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NFATc1 Activity Regulates the Expression of Myocilin Induced by Dexamethasone

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Abstract

Mutations in the myocilin gene (*MYOC)* account for 10% of juvenile open-angle glaucoma cases and 3–4% of adult onset primary open-angle glaucoma cases. It is a secreted glycoprotein found in many ocular and non-ocular tissues and has been linked to elevated intraocular pressure. In human trabecular meshwork (HTM) cells, *MYOC* expression can be induced by the glucocorticoid dexamethasone (DEX). In this study we examined the role of the calcineurin/NFATc1 (Nuclear Factor of Activated T-cells) pathway in the DEX induction of *MYOC* in HTM cells. In post-confluent HTM cells treated with either 500 nM DEX or 0.1% ethanol (EtOH; vehicle control) for 0–6 days both protein and mRNA levels of MYOC were increased while DEX was present. The protein and mRNA levels remained elevated for an additional 12 days after the removal of DEX. Only 1 day of DEX treatment was sufficient to trigger a sustained increase in *MYOC* mRNA that lasted for 4 days after the removal of DEX. Similar to other studies, myocilin protein expression was not seen until the second day of DEX treatment while mRNA increased within one day of DEX indicating that this is a secondary glucocorticoid response. To determine if *MYOC* gene expression was regulated by calcineurin/NFATc1, HTM cells were pre-treated for 1 h with the calcineurin inhibitors cyclosporin A or INCA-6 prior to the addition of DEX or EtOH for 2 days. NFATc1 siRNA was used to determine if NFATc1 was required for *MYOC* mRNA expression. Cells were also treated with the ionophone ionomycin to determine if increased cytosolic calcium affected *MYOC* expression. These studies showed that the DEX induced increase in *MYOC* mRNA could be inhibited with either CsA or INCA-6 or by transfection with NFATc1 siRNA and that ionomycin was unable to increase *MYOC* mRNA. Immunofluorescence microscopy was also performed to determine if DEX caused the nuclear translocation of NFATc1. Immunostaining showed that NFATc1 relocated to the nucleus within 15 min of DEX treatment and remained there for up to 2 h. The data suggest that the DEX-induced increase in *MYOC* expression activates a calcineurin and NFATc1 pathway in a calcium independent mechanism.

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Keywords

glucocorticoids; glaucoma; trabecular meshwork; calcineurin; NFATc1; myocilin

1. INTRODUCTION

Glaucoma is a heterogeneous disease characterized by the progressive degeneration of the optic nerve that eventually leads to irreversible blindness. The most common form of glaucoma in the United States is primary open angle glaucoma (POAG), affecting more than 60 million people worldwide (Quigley, 1996). A major risk factor for POAG is increased intraocular pressure (IOP). Although numerous studies indicate that alterations in the conventional outflow pathway are largely responsible for the elevation in IOP, the molecular and cellular mechanisms responsible are still unknown. To date genetic studies have indicated that there are 4 genes linked to adult onset POAG: *MYOC* (myocilin), *WDR36* (WD Repeat Domain 36), *OPTN* (optineurin), and *NTF4* (neutrophin-4) (Fan and Wiggs, 2010; Takamoto and Araie, 2014).

MYOC was one of the first proteins to be linked to glaucoma. It was originally identified because its expression in human trabecular meshwork (HTM) cells can be increased with the glucocorticoid dexamethasone (DEX) (Nguyen et al., 1998; Polansky et al., 1997). Thus, it is thought to play a role in both POAG and steroid-induced glaucoma which clinically mirrors POAG. Mutations in *MYOC* occur in 10% of juvenile open-angle glaucoma cases and in 3–4% of adult onset POAG cases (Fingert et al., 1999; Fingert et al., 2002; Kwon et al., 2009; Stone et al., 1997). Increasing evidence suggests that mutations in the *MYOC* gene cause glaucoma through a gain of pathogenic function (Kim et al., 2001; Lam et al., 2000) which prevents MYOC from being secreted from the cell. As a result MYOC accumulates within the endoplasmic reticulum of the cell where it causes endoplasmic reticulum stress, impairing trabecular meshwork cell function and viability (Joe et al., 2003; Wang et al., 2007; Zode et al., 2011).

MYOC is a secreted glycoprotein that is expressed in many structures of the eye, including the trabecular meshwork, iris, ciliary body, sclera, choroid, cornea, lamina cribosa, retina and optic nerve (Adam et al., 1997; Kubota et al., 1997; Ortego et al., 1997; Ricard et al., 2001; Tamm et al., 1999). The function of MYOC is not clear but it may play a role in cell-extracellular matrix interactions (Goldwich et al., 2009; Peters et al., 2005), cell migration (Kwon and Tomarev, 2011) and mitrochondrial function (Sakai et al., 2007). In skeletal muscle, MYOC is part of the dystrophin-associated protein complex by binding α1-syntrophin and may play a role as a regulator of muscle hypertrophy pathways (Joe et al., 2012). Recently, it was shown that MYOC can bind and activate ErbB2/ErbB3 in the sciatic nerve implicating a role for MYOC in myelination in the peripheral nervous system (Kwon et al., 2013).

In addition to DEX, *MYOC* expression can also be induced in HTM cells with transforming growth factor-β1 (TGF-β1) (Tamm et al., 1999), optineurin (Park et al., 2007), and mechanical stretch (Tamm et al., 1999). The induction of *MYOC* by both DEX and TGF-β1 is a delayed response, taking days rather than hours to see both *MYOC* mRNA and protein

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levels increase (Shepard et al., 2001; Tamm et al., 1999). This delayed response to stimuli is thought to be a secondary response as it requires new protein synthesis of an unidentified factor(s) for induction. Analysis of nucleotides upstream of the *MYOC* transcription start site support this idea because it failed to identify a functional glucocorticoid response element (Kirstein et al., 2000; Shepard et al., 2001). Recent studies examining how DEX regulates the expression of proteins in the TM show that MYOC is not the only protein up regulated as a result of a secondary glucocorticoid response. The β3 integrin subunit in HTM cells is also up regulated by DEX (Faralli et al., 2013) and this study showed that a calcineurin/NFAT (nuclear factor of activated T-cells) pathway may be involved. Calcineurin is a serine/threonine phosphatase that is modulated by intracellular calcium levels. Upon activation, calcineurin can activate the NFAT family of transcription factors through dephosphorylation (Clipstone and Crabtree, 1992; Emmel et al., 1989). Once dephosphorylated, the NFATs translocate from the cytoplasm to the nucleus where they bind DNA in conjunction with other transcription factors such as AP-1 to induce gene transcription (Chen et al., 1998). There are 5 NFAT family members, NFATc1-5, but only NFATc1-4 are regulated by calcium signaling.

