The Role of Insulin and Insulin Signaling in Eye Growth Regulation

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MAR PORTUGUÊS

Ó mar salgado, quanto do teu sal
São lágrimas de Portugal!
Por te cruzarmos, quantas mães choraram,
Quantos filhos em vão rezaram!

Quantas noivas ficaram por casar
Para que fosses nosso, ó mar!
Valeu a pena? Tudo vale a pena
Se a alma não é pequena.

Quem quer passar além do Bojador
Tem que passar além da dor.
Deus ao mar o perigo e o abismo deu,
Mas nele é que espelhou o céu.

Mensagem (1934), Fernando Pessoa

Portuguese Sea

O sea of salt, how much of all your salt
Contains the tears of Portugal?
So we might sail, how many mothers wept,
How many sons have prayed in vain!

How many girls betrothed remained unwed
That we might posses you, Sea!
Was it worth the effort? Anything 's worth it
if the soul's not petty.

If you'd sail beyond the cape
Sail you must past cares, past grief.
God gave perils to the sea and sheer depth,
But mirrored heaven there.

in Message (1934), Fernando Pessoa

Ao meu pai, mãe e irmão

To my father, mother and brother

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A. Introduction

1. The Process of Emmetropization

Starting from the first stages of embryogenesis and continuing afterwards, most body parts are programmed to achieve a certain size and shape. The eye shares a similar homeostatic mechanism but, in addition it needs vision as input to growth control (Wallman and Winawer, 2004). However, for still unknown reason the eye often grows too long and becomes myopic this way (shortsighted), or it does not grow long enough and consequently gets hyperopic (farsighted).

An eye to be called emmetropic (normal sighted) must match the axial length with the optical power of cornea and lens to achieve that images of distant objects fall on the retina, rather than in front or behind it (Wallman and Winawer, 2004). Some species are born with too short eyes, and thus hyperopia, such as macaque monkey (Smith, 1998) and marmosets (Troilo and Judge, 1993; Graham and Judge, 1999). In others, for instance ostriches (Ofri, Millodot et al., 2001) and falcons (Andison, Sivak et al., 1992) eyes are too long and therefore myopic at birth whereas, in yet other species, such as humans (Curtin, 1985) and owls (Schaeffel, 1998) some individuals are myopic others hyperopic. The emmetropization process occurs mainly during early childhood but there is evidence that ocular dimensions may still change in young adults (McBrien and Adams, 1997).

2. Refractive Errors

According to the World Health Organization, refractive errors (myopia, hyperopia and astigmatism) are one of the most common visual impairments, affecting a large proportion of the worldwide population, independently of age, sex and ethnic group. The prevalence of myopia in the human population has dramatically increased in developed regions of Asia (Lin, Shih et al., 1999), but also in Western societies (Vitale, Sperduto et al., 2009) during the last decades. It is estimated that approximately 30% of the worldwide population are currently myopic (Dirani, Chamberlain et al., 2006). Genetic as well as environmental factors have been implicated in the development of myopia but the relative importance of genes *versus* environment remains controversial (Morgan and Rose, 2005). Twin studies (Hammond, Snieder et al., 2001) found high heritability for

myopia and a number of other studies (Mutti, Mitchell et al., 2002; Mutti, Semina et al., 2002) showed a strong association of myopia in families. These studies thereby support the importance of genes on refractive error development. On the other hand, numerous investigations supported the role of environmental factors and the importance of the nature of visual input on eye growth (Morgan and Rose, 2005). An older study from Angel and Wissman (Angle and Wissmann, 1980) suggested that excess of near-work was a cause of myopia onset. Near work was shown to be associated with myopia in a number of succeeding epidemiological studies, but not in several others (Saw, Hong et al., 2001). It was found that myopia that appears during childhood, especially during school years, is more likely to start in economically developed societies (Morgan and Rose, 2005). In addition, recent studies showed that the amount of outdoor activity might influence the time of onset of myopia (Jones-Jordan, Sinnott et al., 2010). However the percentage of each factor, genetics and environment, for the onset and development of myopia is not entirely clear.

2.1. Myopia

Among the refractive errors, myopia is the most common one and it is, in most cases, characterized by an excessive elongation of the posterior pole of the eye, together with a thinning of the choroid and sclera. As a result, distant objects are focused in front of the retina (Wallman, Turkel et al., 1978; Wallman, Adams et al., 1981; Hodos and Kuenzel, 1984; Gottlieb, Fugate-Wentzek et al., 1987; Christensen and Wallman, 1991). The refractive errors can be easily diagnosed, and corrected with spectacles or contact lenses to enable normal vision (Figure A.1). Positive lenses are used to correct hyperopic eyes (Figure A.1, B1 and B2), on the other side, negative lenses are worn to correct myopic eyes (Figure A.1 C1 and C2).

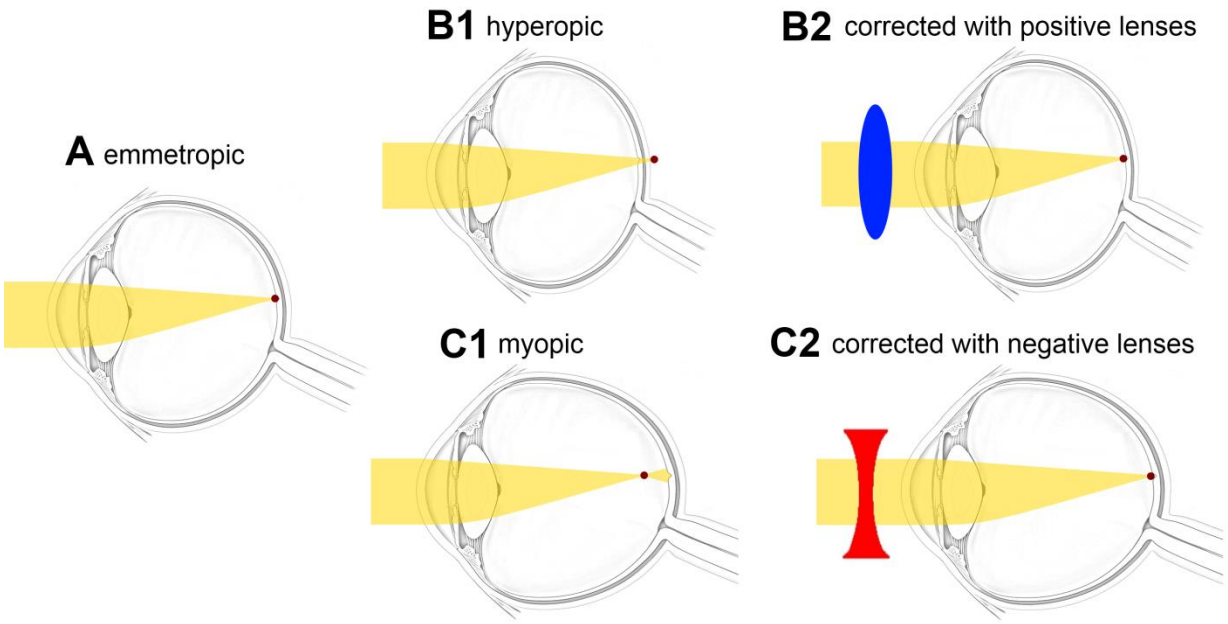


Figure A.1. Diagram showing corrected and uncorrected refractive errors**.** A – emmetropic eye; B1 – uncorrected hyperopic eye; B2 – corrected hyperopic eye with positive lenses; C1 – uncorrected myopic eye; C2 – corrected myopic eye with negative lenses.

2.2. The control of eye growth in animal models

Animal models of myopia have been developed and have shown that active, visually-guided emmetropization exists in vertebrate eyes (Edwards, 1996). Animals respond and compensate for imposed defocus by adjusting the axial eye growth rate such that the focal plane and photoreceptor plane achieve a close match. Myopia can be artificially induced in a number of species like chicks (Wallman, Turkel et al., 1978), tree shrews (McBrien and Norton, 1992), monkeys (Wiesel and Raviola, 1977; Troilo and Judge, 1993) and guinea pigs (Howlett and McFadden, 2009), by placing negative lenses, which induce hyperopic defocus in front of the animal's eye (Schaeffel, Glasser et al., 1988). The shift of the focal plane behind the photoreceptor layer triggers substantially increased eye growth (Figure A.2, C1 and C2). Furthermore, the choroid thins. On the contrary, positive lenses, imposing myopic defocus, slow the rate of ocular elongation or even cause a small amount of shrinkage (Wallman, Zhu et al., 2009) and led to an increase in choroidal thickness by up to a factor of 3 in the chick (Wallman, Wildsoet et al., 1995) (Figure A.2 B1 and B2). Also in marmosets, the choroidal

thickness changes are correlated with the induced changes in eye size (Troilo, Nickla et al., 2000).

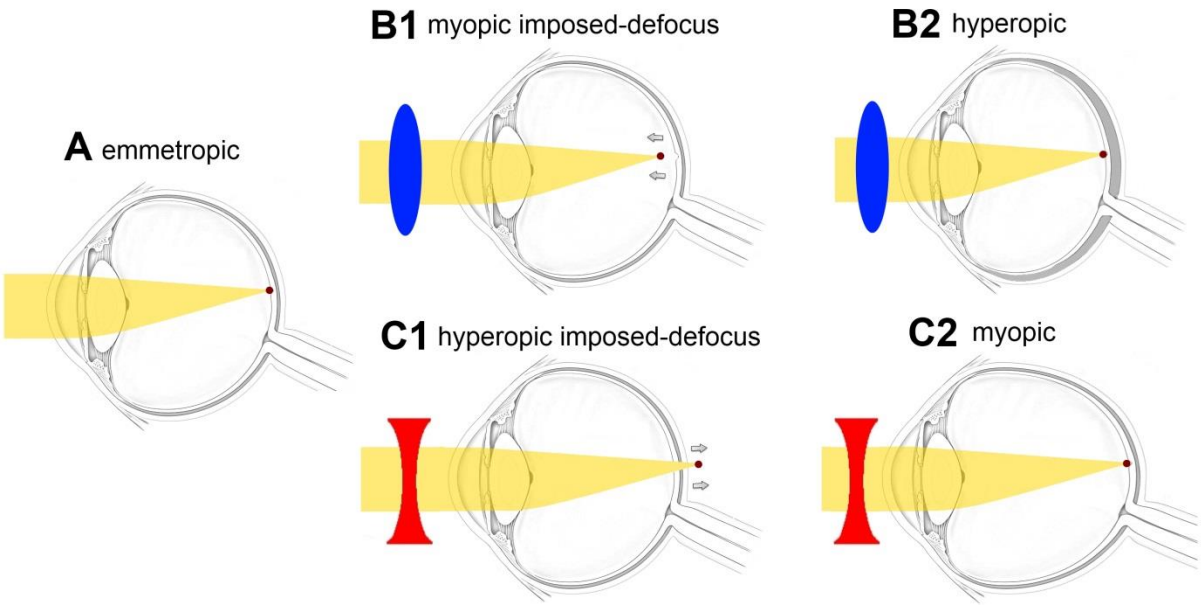


Figure A.2. Diagram showing control of eye growth in animal models**.** A – emmetropic eye; B1 – emmetropic eye before treatment with positive lenses to induce hyperopia; B2 – hyperopic eye as a result with treatment with positive lenses treatment; C1 – emmetropic eye before treatment with negative lenses; C2 – myopic eye as a result with treatment with negative lenses.

Regulation of eye growth was demonstrated to be largely independent of processing in the brain, as shown in optic nerve lesion studies (Troilo, Gottlieb et al., 1987; Wildsoet, 2003 Miles and Wallman, 1990), and in eyes unable to accommodate because of Edinger-Westphal nuclei lesions (Schaeffel, Troilo et al., 1990). Even blockade of retinal cell action potentials did not prevent deprivation induced myopia development (McBrien, Moghaddam et al., 1995), again supporting the idea of a local control of eye growth. The brain seems to play a role under certain conditions, mainly in the fine-tuning of the growth regulation process (Troilo, Gottlieb et al., 1987). Moreover, deprivation of vision limited to a certain region of the retina using hemi-field diffusers produces myopia and axial elongation only in the affected part of the eye, as shown in chickens (Wallman, Gottlieb et al., 1987). Same was true, if hemi-field spectacle lenses were used (Diether and Schaeffel, 1997), once more showing that eye growth is

controlled locally. Similar results were obtained in studies using tree shrews, guinea pigs and rhesus monkeys (Smith, Huang et al., 2009).

2.3. Techniques to measure axial length

To measure changes in axial length (AL), anterior chamber depth (ACD), lens thickness (LT) and vitreous chamber depth (VCD) that are induced by alterations in visual experience, A-scan ultrasonography is a commonly used technique (Wallman and Adams, 1987; Schaeffel and Howland, 1991). High frequency sound waves (typically 10 MHz) are emitted by the probe tip at short pulses, and the transducer is rapidly switched into receive mode to record echoes that bounce back as the sound beam strikes different ocular interfaces. Because sound waves travel the faster the denser the media, the time delay until the echo is received is not directly proportional to the intraocular distances (Byrne, 1995). The A-scan ultrasound device has to be corrected based on the knowledge of where the echoes come from and through which media in the eye the sound has traveled. This is automatically done in any A-scan ultrasound machine that is used in humans, but needs to be individually done for different animal eyes. To measure chicken eyes, a soft rubber tube, filled with water and covered with paramount film, with about 8 mm length and 3 mm diameter has to be attached to the transducer (Schaeffel and Howland, 1991). One disadvantage of the method is that this ultrasound transducer touches the cornea, and a minor indentation of the cornea can therefore not be excluded. Recently, Haag-Streit (Haag-Streit, Koeniz, Switzerland) developed a new non-invasive and non-contact optical low-coherence interferometer (OLCI), the Lenstar LS 900. In addition to ACD, LT, VCD and AL, the Lenstar also provides pupil diameter and central cornea curvature, as well as corneal thickness, white-to-white distance (horizontal iris width), retinal thickness and, sometimes, choroidal thickness, at least in the human eye. Since the measurements are optical no contact is necessary to the cornea.

3. Pharmacology in Myopia

So far the treatment of myopia has focused on correcting the mismatch between the optical power of the eye and its length using either spectacles or contact lenses, or refractive surgery. Even using those approaches there is no good control of myopia progression. There are currently three drugs approved by the US FDA for use as myopia treatment in clinical trials, such as atropine, pirenzepine and 7-methylxanthine (7-MX) (Fan, Lam et al., 2007; Liang, Ho et al., 2008; Siatkowski, Cotter et al., 2008; Trier, Munk Ribel-Madsen et al., 2008; Tong, Huang et al., 2009). The usage of these drugs still has not been entirely successful. Clinical trials performed in Asia used these drugs for treatment of early-onset myopia. However, the results suggested that high doses of the unspecific muscarinic antagonist atropine (1% eye drop solution), administered to children slowed down myopia progression but at the same time caused severe local side-effects, for instance mydriasis, photophobia, blurred vision and allergic dermatitis (Shih, Chen et al., 1999). The long-term effects of atropine usage, such as ultraviolet light-related retinal damage and cataract formation, due to chronic pupillary dilation, still remain unknown. Moreover, after stopping eye drop application, eyes treated with atropine demonstrated even higher rates of myopia progression compared with eyes treated with placebo (Tong, Huang et al., 2009). In other clinical trials using pirenzepine, a M1 specific muscarinic antagonist (Bartlett, Niemann et al., 2003; Tan, Lam et al., 2005; Siatkowski, Cotter et al., 2008), it was shown that pirenzepine is less effective than atropine at reducing the progression of myopia and also causes side-effects, although less severe (Ganesan and Wildsoet, 2010). Later, the adenosine antagonist 7-MX was used in a European clinical trial. The oral therapy showed fewer side effects, but its effect on myopia progression was only very small (Trier, Munk Ribel-Madsen et al., 2008).

As mentioned above, several attempts have been made to stop the progression of myopia. Nevertheless, the questions “why does the eye keep growing?” and “which molecular signals are involved?” so far remain unsolved and are up till now. Recent microarray studies in animal models of myopia (Schippert, Schaeffel et al., 2008; Stone and Khurana, 2010) and genome-wide association studies in humans (Hysi, Young et

al., 2010; Solouki, Verhoeven et al., 2010) have discovered a number of candidate genes that might be involved in eye growth regulation and myopia development. Several retinal transmitters have been suggested to be implicated in the modulation of eye growth such as: vasoactive intestinal polypeptide (VIP) (Stone, Laties et al., 1988; Seltner and Stell, 1995), dopamine (Stone, Lin et al. 1989; Iuvone, Tigges et al., 1991; Schaeffel, Hagel et al., 1994), retinoic acid (Seko, Shimizu et al., 1998; Bitzer, Feldkaemper et al., 2000; Mertz and Wallman 2000), glucagon (Feldkaemper and Schaeffel, 2002; Buck, Schaeffel et al., 2004; Vessey, Rushforth et al., 2005), insulin (Feldkaemper, Burkhardt et al., 2004; Zhu and Wallman, 2009), gama-aminobutyric acid (GABA) (Stone, Liu et al., 2003), and growth factors, such as transforming growth factor and basic fibroblast growth factor (Rohrer and Stell, 1994; Honda, Fujii et al., 1996). In addition, it was shown that the transcription factor Egr-1 (called ZENK in chicks) may be involved (Fischer, Morgan et al., 1999; Bitzer and Schaeffel, 2002; Schippert, Burkhardt et al., 2007). However, the mechanism and the signaling pathways are not yet known or at least not known in detail. Because some of these modulators were found to be up-regulated under conditions that inhibit eye growth, they were considered as STOP signals, like glucagon and ZENK, in the chicken model (Bitzer and Schaeffel, 2002; Feldkaemper and Schaeffel, 2002). These STOP signals for eye growth are interesting candidates for myopia treatment. Unfortunately, glucagonergic cells were only detected in the chicken retina. Although several members of the glucagon super family are expressed in the mouse retina, glucagon itself was not detected (Mathis and Schaeffel, 2007).

3.1. Glucagon *versus* Insulin and IGF-1

Glucagon and insulin have opposite effects on metabolic functions in the body, and also on cell proliferation of progenitor cells in the periphery of the retina (Fischer, Omar et al., 2005), and on axial eye growth (Feldkaemper, Neacsu et al., 2009; Zhu and Wallman, 2009). While intravitreal glucagon injections inhibit negative lens induced growth towards myopia in chicks, by slowing axial eye growth rates, insulin further increases eye growth when negative lenses are worn. In addition, insulin not only blocks positive lens induced hyperopia development, but rather induces high amounts of axial

myopia (Feldkaemper, Neacsu et al., 2009). Additionally, both insulin und IGF-1 increase the rate of ocular elongation also in eyes not wearing any lenses (Zhu and Wallman, 2009). Glucagon agonist injections prevent experimentally induced myopia development in a dose-dependent manner (Feldkaemper and Schaeffel, 2002; Vessey, Lencses et al., 2005), largely by increasing choroidal thickness (Zhu and Wallman, 2009). On the contrary, insulin injections cause choroidal thinning in chicks wearing positive lenses, but have no effect on choroidal thickness in animals that have normal visual experiences. When both glucagon and insulin are injected as a cocktail, the growth promoting effect of insulin is reduced while also the effect of glucagon on choroidal thickness is suppressed (Zhu and Wallman, 2009).

4. Insulin and IGF-1 in the Eye

Insulin and Insulin-like growth factor-1 (IGF-1) belong to a family of polypeptide hormones common in all multicellular organisms, whose main tasks is to control essential functions, such as cell growth, metabolism, reproduction and longevity. Any dysfunction of these hormones in humans is associated with several pathological disorders including diabetes and dwarfism (Kimura, Tissenbaum et al., 1997; Efstratiadis, 1998; Tissenbaum and Ruvkun, 1998; Bitzer, Accili, Kido et al., 2001; Brogiolo, Stocker et al., 2001; Saltiel and Kahn, 2001; Allan, Zannoni et al., 2003; Nef, Verma-Kurvari et al., 2003).

So far, only a few studies targeted the function of IGF-1 and insulin in the eye, besides their role in embryogenesis. The human interphotoreceptor matrix displays IGF-1 immunoreactivity and cultured human retinal pigment epithelial (RPE) cells synthesize and release IGF-1, raising the possibility that the RPE may serve as a source of IGF-1 *in vivo* (Waldbillig, Pfeffer et al., 1991). Moreover, cultured embryonic retinal chicken explants contain, synthesize and release IGF-1 which can stimulate DNA synthesis of retinal explants (Calvaruso, Vento et al., 1996). Insulin-like immunoreactivity was demonstrated in glial cell culture, but it remains unclear whether this immunoreactivity was due to binding of circulating pancreatic insulin to insulin receptors and/or uptake and storage in these cells; or if insulin is indeed locally synthesized. In situ hybridization

studies showed that Müller cells contain mRNA necessary for de novo synthesis of insulin or a closely homologous peptide (Das, Pansky et al., 1987). Because Müller cells contain glycolytic enzymes and can synthesize and store glycogen (Kuwabara and Cogan, 1961), it has been suggested that insulin produced in the retina may play a role in glucose or amino acid metabolism. The evidence that retinal cells are capable of synthesizing preproinsulin mRNA also raises the possibility that insulin may have a local physiolgocial role (Budd, Pansky et al., 1993). Moreover, it was speculated that insulin acts as a growth hormone during development to control retinal differentiation and that insulin may act as a modulator of neurotransmission within the retina (Budd, Pansky et al., 1993). The presence of insulin in the developing retina before pancreatic insulin synthesis is initiated (Meimaridis, Morse et al., 1990) suggests an important role of the hormone in the retina. From the rat brain, it is already known that insulin can modulate neurotransmission by increasing the efficiency of neuroactive amino acid re-uptake (Rhoads, DiRocco et al., 1984). In addition, insulin has been shown to affect brain monoamine metabolism (Shimizu and Bray, 1990) and dopamine release (Amoroso, Taglialatela et al., 1990).

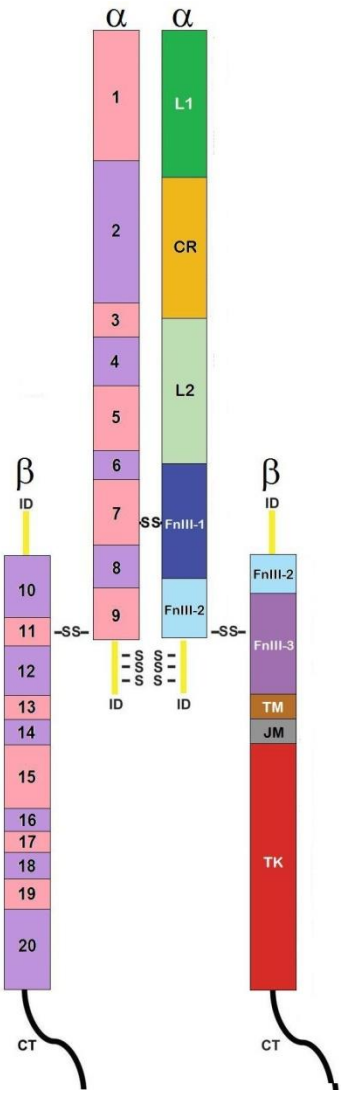
5. Insulin Receptor

5.1. The human IR gene

The human insulin receptor (IR) is alternatively spliced and produces 2 isoforms, *IR-A* and *IR-B*. Subtle differences between both IR isoforms in terms of receptor activation and signaling were demonstrated, suggesting different functions (Mosthaf, Grako et al. 1990; Yamaguchi, Flier et al., 1991; Yamaguchi, Flier et al., 1993). This conclusion was confirmed by a recent study (Frasca, Pandini et al., 2009) showing that the IR-A is a high-affinity receptor for IGF-II whiles the IR-B is not. IR-A is predominantly expressed during prenatal life and enhances the effects of IGF-II during embryogenesis and fetal development, whereas IR-B is predominantly expressed in adult tissues, where it enhances the metabolic effects of insulin (Belfiore, Frasca et al., 2009). The *Ir* and IGF-1 receptor (*Igf1r*) genes have evolved from a common ancestral gene and are highly conserved in vertebrates and invertebrates (Brogiolo, Stocker et al., 2001; Drakas, Tu et

al., 2004). The degree of homology varies between 45-65% in the ligand binding domain and 60-85% in the tyrosine kinase and substrate recruitment domains (Ullrich, Gray et al., 1986; Whittaker, Groth et al., 2001). The most conservative sites are located in the tyrosine kinase domain demonstrating that this part is responsible for maintenance of the general IR signal transduction functions (Hubbard and Till, 2000). Both receptors are oligomers composed of two types of subunits: alpha-subunits and beta-subunits which are encoded by a single gene.

