Melatonin and Its Analog 5-Methoxycarbonylamino-N-Acetyltryptamine Potentiate Adrenergic Receptor-Mediated Ocular Hypotensive Effects in Rabbits: Significance for Combination Therapy in Glaucoma

Almudena Crooke, Fernando Huete-Toral, Alejandro Martínez-Águila, Alba Martín-Gil, and Jesús Pintor

Departamento de Bioquímica, Facultad de Óptica y Optometría, Universidad Complutense de Madrid, Madrid, Spain

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ABSTRACT

Melatonin is currently considered a promising drug for glaucoma treatment because of its ocular hypotensive and neuroprotective effects. We have investigated the effect of melatonin and its analog 5-methoxycarbonylamino-N-acetyltryptamine, 5-MCA-NAT, on β2/α2A-adrenergic receptor mRNA as well as protein expression in cultured rabbit nonpigmented ciliary epithelial cells. Quantitative polymerase chain reaction and immunocytochemical assays revealed a significant β2-adrenergic receptor down-regulation as well as α2A-adrenergic receptor up-regulation of treated cells (P < 0.001, maximal significant effect). In addition, we have studied the effect of these drugs upon the ocular hypotensive action of a nonselective β-adrenergic receptor (timolol) and a selective α2-adrenergic receptor agonist (brimonidine) in normotensive rabbits. Intraocular pressure (IOP) experiments showed that the administration of timolol in rabbits pretreated with melatonin or 5-MCA-NAT evoked an additional IOP reduction of 14.02% ± 5.8% or 16.75% ± 5.48% (P < 0.01) in comparison with rabbits treated with timolol alone for 24 hours. Concerning brimonidine hypotensive action, an additional IOP reduction of 29.26% ± 5.21% or 39.07% ± 5.81% (P < 0.001) was observed in rabbits pretreated with melatonin or 5-MCA-NAT when compared with animals treated with brimonidine alone for 24 hours. Additionally, a sustained potentiating effect of a single dose of 5-MCA-NAT was seen in rabbits treated with brimonidine once daily for up 4 days (extra IOP decrease of 15.57% ± 5.15%, P < 0.05, compared with brimonidine alone). These data confirm the indirect action of melatoninergic compounds on adrenergic receptors and their remarkable effect upon the ocular hypotensive action mainly of α2-adrenergic receptor agonists but also of β-adrenergic antagonists.

Introduction

Glaucoma is an optic neuropathy in which a progressive death of the retinal ganglion cells and loss of their axons in the optic nerve lead to blindness (Quigley, 2011). Chronic high intraocular pressure (IOP) produces an excessive release of the neurotransmitter glutamate that evokes an increase in free radicals and finally retinal ganglion cell death (Moreno et al., 2005; Rosenstein et al., 2010; Almasieh et al., 2012). Glaucoma is, therefore, commonly treated with ocular hypotensive agents. Recent studies suggest that the desirable approach for this disease would be a combination of hypotensive and neuroprotective treatments (Cordeiro and Levin, 2011; Pascale et al., 2012).

One molecule with interesting neuroprotective actions and ocular hypotensive effects is melatonin (N-acetyl-5-methoxytryptamine) (Rowland et al., 1981; Siu et al., 1999; Siu et al., 2006; Alarma-Estrany et al., 2007). This molecule and some of its derivatives produce a marked reduction in IOP when topically applied (Pintor et al., 2001, 2003; Serle et al., 2004; Ismail and Mowafi, 2009; Alarma-Estrany et al., 2008, 2011). This ocular hypotensive action of melatonin and its derivatives seems to be mediated by ciliary body melatonin receptors such as melatonin (MT2) receptor (Alarma-Estrany et al., 2008) together with an unidentified melatonin receptor classically named MT3 (Alarma-Estrany et al., 2009; Vincent et al., 2010). However, the underlying molecular mechanisms of these receptors reducing IOP are still unknown.

A connection between the sympathetic nervous system and the hypotensive effect of melatonin as well as its analog 5-methoxycarbonylamino-N-acetyltryptamine (5-MCA-NAT), the
selective MT$_3$ receptor agonist, has been reported by Alarma-Estrany et al. (2007). Chemical sympathomimetics by means of reserpine or 6-hydroxydopamine significantly inhibits the hypertensive effect of melatonin and 5-MCA-NAT (Alarma-Estrany et al., 2007). Moreover, we have recently shown preliminary evidence to indicate that 5-MCA-NAT regulates the gene expression of ciliary adrenergic receptors (Crooke et al., 2011).

