Effects of Timolol on MYOC, OPTN, and WDR36 RNA Levels

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Objectives: To evaluate if timolol affects expression of 3 open-angle glaucoma genes and to study its ability to modulate dexamethasone-induced up-regulation of MYOC.

Methods: We used quantitative polymerase chain reaction assay of glaucoma gene transcript levels from human trabecular meshwork (HTM) cultures exposed to 3 different doses of timolol. Three HTM cell cultures were grown with or without 1 of 3 timolol doses in the presence or absence of dexamethasone.

Results: All 3 concentrations of timolol reduced MYOC RNA levels in 1 HTM culture compared with an untreated control and showed negligible effects in the other 2 cultures. Timolol had no effect on dexamethasone-induced MYOC transcript levels in any of the 3 cultures. Timolol, dexamethasone, and dexamethasone plus timolol had a negligible effect on OPTN and WDR36 RNA levels.

Conclusions: Timolol can reduce MYOC RNA levels in HTM cultures from some individuals. Timolol does not alter OPTN or WDR36 levels or ameliorate MYOC induction by dexamethasone in vitro.

Clinical Relevance: It remains to be determined whether timolol could reduce production of misfolded myocilin protein by HTM cells in individuals with MYOC missense mutations. A broader survey of interindividual variation in response to timolol as well as mechanistic studies are still needed before potential therapeutic implications can be explored.

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GLAUCOMA IS A MAJOR cause of blindness worldwide. Although the pathogenesis of glaucoma is not known, this disease is characterized by excavation of the optic nerve, resulting in progressive visual field loss that can lead to blindness. Four major prospective glaucoma clinical trials have demonstrated that elevated intraocular pressure (IOP) is a risk factor for glaucoma progression. Both lowering IOP to an appropriate target level and minimizing IOP fluctuation while receiving glaucoma treatment are important glaucoma treatment outcomes.

A glaucoma medical therapy commonly used to lower IOP is timolol, a non-selective β-adrenergic receptor antagonist or β-blocker. The main mechanism of action of the β-blockers is to decrease aqueous humor flow, resulting in lowering IOP. Although the primary IOP-lowering effects of timolol do not operate on trabecular outflow, β-adrenergic receptors are present on trabecular meshwork cells. Long-term use of timolol causes functional and morphological effects on trabecular meshwork cells. Although the clinical effects of timolol on IOP reduction are well established, little is known about the potential effects of timolol on expression of glaucoma genes. If induction of glaucoma genes has the potential to affect disease pathologic features, then it is important to know whether glaucoma medications used to treat patients can affect expression levels of glaucoma gene transcripts or products in relevant tissue types.

There are 3 known open-angle glaucoma (OAG) genes, myocilin (MYOC, Mendelian Inheritance of Man [MIM] 601652), optineurin (OPTN, MIM 602432), and WD repeat-containing protein 36 (WDR36, MIM 609669). Induction of MYOC has been observed in a cell culture model of steroid glaucoma and in native human trabecular meshwork (HTM) from patients with primary and secondary OAG; however, it is not known whether problems resulting from misfolded myocilin protein can be aggravated by increased expression in individuals with MYOC missense mutations. Little is known about conditions that might influence levels of expression of OPTN and
WDR36 or whether individuals with specific glaucoma gene mutations respond differently to specific therapies.

Herein we present data on transcript levels of 3 OAG genes, MYOC, OPTN, and WDR36, in response to timolol in primary cultures of HTM cells. We also evaluate the effect of timolol on transcript levels in the presence of dexamethasone. We present evidence that timolol has a variable effect on MYOC levels in the absence of dexamethasone and that expression of OPTN and WDR36 undergo little change in the presence of either of the therapeutic agents tested.

**METHODS**

**CELL CULTURES**

Eyes were obtained from the Michigan Eye Bank (Ann Arbor), which carried out informed consent and confirmed that none of the donors had been diagnosed with glaucoma. These studies were carried out according to a protocol approved by the University of Michigan institutional review board for human subjects research.

Primary cell cultures of HTM cells, designated HTM10M, HTM12F, and HTM17F, were grown from HTM tissue samples from a 10-year-old white boy (HTM10M), a 12-year-old white girl (HTM12F), and a 17-year-old white girl (HTM17F).26 The subject previously listed as being 16 years old26–27 has been reassigned an age of 10 years based on review of records. Corneal buttons28 were transferred to Optisol29 at 9 hours, 6 days, with media changes 3 times per week. The 21-day period was selected as a model for long-term treatment. A 1555–base pair (bp) region containing 1400 bp immediately 5’ to the reported MYOC translation start site was amplified by polymerase chain reaction (PCR) (Table 1) and sequenced as previously reported.26 No sequence changes were detected in the MYOC promoter (data not shown). Two replicates were performed for each condition assayed, except for the MYOC gene where 6 replicates were carried out from each of 2 different culture wells for each timolol concentration in the timolol-only experiment. Cycle threshold (Ct) values were determined using iCycler Optical System Software 3.0 (BioRad) with the default settings for a PCR baseline subtraction curve fit model. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as the control.