5.2. The chicken IR gene



Several studies described the existence of the insulin receptor in chicken tissues (Simon and Leroith, 1986; Scavo, Shuldiner et al., 1991; Taouis, Taylor et al., 1996), and reported on ligand-stimulated phosphorylation (Taouis, Taylor et al., 1996; Dupont, Derouet et al., 1998). Nevertheless, the structure of the chicken IR has not been completely characterized, and its signaling capabilities have also not been entirely studied. The human insulin receptor gene (*Ir*) is encoded by 22 exons spanning 120 kilobases (kb) and is located on chromosome 19. The chicken *Ir* is located on the chromosome 28 and contains 20 exons (Figure A.3). Despite some differences between the human and the chicken *Ir* gene, the intracellular domain of the chicken is highly similar to that of the human IR (Ebina, Ellis et al., 1985; Ullrich, Bell et al., 1985).

Figure A.3. Model of the alpha2/beta2 quaternary structure of the chicken IR. The left half of the diagram illustrates the boundaries of the 20 exons and on the right the corresponding protein domains are shown. L1 and L2 - large domains 1 and 2 (leucine-rich repeats); CR – furin-like cystein rich domain; FnIII-1, FnIII-2 and

FnIII-3 – fibronectin type 3 domains; TM – transmembrane domain; JM – juxtamembrane domain; TK – tyrosine kinase domain; CT – carboxy-terminal tail. Disulfide bounds are shown. Adapted from Hernandez-Sanchez, 2008 and De Meyts, 2002.

5.3. IR protein structure and function

The insulin receptor family includes the IR, the IGF-1R and the insulin receptor related receptor (IRR) (Lawrence, McKern et al., 2007). Analysis of the protein structure revealed that these receptors belong to a family of cell surface glycoproteins that share a cytoplasmic tyrosine-kinase function (Yarden and Ullrich, 1988; Garcia-de Lacoba, Alarcon et al., 1999). Both IR and IGF-1R proteins are heterodimers including two extracellular alpha-subunits, which contain the ligand-binding domain, and two transmembrane beta-subunits which enclose the tyrosine kinase domain. These subunits are connected together by disulfide bounds (De Meyts and Whittaker, 2002; Lawrence, McKern et al., 2007). From the N-terminus to the C-terminus, each receptor ectodomain monomer include a leucine-rich repeat domain (L1), a cysteine-rich region (CR), a second leucine-rich repeat domain (L2), and three fibronectin type III domains (FnIII-1, FnIII-2 and FnIII-3). The FnIII-2 contains the large insert domain (ID), which in turn holds the furine cleavage site that generates the alpha- and beta-chains of the mature receptor monomer. The tyrosine kinase (TK) domain is flanked by two regulatory regions: the juxtamembrane region (JM), which is involved in docking IR substrates (IRS) and Shc, as well as in receptor internalization; and the C-tail that has phosphotyrosine binding sites for signaling molecules (Hubbard, 2004). Insulin and IGF-1 receptors are highly homologous. Only the carboxy-terminal receptor domain shows a higher degree of heterogeneity and may be responsible for receptor-specific, ligand-induced, intracellular signal generation (Ullrich, Gray et al., 1986).

The three-dimensional structure of the IR ectodomains dimmers has allowed a better understanding of the localization of the insulin binding sites 1 and 2, and may explain the contribution of each domain to insulin binding and the complexity of the binding characteristics of the IR isoforms and the formation of IR/IGF-1R hybrids (Belfiore, Frasca et al., 2009). Located at the alpha-chain of the IR, there are two different ligand-binding regions according to affinity levels. These regions are named site 1 (low-affinity

site) and site 2 (high-affinity site) (Taniguchi, Emanuelli et al., 2006; Lawrence, McKern et al., 2007; Pierre, 2008; Zaid, Antonescu et al., 2008). Alanine scanning and photo-affinity labeling assays demonstrate that insulin first binds to the low-affinity site (site 1) composed of the L1 domain and the alpha-subunit C-terminal peptide sequence, and then to the high-affinity site (site 2) within the C-terminal part of the L2 and the first FnIII domain of the other IR alpha-subunit (McKern, Lawrence et al., 2006; Pierre, 2008). When the receptor is in an insulin-free state, the IR maintains an inhibitory conformation with a minimum separation between the two intracellular tyrosine kinases at the beta-subunits. This distance prevents the tyrosine kinase activation loop of one tyrosine kinase from reaching the catalytic transphosphorylation site of the other tyrosine kinase at the other subunit. The binding of one molecule of insulin to the receptor brings together the two ectodomains reducing the distance between the tyrosine kinases in both subunits; consequently, the transphosphorylation of the tyrosine kinase activation loops occurs at the catalytic loci of the opposing tyrosine kinase domains (McKern, Lawrence et al., 2006; Belfiore, Frasca et al., 2009). According to Scavo, et al. (1991) the amino acid sequence of the tyrosine kinase domain between the human and chicken IR is 93% identical. Moreover, the similarities between the juxtamembrane and C-terminal domains between both species are 79% and 69%, respectively. *In vitro* assays demonstrated that the activity of the intracellular domain of the chicken IR is essentially the same as that of the human IR concerning both ligand-induced autophosphorylation and the phosphorylation of downstream substrates (Kato, Okubo et al., 2000).

5.4. IR and IGFR in neuronal and non-neuronal tissues

The expression level of the IR is regulated during development and it is likely that changing receptor content while keeping the level of insulin constant may be a regulatory mechanism (Holdengreber, Ren et al., 1998). The presence of IR/IGFR hybrids was demonstrated in proliferative neuroretina. These receptors were discussed to be physiologically relevant for the action of the locally produced proinsulin found in early neurogenesis (Garcia-de Lacoba, Alarcon et al., 1999). Two types of IGF-I

receptor were identified based on their binding specificity, subunit structure, kinase activity and interaction with antibodies to IR the murine and human central nervous system (CNS) (Gammeltoft, Haselbacher et al., 1985). In the CNS, like in all other tissues, insulin receptors are also composed of two types of subunit, but the size of the alpha-subunit is significantly smaller, whereas the beta-subunit is similar to that of other cell types (Heidenreich, Zahniser et al., 1983; Rees-Jones, Hendricks et al., 1984; Gammeltoft, Fehlmann et al., 1985). The differences in the composition of IR in neuronal and non-neuronal cells suggest a unique function for IR in neural networks (Lowe, Boyd et al., 1986).

5.5. IR signaling transduction pathways

While insulin is known to be a key regulator of physiological processes such as glucose transport and glycogen and fat biosynthesis (Kahn, 1985), IGF-1 is believed to mediate the effects of growth hormone and also to play a role as a paracrine growth factor (Nilsson, Isgaard et al., 1986). The stimulatory effects of insulin and IGF-1 are regulated through a wide range of complex intracellular responses, beginning with ligand binding to the corresponding receptors leading to a conformational change that induces the dimerization of the receptor, bringing the two beta-subunits into close opposition and increasing tyrosine kinase activity on the intracellular portion of the receptor. Much of the tyrosine kinase activity is due to autophosphorylation that occurs within the two subunits of the receptor, where one acts as a kinase and the other as substrate. This receptor activation induces a cascade of phosphorylation of a number of membrane and cytosolic proteins substrates, and their subsequent phosphorylation (de la Monte and Wands, 2005; Belfiore, Frasca et al., 2009). Among these intracellular substrates activated by the IR tyrosine kinase are the IR substrates (IRS-1, -2, 3 and -4), IRS-5/DOK4, IRS6/DOK5, Shc, Gab1, Cbl, associate protein substrate (APS) and the signal regulatory family members (Cohen, 2006; Taniguchi, Emanuelli et al., 2006; Taguchi and White, 2008). These phosphorylated proteins act as docking sites for effector containing Src homology 2 (SH2) domains that specifically recognize different phosphotyrosine residues, such as regulatory subunit p85 of type 1A

Phosphatidylinositol 3-kinase (PI3K); the protein tyrosine phosphatase SHP2; the Src family of non-receptor-type tyrosine kinases, including Fyn and Csk; the adaptor proteins Grb2; and the GTPase activating protein of Ras (Taguchi and White, 2008; Belfiore, Frasca et al., 2009). In this complex biochemical cascade of signals, depending on the phosphorylated downstream molecules, two different pathways can then be activated, mediating either prevalent metabolic or mitogenic effects originated by the activation of the PI3K or Ras, respectively (Belfiore, Frasca et al., 2009).

5.5.1. The Phosphatidylinositol 3-kinase (PI3K)/Akt pathway

Activation of the PI3K pathway results in the control of cell growth and survival, and any disturbance contributes to a competitive growth advantage and metastatic capability (Hennessy, Smith et al., 2005; Figure A.4). The PI3K family comprises a large family of lipid and serine/threonine kinases, which includes several classes of phosphatidylinositol kinases (Fruman, Meyers et al., 1998). Class 1A PI3Ks are composed of tow subunits: an inhibitory adaptor/regulatory (p85) and a catalytic (p110). The p85 subunit binds and integrates signals from several cellular proteins, such as transmembrane tyrosine-kinase-linked receptors and intracellular proteins, including protein kinase C (PKC), SHP1, Rac, Rho, hormonal receptors, mutated Ras and Src, providing an integration point for the activation of the catalytic subunit p110 and downstream molecules. Moreover, the p85 contains a SH2 domain which has two major divergent functional activities: activation of small G-proteins and relief of *trans*-inhibition of p110 (Hennessy, Smith et al., 2005). Following activation, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to produce the second messenger PIP3 which binds to a subset of plecktrin homology (PH) domains recruiting other lipid-binding domains, proteins, substrates, adaptors and cytoskeletal molecules. Among these downstream targets, the phosphotinositide-dependent kinase 1 phosphorylates and activates subsequent downstream enzymes, including the serine/threonine kinase Akt (protein kinase B – PKB) and protein kinase C (PKC) (Mora, Komander et al., 2004; Hennessy, Smith et al., 2005; Belfiore, Frasca et al., 2009). There are three known Akt isoforms and all of them are derived from distinct genes: Akt1/PKBα, Akt2/PKBβ and

Akt3/PKBγ. The recruitment of Akt to the membrane results in a conformational change that exposes two crucial amino acids that are phosphorylated and necessary for activation: one in the kinase domain (threonine 308 in the Akt1) and the other in the hydrophobic C-terminal domain (serine 473 in Akt1) for full activation (Alessi, Andjelkovic et al., 1996; Blume-Jensen and Hunter, 2001). Consequently, this activation regulates metabolic enzymes, for instance glycogen synthase kinase-3 (GSK-3) and 6-phosphofructo-2-kinase, controlling this way the glucose metabolism by a consequent induction of glucose transporter (Glut-4) translocation from the intracellular storage compartments to the plasma membrane (Taguchi and White, 2008). Bcl-2 family member BAD, an important propapoptotic protein (Datta, Dudek et al., 1997) is also phosphorylated by the activated Akt, as a result Bcl-2 is no longer able to exert its proapoptotic function (Belfiore, Frasca et al., 2009). Furthermore, activated Akt phosphorylates forkhead box “Other” (FoxO) proteins (FoxO1, FoxO3 and FoxO4), which are signaling molecules capable of regulating several cell functions (Barthel, Schmoll et al., 2005), including metabolism, apoptosis promotion (via the proapoptotic member of Bcl-2) and inhibition of cell growth (Myatt and Lam, 2007; Zou, Tsai et al., 2008).

The PI3K pathway at many points bifurcates resulting in diverse functional outcomes that probably represent the effect of the variety, level and duration of particular machinery of the PI3K pathway activation, suggesting that different branches in the pathway may play a central role regarding particular results (Hennessy, Smith et al., 2005). Another pathway regulated by the PI3K-Akt activation is the regulatory-associated protein of raptor-mammalian target of rapamycin (raptor-mTOR) pathway which regulates cell growth and metabolism (Taniguchi, Emanuelli et al., 2006) and combines signals coming from insulin, as well as other growth factors. The activation of the mTOR is not direct. The activated Akt phosphorylates and inactivates the tuberous sclerosis complex 2 (TSC2), that on the other hand forms a complex with the TSC1, and this one is then going to increase a Ras-family GTP-binding protein that binds and activates the raptor-mTOR complex (Belfiore, Frasca et al., 2009). Signaling downstream of the raptor-mTOR complex link IR to the control of mRNA translation regulating factors involved in protein synthesis (Wang and Proud, 2006). A proto-

oncogene c-Abl encodes a protein kinase that is localized in the cytoplasm and the nucleus, and is an important component of the IR signaling. Insulin stimulates the c-Abl tyrosine and consequently dephosphorylates the focal adhesion kinase (FAK), mediating this way the metabolic effects of insulin (Belfiore, Frasca et al., 2009). Therefore, when c-Abl tyrosine kinase is inhibited FAK is phosphorylated in response to insulin, which promotes cell proliferation and migration, instead of cell metabolism (Frasca, Pandini et al., 2007).

5.5.2. The Mitogen-activated protein kinase (MAPK)/ERK pathway

Depending on the requirement of the cell, binding of insulin to the receptor can also activate a family of protein kinase cascades known as mitogen-activated protein (MAP) kinase modules. This cascade contains at least three protein kinases in series that culminate in the activation of a multifunctional MAP kinase (MAPK). The activation of this pathway controls embryogenesis, cell differentiation, and cell death (Puro and Agardh, 1984; Mill, Chao et al., 1985; Heidenreich and Toledo, 1989; Figure A.4). The pathway starts with the activation of a previous complex before it reaches the MAP kinases. Phosphorylated IRS proteins, in response to insulin, attract Grb2. In turn, Grb2 activates Ras guanidine nucleotide exchange factor m-son of seveless (SOS), which consecutively activates $p21^{Ras}$, a GTP-binding protein with GTPase activity toward the active (GTP-bound) or inactive (GDP-bound) $p21^{Ras}$ form (Ceresa and Pessin, 1998). Phosphorylated insulin receptors can also activate the protein $p21^{Ras}$ through Shc proteins. In particular, Tyr-972 of IR binds to the N-terminal phosphotyrosine binding domain of Shc. Consequently, both isoforms of Shc are phosphorylated at Tyr-317, which recruits the Grb2/SOS/$p21^{Ras}$ complex (Belfiore, Frasca et al., 2009). Upon $p21^{Ras}$ activation, there is a subsequent recruitment and activation of the serine/threonine kinase Raf, which, sequentially, phosphorylates the dual specificity kinase MAPK kinase (MAPKK or MEK1), which then phosphorylates ERK1/2, a kinase of the MAP family. When inactive, the ERK1/2 is mainly located in the cytoplasm, where it binds to the MEK1 forming a heterodimer MEK/ERK (Cohen, 2006; Taguchi and White, 2008). When phosphorylated, ERK1/2 dissociates from MEK1 and translocates

to the nucleus, where it phosphorylates a number of substrates involved in the activation of a complex transcription program, such as SRC-1, Pax-6, NF-AT, Elk-1, MEF2, c-Fos, jun, c-Myc and STAT3 (Ceresa and Pessin, 1998; Roux and Blenis, 2004). Furthermore, activated ERK1/2 phosphorylates several substrates in the cytoplasmatic compartment. Given that ERK1/2 transports between the cytoplasm and the nucleus and has numerous targets in both compartments its spatial distribution is fundamental for determining the biological responses (Belfiore, Frasca et al., 2009).

The small GTPases of the Rho family, Rac and CDC42, have also been implicated in insulin action. Both are activated by the exchange of GDP to GTP, which activates a conformational change that induces the interaction of GTPases with downstream molecules, many of which are involved in the rearrangement of the actin cytoskeleton. This quality is thought to link Rac to GLUT4 translocation to the skeletal muscle (Marcusohn, Isakoff et al., 1995), whereas CDC42 might have this role in adipocytes (Usui, Imamura et al., 2003).

Other MAPKs, such as Jun N-terminal kinase and p38 kinase, are also activated by insulin and contribute to the complexity of its biological responses (Roux and Blenis, 2004). IRS proteins also go through serine phosphorylation in response to insulin and other stimuli (Zick, 2005). The IRS1 that, in general, seems to negatively regulate IR signaling, contains around 70 potential serine-phosphorylation sites. Both p70S6K and Jun N-terminal kinase participate in a negative feedback loop involved in the termination of insulin signaling by phosphorylation of serine residues in IRS proteins (Cohen, 2006; Taguchi and White, 2008). p38 MAPK is also an intracellular target of insulin and its activation is necessary for full GLUT4 translocation (Furtado, Somwar et al., 2002). Nevertheless, p38 MAPK activation has also been reported to have opposing roles GLUT4 expression in various tissues (Carlson, Koterski et al., 2003). Whether insulin differentially affects the activation of a variety of isoforms of p38 MAPK, or if these variants specifically modulate distinct cellular processes remains unknown.

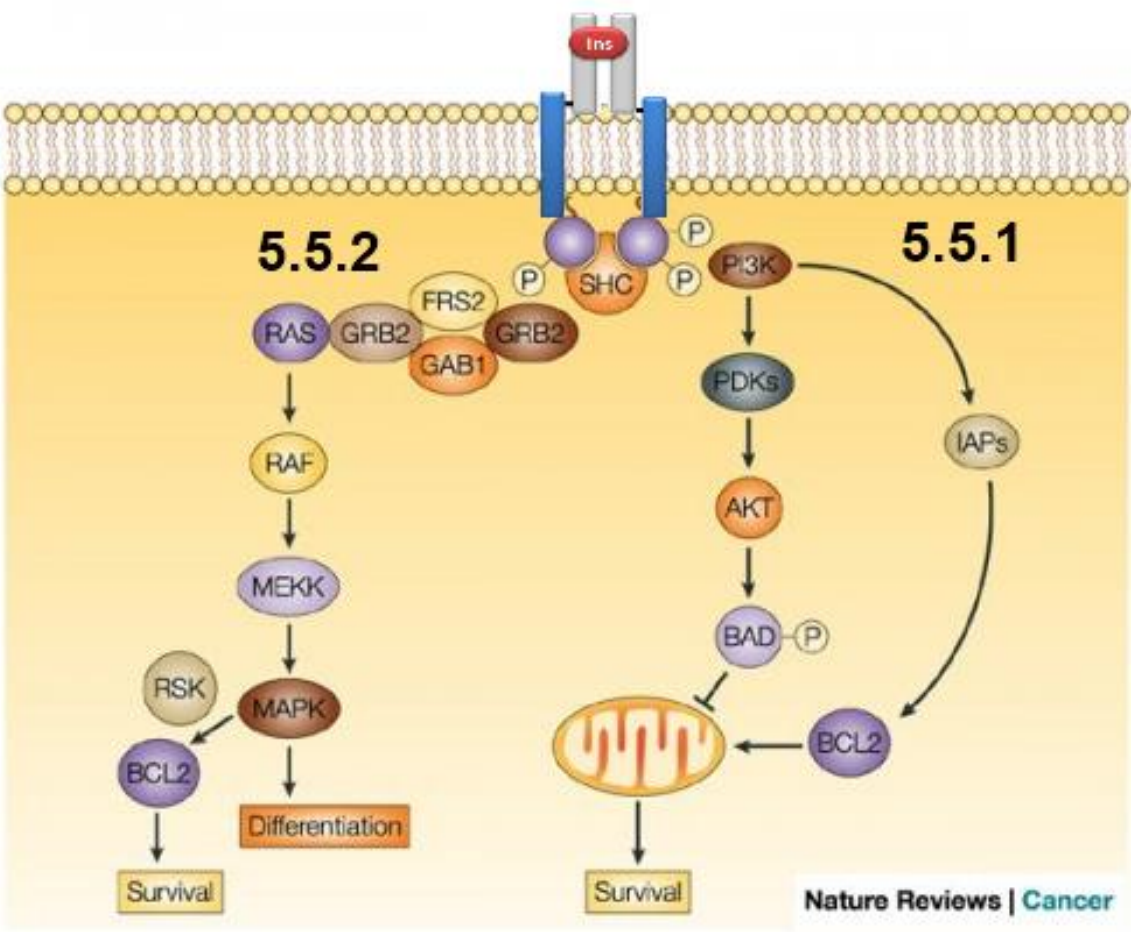


Figure A.4. Diagram of the PI3K-Akt signaling cascade (see text section 5.5.1) and MAPK pathway (see text section 5.5.2) when insulin and/or IGF-1 receptors are activated. For description of pathway and abbreviations see text above. Figure adapted from http://www.humpath.com/ - Nature Reviews cancer.

B. Purpose of the study

PURPOSE OF THE STUDY

The prevalence of myopia in the human population has dramatically increased in developed regions of Asia (Lin, 1999) but also in Western societies during the last decades (Vitale, 2009) and it is estimated that more than 30% of the worldwide population is currently myopic (Dirani, 2006). Although myopia is not a life-threatening disease, high myopia (more than 6 diopters) increases the risk of secondary diseases, such as myopic choroidal neovascularization, retinal degeneration, retinal detachment and glaucoma and thus the risk of becoming blind. It is therefore important to study the mechanisms underlying eye growth regulation.

The interest in studying the role of insulin in myopia development and to investigate the underlying molecular pathways is mainly based on three different key findings. First, it was previously found that glucagon, a peptide hormone with opposite metabolic effects to those of insulin, acts as a “STOP” signal for myopia development in the chicken model of myopia (Bitzer, 2002). Two parallel studies proved that, in fact, glucagon and insulin have opposite effects on axial eye growth as well (Feldkaemper, 2009; Zhu, 2009), with insulin acting as a “GO” signal. In 2002, Cordain and collaborators stated that “during the last years diet has changed. Diets high in refined starches such as bread and cereals increase insulin levels in humans". It was further hypothesized that “high levels of insulin lead to a fall of insulin-binding protein 3 which could disturb the coordination of the eye ball lengthening and lens growth…” Second, Reitner and collaborators (2003) stated that “in the rat retina insulin receptor kinase activity does not fluctuate with the feeding/fasting cycle which is different from the systemic insulin suggesting a tissue-specific signaling pathway in the retina tissue”. Third, Heidenreich et al., (1983) observed fundamental differences especially in the subunits structure between insulin receptor molecules in brain and peripheral target tissues supporting the idea that insulin might have additional functions in neuronal tissues.

Based on these findings, the purpose of **Project 1** of this thesis was to investigate which transcript variants of the insulin receptor exist in the fundal layers of the eye (retina, RPE, choroid and sclera) compared to those found in liver and brain, and to

elucidate whether these variants are involved in eye growth regulation. Another purpose of Project 1 was to quantify changes in mRNA levels for insulin, IGF-1, insulin receptor (IR) and IGF-1 receptor (IGF-1R), all present in all fundal layers in the eye, after treatment with negative spectacle lenses which induce excessive eye growth and positive spectacle lenses, which stopped the eye from growing. Different treatment durations were chosen to investigate the time course of the transcriptional changes.

Project 2 was based on the results of Project 1, in which it was shown that the regulation of IR and IGF-1R mRNA expression levels is specific to the type and duration of the imposed defocus. Since the binding of insulin to IR or IGF-1R can activate two different pathways, the downstream signaling cascades were analyzed in more detail. The objective of Project 2 was to study the effect of intravitreal insulin injections on the activation of the PI3K/Akt or MEK/Erk signaling pathways in the retina during imposed myopic or hyperopic defocus. In addition the effects of two pathway- specific inhibitors on refractive development were investigated.