The sympathetic nervous system controls the production of the aqueous humor (AH) by the $\beta_2$- and $\alpha_2$-adrenergic receptors present in ciliary epithelium (McLaren, 2009; Pintor, 2009). The balance between ciliary production of the AH (Civan and Macknight, 2004) and its drainage through the trabecular meshwork pathway (Ferrer, 2006) or the uveoscleral pathway (Alm and Nilsson, 2009) determines IOP.

Drugs targeting the ocular adrenergic receptors, including $\beta$-adrenergic receptor antagonists and $\alpha_2$-adrenergic receptors agonists, reduce IOP by decreasing AH secretion. In addition, $\alpha_2$-adrenergic receptors agonists also reduce IOP by increasing uveoscleral AH outflow (Adkins and Balfour, 1998). Moreover, a neuroprotective role independent of the IOP lowering effect has been described for the $\alpha_2$-adrenergic receptor agonist brimonidine [5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)quinolin-6-amine] in experimental models of glaucoma and more recently in patients with normal tension glaucoma (Wheeler et al., 2003; Krupin et al., 2011; Pinar-Suero et al., 2011). Therefore, these compounds are currently used as effective drugs for glaucoma treatment.

When first-choice drugs are not sufficiently effective or produce adverse effects, the combination of two or three first-choice drugs is a common strategy (Webers et al., 2008). The most often fixed combinations used to treat glaucoma, combine a $\beta$-adrenergic receptor antagonist with another drug, including $\alpha_2$-adrenergic receptors agonist (Combigan), carbonic anhydrate inhibitors (Cosopt), or hypotensive lipids (Xalcom, Dutraov, and Gatifort) (Webers et al., 2008). Fixed drug combinations cause an additional IOP decrease without the disadvantages of adverse effects (Webers et al., 2008).

In this study, we investigate the effect of melatonin and 5-MCA-NAT on ciliary nonpigmented epithelium $\alpha_2A$- and $\beta_2$-adrenergic receptor mRNA as well as protein expression. In addition, we have studied the possible potentiating effect of melatoninergic drugs upon the ocular hypertensive action of brimonidine, a selective $\alpha_2$-adrenergic receptor agonist (Alphagan), and timolol, (2S)-1-(tert-buty lamino)-3-[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]propan-2-ol (Timolol), a nonselective $\beta$-adrenergic receptor antagonist.

Materials and Methods

Animals and Cultured Cells. Male New Zealand white rabbits, weighing 3–4 kg, were used for IOP studies. The animals were kept in individual cages with free access to food and water. They were provided with controlled 12-hour light/dark cycles. All the protocols herein comply with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and also are in accordance with the European Communities Council Directive (86/609/EEC). Every attempt was made to minimize the number of animals tested.

Studies of mRNA and protein expression were conducted using immortalized rabbit nonpigmented ciliary epithelial (NPE) cells, kindly provided by Dr. Coca-Prados (Department of Ophthalmology and Visual Science, Yale University School of Medicine, New Haven, CT). Cells were seeded (30,000 cells/well) in triplicate 24-well plates and grown at 37°C in high glucose Dulbecco’s modified Eagle’s medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), under a humidified atmosphere of 5% CO$_2$/95% air. Gentamicin (50 mg/ml; Sigma-Aldrich) was added to medium to maintain constant concentration of these compounds.

RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction. At the indicated time points, 240,000 cells (approximate) were washed with phosphate-buffered saline (PBS), and the total RNA was isolated using the RNeasy Mini kit (Qiagen, Madrid, Spain), according to the manufacturer’s protocol. To obtain 240,000 cells at 24, 48, and 72 hours, we used cells from eight, four, and three wells, respectively. To assay adrenergic gene expression in vivo, we sacrificed animals treated with melatonin or 5-MCA-NAT plus brimonidine (24 hours after the treatment) or timolol (12 hours after the treatment) with an overdose of sodium pentobarbital (n = 2 eyes for each treatment). Afterward, eyes were enucleated and intact rings of NPE were isolated following the protocol described by Shahidullah et al. (2007). Afterward, the total RNA was extracted from NPE cells as we mentioned earlier. The amount of total RNA isolated was quantified using the Quant-iT RiboGreen RNA kit (Invitrogen). For first-strand cDNA synthesis, 1 μg of total RNA was retrotranscribed using a High Capacity cDNA RT kit with random hexamer primers (Applied Biosystems, Carlsbad, CA). Real-time quantitative PCR (qRT-PCR) was performed in duplicate using the Quantitect SYBR Green Kit (Qiagen) with gene-specific PCR primers on an ABI Prism 7300 Real-Time PCR System (Applied Biosystems).

