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Effect of Hyperthyroidism on the Orbicularis Oculi Muscle in Rabbits

Andrew R. Harrison, M.D., and Linda K. McLoon, Ph.D.

Department of Ophthalmology, University of Minnesota, Minneapolis, Minnesota, U.S.A.

Purpose: To determine the effect of hyperthyroidism on both myofiber number and myosin heavy-chain isoform composition within the palpebral orbicularis oculi muscle in rabbits.

Methods: Four New Zealand White rabbits were made hyperthyroid by injection of 3,3,3'-triiodothyroinine intraperitoneally every other day for 1 month. Four rabbits were used as control animals. After 1 month the rabbits were euthanized, and the eyelids were excised and sectioned in a cryostat. The sections were immunostained to determine the presence of fast, slow, and neonatal myosin heavy-chain isoforms. To determine alterations in myofiber number, differential counts of myofiber number and the cross-sectional areas of the muscle fibers were performed with the use of computerized morphometry.

Results: The orbicularis oculi muscle in the palpebral portion of the eyelids from hyperthyroid rabbits had significantly fewer myofibers compared with control eyelids, predominantly as the result of a loss of myofibers in the preseptal region. The remaining fibers showed continued expression of fast myosin but upregulated coexpression of slow myosin isoform.

Conclusions: Hyperthyroidism led to reduced orbicularis oculi muscle in the rabbit model and an alteration in the myosin heavy-chain isoform composition. This finding may help explain the clinical finding of eyelid retraction in patients with Graves orbitopathy.

Graves disease is an autoimmune process often associated with an endocrinopathy that leads to an inflammatory orbitopathy with proptosis, strabismus, and eyelid retraction.[1,2] The histologic changes in the extraocular muscles, orbital fat, levator superioris, and the Müller muscle have been well described.[3–5] To the best of our knowledge, no prior histologic studies have examined the effects of hyperthyroidism on the orbicularis oculi muscle. The current study begins to fill that void.

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Address correspondence and reprints to Dr. Andrew R. Harrison, Department of Ophthalmology, University of Minnesota, Box 493, UMHC, Minneapolis, MN 55455. E-mail harri060@tc.umn.edu

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There is no satisfactory animal model for the autoimmune process that leads to Graves disease. Although hyperthyroidism is not always associated with Graves ophthalmopathy, its frequent association lends credibility to the study of the effects of hyperthyroidism on the ocular and nonocular skeletal muscles in other species. A number of studies have been performed with animal models of elevated thyroid hormone to study various muscle-specific aspects caused by this condition.[6–15] The purpose of this study was to evaluate the effect of elevated thyroid hormone levels on orbicularis oculi muscle in a rabbit model.

METHODS

Eight New Zealand White rabbits obtained from Birchwood Valley Farms (Red Wing, Minn.) were housed with Research Animal Resources at the Univer-

sity of Minnesota. All research conformed to the Guidelines of the National Institutes of Health for the Use of Animals in Research.

Four rabbits were made hyperthyroid by intraperitoneal injection of a sterile solution prepared at a concentration of 0.1 mg 3,5,3'-triiodothyroinine (T3) (Sigma, St. Louis, Mo.) in 1 mL of sterile isotonic saline every other day for 1 month. Rabbits received a dose of 0.2 mg/kg body wt. This dose was based on the standard protocol for producing clinical hyperthyroidism in laboratory animals.[6–11] At this point, significance was obtained in the morphometric analyses being performed. Baseline and treated levels of T3 and T4 were determined at 1 week, 2 weeks, 1 month, and at the time of euthanasia for control rabbits and for those receiving T3 injections. All animals showed significant elevations of T3 and T4 levels over baseline and over levels in control rabbits. As required by the institutional review board at the University of Minnesota, the rabbits were monitored daily for signs of hyperthyroidism including weight, food intake, water intake, diarrhea, and irritability. Rabbits were given access to food ad libitum. Rabbit weight dropped from an average of 2.23 kg to 1.37 kg (Fig. 1). The majority of the weight loss occurred within the first 2 weeks of treatment. The weight stabilized over the next 2 weeks of treatment, and during this interval the average weight of the treated rabbits fluctuated between 1.37 and 1.48 kg.