The purpose of **Project 3** was to compare biometric measurements in the eye with A-scan ultrasonography (the “3M Echo Rule”) and a new commercially available low coherence interferometer (the “Lenstar LS-900” by Haag Streit, Switzerland). This project was relevant because the chicken model is frequently used in myopia research and no gold-standard technique is available for biometry in the chicken.

C. Results and Discussion

PROJECT 1:

Insulin, IGF-1, Insulin- and IGF-1 receptor expression in the chick eye and their regulation with imposed myopic or hyperopic defocus

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Purpose: Insulin stimulates eye growth in chicks and this effect is greatly enhanced if the retinal image is degraded by defocus of either sign. However, it is unclear whether the insulin receptor (IR) is expressed at all in the chicken retina in animals 1-2 weeks post-hatching. We have investigated IR expression and whether IR transcripts abundance varies in the fundal layers. To elucidate the possible role of insulin and IGF-1 signalling in eye growth regulation, mRNA levels were measured for insulin, IGF-1, IR and IGF-1R receptor (IGF-1R) during imposed negative or positive defocus.

Methods: Chicks were treated binocularly with positive or negative spectacle lenses for 4 or 24 hours, or they remained untreated (n=6, for each treatment group). Northern blot analyses were performed to screen for transcription variants in the different fundal layers of untreated animals. Real-time PCR was used to quantify insulin receptor, IGF-1 receptor, IGF-1 and insulin mRNA levels in the different fundal layers of the chick eye in the three treatment groups.

Results: Insulin receptor mRNA was found in all the studied tissues, although there is evidence for tissue-specific transcript variations. Three major transcripts were detected for the IR. Brain, retina and choroid showed the longest transcript (4.3 kb), which was not present in the liver. Nevertheless, the liver and brain showed a second transcript (2.6 kb) not present in the retina and choroid. A short transcript (1.3 kb) was the predominant form in liver and choroid, and it seems to be present also in the RPE and sclera. In the retina, no significant gene expression changes were found when defocus was imposed. Interestingly, in the RPE, both IR and IGF-1R were down-regulated already after short periods (4 hours) of positive lens wear. In contrast, IR and IGF-1R were up-regulated in the choroid and fibrous sclera during treatment with negative, but not positive lenses.

Conclusions: Differences observed in the IR transcript length in different tissues suggest possibly different functions. The differential regulation of IR and IGF-1R in RPE, choroid and fibrous sclera is consistent with their involvement in a signalling cascade for emmetropization.

INTRODUCTION

The prevalence of myopia in the human population has dramatically increased in developed regions of Asia[1], but also in Western societies[2] during the last decades. It is estimated that approximately 30% of the worldwide population are currently myopic[3]. Genetic as well as environmental factors have been implicated in the development of myopia but the relative importance of genes versus environment remains controversial[4]. Myopia can be artificially induced in animal models like chick[5], tree shrew[6], monkey[7],[8] and guinea pigs[9], by placing negative lenses, which induce hyperopic defocus[10], in front of the animal's eye. The shift of the focal plane behind the photoreceptor layer triggers substantially increased eye growth. Furthermore, the choroid thins. On the contrary, positive lenses, imposing myopic defocus, slow the rate of ocular elongation and the choroid thickens by up to a factor of 3 in the chick[11].

Among the retinal transmitters and modulators implicated in eye growth regulation are vasoactive intestinal polypeptide (VIP)[12, 13], dopamine[14-16], retinoic acid[17-19], glucagon[20-22], insulin[23, 24], □-aminobutyric acid (GABA)[25], and growth factors, such as transforming growth factor and basic fibroblast growth factor[26, 27]. In addition, it was shown that the transcription factor Egr-1 (called ZENK in chicks) may be involved[28-30].

It was previously found that glucagon and insulin have opposite effects on cell proliferation in the retina[31], and on axial eye growth[24, 32]. While intravitreal glucagon injections inhibit growth towards myopia in chicks, by slowing axial eye growth rates, insulin not only blocks hyperopia development which is normally induced by positive lenses, but rather induces high amounts of axial myopia that is further increased when negative lenses are worn [32]. In addition, both insulin und IGF-1 increase the rate of ocular elongation also in eyes not wearing any lenses[24]. Glucagon agonist injections prevent deprivation myopia in a dose-dependent manner[20, 33], largely by increasing choroidal thickness[24]. On the contrary, insulin injections cause choroidal thinning in chicks wearing positive lenses, but have no effect on choroidal thickness in animals that have normal vision[32]. When both glucagon and insulin are injected as a cocktail, the growth promoting effect of insulin is blocked while also the effects of glucagon on choroidal thickness are suppressed[32]. Interestingly, a very recent study [34] demonstrated

a genetic association between IGF-1 and high-grade myopia in an international family cohort. These findings are in line with experimental data from the chicken model of myopia, showing that IGF-1 can promote ocular growth and axial myopia.

So far, only a few studies targeted IGF-1 and insulin in the eye, besides their roles in embryogenesis. The human interphotoreceptor matrix displays IGF-1 immunoreactivity and cultured human RPE cells synthesize and release IGF-1, raising the possibility that the RPE may serve as a source of IGF-1 in vivo[35]. Moreover, cultured embryonic retinal chicken explants contain, synthesize and release appreciable amounts of IGF-1 which can stimulate DNA synthesis of retinal explants[36]. Insulin-like immunoreactivity was demonstrated in glial cell culture, but it remains unclear whether this immunoreactivity was due to binding of circulating pancreatic insulin to insulin receptors and/or uptake and storage in these cells; or if insulin is indeed locally synthesized. In situ hybridization studies showed that Müller cells contain mRNA necessary for de novo synthesis of insulin or a closely homologous peptide[37]. Because Müller cells contain glycolytic enzymes and can synthesize and store glycogen[38], it has been suggested that insulin produced in the retina may play a role in glucose or amino acid metabolism. There is evidence that retinal cells are capable of synthesizing preproinsulin mRNA, raising the possibility that insulin is involved in intracellular (autocrine) and intercellular (paracrine) signalling[39]. Moreover, it was speculated that insulin acts like a growth hormone during development to control retinal differentiation. Later, it may act as a modulator of neurotransmission within the retina[39]. The presence of insulin in the developing retina before pancreatic insulin synthesis was initiated[40] suggests an important role of insulin in the retina, perhaps as a growth or trophic factor. From the rat brain, it is already known that insulin can modulate neurotransmission by increasing the efficiency of neuroactive amino acid re-uptake[41]. In addition, insulin has been shown to affect brain monoamine metabolism[42] and dopamine release[43].

The polypeptide hormones, insulin and insulin-like growth factor 1 (IGF-1), exert their biological effects by binding to distinct transmembrane receptors on the surface of the target cells. Although the receptors for insulin and IGF-1 are, like their ligands, highly homologous[44, 45], they are known to have different, but partially overlapping, physiological functions[46]. While insulin is known to be a key regulator of physiological

processes such as glucose transport and glycogen and fat biosynthesis[47], IGF-1 is believed to mediate the effects of growth hormone and also to play a role as a paracrine growth factor[48]. The levels of the insulin receptor are regulated during development and it is likely that changing receptor level while keeping the level of insulin constant may be a regulatory mechanism[49]. Analysis of the protein structure has revealed that receptors for IGF-1 and insulin belong to a family of cell surface glycoproteins which share a cytoplasmatic tyrosine kinase function [50, 51]. Both are oligomers composed of two types of subunits: alpha-subunits containing the hormone-binding site, and beta-subunit which are phosphorylated after binding of the ligand. The alpha and beta subunits are encoded by a single gene. Ligand interaction with the extracellular portions of these receptors activates intracellular tyrosine kinase activity, and generates a biological signal that is thought to be specified by structural determinants in the cytoplasmatic domain. The presence of IR/IGFR hybrids was demonstrated in proliferative neuroretina. These receptors were discussed to be physiologically relevant for the action of the locally produced proinsulin found in early neurogenesis [52]. Two types of IGF receptor on nerve cell membranes from the murine and human central nervous system (CNS) were identified based on their binding specificity, subunit structure, kinase activity and interaction with antibodies to insulin receptor [53]. In the CNS, insulin receptors are also composed of two types of subunit, but the size of the α-subunit is significantly smaller, whereas the β-subunit is similar to that of other cell types [54-56]. The differences in the composition of IR in neuronal and non-neuronal cells suggest a unique function for insulin receptor in neural networks[57].

Because of these fundamental differences between insulin receptor molecules in brain and peripheral target tissues[54], the first objectives of this study was to investigate which transcript variants of the insulin receptor exist in the fundal layers of the eye, compared to liver and brain to learn more about which variants might be involved in eye growth regulation. The second objective was to study changes in mRNA levels for insulin, IGF-1, insulin receptor and IGF-1 receptor, after defocus was imposed in the retinal image for 4 or 24 hours, a condition that is known to induce axial refractive errors.

METHODS

Treatment of animals

Ten days old male White Leghorn chickens were raised under a 12-hour light-dark cycle, and treated binocularly either with plus (+7D) or minus (-7D) lenses for 4 (n=6) and 24 hours (n=9), respectively. In addition, a separate control group was used for each treatment duration. To attach the lenses, Velcro rings were glued onto the feathers around the eyes a few hours before the lens treatment was started. The experimental treatment was in accordance with the ARVO Statement for Care and Use of Animals in Ophthalmic and Vision Research and was approved by the university commission for animal welfare.

Tissue preparation

The chicks were sacrificed by an overdose of diethylether between 1 and 3 p.m. Eyes were enucleated and vertically cut with a razor blade, discarding the anterior part containing the lens. The vitreous body was removed and the pecten was cut out. From the posterior part of the eye a biopsy punch of 8 millimetres was made and placed in a Petri dish that was filled with ice-chilled saline. The different fundal layers were carefully separated under visual control of a dissecting microscope. In addition, forebrain and liver samples were dissected from 3 untreated animals. All the tissues were immediately collected in RNAlater (Qiagen, Hilden, Germany), immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction. In general, the right eyes were taken for further analysis. Only when the separation of the fundal layers was not optimal, the left eye was studied instead.

Total RNA extraction and cDNA synthesis

Different RNA extraction methods were used for Northern blot and Real-time PCR analyses. For Northern blot analysis, total RNA from liver, brain, retina, RPE, choroid and sclera was isolated using TRIzol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instruction. For Real-time PCR analyses, the RNeasy Mini kit (RNeasy

Mini Kit; Qiagen, Hilden, Germany) was used following the manufacturer's instructions. All tissues were homogenised in the respective lysis buffer for 1 minute, at a range of speed that increased in four steps from 11,000 to 20,000 rpm (Diax 900 Homogenizer; Heidolph, Ketheim, Germany). All RNA samples were treated with DNase I (DNA-*free* Ambion, Darmstadt, Germany) and the respective yield was measured by spectrophotometry at 260 and 280 nm. The optical density (OD_{260}/OD_{280}) ratios were calculated to ensure the quality of the isolated RNA and samples with a ratio between 1.8 and 2.0 were used for further analysis. The integrity of this RNA samples was confirmed by agarose-gel electrophoresis. Thereafter, 1 µg of brain, liver, retina, RPE and choroid and 0.5 µg of both sclera layers was reversed transcribed by the M-MLV reverse transcriptase (Promega, Mannheim, Germany) using 0.25 µg oligo$(dT)_{15}$ and 0.025 µg random hexamer primers (Invitrogen, Karlsruhe, Germany) in a final volume of 15 µl.

Semi-quantitative Real-time polymerase chain reaction (RT-qPCR)

Table 1 shows all specific primer sequences used for quantitative real-time PCR, the respective amplicon size and the NCBI accession number. Primer design was performed using the web-based program Oligo Explorer 1.4 (Gene Link, Hawthorne, NY, USA). The specificity of the PCR reactions was verified by melting-curve analysis, agarose-gel electrophoresis and the PCR products were sequenced to verify their identity. The PCR reactions were performed in a thermocycler (iCycler iQTM Multicolor Real time PCR Detection system; Bio-Rad, Hercules, CA) using a fluorescence detection kit (QuantiTect SYBR Green PCR kit, Qiagen, Hilden, Germany). Primer annealing was executed at 59ºC for 30 seconds and elongation at 72ºC for 20 seconds. Every single reaction, with a final volume of 15 µl, contained a primer concentration of 0.6 µM and 2 ng of each template mixed with a master mix of the fluorescence kit. Additionally, a hydrolysis probe was used to verify the specificity of insulin mRNA expression (table C1.1). In order to compare the amplification of different regions from the insulin receptor mRNA sequence, different pairs of primers comprising different exons were design based on the sequence provided by the ENTREZ and ENSEMBL data bases (Table C1.2).

Table C1.1 Sequences of the specific primers used for real time-PCR amplification.

Gene	*Forward primer (5'-3')* *Reverse primer (5'-3')*	*Amplicon size*	*NCBI accession*
Beta-Actin	CTGAACCCCAAAGCCAAC CACCATCACCAGAGTCCATCAC	147 bp	**NM_205518**
HPRT	TGGCGATGATGAACAAGGT GCTACAATGTGGTGTCCTCCC	162 bp	**NM_204848**
Insulin	CTTCTGGCTCTCCTTGTCTTTT CAAGGGACTGCTCACTAGGGGC	172 bp	**NM_205222.2**
Insulin Receptor	CGCTGAGAATAACCCTGGTC GCTGCCATCTGGATCATTTC	60 bp	**XM_001233398.1**
IGF-1	CTTCAGTTCGTATGTGGAGACA GATTTAGGTGGCTTTATTGGAG	167 bp	**NM_001004384.1**
IGF-1 Receptor	TCCAACACAACACTGAAGAATC ACCATATTCCAGCTATTGGAGC	167 bp	**NM_205032.1**
Insulin (hydrolysis probe)	GGCTCTCTACCTGGTGTGTG CTCGCTTGACTTTCTCGTATTCC	149 bp	**NM_205222.2**
Insulin-hydrolysis probe	CACTCCTGCCTCGCCACGC		

Table C1.2 Primer sequences used to compare the amplification of different regions of the insulin receptor mRNA

Gene	*Region on the sequence*	*Forward primer (5'-3')* *Reverse primer (5'-3')*	*Amplicon size*
IR-LD	L2- (binding) domain	GGTCGTATGCCTTGGTTTC AGCTGGCGAAGATTCTGG	118 bp
IR-TK	Tyrosine kinase domain	CGTCCACCACCAACACTG TGCCATCAGCGATCTCTG	58 bp
IR-TK2	Tyrosine kinase domain	GTTCACAGAGACCTGGCA AATAATCCGTCTCGTAGAT	103 bp

Northern blot analysis

Differences in transcript size were analysed by Northern blotting. Biotin-labelled antisense probes were designed using Oligo Explorer 1.4 (Gene Link, Hawthorne, NY, USA) based on the published mRNA chicken sequences in ENTREZ and ENSEMBL data bases. Two specific probes for insulin receptor were used for Northern blot analysis (Table C1.3). Approximately 1 μg of RNA was run in a 0.8% formaldehyde-agarose gel, blotted overnight onto a positively charged nylon membrane (Roche, Mannheim, Germany) and UV cross-linked. Blots were hybridized overnight with 100 ng/ml of biotin labelled insulin receptor probe at 50ºC. At the next day, the membranes were washed twice, for 5 minutes each, with 2×SSC/0.1% sodium dodecyl sulphate (SDS) at 42°C, followed by two additional washes, for 15 minutes each, with 0.5×SSC/0.1% SDS, at the same temperature (1xSSC buffer contains 0.15 M NaCl and 15 mM Na_3-citrate*2 H_2O, pH 7.0). Chemiluminescence detection was performed with the Chemiluminescent Nucleic Acid Detection Mode kit (Thermo Scientific GmbH, Ulm, Germany). Blots were exposed to x-ray films Curix HT1 (AGFA, Leverkusen, Germany) and the time of exposure was adjusted as needed to obtain the desired signal strength. Liver RNA was used as positive controls for insulin receptor expression, and the brain was used as a nervous tissue control. Two to 4 samples per tissues were used for Northern blot analysis with probe 1 (Table C1.4), but only one retina, brain and liver sample for Northern blot analysis with probe 2.

Table C1.3 Sequences of the specific probes used for Northern blot analysis.

Gene	*Probe*	*Region*	*Probe sequence*
Insulin receptor	1	Tyrosine kinase domain	AGCCATCTGGATCATTTCTCTCAGTGTTGGTGGTGGACG
	2	L2-binding domain	TTCCTCCACGGATATTTATAACCAAGCTCCCATTAACAAC TGTGCAGCCA

Table C1.4 Summary of treatment groups.

Title of the experiment	*Lens treatment*	*Duration of treatment*	*Tissues*	*N*
Northern blot analysis of IR expression	Without lenses[1]	No treatment	Liver Brain Retina RPE Choroid Sclera (both layers)	4 4 4 2 4 2
Comparison of amplification of different regions of IR	Without lenses[1]	No treatment	Liver Brain Retina RPE Choroid Fibrous sclera Cartilaginous sclera	4
Insulin, IGF-1, IR and IGF-1R mRNA expression in the ocular fundal layers	Without lenses Plus lenses Minus lenses	4 hours	Retina RPE Choroid	6
	Without lenses[1] Plus lenses Minus lenses	24 hours	Retina RPE[2] Choroid Fibrous and Cart sclera	9

IR - insulin receptor; IGF-1 – insulin-like growth factor-1; IGF-1R – IGF-1 receptor

[1] Same animals

[2] The RPE tissues from both eyes were analyzed separately for all 24 hours treatment groups. We then calculated the mean of both eyes before calculating the grand mean. (See section "Tissue preparation")

Statistics and data analysis

Statistical analysis was done based on the quantification cycle (C*q*) values of the PCR products. To test the primers efficiency, a dilution curve was made using template amounts ranging from 0.5 to 16.0 ng per well. The efficiency (*E*) for each primer was calculated according to the formula: $E=10^{(-1/\text{slope})}$, giving a value between 1 and 2, whereby 1 corresponds to 0% efficiency and 2 to 100%. The slope (*m*) was determined by plotting the mean of C*q* of each of the cDNA dilution samples *versus* the logarithm of the sample concentration. The efficiencies were 2.03 for b-actin, 2.11 for HPRT, 2.02 for

the insulin receptor, 1.97 for IGF-1and 1.98 for the IGF-1 receptor. The mean normalized expression (MNE)[58] was used to compare relative expression levels among different groups and was calculated according to the following formula where *E* is the primer efficiency, reference corresponds to □-actin and the targets are insulin receptor, IGF-1 and IGF-1 receptor:

$$MNE = \frac{(E_{\text{reference}})^{Cq_{\text{reference},mean}}}{(E_{\text{target}})^{Cq_{\text{target},mean}}}$$

Mean normalized expression values were first analysed using an outlier calculator (GraphPad, La Jolla, USA). Then, one-way ANOVAs were applied for statistical comparison between the different treatment groups. A significant ANOVA ($p<0.05$) was followed by each pair Student's t-test as post-hoc analysis. Statistical tests were performed using JMP version 7 software (SAS Institute, Cary, USA).

RESULTS

Northern blot analysis of insulin receptor expression in untreated tissues

Northern blots were used to compare the transcript length of the insulin receptor (Figure C1.1) in neuronal and non-neuronal tissues. The emphasis in the Northern blot analyses was placed on the investigation of transcript variants among different tissues and not on the quantification of the insulin receptor mRNA levels in those tissues. Therefore, a loading control was not used. Figure C1.1 shows a Northern blot result for insulin receptor expression in liver, brain, retina, choroid and RPE.

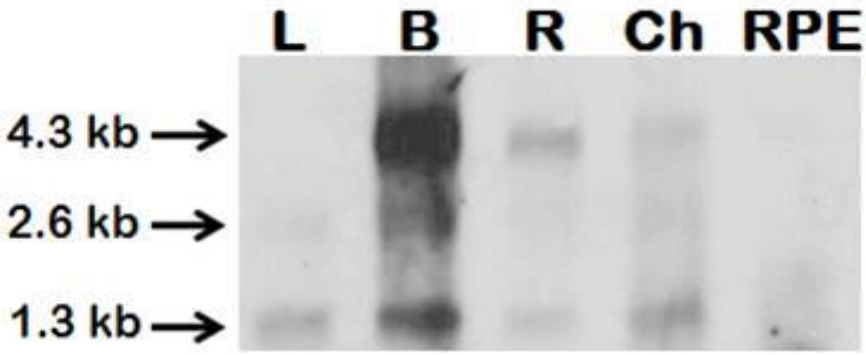

Figure C1.1 Northern blot showing the expression pattern of the insulin receptor mRNA (probe 1) in the liver (L), brain (B) and different fundal ocular layers: retina (R), choroid (Ch) and RPE. Three major transcripts with 4.3, 2.6 and 1.3 kb were found, although the pattern was different among the studied tissues.

With probe 1, the brain and the retina showed two transcripts, approximately 4.3 kb and 1.3 kb long, with the longer transcript being more abundant. The brain in addition showed a third band of about 2.6 kb. This transcript was also found in the liver together with the more abundant 1.3 kb transcript and a small transcript of 0.4 kb, which might be a degradation product (not shown). The choroid, like brain and retina, also expressed two transcripts of 4.3 and 1.3 kb, but in this tissue, the shorter transcript was more prominent than the longer one. The RPE contained only very small amounts of insulin receptor mRNA, and mainly the shorter variant. In addition, we analysed two scleral samples (combined fibrous and cartilaginous layer). They expressed the 1.3 kb transcript and two smaller transcripts of approximately 0.8 kb and 0.4 kb (results not

shown). With probe 2, which was complementary to a part of the L2-binding domain, the retina and the brain only showed one very strong band, corresponding to 5.3 kb. The same band was found in the liver, although very faint, in combination with a 2.0 kb band.

Comparison of amplification of different regions of the insulin receptor mRNA sequence, by real time RT-PCR

Three pairs of primers were designed to amplify different parts of the insulin receptor sequence. The IR-LD primer amplified a part of the sequence corresponding to the L2 domain in the receptor protein. The L2 leucine-rich domain is involved in the ligand binding and is encoded by exon number 4 and 5. The second primer pair, IR-TK, was designed to amplify a fragment that after translation belongs to the Tyrosine-kinase domain which is a catalytic domain with a phosphotransferase activity, and comprises the exons 16 and 17. The mentioned exons are localized on the longest transcript sequence for the insulin receptor mRNA based on ENSEMBL data base. Based on the results, (Figure C1.2) all tissues expressed mRNA for the tyrosine-kinase domain and the L2 (binding domain), although in different amounts. For both the IR-TK and for IR-LD, the brain showed the highest amount, followed by the retina and liver, RPE, choroid, cartilaginous and fibrous sclera. The third primer pair also amplified a part of the tyrosine-kinase domain and comprises exon 17 and 18. Concerning the IR-TK2 region no specific PCR product was obtained in most tissues, only the retina and liver showed a very low expression (data not shown).