In this study we examined whether the DEX-induced *MYOC* expression in HTM cells also involved a calcineurin/NFAT pathway. We showed that DEX increased MYOC protein and mRNA levels within 1–2 days and that just one day of DEX treatment was sufficient to induce the increase in mRNA. The increase in *MYOC* mRNA expression by DEX was dependent on NFATc1 activation by calcineurin, suggesting that NFATc1 may induce the transcription of a gene necessary for *MYOC* expression in HTM cells. Understanding how *MYOC* expression is regulated provides potential targets for the treatment of glaucoma.

2. METHODS

2.1 Cell Culture

HTM cells were isolated from cadaver eyes of a 17 (HTM17), 25 (HTM25) and 27-year old (HTM27) donor with no known history of ocular disease in accordance to the tenets of the Declaration of Helsinki. The cells used for each experiment is indicated in the figure legend. The cells were characterized to be HTM cells based on several criteria, as previously described (Alvarado et al., 1982; Filla et al., 2004). These include a cobblestone morphology in stable differentiated postconfluent monolayers, the ability to form cross-linked actin networks (CLANs), the upregulation of MYOC by DEX and the presence of several proteins (fibronectin, collagen IV, laminin, ZO-1, β-catenin and smooth muscle actin) Cells were cultured as previously described in low glucose DME supplemented with 15% FBS (Atlanta Biologicals), 2% L-glutamine, 1% amphotericin B (Thermo Fisher Scientific), 0.05% gentamycin and 1 ng/ml FGF-2 (Peprotech) (Filla et al., 2004). One week after reaching confluency and for all subsequent experiments, serum was reduced to 10% FBS and FGF-2 treatment was stopped. Cells were then treated with either 500 nM DEX or 0.1% ethanol (EtOH; vehicle control). In some experiments, cells were incubated with 1 or 10 μM cyclosporin A (CsA) or 40, 80 or 120 μM INCA-6 (Tocris Bioscience) for 1 h prior to the addition of DEX or EtOH and incubated for 2 days. Fresh CsA, INCA-6, DEX and EtOH

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were always added after 24 h. Other times 1 or 5 μM ionomycin was added to the media for 2 days. All reagents were obtained from Sigma-Aldrich unless otherwise noted.

2.2 Western Blot Analysis

Western blot analyses were performed as previously described (Faralli et al., 2013) with a few modifications. Briefly, HTM cells were lysed with lysis buffer (25 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1% NP-40, 0.25% deoxycholate, HALT phosphatase inhibitor cocktail and HALT protease inhibitor cocktail (Thermo Fisher Scientific)). Proteins in the cell lysate (10 μg) were separated on a 10% SDS-PAGE and transferred to Immobilon-FL membrane (Millipore Corp.). The membrane was blocked overnight with 3% bovine serum albumin (BSA) in Tris buffered saline (TBS) containing 0.1% Tween-20 and then incubated with either MYOC monoclonal antibody clone 1.1 (1:1000), or succinate dehydrogenase complex, subunit A (SDHA) monoclonal antibody (Abcam; 1:5000). Antibodies were diluted in 1% BSA/TBS/0.1% Tween-20 and incubated for 1 h at room temperature. The MYOC monoclonal antibody was kindly provided by Dr. Michael Fautsch (Dept. Ophthalmology, Mayo Clinic, Rochester, MN). Membranes were washed and then incubated for 1 h with IRDye 800 conjugated goat anti-mouse or anti-rabbit secondary antibody (Licor; 1:15000) in 1% BSA/TBS/0.1% Tween-20/0.01% SDS. Bound antibody was detected using the Licor Odyssey infrared imaging system using Image Studio Ver. 2.1 (Licor).

2.3 RNA Isolation, Reverse Transcription and Real-Time (RT)-qPCR

Total RNA was isolated using the QIAshredder and RNeasy Plus Mini Kits (Qiagen Inc., Valencia, CA) and RNA concentration was determined using a NanoDrop spectrophotometer. Total RNA (1 μg) was reverse transcribed and RT-qPCR experiments using the synthesized cDNA were performed as previously described (Clark et al., 2013). Primers pairs used were: *MYOC* forward 5′-GCCCATCTGGCTATCTCAGG-3′ and reverse 5′-CTCAGCGTGAGAGGCTCTCC-3′ and *ITGB1* (β1 integrin) forward 5′-GTGGAGAATCCAGAGTGTCCCA-3′ and reverse 5′-GACCACAGTTGTTACGG-3′. Relative quantification of the RT-qPCR data was performed according to Pfaffl (Pfaffl, 2001), using *ITGB1* mRNA for normalization. We have determined from previous experiments that *ITGB1* mRNA levels are unaffected by the treatments used in this study (Faralli et al., 2013). Statistical comparisons were done using the Student T-test and a p value < 0.05 was considered significant.

