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Dexamethasone increases αvβ3 integrin expression and affinity through a calcineurin/NFAT pathway

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ABSTRACT

The purpose of this study was to determine how dexamethasone (DEX) regulates the expression and activity of αvβ3 integrin. FACS analysis showed that DEX treatment induced expression of an activated αvβ3 integrin. Its expression remained high as long as DEX was present and continued following DEX removal. FACS analysis showed that the upregulation of αvβ3 integrin was the result of an increase in the expression of the β3 integrin subunit. By real time qPCR, DEX treatment induced a 6.2-fold increase ($p < 0.04$) in β3 integrin mRNA by day 2 compared to control and remained elevated for 6 days of treatment and then an additional 10 days once the DEX was removed. The increase in β3 integrin mRNA levels required only 1 day of DEX treatment to increase levels for 4 days in the absence of DEX. In contrast, DEX did not alter β1 integrin mRNA or protein levels. The DEX-induced upregulation of β3 integrin mRNA was partly due to an increase in its half-life to 60.7 h from 22.5 h in control cultures ($p < 0.05$) and could be inhibited by RU486 and cycloheximide, suggesting that DEX-induced de novo protein synthesis of an activation factor was needed. The calcineurin inhibitors cyclosporin A (CsA) and FK506 inhibited the DEX induced increase in β3 integrin mRNA. In summary, the DEX-induced increase in β3 integrin is a secondary glucocorticoid response that results in prolonged expression of αvβ3 integrin and the upregulation of the β3 integrin subunit through the calcineurin/NFAT pathway.

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1. Introduction

The αvβ3 integrin has been shown to be involved in the pathogenesis of a number of diseases including cancer, diabetes and osteoporosis [1–6]. More recently, αvβ3 integrin has been implicated as having a role in steroid-induced glaucoma (SIG) [7–9]. SIG results from the long term use of glucocorticoids (GCs) such as dexamethasone (DEX), most commonly through topical ocular application. About 30–40% of the normal population exhibit elevated intraocular pressure (IOP) when treated with GCs [10,11] with about 6% of these patients developing SIG. The cause of this increase in IOP is believed to be a restriction in the movement of aqueous humor through the trabecular meshwork (TM) of the anterior segment of the eye.

A number of cytoskeletal events known to be regulated by integrins have been implicated in glaucoma [12,13] including changes in contractility and a reorganization of the actin cytoskeleton into a unique structure of cross linked actin networks called CLANs. CLANs are believed to alter the contractile properties of TM cells by making them more rigid and unable to respond to pressure changes in the eye [14]. They are observed in higher frequency in glaucomatous and DEX-treated tissues [15] and DEX-treated TM cell cultures [7,16]. In TM cell cultures, these networks can be formed by the activation of an αvβ3 integrin/Src/Trio/Rac1 signaling pathway [7–9] that may involve CD47 and requires crosstalk with a β1 integrin/Src/PI-3 kinase pathway. Inhibition of this pathway impairs CLAN formation in DEX-treated cells suggesting that αvβ3 integrin signaling may be responsible for some of the cellular changes observed in glaucoma. Recent work supports this idea and shows that DEX increases the expression of αvβ3 integrin in cells from the TM [7].

Despite the preponderance of studies implicating αvβ3 integrin in disease processes we know very little about how the expression of αvβ3 integrin is regulated. In bone marrow macrophages, IL-4 and to a lesser extent IL-6, GM-CSF and TNFα, increase β3 integrin mRNA [17]. During angiogenesis, the Foxc1 and Foxc2 transcription factors has been shown to regulate expression of the β3 integrin subunit in endothelial cells via Forkhead-binding elements in the promoter region of the β3 integrin subunit [18,19]. In osteoblasts, β3 integrin expression is transiently increased by DEX [20]. Studies show that expression of the β3 integrin subunit in osteoclasts is regulated by calcineurin and the transcription factor NFATc1 [21,22]. The NFAT family of transcription factors is activated through dephosphorylation by calcineurin. Calcineurin is a ubiquitously expressed serine/threonine phosphatase that is activated by Ca^{2+} ion and calmodulin binding. To date, calcineurin is the only

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protein phosphatase that dephosphorylates NFAT [23], making it essential for the activation of genes by NFATs.