At the end of 1 month, the rabbits were euthanized with an overdose of barbiturate anesthesia. The eyelids were removed and placed in embedding molds containing tragacanth gum. The specimens were frozen in methylbutane and chilled to a slurry on liquid nitrogen. Serial sections at 12 μm were prepared in a cryostat in preparation for immunohistochemistry. Sections were immunostained for either fast, slow, neonatal, or developmental myosin heavy-chain (MHC) isoforms (NovoCastra Labs, Vector, Burlingame, Calif.). After incubation in blocking serum, the sections were incubated in the primary antibody for 1 hour at a dilution of 1:40 for the antibodies to fast and slow MHC isoforms and 1:20 for the antibodies to neonatal and developmental MHC isoforms. Control slides were processed by use of the same protocol except that the primary antibody step was omitted. These slides were negative for myosin staining. The sections were rinsed and incubated with the reagents of the Vectastain Elite ABC kit (Vector Labs, Burlingame, Calif.) containing biotin-avidin-peroxidase complexes. The reacted tissue sections were processed with the use of the heavy metal–intensified diaminobenzidine procedure.

The individual muscle fibers were traced and quantified with the use of the Bioquant digitizing morphometry program (R and M Biometrics, Inc., Nashville, Tenn.) to determine the number of muscle fibers in each eyelid section. Previous studies have demonstrated that orbicularis oculi myofibers do not span from the medial to the lateral canthus[16,17]; thus eyelid samples were prepared from the medial, central, and lateral regions of each eyelid. All myofibers were individually traced in 3 different cross sections in each of the three regions sampled. Since the orbital portion of the orbicularis oculi muscle is difficult to define, only the palpebral portion of the eyelids was counted. We defined the palpebral portion from the eyelid margin to the end of the palpebral conjunctiva. Averages were then obtained from the average number of myofibers in each of the 9 sections counted. The average of these was determined for the eyelids from the 4 control and 4 treated rabbits. Differential counts were made of the number of cross-sectional areas of myofibers positive for each of the three MHC isoforms examined (fast, slow, and neonatal myosin). These data were used to determine the percent positive for each myosin isoform from the total number of myofibers for that particular specimen. Averages were deter-

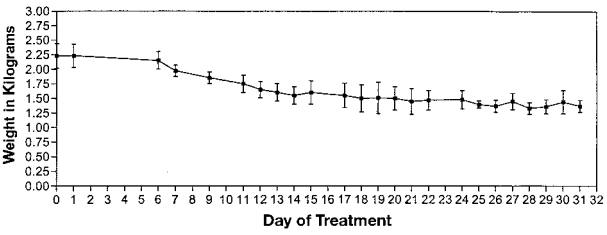

FIG. 1. Daily weights of adult rabbits during course of thyroid hormone treatments. Each point is the average of 4 treated rabbits (±SEM).

TABLE 1. *Palpebral orbicularis oculi fiber counts and area measurements*

Control fiber number	Hyperthyroid fiber number	Control fiber area	Hyperthryroid fiber area
2100	374	556	650
2259	577	600	576
1979	524	809	899
1880	368†	708	972†
2400	422†	677	910†
Mean = 2194	Mean = 453*	Mean = 670	Mean = 626
SEM = 94	SEM = 42	SEM = 44	SEM = 45

Hyperthyroid indicates hyperthyroid rabbit eyelid; Control, control rabbit eyelid.

*Statistically significant difference from control.

†Right and left lids of the same rabbit.

mined for each rabbit, and these were used to determine the averages of the 4 control and 4 experimental eyelids. Data were analyzed with the use of Prism software (Graphpad, San Diego, Calif.). Results were considered significantly different at $P<0.05$.