To detect *NFATc1-4* mRNA expression, cDNA synthesized from untreated HTM cells as described above was used. The cDNA was PCR amplified using 1 U DNA polymerase (Platinum *Taq*; Invitrogen) with 1.5 mM $MgCl_2$, 125 μM each dNTP and 5 μM gene-specific primer pairs. Primers used were: *NFATc1* forward 5′-TGCAAGCCGAATTCTCTGGT-3′ and reverse 5′-CTTTACGGCGACGTCGTTTC-3′ (227 bp fragment), *NFATc2* forward 5′-GAGGGGCTGTCAAAGCTCC-3′ and reverse 5′-ACAGTTTTCCCCGTGATTCGG-3′ (162 bp fragment), *NFATc3* forward 5′-GCTCGACTTCAAACTCGTCTT-3′ and reverse 5′-GATGCACAATCATCTGGCTCA-3′ (95 bp fragment), and *NFATc4* forward 5′-CTTCTCCGATGCCTCTGACG-3′ and reverse 5′-CGGGGCTTGGACCATACAG-3′ (172 bp fragment). The PCR program used was 95°C

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for 1 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The products were extended for an additional 5 min at 72°C then separated on a 3% agarose gel and stained with ethidium bromide.

2.4 NFATc1 Knockdown

HTM cells were plated into 6 well plates and grown to confluency as described above. Human NFATc1 siRNA (ON-TARGETplus SMART pool L-003605-00-005) and control siRNA (ON-TARGETplus SMART pool Non-targeting D-001810-10-005) were obtained from Dharmacon/Thermo Fisher Scientific. HTM cells were transfected with 25 nM siRNA using a siRNA transfection reagent (TransIT-TKO reagent; Mirus) according to the manufacturer's protocol. After 24 h, the media was replaced and DEX or EtOH was added to the appropriate wells and cells were incubated for an additional 48 h. Total RNA was isolated and cDNA was generated as described above. RT-qPCR experiments using the synthesized cDNA were performed as previously described (Clark et al., 2013). The same *MYOC and ITGB1* primers were used as described above in addition to *NFATc1*-specific primers: forward 5′-TGCAAGCCGAATTCTCTGGT-3′ and reverse 5′-CTTTACGGCGACGTCGTTTC-3′. Data were analyzed as described above.

2.5 Immunofluorescence Microscopy

Confluent monolayers of HTM27 cells were treated for 10 days with 500 nM DEX or 0.1% EtOH for 10 days then washed with ice cold PBS and fixed for 15 min in 100% MeOH at −20°C prior to blocking for 1 h with 1% BSA in PBS. Cells were then incubated with a rabbit polyclonal antibody against MYOC followed by Alexa 488 conjugated goat anti-rabbit antibody (Molecular Probes, 1:500). Coverslips were mounted onto slides using Immu-Mount (Shandon) and viewed using an epifluorescence microscope (Axioplan 2; Zeiss) equipped with a digital camera (Axiocam HRm; Zeiss) and image acquisition software (Axiovision ver. 4.5; Zeiss).

For the NFAT localization studies, subconfluent, proliferating HTM cells were plated onto glass coverslips in a 24 well plate at 30,000 cells per well. The next day cells were treated with 500 nM DEX or 0.1% EtOH for 0, 15 min, 30 min, 1 h or 2 h. As a positive control, some cells were treated with 1 μM ionomycin (Sigma-Aldrich) for 1 h. The cells were then washed, fixed and blocked as above and incubated with an NFATc1 monoclonal antibody (Millipore clone 7A6; 1:50) diluted in 0.1% BSA/PBS overnight at 4°C. Cells were then incubated with Alexa 546 conjugated goat anti-mouse antibody (Molecular Probes; 1:500) in 0.1% BSA/PBS for 1 h, mounted and viewed as described above. NFATc1 nuclear localization was quantified by counting the number of cells with nuclei positive for NFATc1 antibody labeling from 15 different fields of view for each condition for each experiment performed (n=3). The percentage of cells with NFATc1 localization versus total number of cells was determined. Data are presented as mean ± S.E.M. Statistical comparisons were done using the Student T-test and a p value < 0.05 was considered significant.

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3. RESULTS

3.1 DEX induces MYOC protein and mRNA expression

Immunofluorescence microscopy of HTM cells show that there was a low basal level of MYOC expression in EtOH treated cells that increased to 100% expression when cells were treated with DEX (Fig. 1A and B). Similar to other studies using HTM cells (Clark et al., 2001; Shepard et al., 2001), DEX induced the expression of MYOC in a time-dependent manner. Figure 1C shows that protein levels of MYOC could be detected starting 2 days after initiation of DEX treatment and remained high as long as DEX was present (10 days total). MYOC was not detected in the EtOH control lysates, presumably because the level of expression was below the detection level on the Western blots when equal amounts of cell lysate were loaded. Upon removal of DEX after 6 days of treatment, Western blot analysis showed that MYOC expression remained high for 4 days after DEX removal (Fig. 1D) then slowly decreased to near baseline levels 12 days after DEX removal.

Using RT-qPCR, we found that DEX significantly increased the expression of *MYOC* mRNA by 13.6 fold ($p<0.05$) after only 1 day of DEX treatment compared to no treatment (Fig. 2A). The amount of *MYOC* mRNA continued to increase significantly each day of DEX treatment. After 6 days of DEX treatment there was a 182 fold increase in *MYOC* mRNA over no treatment, which was significantly higher than the EtOH control ($p<0.02$). In addition, *MYOC* mRNA levels remained elevated for up to 14 days after removal of DEX from the medium (Fig. 2B). Interestingly, just one day of DEX exposure was sufficient to induce a prolonged elevation in *MYOC* mRNA levels in the absence of DEX. As shown in Figure 2C, *MYOC* mRNA levels increased 17-fold over no treatment after 1 day of DEX treatment (Day 0) and was 30-fold higher than no treatment ($p<0.02$ compared to EtOH control) 3 days after DEX removal. *MYOC* mRNA levels remained 6.3 fold higher compared to no treatment 4 days after DEX removal, but this was not significantly different from the EtOH control.

