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Expression of Insulin-Like Growth Factor 1 Isoforms in the Rabbit Oculomotor System

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Abstract

Objective—The insulin-like growth factor-1 (IGF-1) gene encodes two isoforms, IGF-1Ea and IGF-1Eb. Both isoforms can regulate skeletal muscle growth and strength. It has been suggested that IGF-1Eb may be more potent in promoting skeletal muscle hypertrophy. Precise contractile force regulation is particularly important in the oculomotor system. However, expression of these isoforms in mammalian extraocular muscles (EOMs) is unknown. Here, we examined their expression in rabbit EOMs and the innervating nerve, two potential sources for myogenic growth factors, and compared isoform expression between EOMs and limb skeletal muscles.

Design—Expression of IGF-1 isoforms was quantified by real-time RT-PCR in adult rabbit EOMs, trochlear and ophthalmic nerves, and compared with expression in rabbit limb skeletal muscles. The presence of mature IGF-1 peptide in the muscles was further examined by Western blot.

Results—Both IGF-1Ea and IGF-1Eb were expressed in the EOM and the trochlear nerve. Both isoforms were expressed at significantly higher levels (9- fold) in EOM than in limb skeletal muscle. Transcripts of IGF-1 isoforms, of IGF-1 receptor and of IGF binding proteins showed a gradient distribution along the EOM from proximal to distal. The mature IGF-1 protein showed the same gradient distribution in the EOM.

Conclusions—Expression of relatively abundant amounts of both IGF-1 splicing isoforms in EOMs, and at a significantly higher level than in limb skeletal muscle, underscores the potential relevance of these myogenic growth factors in EOM plasticity and force regulation.

Keywords

Extraocular Muscle; Insulin-like Growth Factor-1; Mechano-Growth Factor; Oculomotor System; Real-time PCR; Splicing Isoform; Housekeeping Gene

1. Introduction

Insulin-like growth factor 1 (IGF-1) is a potent myogenic growth factor that stimulates myoblast cell proliferation, differentiation and fusion, and myofiber hypertrophy (Coleman et al., 1995; Engert et al., 1996). Unlike mature limb skeletal muscles where muscle satellite

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cells usually remain quiescent, satellite cells in the adult extraocular muscles (EOMs) continue to divide and fuse in normal uninjured EOMs (McLoon and Wirtschafter, 2002; McLoon and Wirtschafter, 2003). IGF-1 receptor is present in adult rabbit EOMs (Anderson et al., 2006). Delivery of IGF-1 protein into the orbit can significantly increase myofiber diameter and contractile force of developing and adult EOMs (Chen and von Bartheld, 2004; Anderson et al., 2006; McLoon et al., 2006; Li et al., 2010), while administration of IGF Binding Protein 4 (IGFBP4) into the orbit significantly decreased EOM contractile force and prolonged contraction time (Li et al., 2011). Thus, IGF-1 peptide appears to be a key regulator of EOM function. However, little is known about the expression of IGF-1 in the mammalian oculomotor system. Knowledge about the expression of IGF-1 in the mammalian EOMs would help to assess the role of this protein in EOM plasticity (McLoon and Christiansen, 2005; von Bartheld et al., 2010).

The discovery of IGF-1 splicing isoforms makes the roles of the IGF-1 gene more complex. The IGF-1 gene was found to encode two mRNA precursors in mouse liver: IGF-1Ea and IGF-1Eb (Bell et al., 1986). IGF-1Eb in non-primate mammals is equivalent to IGF-1Ec in humans (Matheny et al., 2010). IGF-1Eb is also called mechano-growth factor (MGF) since the increase of this precursor was seen in mechanically stimulated or injured limb skeletal muscles (Goldspink, 2005). In contrast to IGF-1Ea, IGF-1Eb mRNA contains an insert from exon 5, which causes a reading frame shift and therefore encodes a different carboxyl terminal with unique biological activity (McKoy et al., 1999; Goldspink, 2005). It has been suggested that transient increase of IGF-1Eb is responsible for muscle satellite cell activation, myoblast proliferation, and stem cell pool replenishment (Goldspink et al., 2010). Here, we quantified expression of IGF-1 pathway components (IGF-1Ea, IGF-1Eb, IGF-1 receptor, IGFBP4 and IGFBP5) in the adult rabbit oculomotor system. Our findings define the spatial expression of IGF-1 isoforms and suggest that IGF-1Eb may have a role in EOM plasticity.