The following primers were used in qRT-PCR amplification: ADRA2A forward 5′-TTCTGGTTCCGGCTACTGCAA-3′ and ADRA2A reverse 5′-GCCGACTAGATCCTTGTGAA-3′ (for amplification of $\alpha_2A$-adrenergic receptor cDNA); and $\beta_2$-adrenergic receptor (ADRB2) forward 5′-CGAACCAGGCTATGCAAT-3′ and ADRB2 reverse 5′-CTTTGTTGGGCAACCTGGAAGA-3′ (for amplification of $\beta_2$-adrenergic receptor cDNA). Nontemplate and non-reverse-transcribed controls were included in all analyses. The melting curves of the amplification products were included for all experiments. The melting of the agarose gel electrophoresis confirmed the specificity of PCR and the absence of primer-dimers. The ATP5B gene (ATP5B forward 5′-GGATGCGACGGAAGAC-3′ and ATP5B reverse 5′-AATTTTG- CATAGGCCACCCAG-3′) was used as the internal control to normalize mRNA relative expression, after its validation for qRT-PCR. Validation of the ATP5B gene as the internal control implies that its expression is unaffected under our experimental conditions. The methodology used to validate internal control gene and to perform the qRT-PCR data analysis have been described in detail elsewhere (Crooke et al., 2012b).

Immunocytochemical Studies. Immunofluorescent staining was performed to evaluate $\alpha_2A$- and $\beta_2$-adrenergic receptor expression in rabbit NPE cells treated with melatonin and 5-MCA-NAT.

NPE cells were seeded on Laboratory-Tek chamber slides (Nunc-Thermo Fisher Scientific, Rochester, NY) and cultured in presence or absence (control cells) of melatonin or 5-MCA-NAT, as described previously, for 72 hours. After this time, the cells were washed in PBS and fixed with 4% paraformaldehyde in PBS for 15 minutes at room temperature.

After several washes in PBS and preincubation in PBS with 3% blocking serum for 1 hour, cells were incubated for 2 hours with the primary goat polyclonal anti-ADRA2A (sc-1478, antibody raised against $\alpha_2A$-adrenergic receptor) or with mouse monoclonal anti-ADRB2-fluorescein isothiocyanate (FITC) (sc-81577 FITC, antibody raised against $\beta_2$-adrenergic receptor) (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:50 in a dark humidified chamber at room
temperature. After this time, cells incubated primarily with anti-ADRA2A were washed with PBS and incubated in a dark chamber with the secondary antibody donkey anti-goat IgG-FITC (Jackson ImmunoResearch, West Grove, PA) at 1:200 for 1 hour at room temperature. Finally, the cells were washed in PBS and mounted in Vectashield mounting media (Vector Laboratories, Peterborough, UK). Then, the samples were observed under a confocal microscope (Axiovert 200M; Carl Zeiss Meditec GmbH, Jena, Germany), equipped with a PASCAL confocal module (LSM 5; Zeiss). All images were managed with the accompanying PASCAL software. Fluorescent staining of α2A- and β2-adrenergic receptors was quantified as the mean fluorescence intensity per cellular surface area (Lyashkov et al., 2007; Grewal et al., 2008; Crooke et al., 2012b), using ImageJ Software (free public domain software; NIH, Bethesda, MD) (Collins, 2007).

Intraocular Pressure Measurements. Melatonin (Sigma-Aldrich) and 5-MCA-NAT (Tocris) were formulated in isotonic saline containing 1% dimethylsulfoxide (DMSO; Sigma-Aldrich) and were tested at a final concentration of 100 μM (10 μl; doses of 2.32 and 2.75%, respectively). Alphagan 0.2% (brimonidine tartrate ophthalmic solution; Allergan, Irvine, CA) and Timofof 0.5% (timolol maleate ophthalmic solution; Frosst, Madrid, Spain) were instilled at a fixed volume of 40 μl.