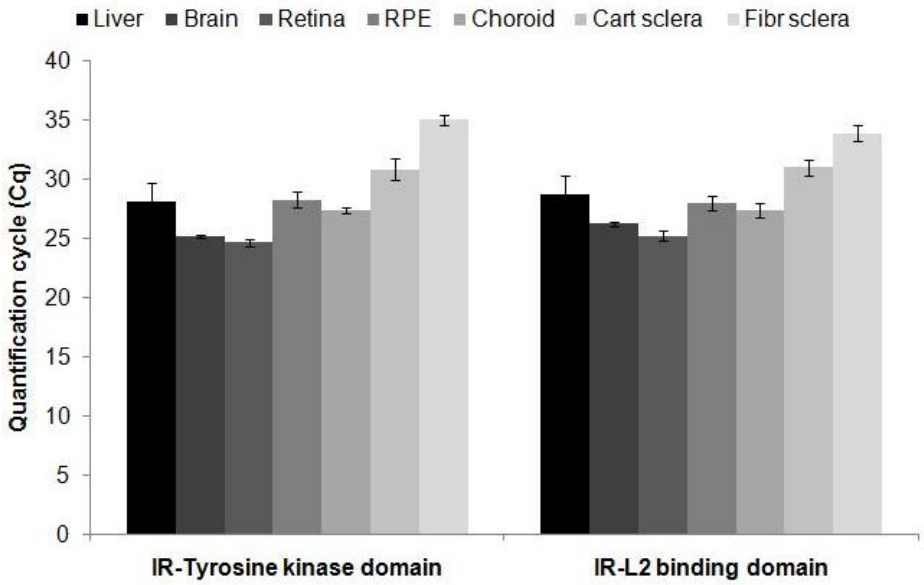

Figure C1.2 Quantification of messenger RNA levels from two different regions of the insulin receptor sequence, in different tissues. IR-TK – tyrosine kinase domain from the insulin receptor mRNA sequence; IR-LD – L2-rich (binding) domain from the insulin receptor mRNA sequence.

Insulin receptor, IGF-1 receptor, IGF-1 and insulin mRNA expression in ocular fundal layers of untreated animals

The expression level of insulin receptor, IGF-1 receptor, IGF-1 and insulin was measured and compared in all fundal layers of untreated chicks. The results are shown in Figure C1.3, with a higher quantification cycle threshold corresponding to a lower amount of mRNA. Both receptors and IGF-1 were detected in all tissues but, besides of the retina, the IGF-1 mRNA amount was very low. In addition, insulin mRNA was detected in the retina, but at very low concentrations, and in the choroid with even lower levels than in the retina. Retinal insulin expression was confirmed when an insulin-specific hydrolysis probe was used. The usage of a hydrolysis probe offers a high specificity, because hybridisation and fluorescence will only occurs if the target DNA sequence does exactly match the hydrolysis probe sequence (for further information see reference [59]). The results for insulin mRNA quantification are not shown in detail, since the expression level was too low to be precisely quantified.

Comparing the mRNA amount between different fundal layers it turned out that all measured genes were most abundant in the retina, followed by the RPE, choroid,

cartilaginous and fibrous sclera. In the fibrous sclera, the C*q* values for all the genes were higher and, therefore, mRNA levels were lower for the reference genes (β-actin and HPRT) as well as for all the other genes.

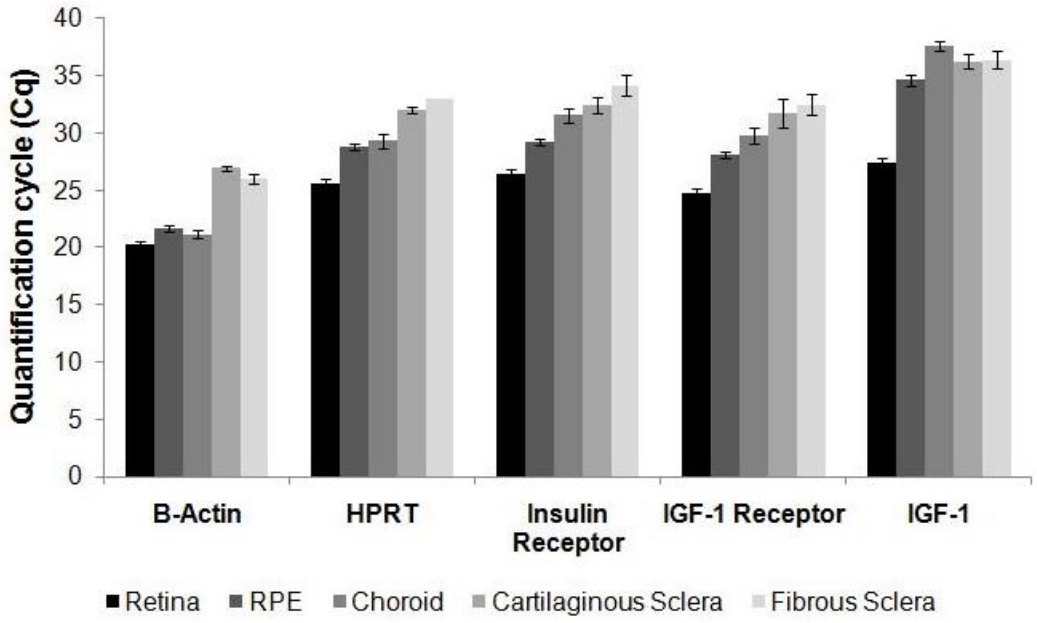

Figure C1.3 Quantification cycle values (Cq-values) for all genes in all fundal layers. Mean ± SEM are shown. Note that a high Cq value corresponds to a low mRNA amount.

Insulin mRNA expression in retina, RPE, and choroid after lens treatment

Although only very low amounts of insulin mRNA were detectable in retina and choroid of untreated animals, lens treatment might up-regulate its amount. The expression level of insulin was therefore measured and compared in the retina, RPE and choroid after 4 and 24 hours of lens treatment. However, no significant increase in insulin mRNA level was detected under any of those conditions

Insulin receptor, IGF-1 receptor, and IGF-1 messenger RNA levels in the retina after lens treatment

Treatment with negative and positive lenses did not significantly alter insulin receptor and IGF-1 receptor mRNA expression levels, neither after 4 and 24 hours of lens

treatment, compared with the appropriate control group (Figure C1.4). In addition, 4 hours and 24 hours of lens treatment had no significant influence on IGF-1 mRNA expression levels.

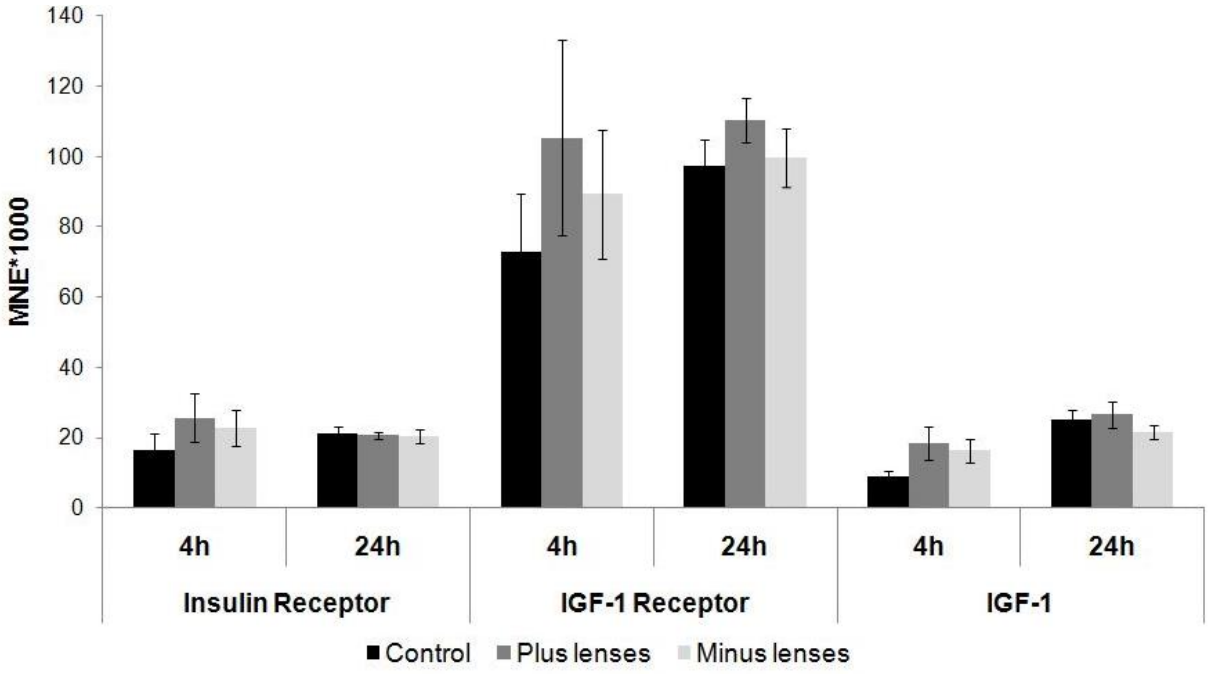

Figure C1.4 Retinal insulin receptor (IR), IGF-1 receptor (IGF-1R) and insulin-like growth factor-1 (IGF-1) mRNA levels after 4 and 24 hours of plus (+7D) and minus (-7D) lens treatment. Results are expressed as the mean normalized expression (MNE) ± SEM. For the 4 hours experiment, 6 animals per groups were used and 9 per group for the 24 hours. IR, IGF-1R and IGF-1 mRNA level were not significantly influenced by lens wear in the retina.

Insulin receptor, IGF-1 receptor, and IGF-1 messenger RNA levels in the RPE after lens treatment

Four hours of myopic defocus induced a 2-fold down-regulation of insulin receptor and an approximately 4-fold down-regulation of IGF-1 receptor mRNA levels, compared to the respective control groups (Figure C1.5, ANOVA, IR, plus lens *vs.* control $p = 0.03$; IGF-1R, plus lens *vs.* control $p = 0.03$). This effect disappeared when the animals were treated with lenses for 24 hours. In comparison to control animals, lens treatment did not significantly influenced IGF-1 mRNA levels, neither after 4 nor after 24 hours.

Nevertheless, IGF-1 mRNA levels were significantly lower after 4 hours of positive lens wear compared to 4 hours of negative lens wear (ANOVA, min *vs.* plus $p = 0.05$).

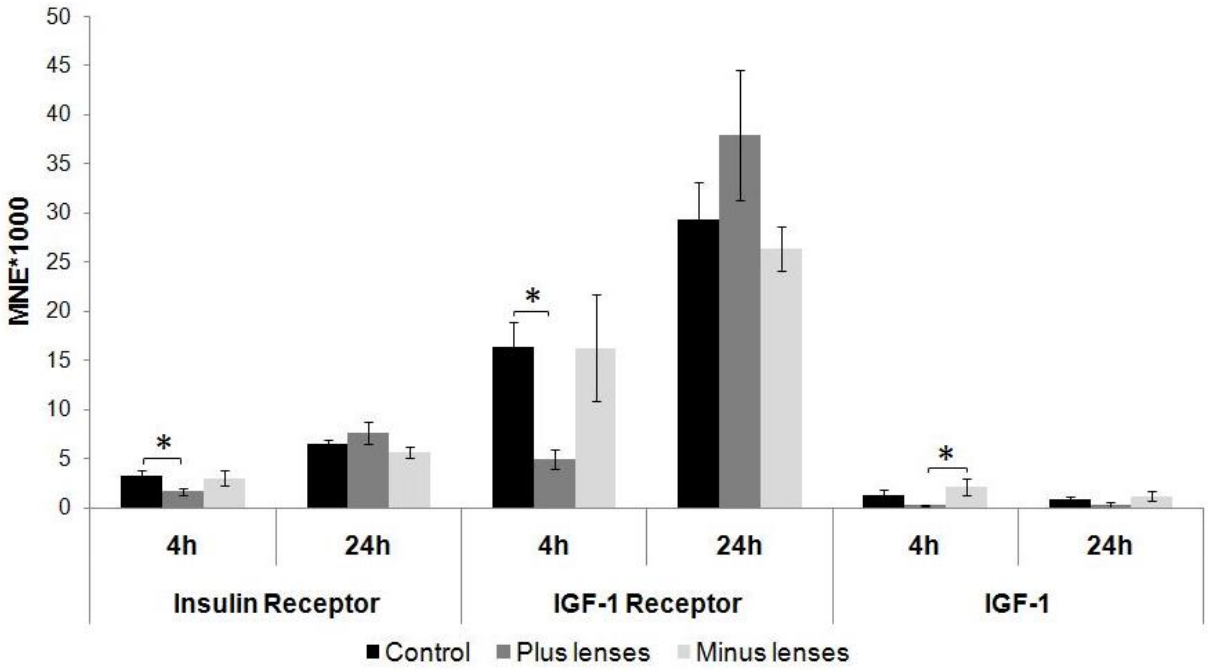

Figure C1.5 Insulin receptor (IR), insulin-like growth factor-1 (IGF-1) and IGF-1 receptor (IGF-1R) mRNA levels after 4 and 24 hours of plus (+7D) and minus (-7D) lens treatment, in the RPE. Results are expressed as the mean normalized expression MNE ± SEM. For the 4 hours experiment, 6 animals per groups were used and 9 per group for the 24 hours. Statistically significant differences, between the treated groups and the control, as determined by one-way ANOVA are denoted in the graph (* for $p < 0.05$). IR and IGF-1R mRNA levels were lower after 4 hours of plus lens treatment compared to untreated control animals. In addition, IGF-1 mRNA levels were significantly lower after 4 hours of plus lens treatment compared to the minus lens treated animals. After 24 hours of lens treatment there were no significant differences in all genes, between all groups.

Insulin receptor, IGF-1 receptor, and IGF-1 messenger RNA levels in the choroid after lens treatment

In the choroid, treatment with negative lenses for 4 hours resulted in an initial 3-fold increase of insulin receptor mRNA concentration, compared to the control group (Figure

C1.6, ANOVA, minus lens *vs.* control $p = 0.03$). These changes in the minus lens treated group stayed after 24 hours, compared with both control and plus groups (ANOVA, minus lens *vs.* control $p = 0.004$; minus lens *vs.* plus lens $p = 0.01$; Figure C1.6). The IGF-1 receptor was also increased in the minus lens treated group, compared to the plus lens group, but only after 24 hours of lens treatment. This effect was not as strong as for the insulin receptor (ANOVA, minus lens *vs.* plus lens $p = 0.05$). IGF-1 mRNA expression in the choroid was very low and difficult to quantify. Within this limitation, no significant changes in IGF-1 mRNA expression were detected.

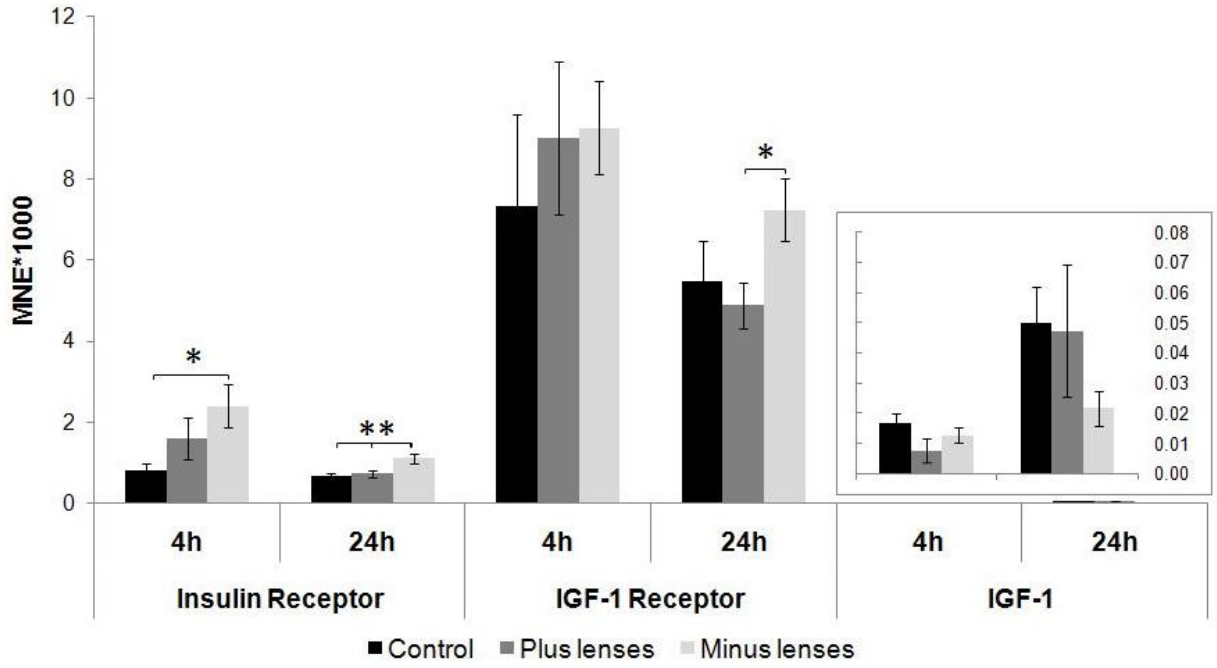

Figure C1.6 Insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) mRNA levels after 4 and 24 hours of plus (+7D) and minus (-7D) lens treatment in the choroid. Results are expressed as the mean normalized expression MNE ± SEM. For the 4 hours experiment, 6 animals per groups were used and 9 per group for the 24 hours. Statistically significant differences, between the treated groups and the control, as determined by one-way ANOVA are denoted in the graph (* for $p < 0.05$ and ** for $p < 0.01$). Messenger RNA levels for IR were significantly increased after 4 hours and 24 hours of minus lens treatment and IGF-1R mRNA level was higher in the minus lens treated group compared to the plus lens treated group after 24 hours of lens wear.

Insulin receptor, IGF-1 receptor, and IGF-1 messenger RNA levels in both scleral layers after lens treatment

In the cartilaginous sclera, lens treatment neither influenced insulin receptor, nor IGF-1 receptor mRNA levels, after 24 hours of lens treatment (Figure C1.7A). However, in the fibrous sclera (Figure C1.7B), 24 hours of positive-imposed defocus induced a 3-fold up-regulation of insulin receptor mRNA levels, compared with the control group and a 4-fold up-regulation for IGF-1 receptor at the same time point (ANOVA, IR, minus lens *vs.* control $p = 0.038$; IGF-1R, minus lens *vs.* control $p = 0.005$). IGF-1 was expressed at low levels, especially in the cartilaginous sclera. Lens treatment did not induce a significant change in IGF-1 mRNA levels in both layers.

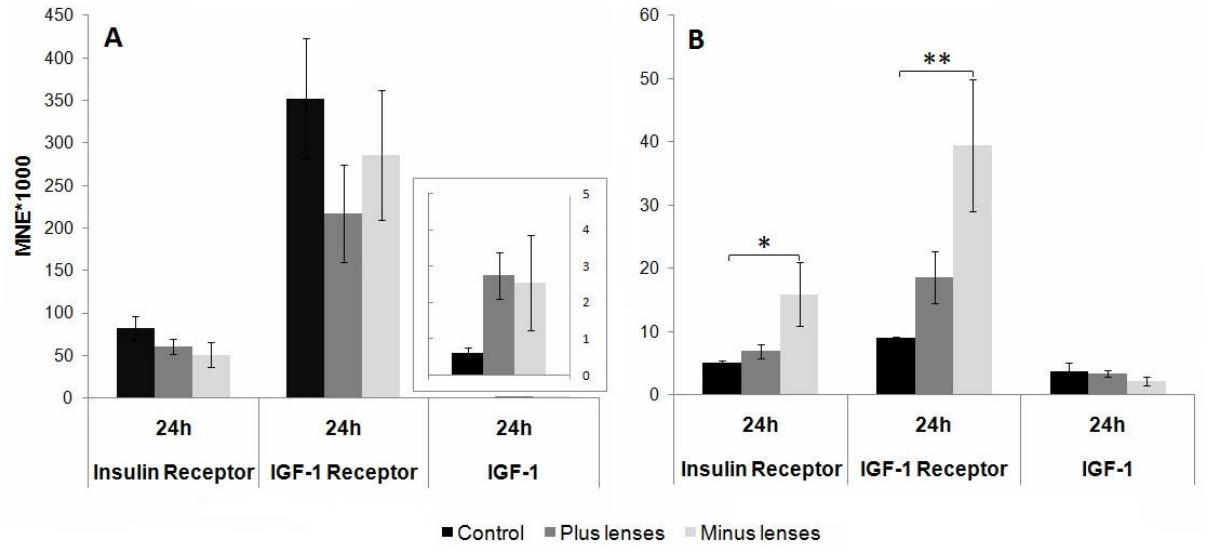

Figure C1.7 Insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) mRNA levels after 24 hours of plus (+7D) and minus (-7D) lens treatment in the cartilaginous sclera (**A**) and fibrous sclera (**B**). Results are expressed as the mean normalized expression MNE ± SEM. Nine animals per group were analysed. Statistically significant differences, between the treated groups and the control, as determined by one-way ANOVA are denoted in the graph (* for $p < 0.05$ and ** for $p < 0.01$). In the cartilaginous sclera the mRNA content of both receptors was not significantly different. In the fibrous sclera, the expression of the insulin and the IGF-1 receptor was higher in the minus lens treated group compared to controls.

DISCUSSION

Insulin receptor transcript variants

Significant differences in transcript sizes of the insulin receptor were previously described in different tissues and animal species. Four different insulin receptor transcript variants were reported in chicks (ENSEMBL database) with transcript lengths varying between 198 and 3220 basepairs. All of them can be translated to a protein product, but in the case of the small transcripts, the functions are unknown. In addition, several proteomic studies showed differences in molecular weight of insulin receptor alpha and beta subunits among different tissues[60-63]. The insulin receptor therefore seems to undergo different transcriptional and translational regulation and post-translational modifications including glycosylation or proteolytic cleavage in the CNS[64]. In the brain, the alpha-subunit of the insulin receptor has a lower molecular size compared to that of other tissues. It was therefore hypothesize that insulin exerts its proposed neuromodulatory effects mediated by the specific insulin receptors in the brain[63]. In our study, we found 4.3 kb, 2.6 kb and 1.3 kb long insulin receptor transcripts when we used a probe that corresponds to part of the sequence for the insulin receptor tyrosine kinase domain. A messenger RNA of 4.3 kb can account for a protein as large as the insulin receptor (1332 amino acids), whereas the smallest transcript can only encode for parts of the protein. We could show that different tissues expressed transcripts of different lengths. RPE and sclera seem to express only short IR transcripts. Moreover, the smaller mRNA transcript was the predominant form in the choroid and liver, whilst the longer transcript was most abundant in the brain and retina. This result may suggest that insulin receptors in the retina and brain possibly have a different function than in the non-neuronal tissues. As expected from the ENSEMBL database, only one long transcript was detected in brain and retina when a L2-binding domain specific probe was used.

One recent publication stated that the chicken retina does not express insulin receptor mRNA[65]. In contrast, we found relatively high amounts in the retina of our chicks. Since different parts of the insulin receptor were amplified in both studies, we used three different primer pairs for the amplification of different parts of the insulin receptor in an

attempt to solve the discrepancy. We found that the mRNA for the insulin binding domain and the tyrosine kinase domain are present at moderate levels in the retina. Nevertheless, one PCR product that was designed to amplify a short sequence between exon 17 and 18 could only be detected in a very small amount in retina and liver, suggesting that this part of the sequence is not efficiently transcribed or different from the one in the literature.

Possible sites of action for insulin and IGF-1 in the eye

Several lines of evidence support a role of insulin and/or IGF-1 in the control of eye growth,[24, 32, 34] including one strong clue coming from chicken studies, in which it was shown that intravitreal injections of both peptides lead to myopia development. The current study aimed to quantify the mRNA expression of both, the receptors and their ligands in all fundal layers to gain a broader insight into their role. Insulin receptor and IGF-1 receptor were expressed in all tissues, being more abundant in the retina, followed by RPE, choroid, cartilaginous and fibrous sclera. Therefore, assuming that the mRNA is translated into protein, all of them are possibly target sites for insulin and IGF-1 action. IGF-1 mRNA expression was only relatively high in the retina, meaning that only here a significant amount of IGF-1 could be produced. This result corresponds with an older study[66] in which IGF-1 specific transcripts were higher in the neural retina than in sclera plus choroid plus pigment epithelium. Although insulin mRNA expression was detected in the retina and choroid, as confirmed with a specific hydrolysis probe and gel electrophoresis, its level was very low. It was therefore impossible to quantify these low amounts reliably. Lens treatment did not increase insulin mRNA levels in retina, RPE or choroid. Taken together, it seems unlikely that changes in the amount of insulin produced by the retina itself may influence eye growth. It is more likely that IGF-1 plays a physiological role in the retina. Insulin and IGF-1 mRNA levels in the retina seem to be developmentally regulated as shown by binding assays, decreasing by about 50% between embryonic to post-hatching stages[67]. In the rat retina it was already shown that Müller cells may contain mRNA necessary for the novo synthesis of insulin or a closely homologous peptide[37]. But the source of insulin in the avascular chicken retina still

remains unclear and, due to the very low amounts, it will be difficult to uncover its function.