2. Methods

2.1. Animals

Twenty New Zealand white male rabbits (2–3 kg) were used (Western Oregon Rabbit Co, Philomath, Oregon), 18 for gene expression studies and 2 for Western blots. Animals were kept in cages with constant temperature and humidity in a 12 hour light/dark cycle ventilated room. Food and water were provided ad lib. Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Nevada, Reno.

2.2. Tissue collection, RNA isolation and reverse transcription

Animals were killed by an overdose of pentobarbital (2 mg/kg). The EOM (superior oblique muscle), the trochlear nerve, and the limb skeletal muscle (extensor digitorum longus, EDL) were dissected, briefly rinsed in ice-cold distilled water, and immediately frozen in liquid nitrogen. The EOM was dissected intact from the bony origin to the distal tendon. The EOM was divided into three segments: proximal (near the orbital bony origin), middle and distal (near the insertion on the sclera, including the tendon) (Fig. 1A). For EDL muscle, only the mid-belly was dissected. The trochlear nerves (exclusively motor axons) and the ophthalmic nerves (exclusively sensory axons) were collected. Four nerves were pooled for each independent experiment. Tissues were homogenized in 1 ml Trizol (Invitrogen, Carlsbad, CA), RNA extracted with 200 μ l chloroform, and then processed with Purelink micro-to-midi total RNA isolation kit (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Isolated RNA was further treated with Turbo-free DNase kit (ABI, Foster City, CA) to remove genomic DNA. RNA concentrations and possible protein contaminations were determined with Nano Drop 1000 (NanoDrop products, Wilmington, DE). The RNA

integrity was evaluated with denaturing gel electrophoresis. RNA was reverse transcribed into cDNA with Superscript III kit and Oligo(dT) primers (Invitrogen, Carlsbad, CA). GoTaq green master mix (Promega, Madison, WI) was used for qualitative PCR. “RT minus” control was performed to detect possible genomic DNA contamination.

2.3. Real-time PCR analysis

Primers were synthesized by Operon Inc, Huntsville, AL. Several sets of primers for IGF-1 pathway components (IGF-1Ea, IGF-1Eb, IGF-1 receptor, IGFBP4, and IGFBP5) were designed with Primer Express (ABI, Foster City, CA). To eliminate binding to the common portion (exon 4) between IGF-1 isoforms, the anti-sense primer spans the exon 4–6 junction (Fig. 1B). Primer sequences of genes were blasted against the rabbit gene bank to confirm their specificity. Five housekeeping genes (EF1A1, EF1A2, HMBS, HPRT1 and GAPDH) were tested in this study. Primer sequences for rabbit EF1A2, HMBS, and GAPDH were designed with Primer Express, and for rabbit EF1A1 and HPRT1 were from the literature (von Smolinski et al., 2006; Mebis et al., 2009). Real-time PCR was performed on an ABI 7300 with SYBR Green PCR Master Mix (ABI, Foster City, CA) according to the manufacturer's default protocol. The best primer pairs (lowest Ct, highest amplification efficiency and stable performance) were selected for real-time PCR. The optimal primer concentrations were determined according to the ABI real-time PCR protocol (Table 1). Dissociation curves were run for each well to check for non-specific amplifications. PCR reaction products were randomly selected for sequencing to further evaluate the amplicon specificity. For reasons explained below, GAPDH was chosen as an internal control for IGF-1 expression. The mRNA level of the target gene was calculated by normalizing its mRNA level with that of GAPDH.

2.4 Western blots

Rabbit superior oblique muscles and extensor digitorum longus were “snap frozen” in liquid nitrogen, and homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). PMSF solution, sodium orthovanadate solution and protease inhibitor cocktail solution were added to the buffer immediately prior to use. Homogenates were centrifuged for 20 min at 4°C. Supernatants were aliquoted and stored at –80°C. Total protein concentrations were determined with a Micro BCA Protein Assay kit (Pierce Biotechnology, Rockford, Ill, USA). Equal amounts of total protein were resolved in precast Tris-HCl polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA) and transferred onto PVDF membranes (0.2 µm, activated in 100% methanol prior to use). To verify equal loading, total myosin in the upper half of the gel was stained with Coomassie blue and evaluated using a myosin standard (prestained SDS-PAGE standards, broad range, Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 0.5% gelatin in TNT buffer, and then probed with a polyclonal goat IGF-1 primary antibody (1:500) (Abcam, Cambridge, MA) and a mouse-anti-goat IgG-AP (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). The blots were visualized using an alkaline phosphatase conjugate substrate kit (Bio-Rad Laboratories, Hercules, CA).