**QUANTITATIVE PCR**

Table levels were evaluated by quantitative PCR using the comparative threshold method. The PCR reactions were carried out on cDNA templates corresponding to 16.66 ng of total RNA using intron-spanning primers (Table 1) and an intercalating dye in iQ SYBR Green Supermix reaction mixture (BioRad Laboratories, Hercules, California). Thermal cycling conditions were 10 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 58°C, 30 seconds at 73°C, and a final extension step for 6 minutes at 72°C performed in an iCycler (BioRad) with an optical module. Each PCR product was confirmed to be a single band by melt curve analysis and visualization on agarose gel (data not shown).

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Orientation</th>
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</tr>
<tr>
<td></td>
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<td>(Reverse)</td>
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<td></td>
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<td>AGGTAGCTAGGCTGCTG</td>
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<td></td>
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Abbreviations: PCR, polymerase chain reaction; qPCR, quantitative PCR.

**RESULTS**

Three HTM cultures were grown with no drug, with timolol, with timolol plus dexamethasone, or with dexamethasone. Levels of gene transcripts were evaluated by quantitative PCR and comparisons were made between transcript levels under the different treatments for each cell culture and for the composite data for each cell culture.
When data from the 3 HTM cultures were pooled, the mean CT values for combined data for each condition did not vary significantly across conditions, except that a much lower CT value for MYOC in the presence of dexamethasone reflected the expected higher level of MYOC RNA in the presence of dexamethasone (Figure 1A). MYOC response to dexamethasone did not appear to differ with the presence or absence of timolol or vary between different timolol concentrations (Figure 1A), while similar GAPDH values across all cultures indicated that comparable amounts of RNA were present in each sample. Inclusion of timolol along with dexamethasone gave a negligible difference in MYOC RNA levels between cells grown with or without dexamethasone, whether evaluated for individual cultures or composite data for all cultures (Figure 1A and Figure 2A and C).

When considered separately, the 3 individual HTM cultures responded differently to timolol (Figure 2). For samples treated with timolol only, MYOC levels changed substantially, relative to untreated samples, for HTM10M but showed no appreciable change for HTM17F or HTM12F (Figure 2A and B). For HTM10M, the MYOC level dropped by more than 11-fold in response to 0.1 µM timolol, 9.2-fold in response to 1 µM timolol, and 5.6-fold in response to 10 µM timolol, with error bars overlapping between the 3 conditions. HTM17F and HTM12F showed less than 2-fold change in response to any timolol concentration. The 3 separate HTM cultures expressed different baseline levels of MYOC RNA as well as different levels of response to dexamethasone (Figure 2A). The selective effect of timolol on HTM10M did not represent an unusually high baseline level being restored to normal since HTM10M started out with the lowest baseline level and then dropped even further in response to timolol (Figure 2). Error bars indicate that the variability in the data for each cell culture and condition is low, with increased variability for the combined data reflecting that data have been combined from cell cultures that individually produce very different values (Figure 1A and Figure 2).

**OPTN AND WDR36 RNA LEVELS**

OPTN CT values for each of the 3 timolol concentrations were comparable with the mean normalized CT values for the untreated cells, with differences in RNA levels between treated and untreated conditions falling with the range of experimental error (Figure 1B). The 3 cell cultures not only showed a similar lack of response but also showed similar baseline values when untreated (Figure 3A). For each HTM culture, there were negligible differences between cell cultures treated with dexamethasone and the same cell culture treated with dexamethasone plus timolol (Figure 3B and C). For all conditions tested, OPTN findings for each of the 3 separate HTM cultures agreed with the findings for the composite data set (Figure 3). We regard the differences observed herein to be within the range of error of the experimental system.

Similarly, we found that mean WDR36 CT values were unchanged by exposure to dexamethasone, timolol, or the combination of the 2 (Figure 1C and Figure 4). In addition, the values for the 3 different cell cultures were all comparable with each other and within the range of experimental error (Figure 4).

**MYOC REGULATORY SEQUENCE VARIANTS**

We carried out PCR amplification of a fragment containing 1550 bp of sequence 5′ of the reported MYOC translation start site and assigned genotypes for previously reported sequence variants at 10 positions across the region (Table 2). We observed 2 apparent haplotypes, designated as haplotype 1, GACC13GTG, and haplotype 2, GCCC15ATG, consisting of variants at the positions of single-nucleotide polymorphisms and 1 length polymorphism shown in Table 2. HTM10M and HTM12F were both heterozygous across the region, each having
both haplotype 1 and haplotype 2, while HTM17F was homozygous for haplotype 1. Thus, across this proximal promoter region the haplotypes are the same for HTM10M and HTM12F and different for HTM17F (Table 2).