Influence of lens treatment on insulin receptor, IGF-1 receptor and IGF-1 expression in the fundal layers of the eye

Lens wear influenced insulin receptor, IGF-1 receptor, and IGF-1 mRNA expression in different fundal layers of the chicken eye with most changes seen in the RPE, choroid and fibrous sclera. In our study, the whole retina was used to measure changes in gene expression after induced positive and negative defocus. Insulin and IGF-1 receptor mRNA are both highly expressed in the retina, but their expression level as well as the IGF-1 mRNA levels were not influenced by defocus of 4 and 24 hours. In contrast, short plus lens treatment periods (4 hours) led to a strong down-regulation of both receptors in the RPE and in addition also IGF-1 mRNA levels were much lower in the plus lens group compared to the minus lens treated animals. The insulin and IGF-1 receptor signalling may therefore be involved in the onset of growth arrest after negative defocus. It is not surprising that the gene expression changes did not persist after 24 hours of treatment, since it is known from microarray studies that only a minority of gene expression changes seem to be common to multiple treatment times[68]. This can be interpreted in terms of different mechanisms, one for the onset of increased (minus lens) or decreased (plus lens) eye growth and the other mechanism for maintaining its persistence. An up-regulation of IGF-1 receptor mRNA expression in the RPE of chicks that were treated with minus lenses for 2 days was recently reported using microarrays (Zhang Z, et al. IOVS 2008;49:ARVO E-Abstract 1741). We did not measure an up-regulation of this receptor after one day, but as already discussed before, time often matters and may explain the different results.

In the present study, we could demonstrate that short treatment with plus lens mainly affected mRNA levels in the RPE, whereas longer and minus lens treatment influenced gene expression level in the choroid and fibrous sclera. The choroid is a thin layer of vascular pigmented tissue with two main physiological functions, the nourishment of the external retina, and the regulation of ocular temperature. Both insulin and IGF-1 mRNA

levels were present at low levels in the choroid, as confirmed with a specific hydrolysis probe and gel electrophoresis, but it was impossible to quantify the low amounts reliably. Nevertheless, the respective receptors levels changed during the treatment. Higher mRNA levels of the insulin receptor were already measured after four hours and insulin and IGF-1 receptors showed higher expression levels than controls and/or plus lens treated animals after 24 hours of lens-wear. Zhu and Wallman (2009)[24] recently hypothesised that although it is unknown whether glucagon and insulin first act at the retina, RPE or choroid, they finally act 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. Our results support this hypothesis. Especially after minus lens treatment the only changes in receptor gene expression were detected in choroid and sclera. Since only low insulin and IGF-1 mRNA levels were detected in the choroid, is seems unlikely that they are synthesised in significant amounts in this tissue. Instead, the tissue could be a potential target for these growth factors action, given that insulin and IGF-1 injections in chicken eyes were shown to induce choroidal thickness changes under some experimental conditions. Insulin increases ocular elongation without thinning the choroid in animals not wearing lenses. Only when plus lenses were attached, which normally cause choroidal thickness, insulin thins the choroid, as well as accelerating ocular elongation. In contrast, IGF-1 injections increase ocular elongation together with thickening rather than thinning the choroid[24, 32].

Different from mammals, the sclera of chicks is composed of two layers: an inner cartilaginous layer, containing collagen type II and IV and aggrecan as the predominant proteoglycans, and an outer fibrous layer (like that in mammals), which contains collagen type I and small proteoglycans such as decorin[69]. When the rate of elongation of the eye is visually manipulated, both scleral layers show opposite modulation[62], with the fibrous sclera getting thinner and the cartilaginous becoming thicker during induced eye growth. Interestingly, we could show that the fibrous sclera showed a similar up-regulation of insulin receptor mRNA expression as the choroid. One of the reasons for this up-regulation of the IGF-1 receptor mRNA expression in the fibrous sclera might be that IFG-1 exerts an effect on the developing ocular tissue by influencing the synthesis

and degradation of the extracellular matrix in chicks[70]. In the guinea pig model, it was already shown that IGF-1 can induce fibroblasts proliferation in a dose dependent manner, through STAT3 signaling transduction pathway[71, 72]. No lens-induced changes in gene expression were detected in the cartilaginous sclera. Compared to the fibrous sclera, the cartilaginous sclera had higher mRNA levels for all measured genes. This result is consistent with a previous study by Schippert et al. (2006). They also showed that the fibrous sclera has in general lower messenger RNA levels than the cartilaginous sclera in untreated chicks[73]. Co-cultures already demonstrated that the choroid can influence the underlying sclera, for example by changing proteoglycan synthesis in the sclera[74]. Retinoic acid, the synthesis of which is influenced in opposite directions by positive and negative defocus in both the retina and choroid, has been shown to affect proteoglycan synthesis in the chick sclera[17]. Moreover, retinoic acid might interact with the IGF-1 signalling by changing the level of IGF-binding proteins and thereby modulating scleral IGF-1 levels[75].

Comparison to studies in humans

Recent epidemiologic and retrospective case series studies in humans underlined a role of IGF-1 as regulator of ocular growth, at least in patients with primary growth hormone insensitivity[76], in children with growth hormone deficiency[77] and in children born preterm[78]. Low IGF-1 serum concentrations were associated with hyperopia in these studies. These results are consistent with our animal study showing an association of reduced IGF-1 mRNA levels with hyperopia development in the RPE of chicks. Interestingly, patients with primary growth hormone insensitivity who received IGF-1 therapy showed a tendency toward mild myopia. This findings point toward a role of IGF-1 as a grow signal in humans as well as chicks.

Implications and summary

In summary, we found that a short exposure to myopic defocus (plus lenses) leads to a down-regulation of insulin receptor and IGF-1 receptor expression in the RPE. In contrast, hyperopic defocus, imposed by minus lenses (but not myopic defocus)

significantly increased their expression levels in the choroid. Similar changes were seen in the fibrous sclera. Taken together, the current study supports a role of insulin and/or IGF-1 signalling during eye growth. Whether different insulin receptor transcript variants as found in retina and choroid are also translated into proteins with different functions needs to be shown in the future.

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PROJECT 2:

Effects of intravitreal insulin and insulin signaling cascade inhibitors on emmetropization in the chick

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Purpose: Intravitreal insulin has been shown to be a powerful stimulator of myopia in chickens, in particular if the retinal image is degraded or defocused. In most tissues, the insulin receptor activates two main signaling pathways: a) the mitogen-activated protein kinase (MAPK) cascade (e.g., mitogen-activated protein kinasem kinase [MEK] and extracellular regulated kinase [ERK]) and b) the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. In the current study, insulin was injected, and these pathways were separately inhibited to determine which is activated when the retinal image is defocused by spectacle lenses.

Methods: Chicks were treated with either +7 D, −7 D, or no lenses. They were intravitreally injected with insulin, the MEK inhibitor U0126, the PI3K inhibitor Ly294002, or a combination of insulin and one of the inhibitors. Refractions and ocular dimension were measured at the beginning and after four days of treatment. The retinal proteins of the chicks were measured with western blots after 2 h and four days of treatment. Incubation occurred with anti-Akt1, anti-Erk1/2, anti-phospho-Akt^{Thr308}, and anti-phospho-Erk1/2$^{(Thr202/Tyr204)}$ antibodies, and the ratio between the relative intensity of the phospho-form and the total-form was calculated.

Results: Chicks wearing positive lenses and injected with saline and with PI3K inhibitor compensated for the imposed defocus and became hyperopic. Insulin injections and insulin plus PI3K inhibitor injections prevented lens-induced hyperopia, whereas the MEK inhibitor alone and insulin plus MEK inhibitor had no effect. Obviously, the MEK inhibitor suppressed the effect of insulin on eye growth in the plus lens–treated animals. Chicks treated with negative lenses and injected with insulin, or with insulin plus MEK inhibitor, overcompensated for the imposed defocus. This effect of insulin was not detected in eyes injected with PI3K inhibitor plus insulin, suggesting that the PI3K inhibitor suppressed the effects of insulin in minus lens–treated animals. Insulin increased the ratio of phospho-Akt/total-Akt in animals with normal visual exposure but even more so in chicks wearing plus or minus lenses. The increase was blocked

by simultaneous PI3K inhibitor injections in control eyes but not in lens-treated eyes. Insulin also increased the ratio of phospho-ERK/total-ERK in animals with normal visual exposure and in animals wearing positive lenses, compared to U0126- and Ly294002-injected eyes. In contrast, no significant activation of the MEK/ERK pathway was observed in the negative lens–treated animals.

Conclusions: Intravitreal insulin promoted axial eye growth and stimulated both signaling pathways. The PI3K/Akt pathway was activated in control and plus and minus lens–treated eyes, but the MEK/ERK pathway was activated only with positive lenses or no lenses. With negative lenses, insulin did not stimulate the MEK/ERK signaling cascade. Independent of the pathway stimulated after insulin binding, the effect on insulin was always the same: an increase in eye growth.

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INTRODUCTION

According to the World Health Organization (WHO), the most common causes of visual impairments are uncorrected refractive errors, such as myopia, hyperopia, or astigmatism, followed by cataract and glaucoma [1]. Animal models of myopia have been developed and have shown that emmetropization in the vertebrate eye is guided by an active, visually guided feedback loop [2]. Animals compensate for imposed defocus by adjusting the axial eye growth rate such that the focal plane and the photoreceptor plane achieve a close match. Regulation of eye growth was demonstrated to be largely independent of processing in the brain, as demonstrated in optic nerve lesion studies [3-7].

Several retinal substances were shown to be implicated in visually guided eye growth regulation, such as vasoactive intestinal peptide (VIP) [8,9], dopamine [10-12], retinoic acid [13-15], glucagon [16-18], insulin [19,20], γ-aminobutyric acid (GABA) [21], and growth factors such as transforming growth factor (TGF) [22,23], basic fibroblast growth factor (bFGF) [22], and insulin-like growth factor-1 (IGF-1) [20]. Moreover, experiments in chickens and mice have implicated the early growth response gene-1 (Egr-1, also called ZENK in chickens) [24-26] in the feedback mechanisms for visual control of axial eye growth and myopia development. However, the mechanism and the signaling pathways are not yet known. Because some of these modulators were found to be upregulated under conditions that inhibit eye growth, they were considered stop signals, like glucagon and ZENK, in the chicken model [16,27]. Glucagon and insulin have opposite effects on metabolic functions in the body, on cell proliferation in progenitor cells at the periphery of the retina [28], and on axial eye growth [19,20]. While intravitreal injections of glucagon or a glucagon agonist can prevent negative lens–induced myopia in chicks, by slowing axial eye growth and increasing choroidal thickness [16,20,29], insulin not only blocks the development of hyperopia, which is normally induced by positive lenses, but also induces axial myopia. In addition, insulin enhances myopia induced by negative lens treatment [19]. Insulin and IGF-1 induce axial myopia even in eyes not wearing any lenses [20].

Insulin binds and acts mainly through the insulin receptor but can also act via the IGF-1 receptor. In addition, IGF-1 can bind to the insulin receptor. In recent years, several studies investigated IGF-1 signal transduction cascades involved in neuroprotection. However, whether the same pathways are activated upon insulin or IGF-1 receptor stimulation during eye growth is currently unknown. Insulin and IGF-1 can mediate their cellular effects by signaling downstream through insulin receptor substrate (IRS) molecules. Ligand binding to the corresponding receptors leads to conformational changes and induces a cascade of phosphorylation of several cytosolic molecules [30]. Depending on the phosphorylated downstream molecules, different pathways are activated. The MEK/ERK pathway involves binding of the tyrosine phosphorylated (TP)-IRS to the growth factor receptor-bound protein 2 (Grb2), which results in sequential activation of p21Ras, mitogen-activated protein kinase (MEK), and mitogen-activated protein kinase (MAPK originally called ERK) [31]. ERK activation directly contributes to insulin- and IGF-1-stimulated mitogenesis, neuritic development, and gene expression [32-34], control of the cell cycle and cell migration, as well as proliferation and differentiation. In addition to this pathway, various cellular stimuli, including activation of the insulin receptor and the IGF-1 receptor, can activate the PI3K pathway. The serine/threonine kinase Akt is a crucial kinase in this pathway. PI3K phosphorylates PI(4,5)P2, generating PI(3,4,5)P3, a potent second messenger required for survival signaling and insulin action [35]. The PI3K pathway controls glucose metabolism [36,37]. This includes glucose uptake and glycogenesis. Furthermore, fundamental cellular functions such as transcription, translation, proliferation, growth, and survival are regulated [38,39]. Generally, the mitogenic signal of the IGF-1 receptor/protein interaction is promoted through the PI3K/Akt pathway, but sometimes is transmitted via either the MEK/ERK pathway or mitochondrial translocation of Raf-1. IGF-1 has been shown to activate the MEK/ERK and PI3K/Akt pathways to induce cellular transformation [40]. The PI3K/Akt pathway often transduces signals that are similar in nature to that of the MEK/ERK pathway, and several studies reported a cross talk between those two pathways on multiple levels [40]. Contrary to mammals, which have two isoforms of ERK, called p44 ERK1 and p42 ERK2, the chick has only one isoform,

the p44 ERK2 [41]. It is not known whether the lack of one isoform determines any differences at the cell signaling level [42].

In the nervous system, most in vitro studies showed that members of the insulin family, especially IGF-1, act as a neuroprotective factor [43-45], and require an initial activation of PI3K as a common step in their intracellular signaling pathway for survival effects. Proinsulin and insulin, before IGF-1, act as survival factors in the chick and mouse retina during development [46-49]. Furthermore, it was shown that proinsulin can act independently of Akt activation [49]. In the embryonic stages, at E5, the insulin survival effects are mediated through MEK/ERK activation, whereas the same effect is mediated through the PI3K/Akt activation at embryonic stage E9 [50].

The role of insulin receptor signaling in neuronal tissues has not been characterized in detail. A recent study [51] suggested that different ocular layers express different insulin receptor transcripts, suggesting that the same receptor probably can activate different signaling cascades, which then depend not only on the stimuli but also on the tissue. The present study analyzed the effect of insulin intravitreal injections on the activation of the PI3K/Akt or MEK/ERK signaling cascades as a possible mechanism responsible for eye growth in the chicken model of myopia.

METHODS

Treatment of animals

Eight-day-old male White Leghorn chickens (n=108) were raised under a 12 h:12 h light-dark cycle. To attach the lenses, Velcro rings were glued onto the feathers around the eyes a few hours before the lens treatment was started. Animals were anesthetized before intravitral injections by exposure to diethyl ether by inhalation. Because insulin binding to the insulin receptor was shown to activate the MEK/ERK pathway and the PI3K/Akt- pathway, selective inhibitors were used to separate the inputs. U0126 was used to block the activation of ERK 1/2 that occurs by inhibiting the activity of MEK. This effect is mediated by antagonizing the AP-1 transcriptional function via noncompetitive inhibition of the dual specificity kinase MEK with a half maximal inhibitory concentration (IC50) of 0.07 µM for MEK1 and 0.06 µM for MEK2 [52]. Ly294002 was used to inhibit PI3-kinases. Ly294002 is a potent and specific cell-permeable inhibitor of PI3K with IC50 values in the range of 1–50 µM, and competitively inhibits adenosine 5′-triphosphate binding to the catalytic subunit of PI3-kinases [53]. The inhibition of PI3K in turn leads to a reduction in the phosphorylation of Akt, as shown in previous studies [50]. The experimental treatment was in accordance with the ARVO Statement for Care and Use of Animals in Ophthalmic and Vision Research and was approved by the University Commission for Animal Welfare. The details of all the treatment groups are summarized in Table C2.1.

Effects of intravitreal insulin and U0126 and Ly294002 injections on refractive development and axial length

At the age of 8 days post-hatching, chicks were intravitreally injected (12.5 µl) in both eyes (Table C2.1). Injections were either saline in one eye and insulin (0.3 µM Insuman Rapid, Aventis, Frankfurt, Germany, groups 1, 4, and 7) in the contralateral eye, or U0126 (50 µM, Calbiochem) in one eye and a combination of insulin (0.3 µM) and the inhibitor U0126 (50 µM; groups 2, 5, and 8) in the contralateral eye, or Ly294002 (50 µM, Calbiochem) in one eye and a combination of insulin (0.3 µM) and the inhibitor

Ly294002 (50 µM; groups 3, 6, and 9) in the contralateral eye. All types of injections were repeated two days later. In addition, from day 8 until the end of the treatment (day 12), chicks were binocularly treated with either positive lenses (groups 4 to 6) or negative lenses (groups 7 to 9), or the chicks did not wear any lenses (groups 1 to 3). Before the injections and the beginning of the lens treatment and four days later, the refractive state and axial length were measured. Six animals were treated per group.

Short- and long-term effects of lens wear and intravitreal injections of insulin, U0126, and Ly294002 on extracellular regulated kinase 1/2 and protein kinase B phosphorylation levels

Signaling cascades triggering myopia or hyperopia onset may differ from those maintaining their progression. Therefore, two different time points were investigated. For the analyses of long-term effect (four days) of lens wear and intravitreal injections of insulin, MEK inhibitor, and PI3K inhibitor, the retinas from groups 1 to 9 (Table 1) were dissected, and the extracted proteins were subjected to western blot analysis. To investigate the short-term effects (2 h) of lens wear and intravitreal injections on ERK1/2 and Akt phosphorylation levels, animals (six per group) were treated for 2 h with no lens (groups 10 to 13), positive lenses (groups 14 to 17), or negative lenses (groups 18 to 21) in combination with the following injections (12.5 µl): saline in one eye and insulin (0.3 µM) in the contralateral eye (groups 10, 14, and 18), U0126 (100 µM) in one eye and Ly294002 (100 µM) in the contralateral eye (group 11, 15, 19), U0126 in one eye and insulin plus U0126 in the contralateral eye (group 12, 16, and 20), and Ly294002 in one eye and a combination of insulin (0.3 µM) and the inhibitor Ly294002 (50 µM) in the other eye (groups 13, 17, and 21; Table C2.1).

Measurements of refractive state and ocular dimensions

Refractive states were measured with automated infrared photoretinoscopy [54]. Axial length, defined as the distance from the surface of the cornea to the vitreoretinal interface, was measured with A-scan ultrasonography [55]. This technique also provided data on anterior chamber depth, lens thickness, and vitreous chamber depth.

Tissue preparation

Chicks were euthanized with an overdose of diethylether by inhalation. Eyes were enucleated and vertically cut with a razor blade, discarding the anterior part containing the lens. The vitreous body was removed and the pecten cut out. From the posterior pole of the eye, a biopsy punch of 8 mm was excised and placed in a Petri dish filled with ice-chilled saline. The retinas from all experimental groups were separated from the rest of the fundal layers under visual control under a dissecting microscope, and immediately frozen in liquid nitrogen and stored at −80 °C until protein extraction.

Total protein extraction

Retinal total protein extraction was performed using T-PER Tissue Protein Extraction Reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL), according to the manufacturer's instructions. To prevent phosphatase action and proteolytic degradation 1 × Phos-STOP Phosphatase Inhibitor Cocktail (Roche, Mannheim, Germany) and 1 × of complete Protease Inhibitor Cocktail (Roche) were added to the extraction buffer. The total protein amount was measured using the Pierce BCA Protein Assay kit (Thermo Scientific, Pierce Technology).

Table C2.1 Summary of experimental plan.

Group number	Binocular lens treatment	Ipsilateral eye injection (12.5 µl)	Contralateral eye injection (12.5 µl)	Concentration [µM] ips. eye/contra. eye	Time of treatment
Effects of lens wear and intravitreal insulin, MEK- (U0126) and PI3K- (Ly294002)-inhibitor injections on refractive development and axial length					
Group 1	Without lenses	Saline	Insulin	0 / 0.3	**4 days***
Group 2		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 3		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	
Group 4	Positive lenses (+7D)	Saline	Insulin	0 / 0.3	
Group 5		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 6		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	
Group 7	Negative lenses (-7D)	Saline	Insulin	0 / 0.3	
Group 8		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 9		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	
Effects of intravitreal insulin, MEK- (U0126) and PI3K- (Ly294002) inhibitor injections on phosphorylation levels					
Group 10	Without lenses	Saline	Insulin	0 / 0.3	**2 hours**
Group 11		U-0126	Ly-294002	100 / 100	
Group 12	Positive lenses (+7D)	Saline	Insulin	0 / 0.3	
Group 13		U-0126	Ly-294002	100 / 100	
Group 14	Negative lenses (-7D)	Saline	Insulin	0 / 0.3	
Group 15		U-0126	Ly-294002	100 / 100	
Group 1	Without lenses	Saline	Insulin	0 / 0.3	**4 days***
Group 2		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 3		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	
Group 4	Positive lenses (+7D)	Saline	Insulin	0 / 0.3	
Group 5		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 6		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	
Group 7	Negative lenses (-7D)	Saline	Insulin	0 / 0.3	
Group 8		U-0126	U-0126 + insulin	50 / 50 + 0.3	
Group 9		Ly-294002	Ly-294002 + insulin	50 / 50 + 0.3	

*Animals used in experiment 1 were also used for experiment 2 (long-term effects).

Western blot analysis

Ten micrograms of protein were mixed with Laemmli buffer, heated at 95 °C for 4 min, fraction-ated by 8% sodium dodecyl sulfate–PAGE and transferred to Trans-Blot Medium Pure Nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked overnight at 4°C with 3% BSA in Tris-buffered saline with 1% Tween-20 (TBST; Roth, Karlsruhe, Germany). The next day, membranes were incubated for 1 h at room temperature with the following primary antibodies: goat anti-Akt1 (1:750, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-ERK1/2 (1:4000, R&D Systems, Wiesbaden-Nordenstadt, Germany), rabbit anti-phospho-Akt^{Thr308}

(1:2000, Cell Signaling, Boston, MA) and rabbit anti-phospho-ERK1/2$^{(Thr202/Tyr204)}$ (1:2000, Cell Signaling). After washing (four times, 4 min each) with 1 × TBST, blots were incubated for 1 h at room temperature with the corresponding peroxidase-conjugated secondary antibodies: rabbit anti-goat (1:10000, Calbiochem, Darmstadt, Germany), goat anti-mouse (1:30000, Calbiochem), and goat anti-rabbit (1:20000, Calbiochem). Following subsequent washing steps, the blots were developed using the Super-Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce Technology) according to the manufac-turer's instructions. Pictures were captured, with different exposure times, using a cooled charge- coupled device (CCD) camera (Raytest, Isotopenmessgeraete GmbH, Germany) and analyzed with AIDA 2.11 software (Raytest, Isotopenmess-geraete GmbH).

Detection of apoptosis with terminal deoxynucleotidyl transferase dUTP nick end labeling staining

Cell death was determined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in retinal sections of saline, insulin (12.5 µl 0.3 µM), U0126 (12.5 µl 100 µM), and Ly294002 (12.5 µl 100 µM) injected chicks (as described in Table 1, for groups 1–3). The posterior part of the eyes, without the vitreous body, was fixed for 20 min in 4% para-formaldehyde, and 12-µm- thick vertical sections were taken. Apoptotic cells containing fragmented DNA were identified with the TUNEL method using the In Situ Cell Death Detec-tion Kit, Fluorescein (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. DN*ase* I-treated retinas (10 min) served as positive control.