2.5. Statistical analysis

Paired two-tailed t-test was used to compare the differences in gene expression between the distal and proximal segments of the rabbit EOMs. To compare the differences between the rabbit EOM and EDL, unpaired two-tailed t-test was applied. In all cases, $p < 0.05$ was considered statistically significant.

3. Results

To measure IGF-1 isoform expression in the adult rabbit oculomotor system, we used real-time RT-PCR. We examined several house-keeping genes (EF1A1, EF1A2, HMBS, HPRT1 and GAPDH) (Table 2). In our experiments, geNorm ranked GAPDH as one of the two most stably expressed genes (GAPDH and EF1A2). We chose GAPDH as the reference gene for two reasons: because of its stable expression and its use for quantifying skeletal muscle IGF-1 expression in previous studies, thus making our data comparable with those generated in other labs (Cheema et al., 2005; Fischer et al., 2005; Heinemeier et al., 2007; Feng and von Bartheld, 2010; Li et al., 2011). The amplification efficiencies of all primers were within 90%–110%. The coefficient of variation R^2 was above 0.99.

To validate our technique, we first measured IGF-1 isoform expression in rabbit EDL muscle, since this tissue was previously examined for IGF-1 isoform expression (Yang et al., 1996; McKoy et al., 1999). Consistent with previous reports, we found that limb skeletal muscles expressed low levels of IGF-1Ea transcripts, and very little IGF-1Eb mRNA (Fig. 1C, D). Since the concentrations of total RNA extracted from different rabbit tissues (nerve vs muscle) varied up to 10-fold, we examined whether differences in RNA concentrations affected results. We made serial dilutions of rabbit liver total RNA up to 100-fold, and then quantified IGF-1 isoform levels. We found that the levels of IGF-1 splicing transcripts (relative to GAPDH) were remarkably consistent (IGF-1Ea, 0.61 ± 0.03 ; IGF-1Eb, 0.11 ± 0.007).

When we measured IGF-1Ea and IGF-1Eb mRNA expression in the rabbit superior oblique muscle, we found that this EOM expressed 8.8 times more IGF-1Ea mRNA than the EDL muscle, as shown in Fig. 1D. The EOM also expressed significantly more IGF-1Eb mRNA (8.7 fold more), compared with the amount expressed in the EDL (Fig. 1D). In previous work (Feng and von Bartheld, 2010), we reported that the innervating motor nerve, mainly composed of Schwann cells, is a major source of IGF-1 mRNA in the juvenile chicken oculomotor system. Therefore, we further examined IGF-1 isoform expression in the adult rabbit trochlear nerves, and compared their expression levels with those in the ophthalmic nerves and EOMs. We found that nerves with exclusive motor fiber contribution (trochlear nerves) expressed considerably more IGF-1Ea and IGF-1Eb transcripts than EOMs and sensory nerves (ophthalmic nerve) (Fig. 1D). These observations indicate that the innervating motor nerves may be a major source of both IGF-1Ea and IGF-1Eb for the adult rabbit EOMs.