RESULTS

The eyelids from hyperthyroid rabbits had 79.4% fewer orbicularis oculi fibers compared with the control rabbit eyelids (Table 1 and Figs. 2 and 3). This was a statistically significant reduction ($P<0.05$). Almost all of the remaining muscle fibers were in the pretarsal region of the eyelids. Although myofiber number was reduced in the orbicularis oculi muscle from the hyperthyroid rabbits, the average myofiber cross-sectional area was not significantly different from control muscles (Table 1). The remaining myofibers within the orbicularis oculi muscle from the hyperthyroid rabbits showed an increase in the proportion that also expressed either slow myosin

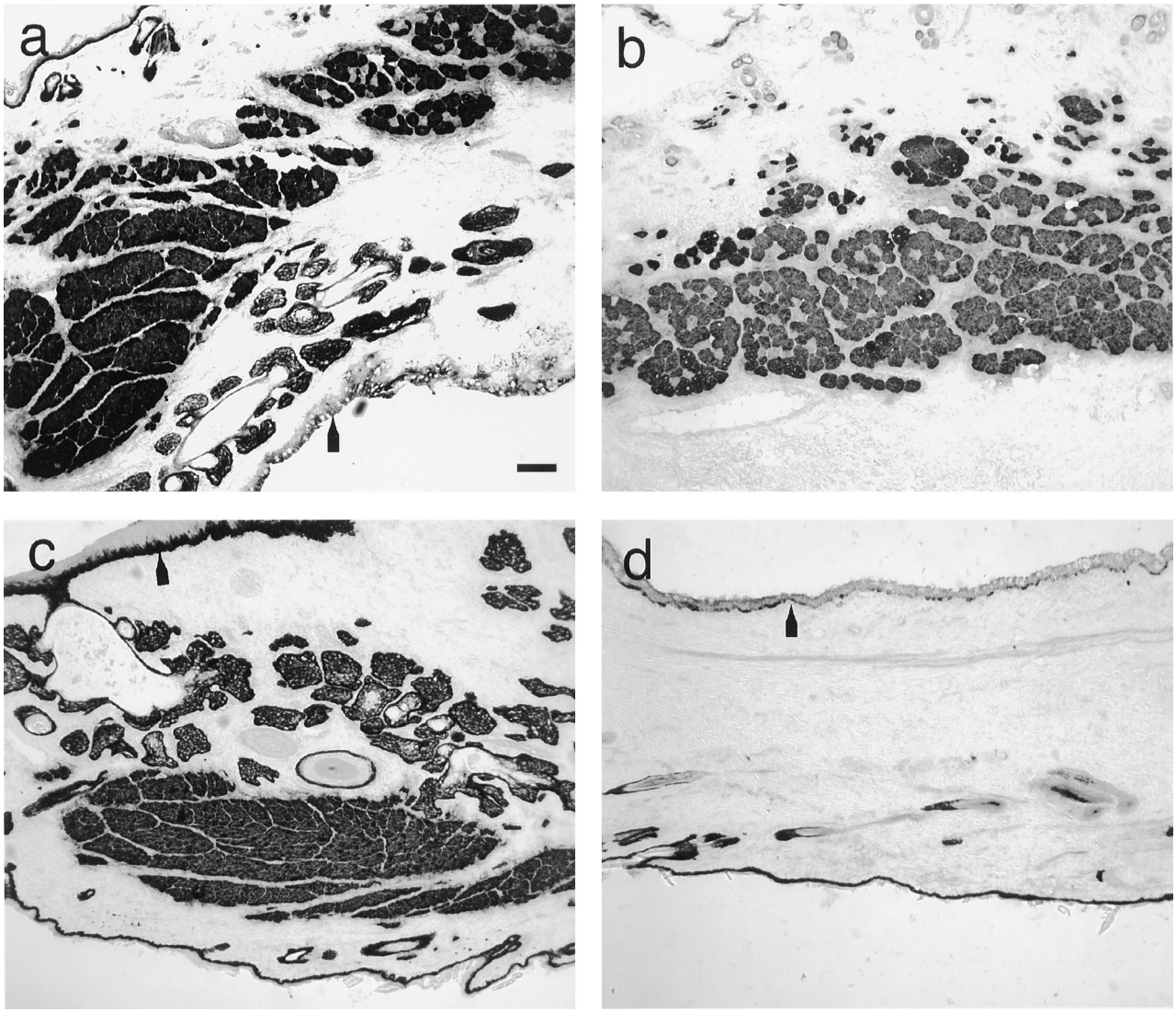

FIG. 2. Cross sections of the palpebral portion of rabbit eyelid stained with the alkaline myosin ATPase procedure. Fast myofibers stain dark in this procedure. **a,** control rabbit pretarsal orbicularis; **b,** control rabbit preseptal orbicularis; **c,** hyperthyroid rabbit pretarsal orbicularis; **d,** hyperthyroid rabbit preseptal orbicularis. All specimens are oriented with the eyelid margin to the left. Arrows indicate conjunctival surface of eyelids. Note reduction of the apparent number of myofibers in both pretarsal and preseptal portions of the hyperthyroid specimen. Although the preseptal portion of this eyelid was totally devoid of myofibers, eyelids from other hyperthyroid rabbits often retained scattered myofibers within this region. Bar is 100 μm.