Statistics and data analysis

All data represent mean values from six animals and standard errors of the mean. For refrac-tive states and ocular dimensions, data represent the differ-ence between the end of the experiment and the baseline levels. For western blot analyses, the relative band intensities were normalized to a control sample. This control sample contained a mixture of two untreated retinas, and one lane per gel was always left for this control sample to control for any variation in signal intensities between different blots and to

control for contralateral effects. The ratio of the phosphorylated-form/total-form was calculated with the normalized values and converted into percentages. All data were controlled for normal distribution with a Shapiro–Wilk test and exposed to analyses of variance (one-way ANOVA). Using a one-way ANOVA, the effects of drug injections in the no lens, positive lens–, or negative lens–treated eyes were compared. A significant ANOVA ($p<0.05$) was followed by a Student *t* test for post hoc analysis. Statistical tests were performed using JMP version 8 software (SAS Institute, Cary, NC).

RESULTS

Effects of lens wear and intravitreal insulin, U0126, and Ly294002 on refractive development and axial eye growth

Effects of intravitreal injections and lens treatment on refractive development

TUNEL staining was employed to control for potential toxicity. However, no apoptotic cells were detected at the doses used for insulin and its inhibitors (data not shown).
In animals not wearing lenses, none of the injections induced significant changes in the refractive state (Figure C2.1). There was only a trend (p=0.06) of an opposite effect of both inhibitors: the MEK inhibitor U0126 induced a trend of a myopic shift compared to the PI3K inhibitor Ly294002-injected eyes.

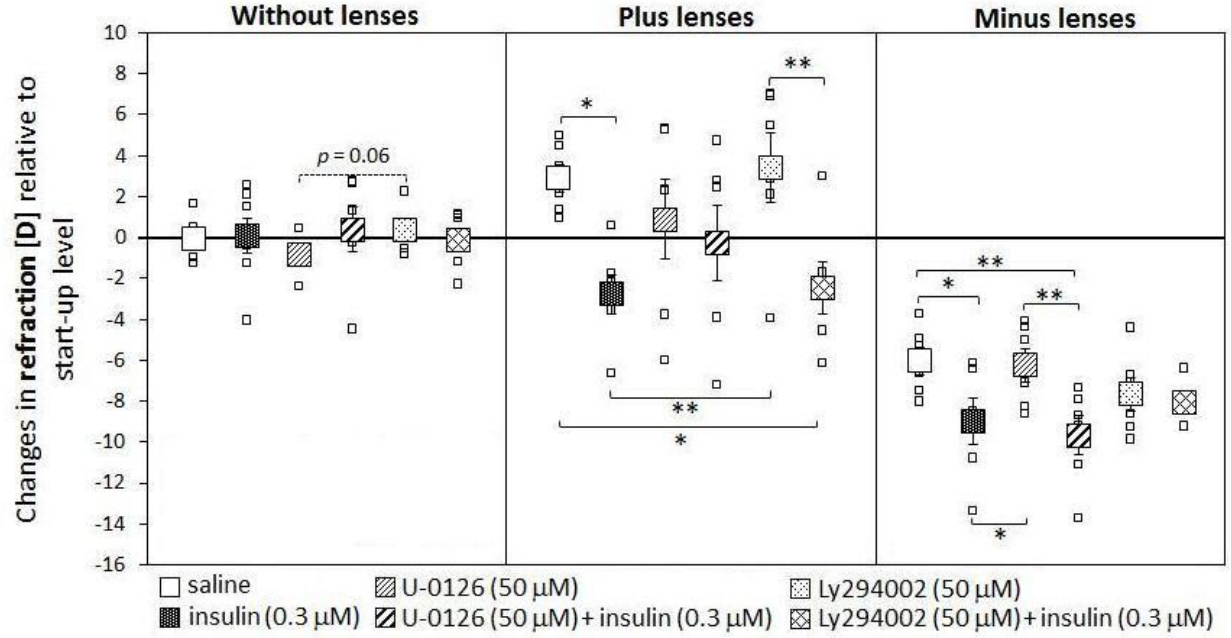

Figure C2.1 Effects of intravitreal saline, insulin, U-0126 and Ly-294002 injections on the development of refractive state in chicks without any lenses, and after bilateral treatment with +7 D lenses and -7 D lenses. The large squares represent the means of the changes in refractive state ± SEM from the beginning of the experiment and 4 days later. The small squares denote data from individual eyes. Six animals per group were used. Insulin and insulin + Ly-294002 prevented hyperopia development in positive lens wearing animals, whereas U-0126 could block the insulin effect. Animals wearing negative lenses and injected with insulin and insulin+U-0126 overcompensate the imposed defocus. In contrast, with the same lens

treatment, Ly-294001 blocked the effect of insulin. Statistically significant differences, as determined by one-way ANOVA, are denoted in the graph (* $p < 0.05$, ** $p < 0.01$).

In accordance with previous studies [19,20], insulin-injected animals wearing positive lenses did not become hyperopic but rather more myopic than age-matched controls without injections (Figure C2.1, Table C2.2). PI3K inhibitor Ly294002 injections had no effect in animals treated with positive lenses, suggesting that the lenses were compensated as normal. Insulin in combination with this PI3K inhibitor was as effective as insulin alone: it prevented hyperopia (refraction after four days with positive lenses: insulin versus insulin and Ly294002+0.49±0.81 D versus +0.61±1.46 D, Table C2.2). Interestingly, refractive states in animals injected with the MEK inhibitor U0126 or insulin plus MEK inhibitor were similar after four days of positive lens wear (U0126 versus U-126 plus insulin: +3.88±2.07 D versus +3.00±2.02 D, n.s.), suggesting that this inhibitor blocked the effect of insulin. Standard errors in MEK inhibitor–injected eyes were higher than in all other groups.

Table C2.2 Effects of lens wear and intravitreal insulin, U-0126 and Ly-294002 injections, after 4 days of treatment, on refractive development and ocular dimensions.

Injection / Lens treatment	Saline	Insulin (0.3 µM)	U-0126 (50 µM)	U-0126 + insulin (50 µM + 0.3 µM)	Ly-294002 (50 µM)	Ly-294002 + insulin (50 µM + 0.3 µM)
4 days WITHOUT LENSES (n=6)						
Refraction [D]	+2.79±0.28	+2.91±0.91	+2.28±0.39	+3.50±1.11	+3.90±0.65	+2.98±0.55
ACD [mm]	1.19±0.05	1.17±0.02	1.20±0.02	1.20±0.02	1.17±0.04	1.21±0.03
LT [mm]	2.21±0.04	2.17±0.02	2.10±0.01	2.13±0.03	2.10±0.04	2.17±0.03
VCD [mm]	5.19±0.05	5.06±0.09	5.20±0.03	5.09±0.06	5.20±0.05	5.09±0.08
Axial length [mm]	8.51±0.08	8.40±0.09	8.50±0.03	8.43±0.05	8.47±0.07	8.47±0.08
4 days with PLUS LENSES (n=6)						
Refraction [D]	+6.25±0.79	+0.49±0.81	+3.88±2.07	+3.00±2.02	+6.40±1.64	+0.61±1.46
ACD [mm]	1.02±0.05	1.18±0.05	1.06±0.05	1.21±0.07	1.15±0.06	1.24±0.09
LT [mm]	2.05±0.06	2.20±0.03	2.12±0.05	2.22±0.02	2.11±0.02	2.21±0.03
VCD [mm]	4.94±0.07	5.01±0.07	5.08±0.08	5.01±0.07	5.02±0.07	5.08±0.10
Axial length [mm]	8.01±0.09	8.39±0.10	8.26±0.11	8.44±0.14	8.28±0.10	8.53±0.17
4 days with MINUS LENSES (n=6)						
Refraction [D]	-3.32±0.90	-6.09±1.11	-3.79±1.05	-6.83±0.88	-4.81±0.89	-6.75±1.59
ACD [mm]	1.20±0.06	1.34±0.04	1.23±0.08	1.36±0.04	1.22±0.07	1.35±0.06
LT [mm]	2.14±0.04	2.25±0.05	2.15±0.04	2.30±0.06	2.18±0.08	2.113±0.04
VCD [mm]	5.48±0.10	5.45±0.07	5.51±0.09	5.50±0.12	5.48±0.13	5.55±0.10
Axial length [mm]	8.82±0.12	9.03±0.07	8.88±0.16	9.15±0.15	8.88±0.13	9.03±0.13

Refractive state (in diopters, D) and ocular dimensions (in mm) on day 4 after the lens and injections treatment. Results are represented as mean±SEM. For all groups n=6. ACD – anterior chamber depth; LT – lens thickness; VCD – vitreous chamber depth; AL – axial length.

In agreement with previous studies [19], animals wearing −7 D lenses and injected with insulin not only became myopic but also overcompensated the negative lenses. The mean change in refraction was −6.01±0.68 D in the saline-injected group and −9.05±1.15 D in the insulin-injected animals, with a difference of 3.04±1.65 D (95% confidence interval [CI], 0.54 D; 5.55 D, p=0.019). This effect was similar when only the MEK inhibitor was injected or the MEK inhibitor in combination with insulin (difference 3.53 D, 95% CI, 1.03 D; 6.04 D, p=0.007). In contrast, insulin plus PI3K inhibitor–injected eyes did not become more myopic than eyes injected with only PI3K inhibitor. In those cases, the eyes compensated for the power of the negative lenses as normal.

Effects of intravitreal injections on ocular dimensions

In animals not wearing any lenses, the injections had no significant effect on the growth of the anterior chamber, the crystalline lens, and axial length (Figure C2.2). Vitreous chamber depth (VCD) was significantly longer in Ly294002-injected eyes than in most other injection groups. Nevertheless, this difference in vitreous chamber depth was small (VCD changes: saline: 0.14±0.03 mm; Ly294002: 0.24±0.04 mm) and was not correlated with a change in refraction (see Figure C2.1).

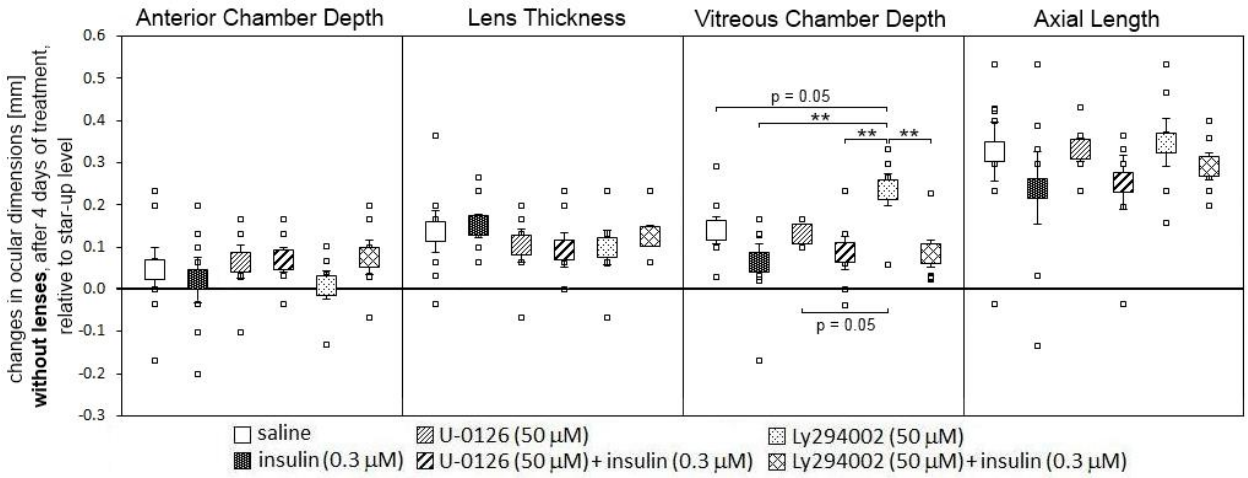

Figure C2.2 Effects of intravitreal saline, insulin, U-0126 and Ly-294002 injections on the ocular compartments in chicks without any lenses. The large squares represent the means of the changes ± SEM from the beginning of the experiment and 4 days later. The small squares denote data from individual eyes. Six animals per group were used. Animals injected with Ly-294002 had deeper vitreous chambers compared with all the other types of injections. No significant changes were observed in anterior chamber depth, lens thickness and axial length. Statistically significant differences, as determined by one-way ANOVA, are denoted in the graph (* $p < 0.05$, ** $p < 0.01$).

No significant changes were detected in the anterior chamber depth (ACD) in animals treated with positive lenses and injected with different drugs (Figure C2.3). Insulin-injected eyes showed a trend of developing thicker lenses, compared with saline-injected eyes, and this effect was even larger and reached significance in eyes that were injected with insulin in combination with U0126 or Ly294002. The same changes

were observed for axial length where the effect of insulin alone was not significant but where insulin in combination with both inhibitors induced a significant increase in axial length compared to saline-injected eyes. Similar to previous studies, positive lens treatment inhibited vitreous chamber growth in saline-injected eyes [19]. When injected with insulin, VCD did not significantly differ from the saline-injected group in this experiment (Figure C2.3). A significant suppression of the effects of positive lenses on VCD was seen only in animals injected with U0126. Those eyes had significantly longer VCDs than saline-injected eyes (Table C2.2, VCD after four days of positive lens treatment; saline versus U0126: 4.94±0.07 mm versus 5.08±0.08 mm, $p<0.05$). The longer axial length in eyes injected with insulin plus Ly294002 compared to saline-injected eyes correlated with a more myopic refraction. However, although the eyes injected with insulin plus MEK inhibitor U-0128 were significantly longer than the saline-injected eyes, their refractions, as shown in Figure C2.2, were not significantly different from the saline group.

Animals treated with negative lenses and injected with a combination of insulin and the MEK inhibitor U0126 had significantly deeper ACDs, thicker crystalline lenses, and longer axial lengths, compared with animals injected with U0126 or Ly294002 alone (Figure C2.4). In addition, they had a trend of a longer ACD compared with insulin-injected animals ($p=0.05$), a thicker lens compared with the eyes injected with insulin and insulin plus Ly294002, and longer axial length than the eyes injected with saline and Ly294002 plus insulin. The highest axial length in the eyes injected with insulin plus U0126 correlated with the highest amount of myopia measured in this group (see Figure C2.1). However, the injections had no significant effect on the growth of the VCD in minus lens–treated animals.

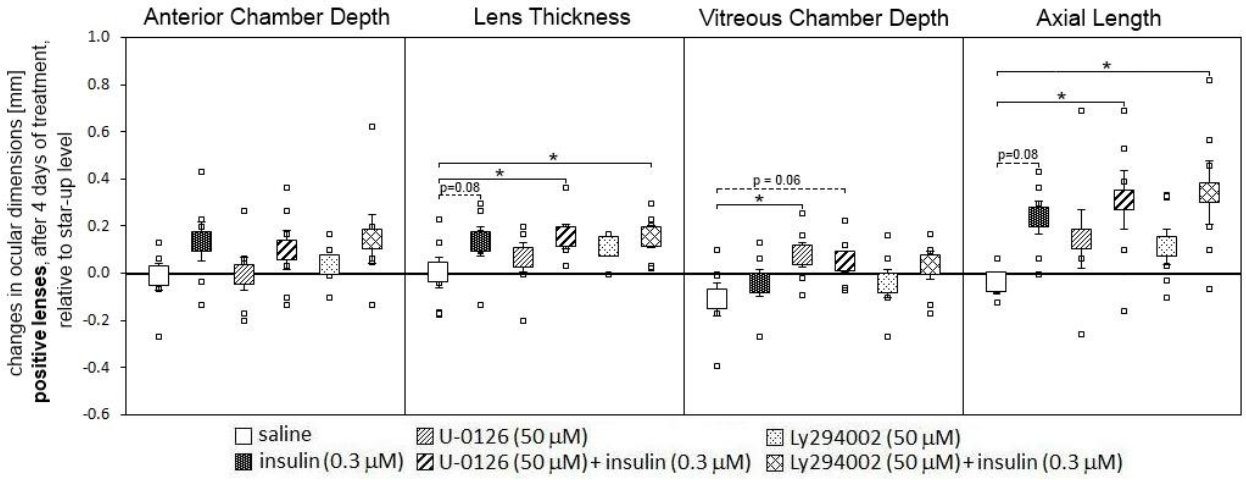

Figure C2.3 Effects of intravitreal saline, insulin, U-0126 and Ly-294002 injections on the ocular compartments in chicks treated with positive lenses during 4 days. The large squares represent the means of the changes ± SEM from the beginning of the experiment and 4 days later. The small squares denote data from individual eyes. Six animals per group were used. Statistically significant differences, as determined by one-way ANOVA, are denoted in the graph (* $p < 0.05$, ** $p < 0.01$).

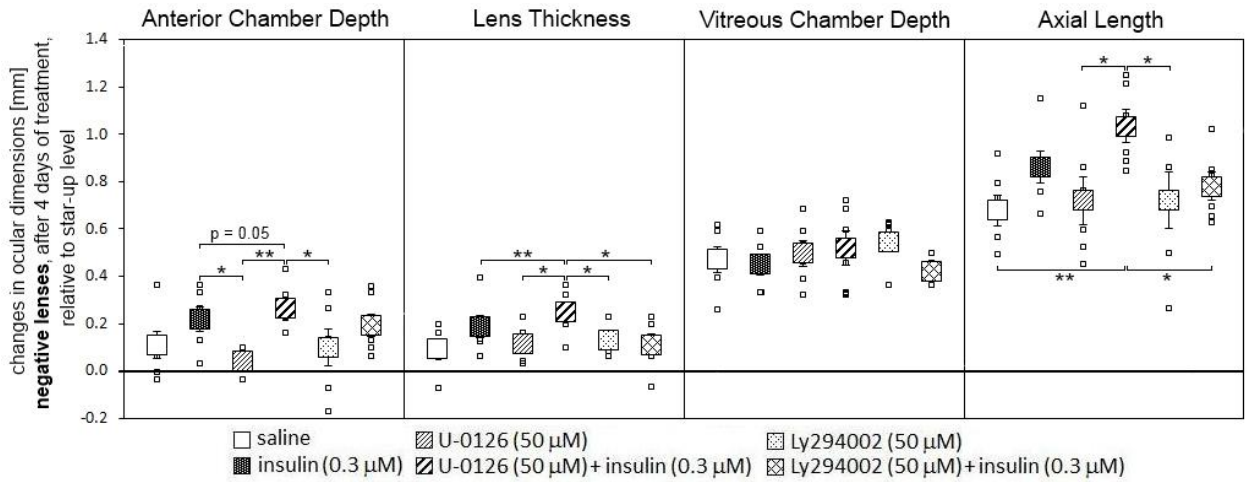

Figure C2.4 Effects of intravitreal saline, insulin, U-0126 and Ly-294002 injections on the ocular compartments in chicks treated with negative lenses during 4 days. The large squares represent the means of the changes ± SEM from the beginning of the experiment and 4 days later. The small squares denote data from individual eyes. Six animals per group were used. Statistically significant differences, as determined by one-way ANOVA, are denoted in the graph (* $p < 0.05$, ** $p < 0.01$).

Effects of short- and long-term lens wear, combined with intravitreal injections of insulin, U0126, and Ly294002 on extracellular regulated kinase 1/2 and protein kinase B phosphorylation levels

Western blots were used to find out whether insulin activates the MEK/ERK and/or PI3K/Akt pathway in the retina. A second question was whether lens treatment per se can activate one of those pathways. The MEK inhibitor U0126 and the PI3K inhibitor Ly294002 were used alone (Figure C2.5A) or in combination with insulin (Figure C2.5B).

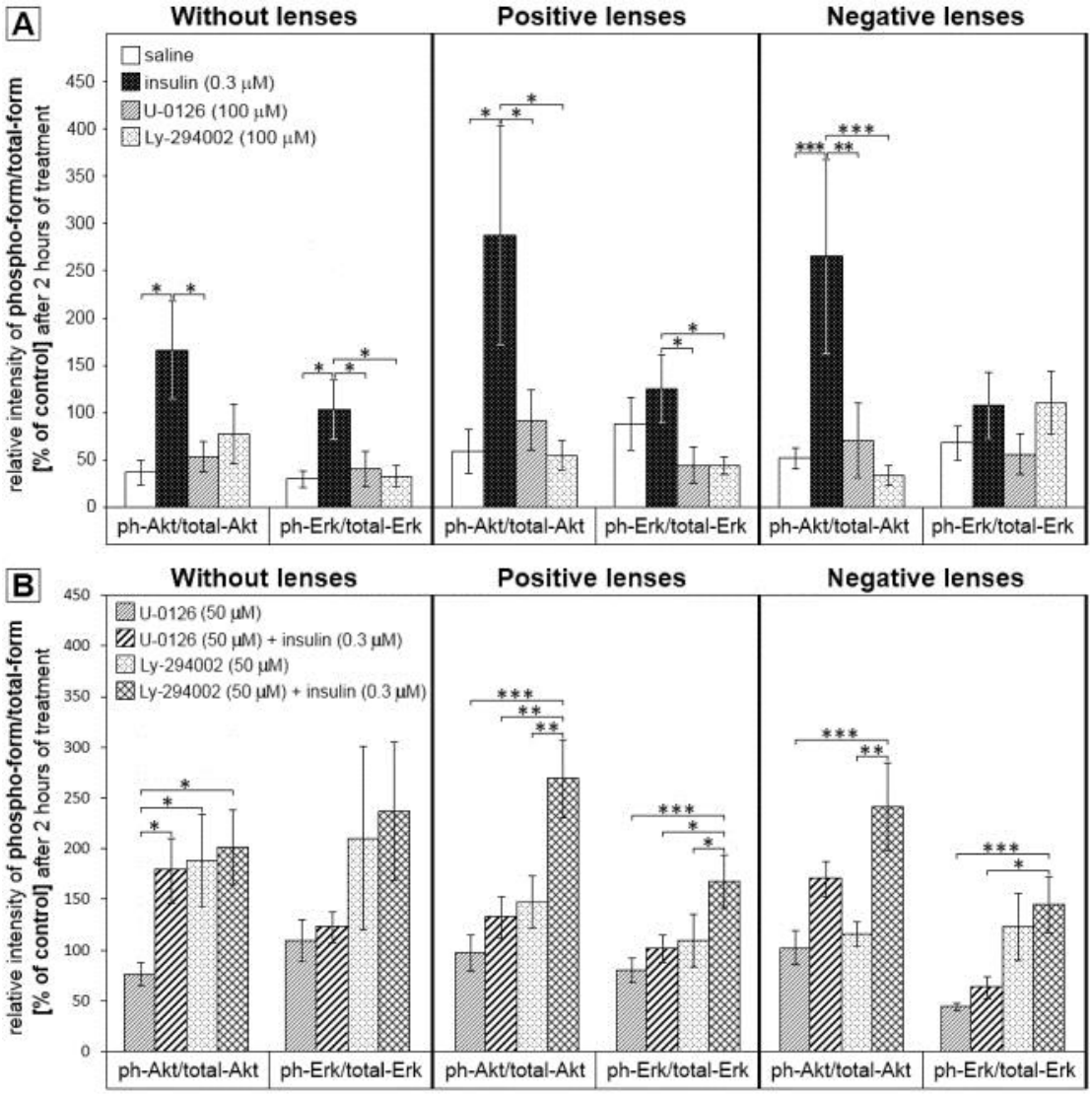

Figure C2.5 Short time effects (2 h) of intravitreal saline, insulin, mitogen-activated protein kinase kinase inhibitor U0126, U0126 plus insulin, phosphatidylinositol 3-kinase inhibitor

Ly294002, or Ly294002 plus insulin injections on phospho-Akt/total-Akt and phospho-Erk/total-Erk levels in chicks not wearing any lenses, and after bilateral treatment with +7 D: lenses and −7 D: lenses. The same independent control sample was analyzed on each blot and used for normalization of the data. Band intensities were converted to percentages of the control sample value. The ratio of phospho-form/total-form was then calculated using the normalized values. The bars represent the mean ratio of relative intensity (mean±SEM) between the phospho-forms and non-phosphorylated proteins in percent. Each group included six animals. In control, positive and negative lens–wearing animals (Figure 5A) insulin injections highly increased the retinal ratio of phospho-Akt/total-Akt. In control and animals wearing positive lenses, insulin injections led to an increase in phospho-Erk/total-Erk levels, compared to either the MEK inhibitor or the PI3K inhibitor (Figure 5A). The PI3K inhibitor blocked the stimulatory effect of insulin in control animals without lenses but not in positive and negative lens–treated animals (Figure 5B). The MEK inhibitor significantly blocked the effect of insulin on ERK phosphorylation (Figure 5B). Significant differences were determined with one-way ANOVA and are denoted in the graph (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

As shown in Figure 5A, intravitreal insulin injections stimulated both pathways. The retinal level of phospho-Akt/total-Akt was significantly elevated, compared to the saline-injected and MEK inhibitor–injected eyes in animals without lens treatment (Figure 5A). The insulin-induced activation of Akt was even more pronounced in animals wearing positive lenses and negative lenses. Here, insulin caused a more than threefold increase in phospho-Akt/total-Akt levels, compared to all other injection groups. Insulin activated not only the PI3K/Akt pathway but also, to a lesser extent, the MEK/ERK pathway. In animals without lenses, significant changes in phospho-ERK/total-ERK levels were induced by insulin compared with all other injection groups. Insulin injections also led to a significant increase in phospho-ERK/total-ERK levels in animals wearing positive lenses, compared to U0126- and Ly294002-injected eyes (Figure 5A). In contrast, no insulin-induced activation of this pathway was detected in negative lens–treated eyes (Figure 5A). The inhibitors U0126 and Ly294002 alone did not significantly influence the retinal level of phospho-Akt and phospho-ERK compared to saline-injected eyes in animals without lenses or in chicks treated with positive and negative spherical lenses (Figure 5A). In addition, positive and negative lens treatment per se did not

significantly alter the amount of phospho-Akt/total Akt or of phospho-ERK/total ERK (one-way ANOVA, saline injections: no lens wear versus plus lens wear versus minus lens wear, n.s.).