3.2 DEX induction of MYOC involves Calcineurin and NFATc1

Our previous work showed that, similar to *MYOC*, *ITGB3* (β3 integrin subunit) mRNA expression induced by DEX was also a secondary glucocorticoid response. This increase was inhibited with cyclosporin A (CsA) (Faralli et al., 2013), an immunosuppressant that forms a complex with cyclophilin leading to an inhibition of calcineurin phosphatase activity (Liu et al., 1991). To determine if CsA could also inhibit the DEX-induced increase in *MYOC* mRNA, we pre-treated HTM cells with 1 or 10 μM CsA for 1 h prior to the addition of DEX or EtOH. For these studies we used HTM cells from three different donors to rule out cell strain variability. Figure 3A shows that CsA significantly reduced the increase in *MYOC* mRNA caused by DEX treatment ($p<0.05$). At the highest dose of CsA tested (10 μM), *MYOC* mRNA expression induced by DEX was significantly reduced by 71% compared to DEX alone ($p<0.05$; Table 1). We also tested a more selective calcineurin inhibitor called INCA-6. INCA-6 specifically prevents calcineurin from binding NFAT, but does not affect calcineurin activity for other substrates (Roehrl et al., 2004). Figure 3B shows that INCA-6 significantly reduced the increase in *MYOC* mRNA caused by DEX treatment with all 3 doses tested (40, 80, 120 μM; $p<0.05$) in all cell strains tested. These

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data suggested that the DEX induced activation of calcineurin was involved in the upregulation of *MYOC*.

Since, calcineurin is known to activate the NFAT family of transcription factors and cause their relocation to the nucleus, we then investigated whether NFAT was involved in the DEX induced expression of *MYOC*. To determine which NFATs were expressed in HTM cells we performed PCR analysis using primers specific for *NFATc1-4*. Figure 4A shows that HTM cells express only *NFATc1*. To demonstrate that NFATc1 is required for DEX induction of *MYOC* transcription, we used siRNA directed against *NFATc1*. As shown in Figure 4B, NFATc1 siRNA decreased *NFATc1* mRNA levels by 94% after 72 h ($p<0.05$), and this decrease was not affected by the presence of DEX or EtOH. This reduction in NFATc1 mRNA levels attenuated the DEX induced increase in *MYOC* transcription by 72% (Figure 4C), while control siRNA had no effect.

We next performed immunofluorescence microscopy to determine if DEX treatment caused the nuclear translocation of NFATc1 over time. We also treated cells with the Ca^{2+} ionophore known as ionomycin as a positive control since calcineurin can be activated by an increase in intracellular calcium. Figure 5 shows that NFATc1 was translocated to the nucleus in cells treated with both ionomycin and DEX (Fig. 5B and D respectively), but not in EtOH treated control cells (Fig. 5C). The number of cells with NFATc1 nuclear localization was quantified and shown in Figure 5E. Only 25% of the no treatment control cells showed a nuclear localization of NFATc1. However, if cells were treated with ionomycin, 60% showed a nuclear translocation of NFATc1. We saw a similar increase in cells treated with DEX for 15 min. A 15 min treatment with DEX resulted in 50% of the cells with nuclear labeling for NFATc1, which was significantly different from the no treatment group ($p<0.02$) and from HTM cells treated with EtOH as a control ($p<0.05$). At 30 min, 58% of cells treated with DEX showed nuclear localization of NFATc1, which was significantly different from the EtOH control ($p<0.02$). After 30 min, the number of cells with nuclear localization of NFATc1 following DEX treatment began to decrease, but was still higher than the EtOH controls. Since DEX treatment did not increase NFATc1 translation or transcription as determined by western blotting and RT-qPCR (data not shown), these data together with the siRNA data, suggests that a DEX induced activation of NFATc1 was involved in the increase in *MYOC* transcription. These also suggest that DEX is utilizing a calcineurin/NFATc1 pathway to upregulate MYOC expression, since calcineurin is the only phosphatase known to activate NFATc1.

Finally, prior studies by Tumlin et al 1997 (Tumlin et al., 1997) showed that DEX can cause an influx in intracellular calcium through the activation of phospholipase C (PLC) and the generation of inositol triphosphate (IP_3) that can activate calcineurin. To determine whether an increase in the cytosolic calcium concentration would be sufficient to activate the calcineurin/NFATc1 pathway and increase *MYOC* RNA expression, we examined MYOC expression in the presence of ionomycin. These studies showed that ionomycin alone was unable to induce an increase in *MYOC* mRNA expression (Fig. 6). This suggests that DEX activates the calcineurin/NFATc1 pathway in a calcium independent mechanism.

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4. DISCUSSION

In this paper we demonstrate that the DEX-induced increase in *MYOC* transcription and expression is due to the activation of the calcineurin/NFATc1 pathway. This is supported by the fact that the increase in *MYOC* expression could be inhibited with the inhibitors CsA and INCA-6 as well as with NFATc1 knockdown using siRNA. A calcineurin/NFATc1 pathway was also recently found to trigger the up regulation of the β3 integrin subunit (Faralli et al., 2013). Since the DEX-induced expression of both proteins is a secondary glucocorticoid response, this suggests that a calcineurin/NFATc1 pathway may be a common mechanism used to regulate a secondary glucocorticoid response in HTM cells and that inhibitors of this pathway may be useful for the treatment or prevention of steroid induced glaucoma.

It is still unclear how DEX activates this pathway. The calcineurin/NFATc1 pathway can be activated by several pathways as shown in Figure 7. Since ionomycin failed to induce *MYOC* expression, a DEX induced increase in cytosolic calcium levels seems to be the unlikely mechanism involved in *MYOC* expression. Rather, we propose that DEX binding to the glucocorticoid receptor may be responsible for the activation of the calcineurin/NFATc1 pathway. Studies have shown that DEX binding to the glucocorticoid receptor causes a release of heat shock proteins, especially HSP90. HSP90 in turn has been shown to directly activate calcineurin in a calcium independent mechanism (Ranta et al., 2008). Alternatively, the activated glucocorticoid receptor could be inducing the transcription of some factor(s) needed to activate calcineurin. This hypothesis is supported by the fact that the glucocorticoid antagonist RU486 can inhibit the DEX induction of *MYOC* transcription (Shepard et al., 2001). In addition, there are no known NFAT family member binding sites in the *MYOC* promoter and the increase in *MYOC* could be inhibited by cycloheximide indicating that de novo protein synthesis was needed.