We further explored whether IGF-1 isoforms were expressed differently along the longitudinal axis of the EOM, because it was reported that myofiber composition varies in different segments along the EOM (McLoon et al., 1999; Kjellgren et al., 2003; Spencer and Porter, 2006), and different segments respond differently to myogenic growth factor stimulation (Anderson et al., 2008; Li et al., 2010). We dissected the EOM into three segments: proximal, middle and distal (Fig. 1A). We found both IGF-1Ea and IGF-1Eb mRNA expression showed a distribution gradient along the EOM, with the distal segment expressing the most (Fig. 2A). There was a significant difference (IGF-1Ea, $p=0.013$; IGF-1Eb, $p=0.012$) in expression levels for both IGF-1 isoforms between the proximal and the distal segments. The distal segment of the EOM contained 1.75 fold more IGF-1Ea and 2.24 fold more IGF-1Eb than those of the proximal segment. We further examined the expression of the IGF-1 receptor, IGFBP4 and IGFBP5 in the EOM. As seen in Fig. 2A, those genes showed a similar distribution pattern with the distal segment containing significantly more transcripts for IGF-1 receptor, and IGFBP4 and IGFBP5 transcripts than the proximal segment ($p=0.038$, 0.011 and 0.021 , respectively). Western blot with an IGF-1 antibody further confirmed the existence of mature IGF-1 peptide in the EOM. Similar to its

mRNA transcripts, the mature peptide distributed consistently in a gradient pattern with unusually high protein levels in the distal segment, and very low levels of IGF-1 protein in the proximal segment (Fig. 2B). The matching of IGF-1 transcript and protein levels was expected based on previous work in skeletal muscle (Adams and Haddad, 1996). IGF-1 peptide in limb skeletal muscle was at or below the threshold of detection, consistent with previous reports of less than 20 pg/μg amounts of IGF-1 in skeletal muscle (Tsai et al., 2007; Shavlakadze et al., 2010). Taken together, these data suggest that different segments and possibly different myofibers or myofiber segments control myogenic plasticity at a local level via differential expression of ligands, receptors, and binding proteins.

4. Discussion

IGF-1 is one of the most important myogenic growth factors in EOM force regulation. Studies have demonstrated the effectiveness of exogenous and endogenous IGF-1 proteins on EOM force regulation (Chen and von Bartheld, 2004; Anderson et al., 2006; Li et al., 2011). Our previous work showed the expression of IGF-1 mRNA in the EOMs of developing chickens (Feng and von Bartheld, 2010). However, whether the IGF-1 gene is expressed in adult mammalian EOMs was unknown. The mammalian IGF-1 gene is more complex than that of birds (Kajimoto and Rotwein, 1989) due to splicing isoforms: IGF-1Ea and IGF-1Eb. IGF-1Eb expression, which can stimulate the replication of myoblast cells in limb skeletal muscles (Goldspink, 2005), has to our knowledge never been studied in mammalian EOMs. We now provide evidence that both IGF-1 transcript isoforms are present in adult rabbit EOMs. They were both expressed at significantly higher levels in EOMs than in the limb skeletal muscles.

4.1 Expression gradients of IGF-1 transcript isoforms in the EOM

The EOM is not a uniform structure. The myofiber number and myosin heavy chain isoform composition differ between proximal, midbelly and distal segments of adult mammalian EOMs (McLoon et al., 1999; Kjellgren et al., 2003; Peng et al., 2010). Consistent with this morphological distinction, EOMs respond to growth factors differently along the muscle length. Transforming growth factor beta 1 decreased the mean cross-sectional area (CSA) only in myofibers within the middle of the muscle belly, while bone morphogenetic protein 4 reduced CSA in the mid region compared to the tendon region (Anderson et al., 2008). Injection of IGF-1 or cardiotrophin-1 in chickens increased the CSA, the myofiber diameter, the myofiber number and the number of proliferating nuclei in proximal but not distal parts of the EOM (Li et al., 2010), even though the exogenous peptides distributed uniformly along the EOM.

Here, we discovered a gradient expression of IGF-1 transcript isoforms and IGF-1 receptor mRNA along the EOMs. Similar to our result in EOM, IGF-1Ea and IGF-1Eb transcripts were found in human and rat patella tendon tissues (Doessing et al., 2010). The tendon expressed 2 times more IGF-1Ea and Eb transcripts than the muscle (Doessing S, personal communication). We found that the distal segment (including the muscle/tendon transition area) of the EOM expressed the most IGF-1Ea, IGF-1Eb, IGF-1 receptor, IGFBP4, and IGFBP5 mRNA, compared with the proximal segment. IGF-1Eb is believed to activate muscle satellite cells (Goldspink, 2005). It was reported that there was an increased percentage of BrdU-positive nuclei toward the EOM tendon than the midbelly (McLoon and Wirtschafter, 2002). Interestingly, IGF-1Eb mRNA also showed an increased gradient toward the EOM tendon in our work. In sum, the mRNA gradients may result from the different myofiber composition or percentage of activated satellite cells. This may explain why the EOM responds differently to exogenous growth factors along the muscle longitudinal axis.