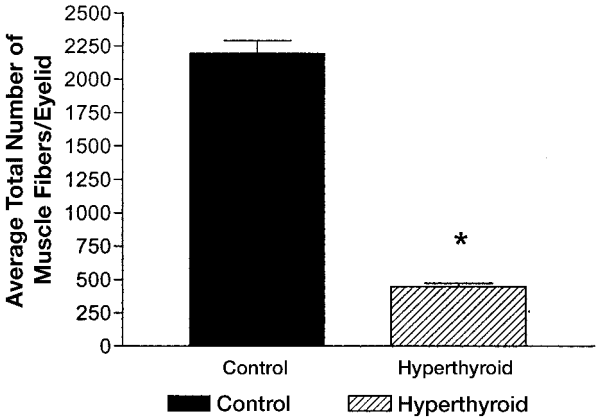

FIG. 3. Palpebral orbicularis oculi myofiber number in control and hyperthyroid rabbit eyelid sections. *Statistically significant.

or coexpressed both slow and fast myosin within single myofibers (Table 2 and Figs. 4 and 5). There was a trend toward increased expression of slow myosin in the pretarsal orbicularis oculi muscle, and this was statistically significant ($P<0.05$) in the preseptal muscle.

TABLE 2. *Myosin heavy chain expression in orbicularis oculi muscle from control and hyperthyroid rabbits*

	Percentage of myofibers positive for each MHC ± SEM	
	Pretarsal orbicularis	Preseptal orbicularis
Fast control	96.00 ± 0.500	76.50 ± 0.500
Fast hyperthyroid	98.70 ± 0.480	96.50 ± 0.808*
Slow control	4.0 ± 0.200	23.5 ± 0.800
Slow hyperthyroid	8.92 ± 3.97	61.167 ± 10.164*
Neonatal control	22.90 ± 0.176	37.60 ± 0.580
Neonatal hyperthyroid	28.00 ± 9.096	37.70 ± 4.000

Percentages do not add up to 100% because muscle fibers coexpress more than one MHC isoform.

*Statistically different from control eyelids.

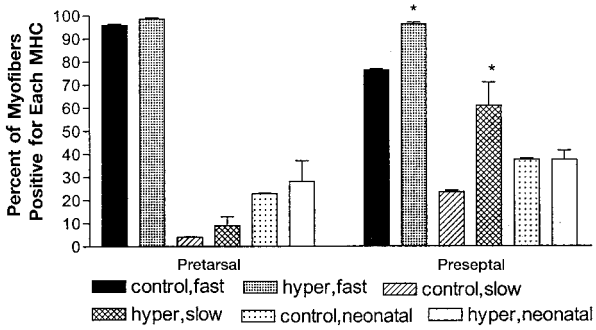

FIG. 4. Myosin heavy chain (MHC) isoform content of all myofibers within orbicularis oculi muscles from both control and hyperthyroid rabbits, including both pretarsal and preseptal regions. *Statistically different from control eyelids. Each bar represents data from 4 eyelids (mean ± SEM).