Together, these data suggest that the glucocorticoid receptor must be inducing the expression of additional factor(s) responsible for the induction of *MYOC* mRNA within the first 24 h. A plausible factor could be the transcription factor AP-1 which is known to activate gene expression in conjunction with NFATc1 (Chen et al., 1998). Clearly additional studies are needed to determine the mechanisms responsible for the activation of calcineurin/NFATc1.

As in the other studies using HTM cells (Clark et al., 2001; Joe et al., 2011; Shepard et al., 2001), it took up to 24 h for *MYOC* mRNA to increase and up to 2 days before protein expression could be detected. This increase in *MYOC* mRNA could be inhibited by cycloheximide indicating that de novo protein synthesis was needed. As previously reported, our studies also showed that the removal of DEX results in a slow decrease in the levels of the MYOC protein and mRNA to control levels. Following a 5 day treatment with DEX, Joe et. al (Joe et al., 2011) showed that about 50% of *MYOC* mRNA was still present 4 days after removal of DEX. In this study, we saw a similar phenomenon and observed a 50% decrease in *MYOC* mRNA approximately 6 days after DEX was removed. This prolonged stability of the *MYOC* mRNA corresponded to the continued expression of the MYOC protein which remained near treated levels for 4 days after DEX removal. This is likely due to the long half-life of *MYOC* mRNA and not the stability of the protein. MYOC protein

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levels in TM cells appear to be short-lived and decreased about 50% after 3 h of cycloheximide treatment. (Qiu et al., 2014).

In summary, this is the first study to demonstrate that activation of a calcineurin/NFATc1 pathway is responsible for the DEX-induced up regulation of *MYOC* and suggests that this may be a common pathway used by DEX to activate protein expression in the TM. Understanding how *MYOC* expression is regulated by DEX can help us design novel strategies for treating myocilin-related glaucoma.

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Abbreviations

CsA	cyclosporin A
DEX	dexamethasone
EtOH	ethanol
HTM	human trabecular meshwork
IOP	intraocular pressure
MYOC	myocilin
NFAT	Nuclear factor of activated T-cells
POAG	primary open angle glaucoma
SDHA	succinate dehydrogenase complex, subunit A
TGF-β1	transforming growth factor-β1

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Highlights

- Myocilin expression induced by dexamethasone is a secondary glucocorticoid response
- Calcineurin activity is required for myocilin expression induced by dexamethasone
- NFATc1 nuclear localization plays a role in myocilin expression

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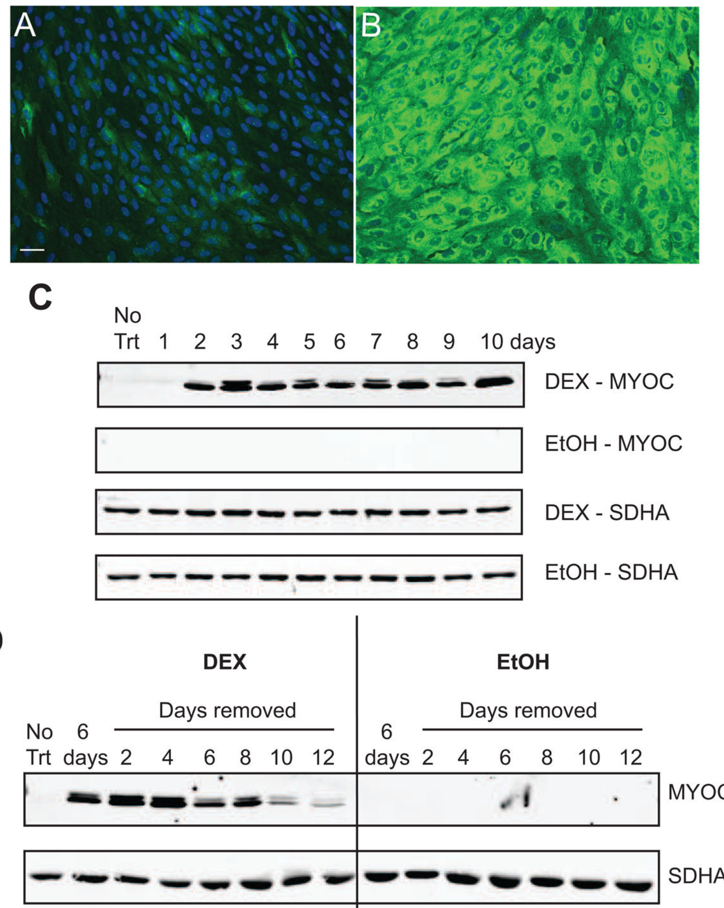

Figure 1.

Please change DEX to EtOH and EtOH to DEX so it should read: DEX increases MYOC expression. HTM27 cells were treated with EtOH (A) or DEX (B) for 10 days prior to methanol fixation and labeled with a rabbit polyclonal antibody against MYOC (green) and Hoescht 33342 to label nuclei (blue). Images were taken with the same exposure time. Images of DEX treated cells were deliberately overexposed in order to show the basal levels of MYOC expression. Scale bar = 50 μm. (C) HTM27 cells were treated with DEX or EtOH for 10 days. Cell lysates were collected daily and analyzed for MYOC and SDHA (loading control) expression. (D) HTM27 cells were treated with DEX or EtOH for 6 days followed by media alone for 12 days. Lysate was collected every other day after DEX or EtOH removal and analyzed for the expression of MYOC or SDHA. Blots are representative of 2 biological replicates.