IOP was measured by use of a TonoVet Contact tonometer supplied by Tiolat Oy (Helsinki, Finland). All compounds were applied bilaterally to the cornea in the treated animals (six rabbits per group). Six control animals received the same volume of saline + 1% DMSO.

Basal IOP was measured twice in each rabbit before treatment with different substances. These values were used to normalize the real changes of IOP in each rabbit. On day zero, rabbits were instilled with melatonin, 5-MCA-NAT, Alphagan, Timofof, or saline + 1% DMSO (control rabbits), and the IOP was measured during 6 hours. These values were taken as the reference for the normal effect of each compound.

In the following days (1–4 days), rabbits which were pretreated with melatonin or 5-MCA-NAT, and were instilled with Alphagan or Timofof once daily. Rabbits that were treated with Alphagan or Timofof received the same compound during the four days. Instillation and IOP measurements were performed all days as described previously.

Statistical Analysis. All data are presented as the mean ± S.E.M. Statistical differences between treatments were calculated using analysis of variance test and Tukey-Kramer test. Plotting and fitting were performed with the computer program GraphPad Prism 5 (GraphPad Software, La Jolla, CA).

Results

Regulation of In Vitro α2A-Adrenergic Receptors Expression by Melatonin and 5-MCA-NAT. We analyzed the effect of the natural ligand melatonin and 5-MCA-NAT on the α2A-adrenergic receptor mRNA as well as its protein levels. As shown in Fig. 1, a statistically significant increase of α2A-adrenergic receptor expression was observed with both compounds in comparison with the corresponding controls. Adrenergic receptor mRNA upregulation (Fig. 1A, left axis) was very similar between both compounds and began after 48 hours of treatment (1.7-fold relative to control, P < 0.05). In addition, α2A-adrenergic receptor mRNA levels were still increased after 72 hours of treatment (2.2-fold versus control, P < 0.01). Furthermore, the results of immunocytochemical studies showed a statistically significant qualitative increase of adrenergic receptor protein levels after 72 hours of melatonin and 5-MCA-NAT treatment (179.53% ± 17.96%, P < 0.001 and 148.59% ± 12.35%, P < 0.01, respectively) in comparison with the untreated cells (Fig. 1A, right axis, and B).

Regulation of In Vitro β2-Adrenergic Receptors Expression by Melatonin and 5-MCA-NAT. Concerning β2-adrenergic receptor expression, a reduction in mRNA and protein levels was observed in NPE cells when they were treated with melatonin as well as 5-MCA-NAT in comparison with the corresponding controls (Fig. 2). The adrenergic receptor mRNA downregulation in treated cells was statistically significant (P < 0.001) at all time points (Fig. 2A, left axis). As shown in Fig. 2 (left axis), the minimal amount of β2-adrenergic receptor transcripts (2.5-fold lower than control levels, P < 0.001) was achieved after 48 hours of 5-MCA-NAT treatment (Fig. 2A, left axis). Melatonin treatment also evoked
a maximal effect on mRNA content after 48 hours, but the reduction was more profound than obtained with 5-MCA-NAT (4.7-fold lower than control levels, \( P < 0.001 \)) (Fig. 2A, left axis).

In addition, \( \beta_2 \)-adrenergic receptor protein levels were qualitatively reduced after 72 hours of melatonin and 5-MCA-NAT treatment but to a lesser extent than the mRNA reduction (79.61% \( \pm \) 2.75% and 78.65% \( \pm \) 4.56%, respectively, versus control) (Fig. 2A, right axis; Fig. 2B).

Melatonin and 5-MCA-NAT Potentiate the Ocular Hypotensive Effect of Brimonidine. To study a possible potentiating effect of melatonergic drugs upon the ocular hypotensive action of brimonidine (Alphagan), a selective \( \alpha_2 \) adrenergic receptor agonist, we performed IOP experiments. The time course of the effect of melatonin, 5-MCA-NAT, and brimonidine on rabbit IOP was observed for 6 hours. The treatment with either of these compounds resulted in a maximal IOP reduction of 18.6% \( \pm \) 1.30%, 53.4% \( \pm \) 2.31%, and 40.6% \( \pm \) 3.52%, for melatonin, 5-MCA-NAT, and brimonidine, respectively, when compared with control (\( P < 0.001 \)) (Fig. 3).