DISCUSSION

These results clearly demonstrate that elevated levels of thyroid hormone lead to significantly reduced numbers of palpebral orbicularis oculi muscle fibers after a 1-month period in rabbits. One possible mechanism is increased proteolysis induced by the hyperthyroidism, which has been previously demonstrated in a rat model.[18] Previous studies have shown other skeletal muscle dysfunction in human beings with hyperthyroidism secondary to Graves disease.[19] This finding was thought to be the result of elevated levels of thyroid hormone and circulating catecholamines. It is also interesting that elevated thyroid hormones were reported to hinder muscle repair and cause skeletal muscle atrophy in a mouse model of muscular dystophy.[20] This increase in thyroid hormone levels directly affects the energy metabolism of muscles, but in a muscle-specific manner.[7] In one study, the soleus muscle, composed largely of slow myofiber types, showed a significant alteration in energy metabolism, whereas the extensor digitorum longus, a muscle composed largely of fast myofiber types, did not.[7] However, neither of these muscles from the treated rabbits showed a decrease in myofiber number. These catabolic effects were seen in extraocular muscle as well, in which elevated thyroid hormones resulted in a decrease in muscle mass of approximately 50%.[21] Interestingly, recent studies demonstrated a direct effect of triiodothyronine on apoptosis in primary muscle cells in vitro.[22,23] These studies demonstrate the catabolic nature of elevated thyroid hormone and support our finding of orbicularis oculi myofiber loss in the hyperthyroid rabbit model in the current study.

Few studies have evaluated the effect of Graves disease on the orbicularis oculi muscle in human beings. Frueh et al.[24] evaluated the posterior force generated by the eyelid protractors of patients with Graves disease and found it to be significantly lower than that found in normal eyelids. They postulated that orbicularis atrophy was occurring in the patients with Graves disease, probably secondary to chronic eyelid inflammation. This study supports our finding of significant orbicularis oculi myofiber loss in hyperthyroid rabbits. Oestreicher and Frueh[25] found that the elastic modulus of orbicularis in patients with thyroid-associated eye disease was similar to that in normal patients. These results supported the fact that there was no functionally significant fibrosis

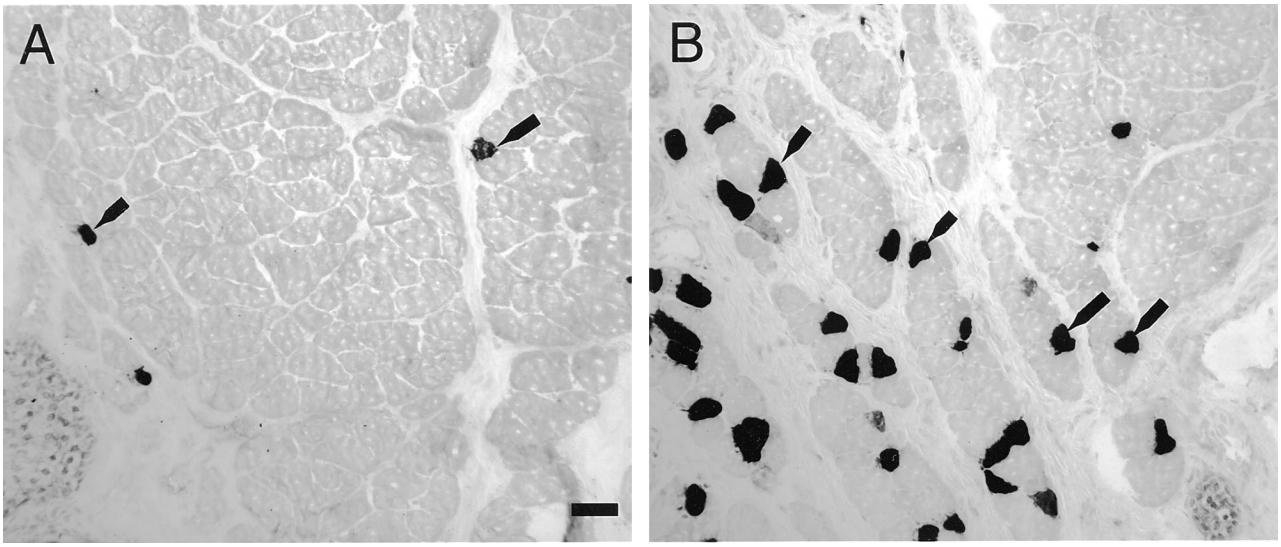

FIG. 5. Serial sections indicating coexpression of myosin heavy chain (MHC) isoforms in individual myofibers from the pretarsal region of the orbicularis oculi of **A,** control, and **B,** hyperthyroid rabbit eyelid. Cross sections are immunostained for slow MHC. Arrows indicate myofibers positive for slow MHC. Bar indicates 100 μm.