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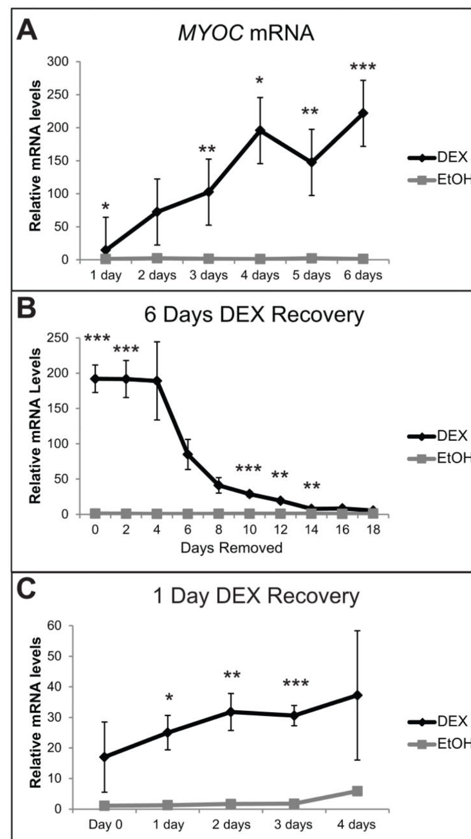

Figure 2.

DEX treatment increases *MYOC* mRNA. RT-qPCR was performed on HTM27 cells treated with DEX or EtOH. Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl (Pfaffl, 2001). Data were then normalized to *ITGB1* mRNA (β1 integrin housekeeping gene). (A) Levels of *MYOC* mRNA in the presence of DEX or EtOH for 1–6 days. (B) Levels of *MYOC* mRNA in HTM cells treated with DEX or EtOH for 6 days (Day 0) followed by 18 days with no DEX or EtOH. (C) Levels of *MYOC* mRNA in HTM cells treated with DEX or EtOH for 1 day only (Day 0) followed by 4 days with no DEX or EtOH. n = 3 biological replicates. DEX significantly different from EtOH *$p<0.05$, **$p<0.04$, ***$p<0.02$.

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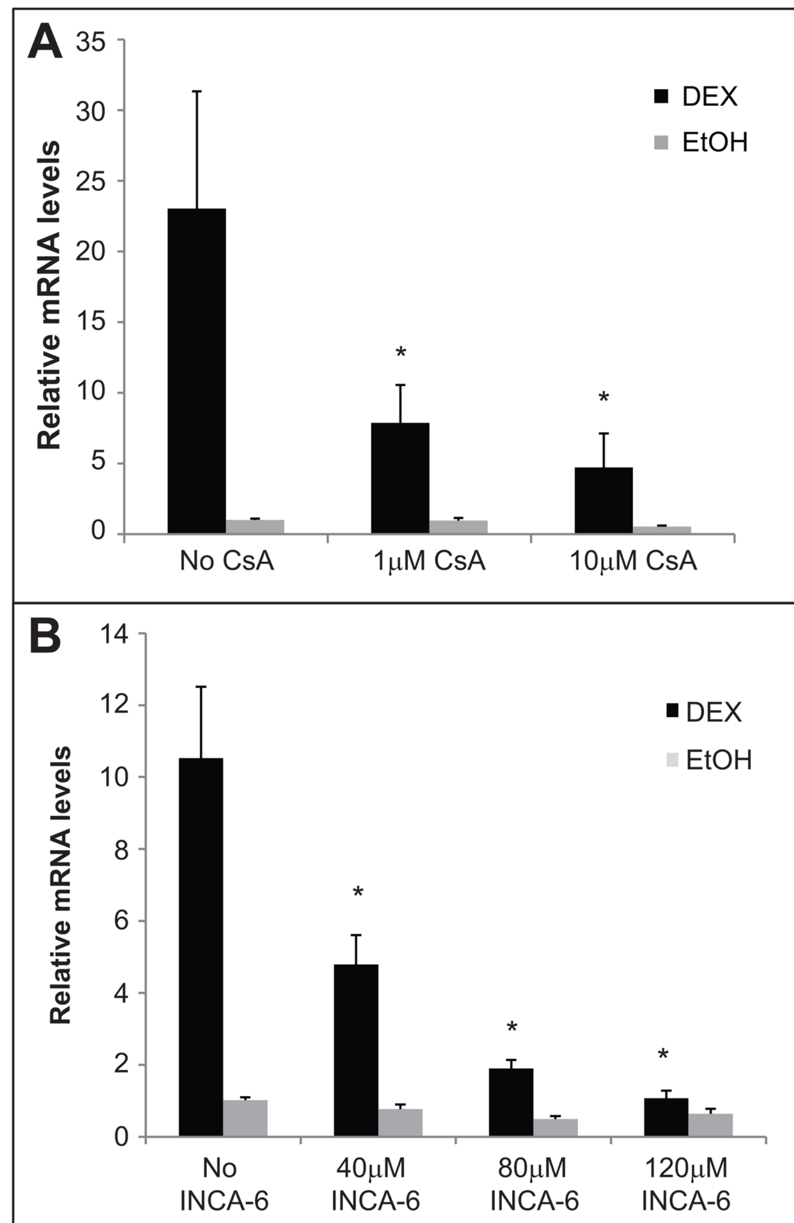

Figure 3.

CsA and INCA-6 inhibit the DEX-induced expression of *MYOC* mRNA. RT-qPCR was performed on 3 separate HTM cell lines (HTM17, HTM25 and HTM27). All cell strains had similar results so the data was averaged together. Individual cell strain data is shown in Table 1. Cells were pre-treated for 1 h with 1 or 10 μM CsA (A) or 40, 80 or 120 μM INCA-6 (B) prior to the addition of DEX or EtOH and incubated for 2 days. Fresh CsA, INCA-6, DEX and EtOH was added after 1 day. Ct values were corrected for primer efficiency and compared to cells with no treatment. Data were then normalized to *ITGB1* mRNA (β1 integrin housekeeping gene). n = 4 biological replicates. DEX significantly different from DEX + 1 or 10 μM CsA (A) and from DEX + 40, 80 or 120 μM INCA-6 (B) $^{*}p<0.05$.