Furthermore, insulin was injected in combination with either U0126 or Ly294002 to test the efficiency and specificity of the inhibitors in vivo (Figure 5B). We already knew from our studies (Figure 5A) that insulin stimulated the PI3K/Akt pathway in chicks without lenses, as well as in chicks with positive lenses, and with negative lenses. We therefore expected that the same amount of phospho-Akt/Akt would be found in eyes injected with PI3K inhibitor plus insulin, compared to the inhibitor-injected fellow eyes, as long as the inhibitor worked. Inhibitor concentrations were chosen based on a study by Chavarria et al. in organotypic chicken retinal cell cultures [50]. With no lens treatment, the amount of phospho-Akt/Akt in the Ly294002-injected eyes versus the insulin plus Ly294002-injected eyes was nearly identical, showing that the PI3K inhibitor blocked the stimulatory effect of insulin (Figure 5B, third and fourth bars). In contrast, Ly294002 did not block the effects of insulin in positive and negative lens–treated animals.

Based on our results (Figure 5A), we also expected to see the same amount of phospho-EKR/ERK in the groups injected with MEK inhibitor plus insulin compared to their contralateral insulin-injected eyes in animals with no lenses, or with positive lenses, if the inhibitor worked. Indeed, the MEK inhibitor U0126 blocked the effect of insulin on ERK phosphorylation (Figure 5B, fifth versus sixth bar, 13th versus 14th bar). Since insulin did not stimulate phosphorylation of ERK in the negative lens–treated group (Figure 5A), we could not study the potency of the MEK inhibitor in those retinas.

An effect of the PI3K inhibitor on insulin-induced ERK phosphorylation and of the MEK inhibitor on insulin-induced Akt phosphorylation was not expected, unless both pathways interacted and controlled the phosphorylation level of each other's components. This was indeed found in some groups. The MEK inhibitor U0126 did not block the insulin-induced increase in Akt phosphorylation in eyes without lenses (Figure 5B, first and second bars). This result therefore does not support a cross-talk between the pathways. In contrast, the MEK inhibitor blocked the insulin effect on Akt phosphorylation in plus and minus lens–treated animals (Figure 5B: phosphoAKt/Akt,

plus lens, U0126 versus insulin plus U0126: n.s., U0126 versus insulin plus Ly294002: $p<0.001$). The MEK inhibitor was even more potent than the PI3K inhibitor itself (Figure 5B: phosphoAkt/Akt, plus lens: insulin plus U0126 versus insulin plus Ly294002, $p<0.01$). The potency of the PI3K inhibitor on ERK phosphorylation depended on the lens treatment. The PI3K inhibitor Ly294002 did not block the insulin-induced increase in ERK phosphorylation in eyes treated with plus lenses but in animals without lenses.

After four days of lens treatment, and injections every other day, the retinal levels of phospho-Akt/total-Akt and phospho-ERK/total-ERK were not significantly different and seemed to have a ceiling (data not shown).

DISCUSSION

After ligand binding, the insulin receptor can recruit and phosphorylate IRS proteins. Depending on the stimuli and the phosphorylated IRS, two different pathways are activated: the PI3K/Akt and MEK/ERK pathways. Although intravitreal insulin [19,20] and insulin-like growth factor-1 (IGF-1) [20] can induce myopia in chicks, it is not known if both or only one of the two signaling pathways is responsible for this effect. Therefore, in the present study, we attempted to find out how inhibiting either pathway affects eye growth. The effects of insulin and two specific pathway inhibitors were investigated under different visual conditions (normal vision or treatment with positive or negative lenses).

The current study showed that insulin and PI3K and MEK inhibitors induced only small changes in refractive states in eyes with normal visual exposure. Both pathways were activated because the levels of phospho-Akt/Akt and phospho-ERK/ERK were significantly increased in insulin-injected eyes, and the specific inhibitors blocked these stimulatory effects of insulin.

As soon as lenses were used to defocus the retinal image, much larger effects of insulin on refractive development and on the biometry of the eye were seen. Insulin prevented the compensation of positive lenses, in line with previous findings [19]. Although the PI3K inhibitor did not block this effect of insulin, the MEK inhibitor did. This suggests that the effect of insulin on positive lens compensation is likely to be mediated by activation of the MEK/ERK pathway. The effects of the MEK inhibitor on the development of the refractive state were correlated with high amounts of phospho-ERK/total-ERK in the insulin-injected, positive lens–treated eyes compared to the PI3K or MEK inhibitor–injected eyes and with an effective block of ERK activation in eyes in which insulin was injected together with the MEK inhibitor. The changes were seen in western blots after 2 h, emphasizing the importance of this pathway when positive lenses are compensated. In addition, the PI3K/Akt pathway was activated in plus lens–treated animals, but the PI3K inhibitor did not completely block this activation.

In agreement with previous studies [19], insulin induced not only myopia but also overcompensation of the power of the lenses in animals wearing negative lenses. Only the PI3K inhibitor reduced the insulin-induced overcompensation. Western blots revealed that insulin did not significantly influence the MEK/ERK pathway but highly increased the amount of retinal phospho-Akt/total-Akt in animals treated with negative lenses for 2 h. These results emphasize the importance of the PI3K/Akt pathway when positive defocus is imposed and insulin concentrations are high. Although the PI3K inhibitor reduced the insulin effect in minus lens–treated chicks, the inhibitor did not completely block the activation/phosphorylation of Akt. Therefore, the doses used in our study may not have been high enough to completely block the pathway. There may also be a direct interaction between both pathways because the MEK inhibitor was more potent in decreasing the phosphorylation of Akt in plus and minus lens–treated animals than the PI3K inhibitor itself.

Changes in refractive errors did not always correlate with changes in biometry. In particular, insulin injections caused a similar increase in axial length and lens thickness in combination with both inhibitors in plus lens–treated animals, but the refractions were different. High variability in the ocular components might have combined to different refractive outcomes, in particular in the data of the MEK inhibitor–injected eyes. In addition, changes in corneal curvature were not considered. In animals treated with negative lenses, the refractive changes were in general in agreement with the changes observed in the biometry of the eyes. The group that developed the highest amount of myopia (insulin plus MEK inhibitor injection) also had the longest axial length, the deepest anterior chamber, and the thickest lens.

In summary, the current study may show that insulin activates the PI3K/Akt pathway in eyes with normal vision and eyes treated with positive and negative lenses but stimulates eye growth through the MEK/ERK pathway in eyes with normal vision and eyes treated with positive lenses. As has been concluded from studies of other signaling pathways, the current study adds support to the idea that positive and negative lenses activate different biochemical pathways.

Based on our findings, we propose the following hypothesis. Since Egr-1 is an immediate early gene that responds to defocus of different sign in opposite directions and has an important role in emmetropization [19,24,26,27], we assume that the changes represent the initial event when changes in eye growth occur. We propose that Egr-1 controls the balance between the PI3K/Akt and MEK/ERK pathways. There is already evidence from other systems that the preferential activation of the PI3K/Akt and MEK/ERK pathways is mediated by Egr-1. In these studies, Egr-1 was hypothesized to be an important regulator of insulin sensitivity by controlling the balance between these pathways in mice [56,57]. Intravitreal injections of insulin had powerful stimulatory effects on Egr-1 mRNA levels in eyes with normal vision and eyes treated with positive lenses. There was increased Egr-1 protein expression in Müller cells and bipolar cells [19]. Furthermore, insulin reduced the number of ZENK-positive glucagon amacrine cells in positive lens–treated eyes. This is opposite of what is normally observed in eyes with positive lenses but seen in eyes that develop myopia.

Recent studies showed that under hyperinsulinism stress, Egr-1 is able to control the balance between the Akt and MAPK pathways in fat tissue by inducing the expression of downstream genes involved in the pathways. Sustained expression of Egr-1 can stimulate phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) and geranylgeranyl diphosphate synthase (*GGPPS*) transcription [56]. Increased *PTEN*blocks PI3K/Akt signaling and thus impairs insulin-induced glucose metabolism. Meanwhile, increased *GGPPS* causes sustained ERK1/2 activation. If the same mechanism operated in the eye, this would support our hypothesis that eye growth induced by insulin injections follows a change in the Egr-1-mediated balance between the PI3K/Akt and MEK/ERK pathways. Since we know that positive lens treatment also leads to an increase in Egr-1, this could cause sustained ERK activation.

In addition, other downstream molecules from both pathways need to be studied under different visual conditions to confirm our hypothesis. A microarray study [58] showed that 24 h of positive lens treatment induced upregulation of growth factor receptor-bound protein 2 (*Grb2*), which is implicated in the insulin pathway, by activating the MAPK pathway through the interaction with Ras and the son of sevenless [59]. In that

study, the authors found upregulation of *Grb2* in negative and positive lenses–treated animals although the upregulation was stronger with negative lenses.

Cordain et al. [60,61] proposed that a change in nutrition toward a higher intake of refined carbohydrate and sugar might be responsible for the dramatic increase in myopia: diets high in refined carbohydrates are increasing glucose levels (hyperglycemia) and consequently insulin levels (hyperinsulinemia), and insulin resistance, which, in turn, induces growth of the eyeball. One could speculate that a sustained hyperinsulinism, stimulated by insulin and enhanced by insulin resistance [56], could impair the balance between PI3K/Akt and MEK/ERK signaling.

CONCLUSION

Our study suggests that insulin stimulates the PI3K/Akt pathway under all visual conditions. In contrast, the MEK/Erk pathway was preferentially stimulated only in chicks with normal visual exposure and in chicks treated with positive spectacle lenses. The MEK/Erk pathway was not stimulated in chickens treated with negative spectacle lenses. Identifying key regulators that control the balance between the two signaling pathways may provide a new strategy for pharmacological intervention of myopia.

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PROJECT 3:

Ultrasonography and optical low-coherence interferometry compared in the chicken eye

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Purpose: To compare ocular biometry (anterior chamber depth (ACD), lens thickness (LT), vitreous chamber depth (VCD) and axial length (AL) using 3M Echorule and the Lenstar LS900 in the chicken eye.

Methods: Two-week old chicks (n=42) were measured. Bland-Altman plots, repeatability and correlation analyses were calculated for both methods.

Results: There was a high correlation between both methods for ACD ($r = 0.6144$, $p < 0.0001$), VCD ($r = 0.9595$, $p < 0.0001$) and AL ($r = 0.9290$, $p < 0.0001$), but not for LT ($r = 0.1604$, $p = 0.144$). Measurements of the Lenstar were more consistent (smaller coefficients of variation, higher intraclass correlation). Bland-Altman plots showed that ultrasound provided larger values for LT, VCD and AL, but not for ACD (differences between Echorule and Lenstar (mean ± SD): ACD = -0.11 ± 0.12 mm; LT = 0.10 ± 0.09 mm; VCD = 0.25 ± 0.08 mm; AL = 0.50 ± 0.16 mm).

Conclusions: A high correlation between both instruments was found for three of the four parameters (ACD, VCD, AL). However, since the absolute values were different, both techniques cannot replace each other mainly because: 1) one is non-contact and the other contact and can induce a minor indentation of the cornea; 2) each device uses different types of waves that cross the ocular interfaces differently. While consistency and repeatability were better with the Lenstar, a disadvantage is that, different from humans, it can only be used in anesthetized chicks.

Keywords: A-scan ultrasound, optical low coherence interferometry, chicken, biometry

INTRODUCTION

The chicken is an important model in myopia research which has been extensively used since the classical paper by Wallman and colleagues[1]. To measure changes in axial length (AL), anterior chamber depth (ACD), lens thickness (LT) and vitreous chamber depth (VCD) that are induced by alterations in visual experience, A-scan ultrasonography is the common technique [2, 3]. High frequency sound waves (typically 10 MHz) are emitted by the probe tip at short pulses, and the transducer is rapidly switched into receive mode to record echoes that bounce back as the sound beam strikes different ocular interfaces. Because sound waves travel the faster the denser the media, the time delay until the echo is received is not directly proportional to the intraocular distances [4]. Velocity of sound is determined by density, compressibility and, to a minor extent, by the temperature of the medium through which the sound travels. Related to these properties, sound travels faster in solids than liquids and there is also a clear difference of the speed of sound in the different ocular media which need to be known to derive the correct linear distances between the intraocular surfaces. The various commercially available ultrasound devices convert time delays into linear distances using assumption about the speed of sound in the ocular media. Since the systems are optimized to provide most reliable data in humans, they need calibrations for different animal eyes. Another drawback is that the ultrasound transducer touches the cornea and may cause a minor indentation.

Optical low coherence interferometry is an alternative method for ocular biometry in vivo. Optical path lengths are determined by the refractive indices of the media. The speed of light in a given medium, in turn, can be derived from refractive index which can be determined from the angle of refraction of a light ray at the border to a medium with known refractive index, like air. Dividing the speed of light in vacuum by the refractive index provides the speed in the given medium. Therefore, to convert optical path length into geometrical path lengths, the travel time of light needs to be multiplied by refractive index. Recently, Haag-Streit (Haag-Streit, Koeniz, Switzerland) developed a new non-invasive and non-contact optical low-coherence interferometer (OLCI), the Lenstar LS 900. The Lenstar LS900 already performs the conversion of optical path length into

geometrical path length, assuming refractive indices that are typical for ocular media. In addition to ACD, LT, VCD and AL, the Lenstar LS900 also provides pupil diameter and central cornea curvature, as well as corneal thickness, white-to-white distance (horizontal iris width), retinal thickness and, sometimes, choroidal thickness, at least in human eyes. Since the measurements are optical, no contact to the cornea is necessary. The accuracy of A-scan ultrasonography in the chicken eye is not known and has been scarcely studied (except for [3]) because the primary interest of most researchers lies on measuring changes in ocular dimensions rather than absolute values. The purpose of the current study was to compare A-scan ultrasonography and optical low coherence interferometry (OLCI) in the chicken eye. Since no gold standard for the measurements is available, no data on the accuracy of either machine can be provided. Bland-Altman statistics was used to evaluate precision and to compare the measurements by both devices.

MATERIAL AND METHODS

Treatment of animals

Two weeks old chicks (n = 42) were monocularly treated with occluders for 5 days and allowed to recover for the following 72 hours. We used eyes that had undergone variable treatments. Therefore the ocular dimensions were variable which made it easier to judge the performance of A-scan ultrasonography in comparison to OLCI. The experimental treatment was in accordance with the ARVO statement for Care and Use of Animals in Ophthalmic and Vision Research and was approved by the university commission for animal welfare. ACD, LT, VCD and AL of experimental and untreated contralateral eyes were measured before the treatment, after 5 days of occluder wear and at six different time points during the recovery period.

A-scan applanation ultrasound and OLCI

Each animal was measured in duplicate by the same person with both techniques. For ultrasound measurements one or two drops Xylocain® 2% (AstraZeneca GmbH, Wedel,

Germany) were administered to the cornea for local anesthesia. In A-scan ultrasonography, a soft rubber tube, filled with water and covered with paramount film, with about 8 mm length and 3 mm diameter was attached to the transducer. This procedure improved the measurement in the chicken eye as previously shown [2]. In young chicks (1-2 weeks old), measurements with OLCI were possible only under anesthesia because awake animals closed their eyes already when the operator tried to align the eye with the device. In older chickens (2-3 weeks), a few measurements could be completed without but, for routine measurements, the success of the procedure appeared too uncertain. Therefore, the animals were intramuscularly injected with Rompun® (Bayer Healthcare, Germany) 1 µl/g of body weight and the eyes were kept open with lid retractors as used in pediatrics. The Lenstar was aligned with the eyes by centering the pupil. During data collection, at least two scans were taken and cursor positions were adjusted manually before averaging. Cursors needed to be set manually because the Lenstar software could not automatically assign the peaks to the different ocular structures.

Data analysis

Pearson's correlation coefficient (r) was used to quantify correlations between both methods. Furthermore, to find out whether there were systematic variations in the differences between both techniques, the method described by Bland and Altman [5] was employed. To this end, differences between the values obtained with both methods were plotted against their averages. The 95% limits of agreement were defined as mean ± 1.96 standard deviations (SD) of the differences between both methods [5]. To estimate repeatability of the measurements, the mean standard deviation between two consecutives measurements (SD^{within}) and the coefficient of variation (CV) (ratio of the SD^{within} to the mean) were calculated. Lower SD^{within} and CV values indicate better repeatability. The consistency of the methods was evaluated by calculating the intraclass correlation coefficient (ICC). The ICCs range from 0 to 1 and a value of 0.9 or more indicates high agreement [5]. A p value of < 0.05 was considered significant.

RESULTS

Reproducibility of measurements with A-scan ultrasound and OLCI

Table 1 shows the intra-observer reliability for measurements of ACD, LT, VCD and AL for both techniques, the A-scan ultrasound and the OLCI. Each eye was measured twice with each instrument. OLCI measurements of the same eyes showed less variation than the A-scan ultrasound in all cases, being smallest for the AL with a CV of 0.22% and the largest for ACD with 0.96%. With the A-scan ultrasound, the smallest variation was detected in VCD measurements, followed by AL, LT and ACD with a CV of 0.58%, 0.71%, 2.35% and 5.48%, respectively. The ICC values showed that especially LT and ACD measurements were more consistent with OLCI than with the A-scan ultrasound.

Table C3.1 Intra-observer reliability from two consecutive measurements using A-scan Ultrasound (3M Echorule) and OLCI (Lenstar LS900).

Variable [mm]	Method	SD^{within} (SD)	CV (SD) [%]	ICC
Anterior Chamber Depth	3M Echorule	0.066 (0.059)	5.48 (4.94)	0.652
	Lenstar	0.013 (0.009)	0.96 (0.69)	0.995
Lens Thickness	3M Echorule	0.056 (0.055)	2.35 (2.28)	0.492
	Lenstar	0.010 (0.016)	0.43 (0.70)	0.953
Vitreous Chamber Depth	3M Echorule	0.033 (0.038)	0.58 (0.67)	0.972
	Lenstar	0.016 (0.029)	0.30 (0.57)	0.994
Axial Length	3M Echorule	0.066 (0.064)	0.71 (0.68)	0.954
	Lenstar	0.019 (0.034)	0.22 (0.39)	0.992

SD – standard deviation; $\mathbf{SD^{within}}$ – mean standard deviation between two consecutives measurements; **CV** – coefficient of variation; **ICC** – intraclass correlation coefficient

Agreement of measurements

The results of the pair-wise comparisons and the correlation between A-scan ultrasound and low coherence interferometry measurements, including mean differences, Pearson

correlation factors (r) and 95% confidence intervals for the correlations, are shown in Table 2. All pair-wise correlations, except for LT, were highly significant ($p < 0.0001$).

Table C3.2 Pair-wise comparisons and correlations between A-scan ultrasound and OLCI measurements, with corresponding 95% confidence intervals and significance levels.

Variable [mm]	Method	Overall mean (SD)	Mean difference* (SD)	Pearson correlation coefficient (r)	95% confidence intervals	*p* value
ACD	3M Echorule Lenstar	1.21 (0.13) 1.33 (0.15)	-0.11 (0.12)	0.6144	0.320 to 0.583	<0.00001
LT	3M Echorule Lenstar	2.36 (0.09) 2.25 (0.05)	0.10 (0.09)	0.1607	-0.023 to 0.151	0.1442
VCD	3M Echorule Lenstar	5.62 (0.29) 5.37 (0.26)	0.25 (0.08)	0.9595	0.600 to 0.747	<0.00001
AL	3M Echorule Lenstar	9.19 (0.41) 8.69 (0.42)	0.50 (0.16)	0.9290	0.454 to 0.624	<0.00001

ACD – Anterior Chamber Depth; **LT** – Lens Thickness; **VCD** – Vitreous Chamber Depth; **AL** – Axial Length; *** mean difference** = overall mean ultrasound – overall mean lenstar

The average ACD was 1.21 ± 0.13 mm with A-scan ultrasound and 1.33 ± 0.15 mm with OLCI (Table 2). Although there was a high correlation between both methods (Pearson correlation coefficient (r) = 0.6144; *p* value < 0.0001; Figure 1A) the Bland-Altman plot (Figure 1B) illustrates that A-scan ultrasound measured shallower ACDs than OLCI. This difference in ACD between both methods was significant (Table 2: paired t-test: ACD A-scan ultrasound vs. ACD OLCI: $p < 0.0001$).

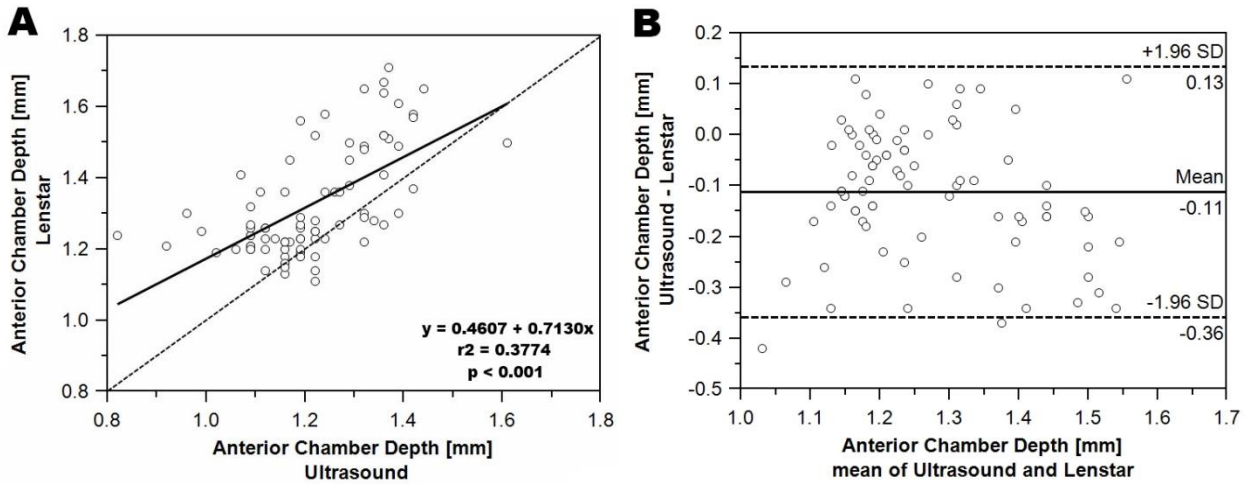

Figure C3.1 Anterior chamber depth as measured by A-scan ultrasound using the 3M Echorule device and by OLCI using the Lenstar LS 900. A – Correlation data between A-scan ultrasonography and OLCI. There was a high correlation between both methods; B – Bland-Altman plot.

