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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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). 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 buttons28 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. Deaths of all 3 donors were accidental and the Michigan Eye Bank considered their medical and ocular histories to be unremarkable. Cells from the fifth passage were grown in Dulbecco modified Eagle medium containing 15% fetal calf serum, supplemented with 1 ng/mL of basic fibroblast growth factor (bFGF) at 37°C under 10% carbon dioxide to confluence. Cells were harvested using Trizol (Invitrogen, Carlsbad, California) according to the manufacturer’s directions. The RNA quality and quantity were evaluated by spectrophotometry and gel electrophoresis. Total RNA was reverse transcribed to complementary DNA (cDNA) using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California) according to the manufacturer’s directions.

**QUANTITATIVE PCR**

Transcript 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 (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, 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). 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) values showed little variation across all samples (absolute CT range, 13.62-17.42; mean, 16.38) and did not vary substantially in response to different treatment conditions when normalized to the eukaryotic translation initiation factor 4E gene (EIF4E) 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\textsubscript{T} values for replicates of each individual cell culture and the composite average of 3 cell cultures were calculated for each gene. The C\textsubscript{T} is exponentially related to copy number, with error bars represented by the fold change calculation (2\textsuperscript{ΔΔC_{\text{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 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).

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 within 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, GACC13GGT, and haplotype 2, GACC15ATT, 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).

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-

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.
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 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 (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 (ΔC) (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 over a 3-week course of treatment. 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,45 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 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 shear stress, oxidative stress, or the misfolded protein response to presence of MYOC missense mutations.29-46-48

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.49-52 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 preymptomatic juvenile glaucoma in individuals with causative MYOC mutations.

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