within the orbicularis muscle in these patients. Our specimens also showed no signs of fibrosis in the orbicularis oculi muscle and surrounding eyelid tissue.

Retraction of the upper eyelid in thyroid eye disease is a well-described clinical finding without a singular pathophysiologic explanation. Multiple authors have suggested a variety of mechanisms including increased sympathetic tone in the Müller and levator muscles,[26] levator muscle contraction and fibrosis, abnormal adhesions between levator muscle and orbital connective tissue and fat,[27] levator muscle hypertrophy,[24,28] and fixation duress secondary to inferior rectus restriction.[29,30] No correlation was shown between the amount of eyelid retraction and enlargement of the levator/superior rectus complex.[6,31] We believe another factor that may contribute to upper eyelid retraction is weakness of the antagonist protractor muscle, that is, the orbicularis oculi. Although not directly determined, a previous study supports this hypothesis as well.[24]

Our study does not address the finding of eyelid retraction in patients with euthyroid Graves opthalmolopathy. Although patients with no prior history of treatment for hyperthyroidism make up 8% to 21% of the patients with Graves ophthalmopathy, it has been demonstrated that between 22% and 88% of these patients have a palpable thyroid abnormality.[32] Another 62% of these patients have blunted responsiveness to thyroid-stimulating hormone.[32] Thus, although thyroid hormone abnormalities of these patients may be subtle, one cannot easily preclude thyroid-associated abnormalities in what otherwise appears to be euthyroid patients. Finally, eyelid retraction probably is a multifactorial process, of which orbicularis oculi weakness is one factor.

Of the myofibers that remained in the eyelids of the hyperthyroid rabbits, many coexpressed fast and slow MHC isoforms. Normal orbicularis oculi is composed primarily of fast myofibers.[17] An increased percentage of myofibers expressing slow myosin would result in muscles that are more fatigue-resistant and have slower contractile properties, which may correlate with the development of eyelid lag seen in patients with Graves ophthalmopathy.

The effect of elevated thyroid hormone levels has been studied in other animal adult skeletal muscles with various results. In soleus, a muscle containing largely slow myofibers, elevated thyroid hormone levels resulted in a shift to an increased number of myofibers positive for fast myosin in rats.[12,13] In plantaris, a muscle containing large fast myofibers, elevated thyroid hormone resulted in a shift to increased numbers of slow myofibers in rats.[14] In other muscles, elevated thyroid hormone levels caused changes in expression of all 6 genes in the MHC multigene family, and the same MHC gene could be regulated in different directions in different muscles.[15] Thus, there is precedence in the literature for elevated levels of thyroid hormone to cause MHC isoform transitions like those in the current study.

Clearly, the rabbit model that we used does not per-

fectly parallel the changes in Graves orbitopathy. This complex autoimmune inflammatory response is not the same as simple hyperthyroidism. Since there is currently no animal model of Graves orbitopathy, we chose to study the orbicularis oculi in hyperthyroid animals because the majority of patients with Graves disease have hyperthyroidism during the course of their illness. Previous studies have demonstrated the use of the hyperthyroid rabbit model.[10,11] An animal model of hyperthyroidism has been used to study the eyelid changes seen in patients with Graves orbitopathy.[6] It is evident from our study that hyperthyroidism itself may lead to significant changes in the orbicularis oculi muscle. The development of eyelid retraction in patients with Graves disease is probably due to a multifactorial effect of elevated thyroid hormones on the orbicularis oculi, Müller muscle, and the levator muscle. In the future, we plan to examine the orbicularis oculi muscle from patients with Graves disease.

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