4.2 IGF-1 gene expression regulation

In our study, we found that the ratio between IGF-1Ea and IGF-1Eb transcripts remains constant across different EOM and limb skeletal muscle samples. The relative ratios of IGF1Ea/IGF1Eb are 11.2 (EDL belly), 11.2 (whole EOM), 12.9 (proximal EOM), 11.5 (middle EOM), and 10.1 (distal EOM). It seems that the IGF-1Ea/Eb expression ratio is relatively fixed in skeletal muscles. A similar phenomenon was also documented by other groups (Iida et al., 2004; Hammers et al., 2011), and also discussed in a recent review article (Matheny et al., 2010). Most previous studies of IGF-1 gene regulation were conducted in the hypothalamo-pituitary-liver axis and musculoskeletal system and indicated that IGF-1 splicing isoform expression is regulated at the transcriptional level (Adamo et al., 1993; Rotwein et al., 1993).

However, several other studies show that IGF-1 splicing isoforms can also be differentially regulated. The IGF-1 gene was reported to initially splice predominantly toward IGF-1Eb and then towards IGF-1Ea in muscle after mechanical stimuli, injury or exercise (Hill and Goldspink, 2003; Hill et al., 2003; Goldspink, 2005) or after growth factor treatment (Iida et al., 2004). In sum, IGF-1 isoform expression can be regulated at both transcriptional and posttranscriptional levels. The significance of the constant ratio of IGF-1 isoform expression in EOMs remains to be determined. Our discovery of relatively abundant IGF-1Eb expression (compared with the limb muscles) in the oculomotor system is consistent with the notion that these proteins play a role in EOM plasticity (McLoon and Christiansen, 2005; von Bartheld et al., 2010). Studies assessing effects of exogenous IGF-1Eb protein products or IGF-1Eb siRNA in the orbit will further define the significance of this IGF-1 isoform in the oculomotor system and EOM force regulation.

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Abbreviations

EDL	extensor digitorum longus
EF1A	elongation factor 1A
EOM	extraocular muscle
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HMBS	hydroxymethylbilane synthase
HPRT1	Hypoxanthine-guanine phosphoribosyl-transferase 1
IGF-1	insulin-like growth factor 1
IGFBP	insulin-like growth factor binding protein
MGF	mechano-growth factor
RT-PCR	reverse transcription-polymerase chain reaction
SOM	superior oblique muscle

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Fig. 1.

A–D. Illustration of the rabbit superior oblique muscle, the design of IGF-1Ea/Eb primers and their expression in oculomotor and limb muscle tissues. (A): Dissected rabbit superior oblique muscle and the trochlear nerve. The muscle was arbitrarily divided into three segments (P, proximal; M, middle; D, distal), as indicated by dotted lines. Black arrow points to the trochlear nerve which is held by forceps. The orientation and relative number of myofibers (1 line represents approximately 1000 myofibers) in rabbit EOMs is depicted in the adjacent schematic. Scale bar = 5 mm. (B): Schematic depiction of IGF-1Ea and IGF-1Eb mRNA structures, primer locations and their amplicons. “B”, “C”, “A” and “D” distinguish four domains in the mature IGF-1 peptide (Daughaday and Rotwein, 1989; Yang et al., 1996). (C): IGF-1 isoform expression in the limb skeletal muscle (extensor digitorum longus - EDL). The IGF-1Eb level in the EDL was set as 1. (D): Comparison of IGF-1 isoform expression in EDL, extraocular muscle (EOM), trochlear nerve (TN) and ophthalmic nerve (ON). The IGF-1 transcript level in EDL was set as 1 for each isoform. Rabbit EOMs expressed significantly more IGF-1Ea (8.8 fold) and IGF-1Eb (8.7 fold) mRNA than rabbit EDLs. $p=0.004^*$, 0.006^{**} . Error bars=SEM. In rabbit, trochlear nerves express substantially more IGF-1Ea and IGF-1Eb than EOMs. The sensory ophthalmic nerve expressed similar or less IGF-1 transcripts than EOMs. $n=3-6$.