The same type of analysis was performed for LT measurements. The mean of LT as measured by A-scan ultrasound was 2.36 ± 0.09 mm and for the OLCI it was 0.10 ± 0.09 mm thinner (Table 2). This difference was significant (Table 2: paired t-test: LT A-scan ultrasound vs. LT OLCI: $p < 0.0001$). Figure 2A shows that there was no significant correlation between the measurements performed with the two methods in the case of LT measurements (Table 2: r = 0.1607, *p* value = 0.1442), and the differences tended to become larger as the measured values increased (Figure 2B).

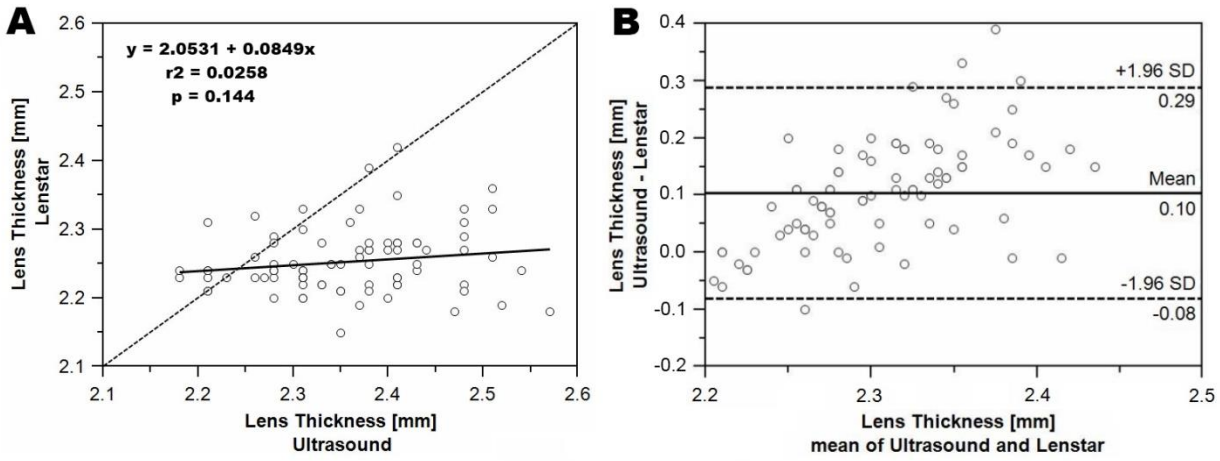

Figure C3.2 Lens thickness as measured by A-scan ultrasound using the 3M Echorule device and by OLCI using the Lenstar LS 900. A – Graph shows the correlation between 3M Echorule and Lenstar. There was no significant correlation between both methods; B – Bland-Altman plot.

For ultrasound measurements the VCD was calculated from the distance of the posterior lens surface to the vitreo-retinal interface. The mean value for VCD using this method was 5.62 ± 0.29 mm. Corneal thickness, anterior chamber depth, and lens thickness had to be subtracted from axial length data to obtain the VCD in the OLCI measurements. Correlation analysis revealed a highly significant correlation between the VCD measurements with both devices (Table 2: r = 0.9595, *p* value < 0.0001). However, linear regression analysis showed that the A-scan ultrasound measured deeper vitreous chambers than the OLCI (Figure 3A). This difference in VCD values was significant (Table 2: paired t-test: $p < 0.0001$).

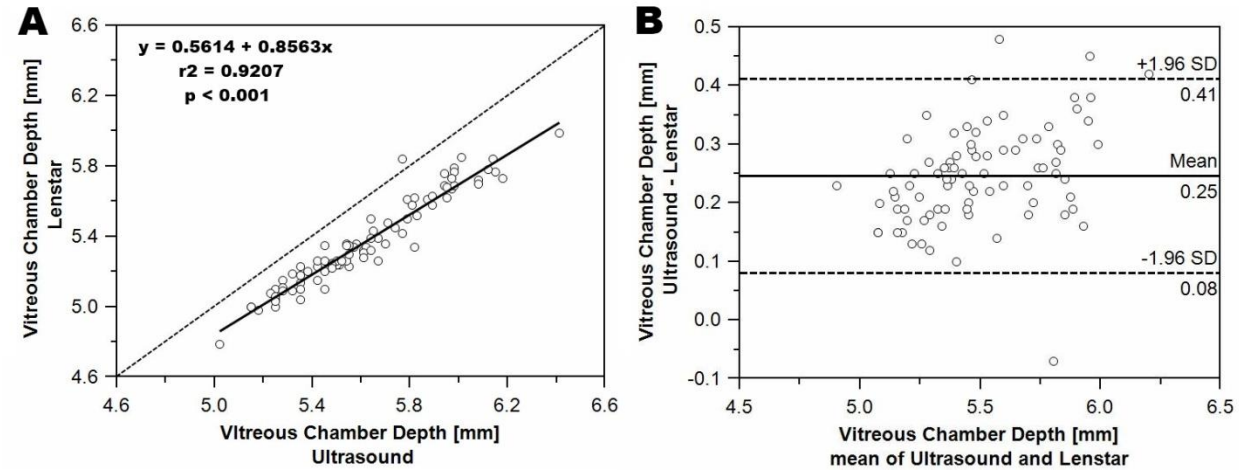

Figure C3.3 Vitreous chamber depth as measured by A-scan ultrasound using the 3M Echorule device and by OLCI using the Lenstar LS 900. A – Correlation data between A-scan ultrasonography and OLCI. There was a tight correlation between both methods. Nevertheless, the 3M Echorule measured deeper vitreous chambers than the Lenstar. B – Bland-Altman plot.

Axial length measurements with the two devices were highly correlated (Table 2, Figure 4A). A mean of 9.19 ±.41 mm was measured with the ultrasound technique compared with a mean of 8.69 ± 0.42 mm obtained with the OLCI (Table 2). This difference in AL was highly significant (paired t-test: $p < 0.0001$, Figure 4B).

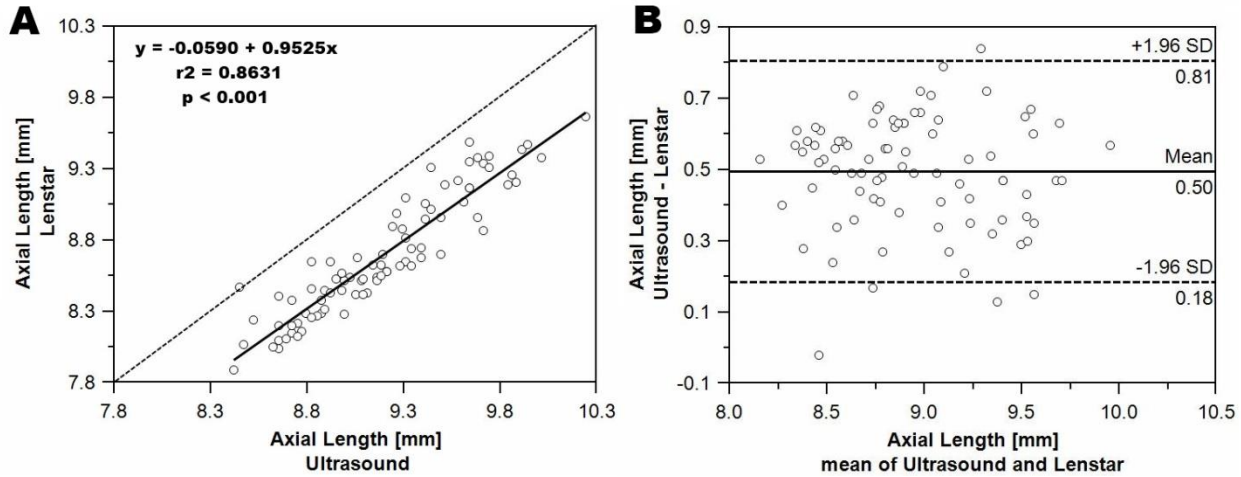

Figure C3.4 Axial lengths as measured by A-scan ultrasound using the 3M Echorule device and by OLCI using the Lenstar LS 900. A – Correlation data between A-scan ultrasonography and OLCI. There was a tight correlation between both methods.. B – Bland-Altman plot.

A-scan ultrasonography is the most commonly used technique to measure changes in ocular parameters that are induced by alterations in visual experience in chicks. Chicks were unilaterally deprived for 5 days and allowed to recover for 3 days. The untreated contralateral eyes served as control. Table 3 shows a comparison of the treatment effects using ultrasonography (3M Echorule) and optical low-coherence reflectometry (Lenstar LS 900).

Table 3 Effect of deprivation (5 days) and recovery (3 days) on ocular parameters as measured by A-scan ultrasound (3M Echorule) and by OLCI (Lenstar LS900).

Variable [mm]	Technique	Mean difference exp – control after 5 days of deprivation (SD)	*t*-test for differences after 5 days of deprivation	Mean difference exp – control after 3 days of recovery (SD)	*t*-test for differences after 3 days of recovery
ACD	Ultrasonography	0.16 (0.05)	0.454	0.11 (0.10)	0.222
	OLCI	0.21 (0.07)		0.20 (0.05)	
LT	Ultrasonography	0.09 (0.03)	0.318	-0.02 (0.07)	0.668
	OLCI	0.03 (0.01)		-0.05 (0.01)	
VCD	Ultrasonography	0.72 (0.11)	0.090	0.18 (0.01)	0.237
	OLCI	0.63 (0.07)		0.15 (0.02)	
AL	Ultrasonography	1.00 (0.13)	0.838	0.27 (0.06)	0.761
	OLCI	1.02 (0.12)		0.28 (0.07)	

ACD – Anterior Chamber Depth; **LT** – Lens Thickness; **VCD** – Vitreous Chamber Depth; **AL** – Axial Length; *** mean difference** = overall mean ultrasound – overall mean OLCI

Consistent with previous studies, diffusers induced an increase in axial length and vitreous chamber depth, which declined during the 3rd day of recovery period. The treatment effect, as measured with A-scan ultrasound and the OLCI (Table 3) was very similar (no significant difference between both methods).

DISCUSSION

A-scan ultrasonography is a well-established biometric technique that has been used for a long time to measure chicken eyes in myopia studies [2, 3]. Although it is the standard technique its accuracy in not known.

We have studied whether OLCI could be an alternative technique because it represents a non-contact measurement and because it is potentially more accurate. To our knowledge this is the first study on reproducibility and agreement with another biometrical technique in the chick. Consistent with studies in humans [6], a better repeatability was found for the OLCI compared to the A-scan ultrasound, especially for anterior chamber depth and lens thickness (lower coefficient of variation, higher intraclass consistency). This difference could be explained by the higher spatial resolution of OLCI compared to sound waves. A-scan is limited by the wavelength of sound which is about 0.15 mm for a 10 MHz probe (speed of sound in water 1484 m/sec). OLCI is limited by the coherence length of the (infrared) light used for measurements, which is determined by its bandwidth: coherence length = $2 * \ln(2) * \lambda^2/(\pi * n * \Delta\lambda)$, which is also in the range of 0.1 mm. However, measurements are much faster and 16 individual waveforms are averaged in a 2 second time window that is used for measurement. Probably for this reason, the variability of readings from subsequent measurements is much less. Another variable affecting repeatability is the alignment of the ultrasound probe which is done by the operator based on experience. This includes also some variability of the mechanical pressure exerted on the cornea. The Lenstar takes measurement only if the pupil is properly centered and, as stated above, no mechanical contact is made to the eye. However, if the proper alignment is lost due to head or eye movements, the device pauses the measurement and resumes again when proper alignment is restored. The collection of the 16 individual scans can in principle be accomplished over multiple pauses and resumptions but we found that long interruptions stopped the measurement procedure. The OLCI device clearly shows potential for use in animal research, and may be useful also for measurements in hand-held alert chicks but only if they are older (2-3 weeks). In our hands, routine measurements in young chicks required mild anesthesia and lid retractors because

awake animals closed their eyes before the measurements could be completed. It is possible that anesthesia of the chickens reduced measurement noise, although the potential effects of lid retractors on the optics of the eye may counterbalance the positive effects of anesthesia.

The present study shows that A-scan ultrasonography (3M Echorule) and OLCI (Lenstar LS 900) measurements differ significantly in the measured anterior chamber depths, lens thickness, vitreous chamber depths and axial lengths. When comparing both methods, the ultrasound always measured longer distances, except for ACD which was shallower– perhaps due to the minor indentation of the cornea that occurred when the transducer touched the eye. The latter result is in agreement with other studies in humans [7-9]. AL measured by the A-scan ultrasound was longer compared to OLCI, in agreement with observations by Buckhurst et al. [7], but not by Gursoy et al. [8] in humans. Since one method uses sound waves and the other light waves, they are differently affected by the material properties. Sound wave become the faster the denser the material whereas light waves slow down. Therefore, the time of flight is changing in the opposite directions, and extensive calibrations (and, basically the material constants, speed of sound, and refractive indices) must be done all along the way. Both A-scan ultrasound and OLCI are calibrated to measure human eyes to achieve best precision, but calibrations from chicken eyes are not available. We used both devises in a human calibration mode, and the differences found in this study between A-scan applanation ultrasonography and OLCI could partially trace back to the inappropriate human calibration. It was striking that LT was measured with no correlation between both measurement principles. Lens measurements would be most affected by different behavior of sound and light waves, because its refractive index varies along the path, and because the speed of sound will also vary. The lens gradient index is not fully known for the chicken eye, leaving an undetermined variable in the measurements that could easily explain the lack of a correlation. Two other comparative studies revealed problems in obtaining a good correlation for the thickness of the crystalline lens [7, 8].

Only a few studies have compared A-scan ultrasound with OLCI and none of them was done in chicks. In line with other studies in humans, OLCI showed a better consistency

in our study in chicks. Nevertheless, despite the differences between both methods, they showed a high agreement in most biometric variables. Perhaps a careful calibration, using data on speed of sound and refractive index of the ocular media, could generate an even better agreement of the data. Changes in ocular dimensions induced by visual deprivation or recovery from induced myopia could be nicely resolved with both instruments. Therefore, low coherence interferometry represents a powerful alternative to ultrasonography.

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D. Summary

SUMMARY

Insulin is a candidate for a growth promoting signal in the retina. So far, only a few studies targeted the function of insulin and the IGF-I in the eye and the down-stream activated signaling cascade. Project 1 and Project 2 aimed at adding new information about the role of insulin in the eye.

Project 1: The insulin receptor undergoes transcriptional and translational regulation (Gammeltoft, Kowalski et al., 1984) and the existence of different isoforms in peripheral tissues and the CNS support the idea that insulin might have a specific function in neuronal tissues (Masters, Shemer et al., 1987).

In the first part of project 1, it was shown that different tissues express transcripts of different lengths. When a probe that corresponds to part of the sequence for the insulin receptor tyrosine kinase domain was used three different transcripts were found: 4.3 kb, 2.6 kb and 1.3 kb long. RPE and sclera seem to express only short IR transcripts. Moreover, the smaller mRNA transcript was the predominant form in the choroid and liver, whilst the longer transcript was most abundant in the brain and retina. These results support the idea that insulin receptors in the retina and brain possibly have a different function than in the non-neuronal tissues.

Insulin receptor and IGF-1 receptor were expressed in all fundal layers, being more abundant in the retina, followed by RPE, choroid, cartilaginous and fibrous sclera. Therefore, assuming that the mRNA is translated into protein, all of them are possibly target sites for insulin and IGF-1 action. In addition, lens wear influenced IR, IGF-1R, and IGF-1 mRNA expression. In the RPE, both IR and IGF-1R mRNA content was already down-regulated after short periods (4 hours) of positive lens wear. In contrast, IR and IGF-1R were up-regulated in the choroid and fibrous sclera during treatment with negative, but not positive, lenses.

Taken together, the results underline a role of insulin and/or IGF-1 signaling during eye growth. Whether different insulin receptor transcript variants, as found in retina and choroid, are also translated into proteins with different function needs to be shown in the future.

Project 2: The experiments in project 2 showed that insulin activates the PI3K/Akt pathway in eyes with normal vision and eyes treated with negative lenses whereas it stimulates eye growth through the MEK/Erk pathway in eyes that are treated with positive lenses. This conclusion was drawn from two types of experiments. In the first approach, the effect of insulin and insulin in combination with inhibitors of the PI3K or MEK/Erk pathways on refractive development was measured in control animals and chicks treated with positive and negative spectacle lenses. In the second approach, western blots were used to measure the phosphorylation level and thereby activation level of two proteins involved in the two pathways under investigation. In line with other studies, the current investigation adds new data in support of the idea that positive and negative lenses activate different biochemical pathways. It would be important to identify key regulators that control the balance between the two signaling pathways in order to provide a new strategy for pharmacological intervention of myopia.

With these new results, in combination with previous studies which showed the involvement of the transcription factor Egr-1 (ZENK in the chicken) in eye growth regulation, a new hypothesis was formulated, proposing that eye growth induced by insulin injections is controlled by an Egr-1 mediated balance between PI3K/Akt and MEK/Erk/MAPK pathways.

Further downstream molecules from both pathways need to be studied under the different visual conditions to confirm the roles of both pathways in the different growth responses.

Project 3: The result of project 3 was that A-scan Ultrasound 3M Echorule and Lenstar LS 900 generally correlated closely, except for measurements of anterior chamber depth and lens thickness. Also the absolute values determined with both techniques differed consistently for vitreous chamber depth and axial length. Ultrasonography always measured longer distances, except for anterior chamber depth. This was the first study in which both techniques were compared in the eye of the chicken.

E. References

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F. Statement of own contribution

Publication 1

Insulin, IGF-1, Insulin- and IGF-1 receptor expression in the chick eye and their regulation with imposed myopic or hyperopic defocus

Molecular Vision **2011; 17:1436-1448** <http://www.molvis.org/molvis/v17/a162>
Received 17 December 2010 | Accepted 26 May 2011 | Published 31 May 2011

Authors: Alexandra Marcha Penha, Frank Schaeffel and Marita Feldkaemper

Contribution of the author AMP:
I performed all the experiments, analyzed the data and wrote the first version of the manuscript and kept improving it based on the ideas of the other authors. In total, I estimate my own contribution to the work of about 90%.
Contribution of MF:
Marita Feldkaemper designed the experiment and corrected the following versions of the manuscript.
Contribution of FS:
Frank Schaeffel helped improving the manuscript versions.

Publication 2

Effects of intravitreal insulin and insulin signaling cascade inhibitors on emmetropization in the chick

Alexandra Marcha Penha, Eva Burkhardt, Frank Schaeffel and Marita Feldkaemper

Molecular Vision 2012; 18:2608-2622 http://www.molvis.org/molvis/v18/a270
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Authors: Alexandra Marcha Penha, Eva Burkhardt, Frank Schaeffel and Marita Feldkaemper

Contribution of the author AMP:

I helped in the design of the experiments and, as in publication 1, I performed all the techniques, analyzed the data and wrote the first version of the manuscript and kept improving it based on the ideas of the other authors. In total, I estimate my own contribution to the work of about 90%.

Contribution of the author EB:

Eva Burkhardt has a lot of experience in measuring the axial length with A-san ultrasonografy; therefore, she helped me with the measurements.

Contribution of MF:

Marita Feldkaemper helped in the design of the experiment and in the analysis of the data and corrected the following versions of the manuscript.

Contribution of FS:

Frank Schaeffel helped improving the manuscript versions.

Publication 3

Ultrasonography and optical low-coherence interferometry compared in the chicken eye

Penha AM*, Burkhardt E*, Schaeffel F*, Feldkaemper MP*.

**Institute for Ophthalmic Research, Section of Neurobiology of the Eye, University Eye Hospital, Tuebingen, Germany.*

Optom Vis Sci. 2012 Jun;89(6):916-21. doi: 10.1097/OPX.0b013e318257a255.

Authors: Alexandra Marcha Penha, Eva Burkhardt, Frank Schaeffel and Marita Feldkaemper

Contribution of the author AMP:

I analyzed the data and wrote the first version of the manuscript and kept improving it based on the ideas of the other authors. In total, I estimate my own contribution to the work of about 60%.

Contribution of the author EB:

Eva Burkhardt performed the measurements.

Contribution of MF and FS:

Both authors helped in analyzing the data and correcting the following versions of the manuscript.

G. Presentations related to this work

Publications:

Penha AM, Burkhardt E, Schaeffel F, Feldkaemper M, *Effects of intravitreal insulin and insulin signaling cascade inhibitors on emmetropization in the chick*, Molecular Vision 2012; 18:2608-2622.

Penha AM, Burkhardt E, Schaeffel F, Feldkaemper M, *Ultrasonography and optical low-coherence interferometry compared in the chicken eye*, Optom Vis Sci. 2012 Jun;89(6):916-21.

Penha AM, Schaeffel F, Feldkaemper M, *Insulin, Insulin-like Growth Factor I, Insulin Receptor and Insulin-like Growth Factor I-Receptor expression in the chick eye and their regulation with imposed myopic and hyperopic defocus*, Mol Vis, 2011; 17:1436-48.

Penha A, *Insulin Receptor Signaling During Eye Growth*, Myopia: Proceeding of the 13th International Conference, Opt Vis Scie, 2011 March, 88: 395-403.

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Presentations at National and International Meetings:

Marcha Penha, A., Burkhardt E., Schaeffel F., Feldkaemper M., (2010) *Insulin receptor signaling during eye growth*, in Procedings of the 13th International Myopia Conference, Tuebingen, Germany, Optom Vis Sci 2011; 88:395-403. (Talk)

Marcha Penha, A., (2010) *Hormones in myopia development*, The Young Research Vision Camp, Castle Wildenstein, Germany. (Talk)

Marcha Penha, A. and Feldkaemper, M., (2010) *The insulin system regulation after negative and positive lens-induced defocus,* in MyEuropia 3rd Annual Meeting, Murcia, Spain. (Talk)

Marcha Penha, A., Vogt, M., Feldkaemper M., (2009) *Insulin receptor expression in different fundal layers of the chick eye and the effect of insulin on visual performance*, ARVO-International Society for Ocular Cell Biology E-Abstract 61-B11, Ericeira, Portugal. (Poster)

Marcha Penha, A. and Feldkaemper M., (2009) Insulin, insulin receptor, IGF-1 and IGF-1 receptor mRNA expression in the chicken eye after positive and negative lens treatment, ARVO Meeting Abstracts April 11, 2009 50:3851, Fort Lauderdale, USA. (Poster)

Marcha Penha, A. and Feldkaemper M., (2009) The *role of insulin and IGF-1 in myopia development*, in MyEuropia Mid Term Review Meeting, Venice, Italy. (Talk and Poster)

Marcha Penha, A. and Feldkaemper M., (2008) The *role of insulin and IGF-1 in myopia development*, in MyEuropia 2nd Annual Meeting, Castle Maurach, Germany. (Talk)

Marcha Penha, A. and Feldkaemper M., (2008) The *role of insulin and IGF-1 in myopia development*, in MyEuropia 1st Complementary Training Course, Tuebingen, Germany. (Talk)

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First of all, I would like again to mention some words of my favorite Portuguese poet, Fernando Pessoa: *"Tudo o que chega, chega sempre por alguma razão…"* (translation: Whatever comes, comes always for some reason) and *"Tudo vale a pena quando a alma não é pequena…"* (translation: All worth it when the soul is not small).

I do believe that I came to this small city, called Tuebingen, for some reason; and the truth is that here I had the best years of my life!

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I. *Curriculum Vitae*

Curriculum Vitae

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