The IOP changes over time after daily dosing of brimonidine during 4 days were studied but no statistically significant differences were observed (unpublished data).

The pretreatment with a single dose of melatonin or 5-MCA-NAT significantly potentiated the hypotensive action of brimonidine for up to 96 hours (Fig. 3). As shown in Fig. 3A, the maximal reduction in IOP in rabbits pretreated with melatonin was evoked by brimonidine 24 hours after the pretreatment with the indole, and it resulted in an additional IOP reduction of 29.3% \( \pm \) 5.21% (IOP reduction of rabbits treated with melatonin plus brimonidine = 69.9% \( \pm \) 1.69%; IOP reduction of rabbits treated with brimonidine alone = 40.6% \( \pm \) 3.52%; \( P < 0.001 \)).

The 5-MCA-NAT pretreatment also evoked a maximal reduction of IOP in rabbits treated with brimonidine for 24 hours, but the reduction was stronger than that obtained with melatonin (IOP reduction of rabbits treated with 5-MCA-NAT plus brimonidine = 79.7% \( \pm \) 2.29%, \( P < 0.001 \)) (Fig. 3B). Furthermore, these extra IOP reductions were statistically significantly maintained for up to 96 hours with values of 7.2% \( \pm \) 4.29% (\( P < 0.05 \)) for melatonin (Fig. 3A) and 15.6% \( \pm \) 5.15% for 5-MCA-NAT (\( P < 0.01 \)) (Fig. 3B).

Melatonin and 5-MCA-NAT Potentiate the Ocular Hypotensive Effect of Timolol. We also performed experiments with IOP with melatonin or 5-MCA-NAT plus timolol (Timofol), a nonselective \( \beta_2 \)-adrenergic receptor antagonist. Timolol alone produced a maximal IOP reduction of 25.8% \( \pm \) 3.39% (\( P < 0.001 \)) in comparison with control values (Fig. 4). The IOP changes over time after daily dosing with timolol over 4 days were studied, but no statistically significant differences were observed (unpublished data). The pretreatment with melatonin or 5-MCA-NAT also significantly potentiated the hypotensive action of timolol, but only for 24 hours (Fig. 4). At this time, melatonergic pretreatment evoked a similar maximal IOP reduction, 39.8% \( \pm \) 2.41% (\( P < 0.01 \)) for melatonin and 42.6% \( \pm \) 2.09% (\( P < 0.01 \)) for 5-MCA-NAT (14% to 17% of extra IOP reduction compared with animals treated with timolol alone, respectively) (Fig. 4).

Regulation of In Vivo \( \alpha_{2A}/\beta_2 \)-Adrenergic Receptor Expression by Melatonin and 5-MCA-NAT. We analyzed the effect of melatonin and 5-MCA-NAT on \( \alpha_{2A} \)-adrenergic receptor mRNA as well as \( \beta_2 \)-adrenergic receptor mRNA in rabbit eyes. As shown in Fig. 5A, a statistically significant increase of \( \alpha_{2A} \)-adrenergic receptor expression was observed in rabbits treated with 5-MCA-NAT plus brimonidine in comparison with the corresponding control (2.3-fold relative to control, \( P < 0.001 \)). In contrast, rabbits treated with timolol showed a downregulation of \( \beta_2 \)-adrenergic receptor mRNA (Fig. 5B).

**Fig. 2.** Effect of melatonin and 5-MCA-NAT treatment on “in vitro” \( \beta_2 \)-adrenergic receptor expression. (A) The left axis shows the fold changes in the mRNA levels of the \( \beta_2 \)-adrenergic receptor gene normalized to the ATP5B gene and relative to control levels (\( t = 0 \) hours, set to 1) in rabbit NPE cells treated with melatonin or 5-MCA-NAT. Each data point represents the mean \( \pm \) S.E.M. of triplicate 24-well plates for one experiment. The right axis shows quantification of the \( \beta_2 \)-adrenergic receptor immunostaining intensity (in %) in the NPE cells treated with melatonin or 5-MCA-NAT versus control intensity (untreated cells, white bar, set to 100%). Each data point represents the mean \( \pm \) S.E.M. of eight visual fields. (B) \( \beta_2 \)-Adrenergic receptor immunostaining of NPE cells treated with melatonin or 5-MCA-NAT for 72 hours. Green fluorescence (FITC) localizes the \( \beta_2 \)-adrenergic receptor. Original magnification, 40×. *\( P < 0.05 \); **\( P < 0.001 \) compared with corresponding control values. RFU, relative fluorescence units.
receptor mRNA level (1.2-fold lower than control, $P < 0.05$).