In this study we used human trabecular meshwork cells, which are specialized smooth muscle-like cells in the eye that regulate the contractile properties of the anterior segment of the eye, to study how DEX regulates αvβ3 integrin expression. Our studies show that DEX increased transcription of β3 integrin mRNA through a secondary glucocorticoid response mechanism and required de novo protein synthesis. This increase was sensitive to the immunosuppressive drugs cyclosporin A (CsA) and FK506 indicating that calcineurin may be involved. Furthermore, we show that the increased transcription of β3 integrin mRNA resulted in increased protein expression of the β3 integrin subunit that persisted even after removal of DEX and that the αvβ3 integrin was in an active conformation. These results suggest that induction of β3 integrin by DEX occurs at both the transcriptional and protein levels and may result in the dysregulation of an activated αvβ3 integrin signaling pathway that can lead to the cytoskeleton changes (i.e., CLANs) observed in glaucoma. Understanding how DEX affects TM cells in the eye is important since many systemic steroid treatments can lead to increases in intraocular pressure and glaucoma.

2. Materials and methods

2.1. Materials

For western blotting, the primary antibodies used were: β3 integrin mAb (EP2417Y, Abcam; 1:500), β1 integrin mAb (HB1.1, Millipore; 1:1000), FKBP51 (also known as FKBP5; 1:1000) pAb (Sigma-Aldrich) and succinate dehydrogenase complex, subunit A (SDHA) mAb (2E3, Abcam; 1:2000). Secondary antibodies used were goat anti-mouse or anti-rabbit HRP conjugated Ab (Santa Cruz; 1:5000). Antibodies used for FACS were: mouse IgG1 (BD Biosciences; 1:100), αvβ3 integrin mAb (LM609, Millipore; 1:100), an activated β3 integrin mAb (CRC54, Abcam; 1:100) and goat anti-mouse Alexa 488 conjugated Ab (Life Technologies; 1:400). All inhibitors were obtained from Sigma-Aldrich, Co.

2.2. Cell culture

The N27TM-2 cell strain of human trabecular meshwork (HTM) cells were isolated from cadaver eyes of a 27-year old donor and cultured as previously described [24] and used between passages 7–8. One week after reaching confluency, cells were treated with either 500 nM DEX or 0.1% ethanol (EtOH; vehicle control). In some experiments, cells were incubated with the RNA polymerase II inhibitor actinomycin D (5 μg/ml). In other experiments, the glucocorticoid inhibitor RU486 (mifepristone; 2.5, 10 or 25 μg/ml), cycloheximide (25 μg/ml) or CsA or FK506 (1 or 10 μM) was added 1 h prior to the addition of DEX or EtOH and incubated for 2 days.

2.3. Cell spreading assay

The cell spreading assay was done as previously described [7]. Briefly, cells were spread for 1.5 h on coverslips precoated with 20 nM fibronectin and co-labeled with anti-αvβ3 integrin mAb and Alexa 488 conjugated phalloidin (Life Technologies) as described [9]. Images were captured with an Axioplan 2 epifluorescence microscope (Carl Zeiss, Inc.) equipped with an Axiocam HRm digital camera using AxioVision image acquisition software.

2.4. Immunoblotting

HTM cells were washed and 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, Inc.)). The cellular debris in the cell lysate was removed by centrifugation at 10,000 ×g. A bicinchoninic acid (BCA) assay (Pierce) was done to determine protein concentration and the lysate (10 μg) was separated on a 10% SDS-PAGE and transferred to Immobilon-P (Millipore Corp.). The membrane was blocked in 3% bovine serum albumin (BSA)/tris buffered saline (TBS) or 5% milk/TBS (FKBP51 pAb) overnight at 4 °C and incubated with the primary antibody in 1% BSA/TBS/0.1% Tween-20 or 5% milk/TBS/0.1% Tween-20 for 1 h. Membranes were washed with TBS/0.1% Tween-20 and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG. Bound antibody was detected with the ECL Plus western blotting detection kit (Amersham Biosciences).