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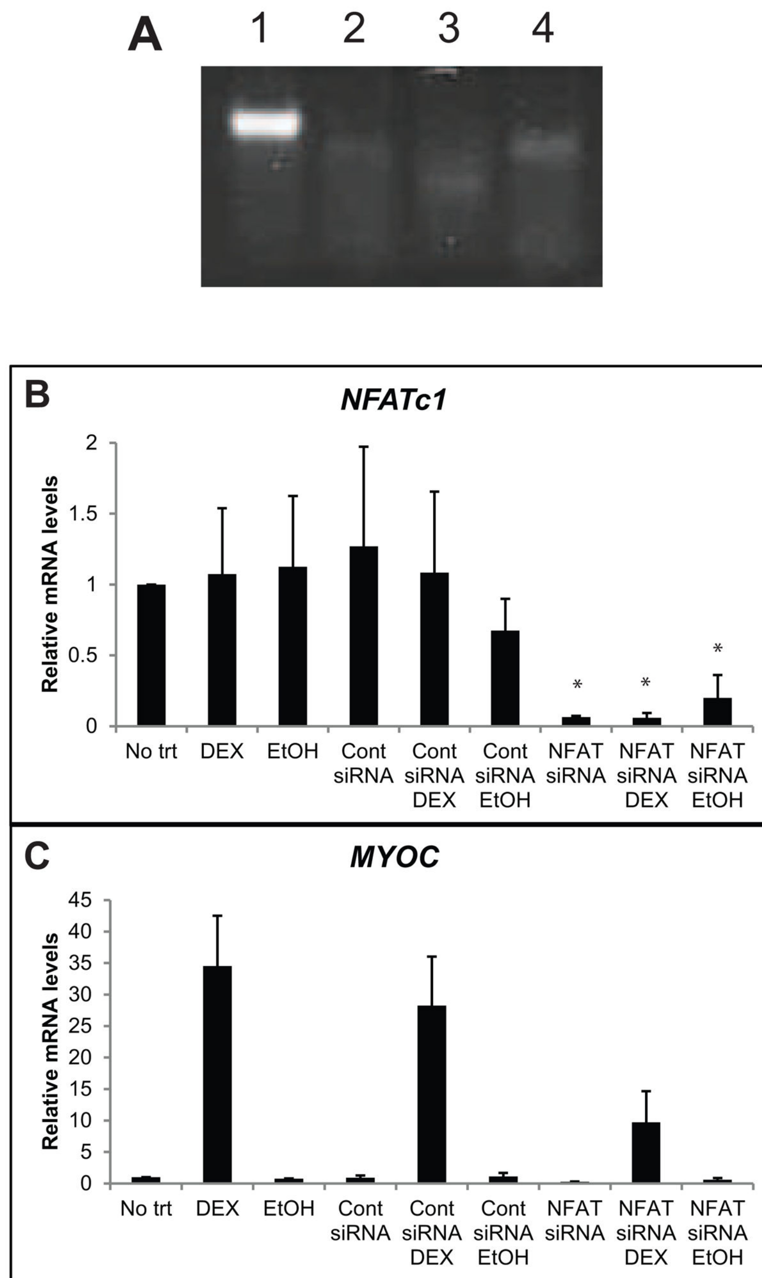

Figure 4.

(A) *NFAT* expression in HTM27 cells. cDNA was made from HTM cell mRNA. PCR was performed using primers specific for *NFATc1* (lane 1), *NFATc2* (lane 2), *NFATc3* (lane 3) and *NFATc4* (lane 4). (B, C) NFATc1 mediates the DEX-induced increase in *MYOC* mRNA in HTM cells. HTM**27** cells were transfected with siRNA directed against *NFATc1*. 24 h later cells were treated with DEX or EtOH for 48 h. *NFATc1* levels (A) or *MYOC* levels (B) were determined using RT-qPCR 72 h after transfection. Ct values were corrected for primer efficiency and compared to cells with no treatment. Data were then normalized to *ITGB1* mRNA. n = 3 biological replicates. Significantly different from no trt, *p<0.05.

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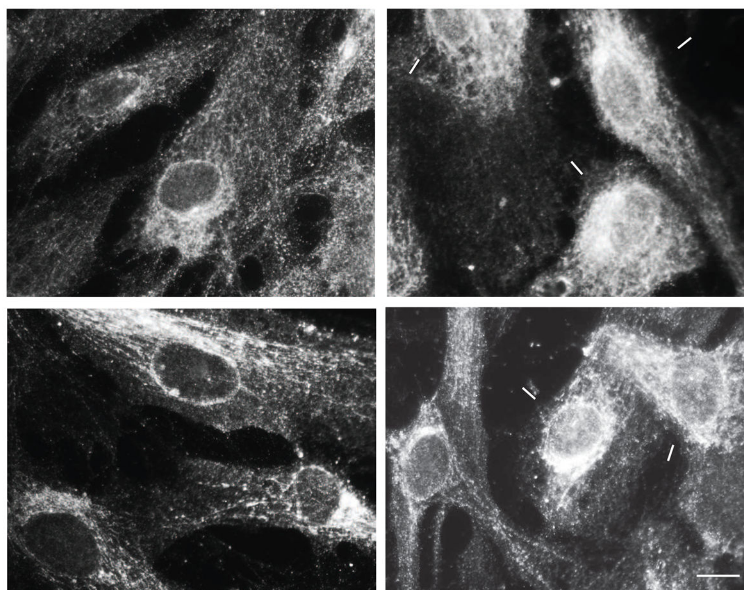

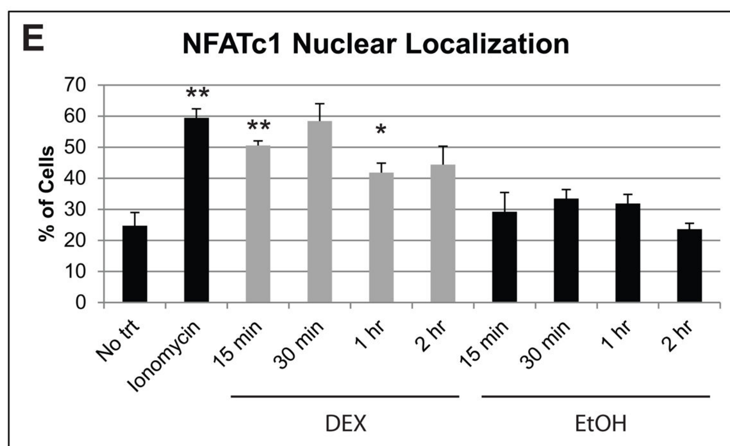

Figure 5.