**Discussion**

Melatoninergic compounds have been identified as hypertensive IOP molecules in normotensive rabbits, glaucomatous monkeys, and non-glaucomatous humans (Pintor et al., 2001, 2003, 2011; Serle et al., 2004; Alarma-Estrany et al., 2008, 2011; Ismail and Mowafi, 2009). Although there are several risk factors for glaucoma, high IOP remains the major risk factor, and it is the only one currently that can be modified (Crooke et al., 2012a). In this sense, melatonin and its analogs can act as potent neuroprotective agents (Belforte et al., 2010) in addition to reducing IOP. These two facts seem to indicate that melatonin and its derivatives are promising drugs to prevent glaucoma (Agorastos and Huber, 2011; Crooke et al., 2012a).

We previously reported that the sympathetic nervous system regulates the hypertensive action of melatoninergic compounds (Alarma-Estrany et al., 2007). More recently, it has been shown that the selective MT$_3$ melatonin receptor agonist 5-MCA-NAT regulated mRNA expression of ciliary adrenergic receptors (Crooke et al., 2011). In our present study, we analyzed the effect of the natural ligand melatonin and also its analog 5-MCA-NAT on ciliary adrenergic receptor mRNA as well as protein levels. We conducted the in vitro...
study using NPE cells of rabbits. The presence of melatonin receptors in the ciliary body has been demonstrated in different species through the use of pharmacologic and molecular tools (Osborne and Chidlow, 1994; Pintor et al., 2001, 2003). Indeed, melatonin receptors have been identified in the NPE cells of humans and frogs (Wiechmann and Wirsig-Wiechmann, 2001; Dortch-Carnes and Tosini, 2013).

The in vitro results confirm the findings that suggested an indirect action of melatonin derivatives on ocular adrenergic receptors. Traditionally, β-adrenergic receptors have been described as modulators of IOP by changing the intracellular levels of cAMP in nonpigmented epithelial cells (Crider and Sharif, 2002). This has permitted the design and discovery of new β-adrenergic receptors antagonists to reduce IOP (Sharif et al., 2001). In this sense, melatonin and 5-MCA-NAT produce an indirect action that seems to be a two-step process, in which the first part is the downregulation of ciliary β2-adrenergic receptors that begins 24 hours after the treatment with melatoninergic compounds (principally by melatonin). This effect was quantifiable at the mRNA level. In addition, immunocytochemical studies revealed a qualitative reduction of β2-adrenergic receptor protein levels after melatoninergic treatment but to a lesser extent than mRNA reduction. This reduced correlation between mRNA and protein content may be explained by a different turnover rate for adrenergic receptor mRNA and its cognate protein in NPE cells treated with melatoninergic substances. In this sense, it has been previously reported that in vitro or in vivo changes at mRNA level of other adrenergic receptors, such as α1B-adrenergic receptors are not accompanied by similar changes at protein level (Coon et al., 1997).

The second step of the melatonergic action is the up-regulation of α2A-adrenergic receptors that begin after 48 hours of melatonin and 5-MCA-NAT treatment. In this case both melatoninergic compounds exert a similar action on mRNA levels as well as protein levels. In addition, there is a high correlation between up-regulation of α2A-adrenergic receptor mRNA and its cognate protein (immunoreactive adrenergic receptor protein).

The action of melatonin on gene expression seems complex and sometimes opposite effects are observed (Fustin et al., 2009; Korkmaz et al., 2012). Thus, melatonin can induce or suppress gene expression under the same conditions (Mayo et al., 2002; Fustin et al., 2009; Korkmaz et al., 2012). Furthermore, differential gene expression regulation of adrenergic receptors has also been observed (Bengtsson et al., 2000; Monjo et al., 2003; Spasojevic et al., 2011). Therefore, it is not strange that the expression of α2A-adrenergic and β2-adrenergic receptors was induced as well as suppressed by melatonin and 5-MCA-NAT treatment.