Figure 2. MYOC expression in treated vs untreated human trabecular meshwork cultures HTM10M, HTM12F, and HTM17F and all cultures (HTM(ALL)). A, Mean difference in cycle threshold (ΔCt) (MYOC – GAPDH). B and C, Fold change in MYOC level (treated vs untreated) is shown in response to timolol (B) and dexamethasone plus timolol (C). Error bars indicate standard deviation.

An important novel finding of this study is the substantial reduction of MYOC expression in response to timolol in 1 of 3 cultures tested. When we look at the aver-
Average level of MYOC RNA in the composite data set, we see little difference in MYOC levels with or without timolol; however, when we consider the data for the separate cell cultures, 2 cultures remained unchanged by timolol exposure while HTM10M showed differences in MYOC RNA levels ranging from more than a 5-fold decrease to more than an 11-fold decrease across the range of timolol concentrations tested. Although this is a large decrease, the MYOC transcript is only decreased and not eliminated.

Figure 3. OPTN expression in treated vs untreated human trabecular meshwork cultures HTM10M, HTM12F, and HTM17F and all cultures (HTM[ALL]). A, Mean difference in cycle threshold (\(\Delta C_T\)) (OPTN–GAPDH). B and C, Fold change in OPTN level (treated vs untreated) is shown in response to timolol (B) and dexamethasone plus timolol (C). Error bars indicate standard deviation.
A curious accompanying observation is that timolol had no effect on dexamethasone-induced MYOC RNA level increases in any of the 3 cell cultures tested, not even the HTM10M culture that showed a decrease in MYOC level in response to timolol alone. Thus, the mechanism by which timolol reduces MYOC expression in culture HTM10M might be unrelated to the mechanism by which dexamethasone induces MYOC in that same culture, but we cannot be sure until the pathways mediating each change are elucidated.

Figure 4. WDR36 expression in treated vs untreated human trabecular meshwork cultures HTM10M, HTM12F, and HTM17F and all cultures (HTM[ALL]). A, Mean difference in cycle threshold (∆C_t) (WDR36−GAPDH). B and C, Fold change in WDR36 level (treated vs untreated) is shown in response to timolol (B) and dexamethasone plus timolol (C). Error bars indicate standard deviation.
Our dexamethasone data are consistent with our previous observations that HTM10M had the largest MYOC changes in response to dexamethasone and that the apparently smaller MYOC response of HTM17F is in part due to the fact that it started out with a higher baseline value in untreated cells. Based on our experience, the variation in dexamethasone-induced MYOC levels, with and without timolol, is consistent with what we usually observe in experiments involving no timolol. 36, 37 All of the MYOC values obtained in both treated and untreated samples fall within the range of values normally seen in our cell cultures from unaffected individuals and suggest that timolol is not blocking or reducing MYOC induction by dexamethasone.

In contrast to the variable effect on MYOC RNA levels, timolol had negligible effects on levels of OPTN or WDR36 RNA. Similarly, dexamethasone had little effect on their RNA levels, and this situation was not altered in the presence of timolol. The very small differences observed represent the range of expected variability of the experimental techniques. Thus, we conclude that within the limits of this particular cell culture and assay system, neither dexamethasone nor timolol altered levels of OPTN or WDR36 RNA over a 3-week course of treatment.

Thus, our results from a limited sample size suggest that timolol exerts consistent but different effects on the RNA levels, with and without timolol, is consistent with what we usually observe in experiments involving no timolol. 36, 37 All of the MYOC values obtained in both treated and untreated samples fall within the range of values normally seen in our cell cultures from unaffected individuals and suggest that timolol is not blocking or reducing MYOC induction by dexamethasone.

### Table 2. MYOC Promoter Region Sequence Variants in 3 HTM Cell Cultures

<table>
<thead>
<tr>
<th>Position Change</th>
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<tr>
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<tr>
<td>T→C</td>
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<tr>
<td>T→C</td>
<td>T/T</td>
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</table>

Abbreviation: HTM, human trabecular meshwork.

In summary, timolol caused reduced expression of MYOC in 1 of 3 HTM cultures. It is unclear whether timolol might be used to limit the induction of MYOC by other mechanisms discussed by other groups such as sheer stress, oxidative stress, or the misfolded protein response to presence of MYOC missense mutations. 30, 40, 48

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Author Contributions: Drs Richards and Rozsa had access to all of the data and take full responsibility for the findings.
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REFERENCES