2.5. Flow cytometry

HTM cells treated with DEX or EtOH were lifted from the plate with Cell Dissociation Buffer (Sigma), washed with ice cold PBS and blocked for 30 min on ice with 2% BSA in PBS (blocking buffer). Cells were labeled with the primary antibodies for 1 h on ice in blocking buffer and then incubated on ice for 30 min with the secondary antibody. Cells were washed then resuspended in 1% paraformaldehyde in PBS. Flow cytometry was done with a FACSCalibur (BD Biosciences) using FlowJo software (Tree Star, Inc.) for analysis.

2.6. RNA isolation, reverse transcription and real-time (RT)-qPCR

Total RNA was isolated using the QIAshredder and RNeasy Plus Mini Kits (Qiagen Inc.) 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 [25]. Data were normalized to no treatment and fold change compared to the β1 integrin housekeeping gene was determined according to Pfaffl [26]. Primer pairs used were: β1 integrin forward 5′-GTGGAGAATCCAGAGTGTCCCA-3′ and reverse 5′-GACCACAGTTGTTACGG-3′, β3 integrin forward 5′-GTGACCTGAAGG AGAATCTGC-3′ and reverse 5′-TTCTTCGAATCATCTGGCC-3′, and FKBP51 forward 5′-CTCCCTAAAATTCCCTCGAATGC-3′ and reverse 5′-CCCTCTC CTTTCCGTTTGGTT-3′.

2.7. Rate of RNA synthesis

Nascent RNA synthesis was determined using the Click-iT® Nascent RNA Capture Kit (Life Technologies). Cells were treated with DEX or EtOH for 2 days after which cells were labeled by incubation with 0.1 mM 5-ethynyl uridine (EU) for 1 h in the presence of DEX or EtOH. Cells were collected and the RNA was isolated as described above. EU labeled RNA (1 μg total RNA) was then biotinylated and isolated using Dynabeads® MyOne™ Streptavidin T1 magnetic beads according to the manufacturer's instructions. RT-qPCR was performed as above using primers for β3 integrin.

2.8. Data analysis

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. Relative quantification of the RT-qPCR data was performed according to Pfaffl [26], using β1 integrin for normalization.

3. Results

3.1. DEX increases β3 integrin protein and mRNA levels

Fig. 1 shows that treating HTM cells with DEX increases the level of αvβ3 integrin in focal adhesions (Fig. 1A vs. C). When stained with phalloidin, a subset of the cells incubated with DEX also exhibited the distinctive geodesic dome actin structure called CLANs thought to be involved in glaucoma and caused by αvβ3 integrin signaling (Fig. 1B vs. D).

