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.1 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.2-5 Both lowering IOP to an appropriate target level6 and minimizing IOP fluctuation while receiving glaucoma treatment2,7 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,8 resulting in lowering IOP. Although the primary IOP-lowering effects of timolol do not operate on trabecular outflow,9 β-adrenergic receptors are present on trabecular meshwork cells.10-12 Long-term use of timolol causes functional and morphological effects on trabecular meshwork cells.13-16 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).17-21 Induction of MYOC has been observed in a cell culture model of steroid glaucoma22 and in native human trabecular meshwork (HTM) from patients with primary and secondary OAG23; however, it is not known whether problems resulting from misfolded myocilin protein24,25 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 re-assigned an age of 10 years based on review of records. Corneoscleral buttons26 were transferred to Optisol29 at 9 hours, 6 hours 45 minutes, and 13 hours 25 minutes post mortem, respectively. Human trabecular meshwork was dissected from the corneoscleral button and cultured as previously described.26 Deaths of all 3 donors were accidental and 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.

RNA using intron-spanning primers (Table 1) and an intercalating dye in iQ SYBR Green Supermix reaction mixture (Bio-Rad 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, 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). Six replicates were performed for each gene and 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 subtracted curve fit model. The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as the control.

### STATISTICAL ANALYSIS

The fold change for MYOC, WDR36, and OPTN levels in drug-treated cell cultures relative to levels in untreated cell cultures used values normalized to GAPDH control levels. The mean (SD) C_{T} values for replicates of each individual cell culture and the composite average of 3 cell cultures were calculated for each gene. The C_{T} is exponentially related to copy number, with error bars represented by the fold change calculation (2^{-ΔΔC_{T}}) plus and minus the standard deviation.

### 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 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 0.1µM timolol, 9.2-fold in response to 1µM timolol, and 5.6-fold in response to 10µM 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 $C_T$ values for each of the 3 timolol concentrations were comparable with the mean normalized $C_T$ 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 $C_T$ 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, GACC13GTT, and haplotype 2, GCC15ATT, 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 ($\Delta C_t$) ($\text{MYOC} - \text{GAPDH}$). 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-
age 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 a 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 (ΔCt) (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 (∆Ct) (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.

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 from 3 OAG genes in HTM. Selective biological effects of β-blocker therapy have been demonstrated in other tissues. For instance, in congestive heart failure, β-blocker treatment improves cardiac function by altering myocardial gene expression with time-dependent biological effects in cardiomyocytes. Interactions between β-adrenergic receptors and nitric oxide signaling have been shown at both biochemical and transcriptional expression levels in various tissues that include endothelium and ocular ciliary processes. The β-blockers have also been shown to increase subconjunctival collagen density by regulating expression of specific matrix metalloproteinases and tissue inhibitors of metalloproteinases.

Evaluation of more than a thousand bases of sequence proximal to the MYOC transcription start site indicated that sequence differences in the proximal promoter region of MYOC are not responsible for the observed timolol-response differences between HTM10M and the other 2 HTM cultures. HTM10M, with its decrease in response to timolol, had the same promoter region diplo-type as HTM12F, which showed negligible reaction to timolol. HTM17F and HTM12F, which had very similar responses to timolol, share one haplotype but differ at the second haplotype. Additional sequencing and functional studies would be needed to evaluate changes in other potential regulatory sequences further from the transcription start site or in unidentified enhancer sequences, but the underlying basis for the response difference of HTM10M could reside elsewhere in the genome.

It is curious that the cell culture that responds to timolol comes from a male donor, HTM10M, while the 2 cell cultures that do not respond come from female donors HTM12F and HTM17F. However, IOP-lowering effects of timolol are not thought to be differential according to sex, and it would be premature to conclude any association of MYOC timolol responsiveness with sex or other characteristics based on so few samples.

Our use of cultured cells has the disadvantage that it might fail to mimic responses in vivo and the advantage that it separates out the HTM cell responses to timolol from responses to secondary factors such as elevated IOP or altered aqueous composition. In addition, the study’s limited scope encompasses only the 3 known OAG genes even though it is clear that there are many additional yet-to-be-identified glaucoma genes whose expression we will eventually want to evaluate. These experiments do not tell us what effect the altered RNA level might have on accumulation of myocilin protein over time.

It would appear that use of timolol might not be of any assistance in bringing MYOC RNA levels back down into the normal range in the presence of dexamethasone. Since the pathways by which MYOC induction occurs are not well characterized, 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.

In summary, timolol caused reduced expression of MYOC in 1 of 3 HTM cultures. It is unclear whether timolol could play a role in preventing or delaying onset of myocilin-based glaucoma by reducing the amount of aberrant myocilin protein in individuals with MYOC mutations; down-regulation of MYOC would not be predicted to be a problem since a reduction or loss of myocilin has been reported to cause no identifiable abnormalities. Mechanistic studies, determination of the prevalence of the timolol-MYOC effect, and evaluation of in vivo responses will all be needed before deciding whether timolol treatment will have valid applications in presymptomatic juvenile glaucoma in individuals with causative MYOC mutations.

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