotransmitter-, neuropeptide- and receptor composition of the glucagon ZENK-immunoreactive cells is necessary and may also lead to new candidates involved in eye-growth-modulating pathways.

References