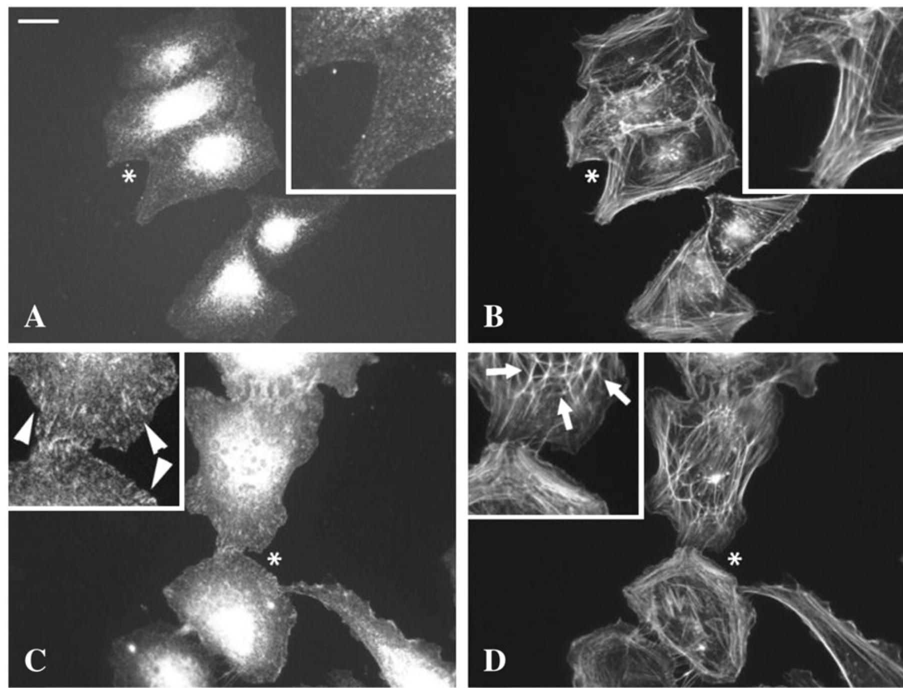

Fig. 1. Localization of αvβ3 integrin in DEX and EtOH treated HTM cells. HTM cells treated with EtOH (A, B) or DEX (C, D) for 4 days were spread on fibronectin-coated coverslips for 1.5 h prior to fixation. The cells were labeled with mAb LM609 against αvβ3 integrin (A, C) and phalloidin (B, D). Asterisks indicate areas enlarged in the insets; arrowheads indicate αvβ3 integrin-positive adhesions; arrows indicate part of a CLAN. Scale bar = 20 μm.

To understand how DEX affected the expression of αvβ3 integrin in HTM cells that leads to CLAN formation, western blot analysis was carried out to detect changes in αvβ3 integrin expression over 6 days of DEX treatment. This prolonged treatment was chosen in order to correspond with the time frame needed to observe CLAN formation in vitro [27] and the time course of patient treatment. As shown in Fig. 2A, DEX induced a time dependent increase in the expression of the β3 integrin subunit starting 2 days after initiation of DEX treatment. By day 5, the β3 integrin subunit level appeared to plateau. This increase was not seen in the EtOH treated controls. Rather, β3 integrin levels appear to diminish the longer the EtOH treated cells were in culture (Fig. 2A). The DEX-induced increase was unique to the β3 integrin subunit as no changes were observed in β1 integrin levels. This upregulation of expression was not limited to this cell strain and was observed in three other HTM cell strains (data not shown). As a positive control we also examined the levels of the FK506-binding protein FKBP51, which is known to be induced by DEX in several cell types [28,29], including TM cells [30]. As expected, FKBP51 also showed a DEX induced increase in expression. However, this increase occurred after one day of treatment compared to the 2 days needed to increase β3 integrin. FACS analysis verified the western blot analysis and showed that DEX treatment increased the expression of β3 integrin at the cell surface 2.4 fold compared to EtOH (Fig. 2B). Although αvβ3 integrin had an active conformation in both DEX-treated and ETOH-treated cells, as determined with the CRC54 mAb that detects activated αvβ3 integrin (Fig. 2B, right panel), the level of activated αvβ3 integrin was ~2 fold greater in the DEX-treated cells compared to the EtOH-treated cells. In contrast, the level of the αv integrin subunit was not changed by DEX, as determined by FACS (data not shown).

Using RT-qPCR, we found that DEX increased the expression of β3 integrin mRNA by 6.2 fold ($p < 0.04$) after 2 days of DEX treatment compared to the EtOH controls. The mRNA levels remained elevated throughout the 6 day treatment (Fig. 3A). In contrast, β1 integrin mRNA was unaffected by DEX (data not shown) and therefore was used as a housekeeping gene to control for normalization. As expected,