Fig. 2.

A–B. Expression of IGF-1 isoforms, IGF-1 receptor and IGF binding proteins (IGFBPs) in different segments of the EOM. The EOM was dissected into three segments: proximal, middle and distal. Relative mRNA levels are expressed as * fold difference compared with that of the proximal segment. (A) Compared with the proximal segment, the distal segment of the EOM expressed significantly more mRNA transcripts of IGF-1Ea ($p=0.013$), IGF-1Eb ($p=0.012$), IGF-1 receptor ($p=0.038$), IGFBP4 ($p=0.011$) and IGFBP5 ($p=0.021$). All three genes showed the same expression gradient. $n=4$ for each muscle segment. Error bars=SEM. IGF-1 R, IGF-1 receptor. (B) Western blots show a gradient distribution of mature IGF-1 peptide in the EOM, while IGF-1 protein was at or below the limit of detection in the EDLs, consistent with known low levels of this peptide in skeletal muscle. Myosin is shown as a loading control. The gradient pattern of the IGF-1 protein is consistent with that of the IGF-1 mRNA.

TABLE 1

List of primers used in this study.

Gene Name	Primer	Amplicon	Conc. pmol
IGF-1Ea	Forward 5'-AGGAGGCTGGAGATGTACTG-3' Reverse 5'-AAATGTACTTCCTTCTGAGTCT-3'	107 bp	900/900
IGF-1Eb	Forward 5'-CATGCCCAAGACTCAGAAAGT-3' Reverse 5'-AAATGTACTTCCTTCTGAGTCT-3'	82 bp	900/900
IGF-1 R	Forward 5'-ACCGCAACTACCGCTTCCCC-3' Reverse 5'-CCGCGGATGACCGTGAGGTT-3'	115 bp	300/300
IGFBP4	Forward 5'-TCACCCCAACAACAGCTTCAG-3' Reverse 5'-CGCCCAATAATCTTCATCTTG-3'	118 bp	300/300
IGFBP5	Forward 5'-GCCGTGTACCTGCCCAACT-3' Reverse 5'-TGTCCACGCACCAGCAGAT-3'	100 bp	300/300
EF1A2	Forward 5'-GGCATCGACAAGAGGACCAT-3' Reverse 5'-ACCCAGGCGTACTTGAAGGA-3'	80 bp	300/300
HMBS	Forward 5'-TGGCCTGCGGTTTGAAAT-3' Reverse 5'-AACAGGCTCTTCTCCCAATC-3'	90 bp	300/300
GAPDH	Forward 5'-ATTGCCCTCAATGACCACTTTG-3' Reverse 5'-TCTTACTCCTTGGAGGCCATGT-3'	110 bp	300/300
EF1A1*	Forward 5'-TGTTGAGAGCTTCTCTGACTATCC-3' Reverse 5'-CTCCAGCAGCCTTCTTGTC-3'	110 bp	300/300
HPRT*	Forward 5'-TGTAGATTTTATCAGACTGAAGAGCTACTGT-3' Reverse 5'-AAGGAAAGCAAGGTCTGCATTGTT-3'	159 bp	300/300

* Primer sequences are from the literature (von Smolinski et al., 2006; Mebis et al., 2009).

TABLE 2
Crossing point (Cp) values of the house-keeping genes for different EOM segments.

Gene*	EF1A1	EF1A2	HPRT1	HMBS	GAPDH
P1	19.74	22.80	24.11	25.60	16.16
M1	19.16	22.75	23.64	25.57	16.23
D1	18.40	23.01	24.51	25.56	16.63
P2	19.52	23.25	24.90	25.77	16.27
M2	19.15	22.61	24.73	25.38	16.20
D2	18.86	23.76	25.73	26.08	17.17
P3	19.60	22.67	24.57	25.53	16.34
M3	19.08	22.96	24.49	25.36	16.10
D3	18.60	22.92	24.46	25.72	16.65
P4	19.25	23.04	25.22	25.49	16.55
M4	19.18	22.61	25.06	25.35	16.21
D4	18.46	23.54	25.36	25.63	17.17

* D, distal, M, middle, P, proximal segments of the extraocular muscle (EOM). 1–4, sample number.