Downstream pathways by which melatonin can modulate gene expression are complex due to the participation of a great number of proteins including kinases, transcription factors, coregulators, nuclear receptors, and epigenetic enzymes (Fustin et al., 2009; Korkmaz et al., 2012). It would be very interesting to investigate the specific underlying mechanism of ocular adrenergic receptors expression regulation by melatoninergic compounds.

To study whether these melatoninergic-genomic actions can potentiate the ocular hypotensive action of β-adrenergic receptor antagonist or α2-adrenergic receptor agonist drugs such as timolol (Timoftol) or brimonidine (Alphagan), respectively, we have also performed IOP experiments. In this context, we have elsewhere described the pharmacologic profile of melatonin and 5-MCA-NAT as ocular hypotensive agents (Pintor et al., 2003). As noted earlier, a combination of two or three antiglaucoma drugs is frequently required when monotherapies are not sufficiently effective or produce adverse effects (Webers et al., 2008). In this regard, an additional IOP reduction of 10 to 25% at peak (at the moment of maximal IOP-lowering drug effect) has been observed in glaucoma patients when brimonidine (administered 2 to 3 times daily) (Simmons, 2001; Solish et al., 2004; Akman et al., 2005), dorzolamide (administered 2 to 3 times daily) (Hutzelmann et al., 1998; Hartenbaum et al., 1999; Kaluzny et al., 2003), or latanoprost (administered once daily) (Orengo-Nania et al., 2001; Akman et al., 2005) were added to a timolol regimen for long-term use (Webers et al., 2008). Moreover, an extra IOP decrease of 19 to 20% at peak has been obtained when timolol were added to a latanoprost or travoprost regimen (both administered once daily) for long-term use (Stewart et al., 1999; Reis et al., 2006).

Our results show that the administration of timolol once daily in normotensive rabbits pretreated with a single dose of melatonin or 5-MCA-NAT evokes an additional IOP reduction of 14 or 17% at peak in comparison with rabbits treated with timolol alone for 24 hours. A possible explanation of this additional IOP reduction could be that the concentration of timolol achieved in the anterior chamber after topical administration was nonsaturating concentration. Thus, the downregulation of β2-adrenergic receptors mediated by melatoninergic compounds combined with timolol antagonism could produce the potentiating effect observed in this work. Nevertheless, the concentration of timolol present in aqueous humor after topical application of 0.5% timolol in rabbits is $8 \times 10^{-6}$ M (Vareilles et al., 1997), and the timolol inhibition constant is $6 \times 10^{-9}$ M (Bartels et al., 1980). On the other hand, it could be the case that in the presence of melatonin or its analog, the remaining receptors become markedly more sensitive to the ordinary levels of the ligand, in this case timolol. This has been described for caffeine and adenosine since the beginning of the 1980s as being one of the mechanisms to explain caffeine withdrawal headache (von Borstel et al., 1983, 1984). More experiments are necessary to confirm if this is the reason for such unexpected result.

To study the possible correlation between IOP results and in vitro gene expression data, we have measured the level of β2-adrenergic receptor mRNA in eyes treated with melatonin or 5-MCA-NAT plus timolol. As in an in vitro assay, a significant downregulation of β2-adrenergic receptors was observed in rabbits pretreated with melatonin. An additional IOP reduction was observed in both rabbits pretreated with melatonin and 5-MCA-NAT. As we commented earlier, the correlation between mRNA and protein levels is not always direct. In addition, the timolol compound acts on β-adrenergic receptor proteins. Thus, the combination of β2-adrenergic receptor downregulation plus the blockade of these receptors could explain the potentiating effect observed in rabbits treated with melatoninergic compounds in the presence of timolol.