DEX treatment causes the translocation of NFATc1 to the nucleus. HTM27 cells were plated onto coverslips then treated with ionomycin for 1 h or DEX or EtOH for 15 min, 30 min, 1 h or 2 h. Cells were fixed with methanol then incubated overnight with anti-NFATc1 antibody followed by Alexa 546 conjugated secondary antibody. (A) No treatment, (B) 1 h Ionomycin, (C) 15 min EtOH, (D) 15 min DEX. Arrows indicate cells positive for nuclear labeling of NFATc1. Scale bar = 20 μm. (E) The number of cells with NFATc1 nuclear labeling was counted from 15 different fields of view for each treatment group and the percentage of total cells was determined. n = 3 biological replicates. Significantly different from no trt, *$p<0.04$, **$p<0.02$.

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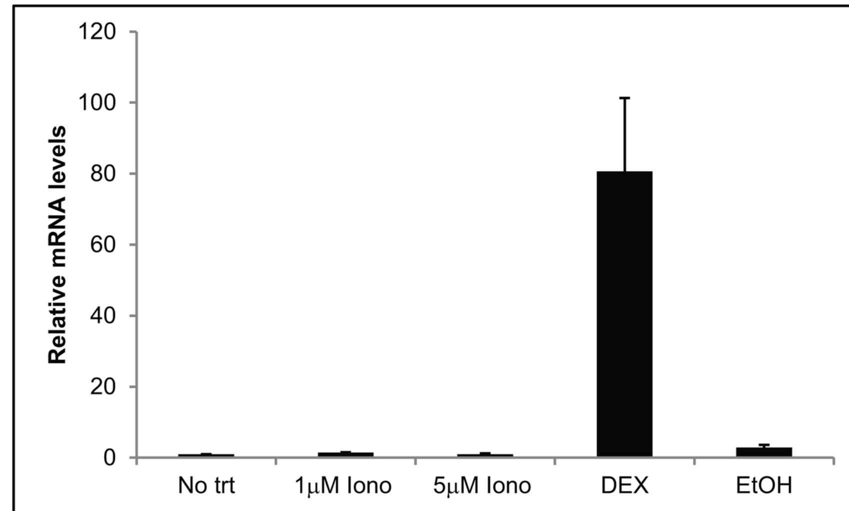

Figure 6.

Ionomycin does not increase *MYOC* mRNA. RT-qPCR was performed on HTM27 cells treated with 1 or 5 μM ionomycin or DEX or EtOH for 2 days. Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl (Pfaffl, 2001). Data were then normalized to *ITGB1* mRNA (β1 integrin housekeeping gene). n = 3 biological replicates.

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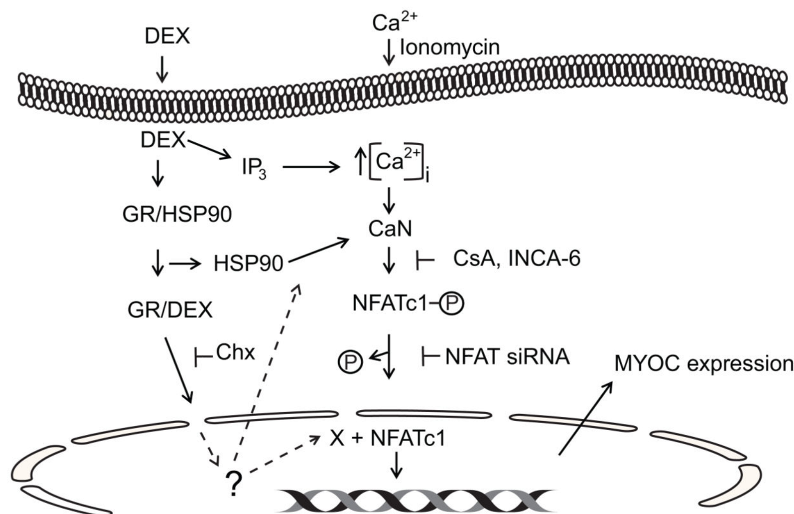

Figure 7.

Model of DEX activation of *MYOC* transcription. Schematic shows possible pathways that DEX can use to activate calcineurin (CaN)/NFATc1. Once activated NFATc1 is dephosphorylated and translocated to the nucleus where it can interact with other factors (X) to control transcription. Dotted lines indicate possible places that the glucocorticoid receptor (GR) could regulate calcineurin/NFATc1 activity. The GR could be promoting the expression of HSP90, cofactor (X) for NFATc1 or other transcription factors need for *MYOC* expression. Sites where the inhibitors cycloheximide (Chx), cyclosporin A (CsA), and INCA-6 target are indicated.

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Table 1

Individual Cell Strain Data

	Cell Strain				
	HTM27	**HTM25**	**HTM17**	**Average**	**Avg. % Decrease**
DEX	100	100	100		
DEX + 40μM INCA-6	43	65	35	48 ± 9%	52%
DEX + 80μM INCA-6	12	27	29	22 ± 5%	78%
DEX + 120μM INCA-6	8	7	22	12 ± 5%	88%
DEX + 1μM CsA	29	77	87	64 ± 18%	36%
DEX + 10μM CsA	19	34	34	29 ± 5%	71%

Table represents the relative amount of *MYOC* mRNA levels compared to DEX treatment alone (set to 100%) for individual cell strains tested. Figure 3 shows the average of all 3 cell strains.