More interesting are the results obtained with brimonidine. The administration of brimonidine once daily in normotensive rabbits pretreated with melatoninergic compounds evokes an additional IOP reduction of 29 to 39% at peak in comparison with rabbits treated with brimonidine alone for 24 hours. These results show a lack of correlation with the in vitro effect of melatonin and its analog 5-MCA-NAT on α2A-adrenergic receptors.
mRNA levels (which was evident at 72 and 48 hours, respectively). This apparent discrepancy may be due to several factors related to the complexity of the eye when studied in vivo. One of them is the fact that melatonin is produced with circadian rhythmicity, with peak levels occurring at night in darkness (Nowak and Zawilska, 1998). Therefore, the effect of melatonin and 5-MCA-NAT on IOP in rabbits may be enhanced by a daily dose of melatonin compounds. Another factor is the wide presence of α2A-adrenergic receptors in other ocular structures related to aqueous humor dynamics, like ciliary arteries and episcleral veins (Reitsamer et al., 2006). Thus, melatoninergic derivatives can also modify the in vivo expression of α2A-adrenergic receptors present in those ocular areas and then facilitate brimonidine action. Indeed, when we studied the levels of ciliary α2A-adrenergic mRNA in rabbits treated with 5-MCA-NAT plus brimonidine we observed a significant upregulation of this transcript. However, as occurs with timolol, an additional IOP reduction was observed in both rabbits pretreated with melatonin and 5-MCA-NAT.

After 96 hours of pretreatment with melatoninergic compounds, an additional IOP reduction of 7 to 16% was observed in comparison with animals treated with brimonidine alone. These data confirm a remarkable potentiating effect of melatoninergic drugs upon the ocular hypotensive action of brimonidine and to a lesser extent of timolol.

Genomic actions of melatonin and 5-MCA-NAT on different target genes have been reported previously elsewhere (Mayo et al., 2002; Anisimov and Popovic, 2004; Crooke et al., 2011). We observed different levels of potentiating effect when brimonidine was administered instead of timolol on rabbits pretreated with melatonergic compounds. Consequently we think that the main molecular mechanism responsible for this effect is the α2A-adrenergic receptor upregulation.

The translation of our results from rabbits to humans should be taken with caution due to the obvious (physiologic, anatomic, etc.) and subtle (possible gene functions and gene networks divergence) differences between these species. In addition, normotensive healthy condition is not the same as glaucoma condition. As has already been mentioned, the potent ocular hypotensive action of melatonergic compounds demonstrated in rabbits has also been confirmed in glaucomatous monkeys as well as in nonglaucomatous humans (Serle et al., 2004; Ismail and Mowafi, 2009). The results obtained in normotensive rabbits and glaucomatous monkeys point in the same direction, but the maximal effects are different. Serle and colleagues (2004) observed a gradual IOP reduction as long as the treatment is applied on consecutive days, rather than a robust reduction of IOP. This indicates a mechanism that matches more with the results from our study (long-term) than with the sharp, short-term IOP reductions we characterized in our previous works (Pintor et al., 2001, 2003). These differences could be related to previously noted factors (specie and normotensive healthy/glaucomatous condition) but also to the vehicle used to solubilize 5-MCA-NAT (DMSO versus propylene-glycol). Also, it is interesting to note that in humans, melatonin is given to patients before cataract surgery because it reduces IOP (Ismail and Mowafi, 2009). The action of melatonin is fast because it is applied before the surgery; this may indicate that the rabbits are closer to the human than the monkey for assaying the effect of melatonin in IOP.

Furthermore, we have recently demonstrated that the application of melatoninergic compounds reduces the IOP of rabbits with ocular hypertension induced by the Trendelenburg position (Martinez-Aguila et al., 2013).

The neuroprotective action of brimonidine has been confirmed in experimental models of glaucoma and even in patients with low pressure glaucoma (Wheeler et al., 2003; Krupin et al., 2011; Pinar-Sueiro et al., 2011), and our results with α2A-adrenergic receptor upregulation mediated mainly by 5-MCA-NAT seem to suggest a possible additional beneficial effect of this melatoninergic treatment. However, confirmation of 5-MCA-NAT neuroprotection and of its ocular hypotensive potentiating effect in glaucomatous animal models is necessary to increase confidence in the use of 5-MCA-NAT as a viable therapy for glaucoma.

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Authorship Contributions

Participated in research design: Crooke, Martinez-Aguila, Martin-Gil

Conducted experiments: Crooke, Martinez-Aguila, Martin-Gil

Performed data analysis: Huete-Toral

Wrote or contributed to the writing of the manuscript: Crooke, Huete-Toral

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