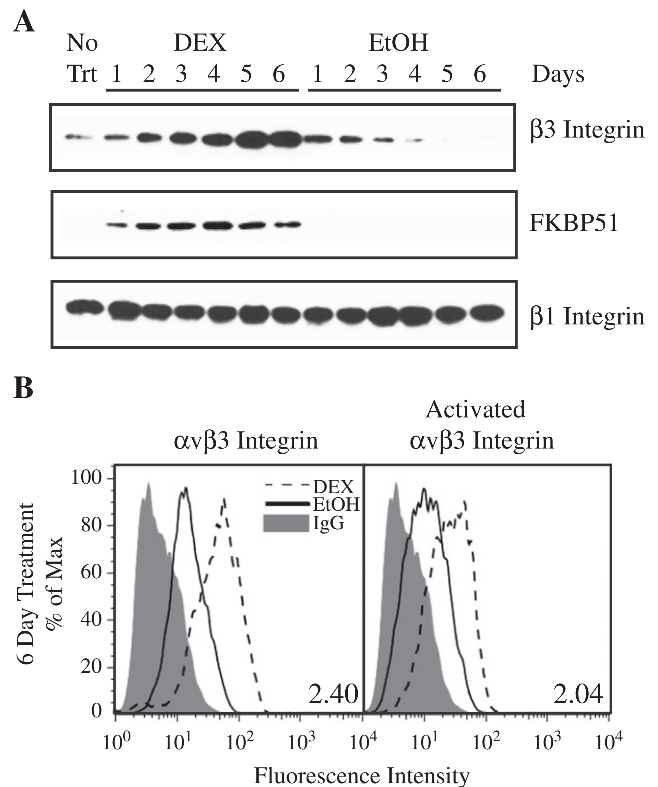

Fig. 2. DEX induced β3 integrin expression and activation. (A) HTM cells were treated with DEX or EtOH for 6 days and cell lysates were analyzed for levels of β3 integrin, β1 integrin and FKBP51. SDHA was included as a loading control. Blots are representative of 3 biological replicates. (B) Cells were treated with DEX or EtOH for 6 days. FACS analysis was done using the mAb LM609 against αvβ3 integrin (left) or mAb CRC54 which detects activated αvβ3 integrin (right). The fold change in geometric mean of DEX vs. EtOH is indicated the panels for each antibody. Data representative of 2 biological replicates.

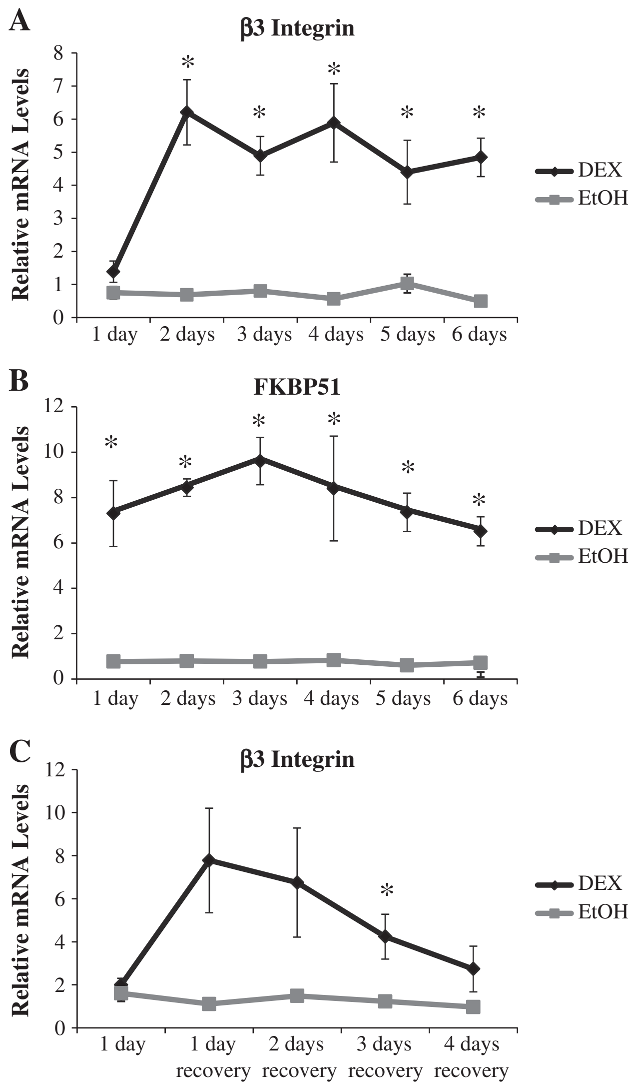

Fig. 3. DEX treatment increases β3 integrin mRNA levels. RT-qPCR was performed on HTM cells treated with DEX or EtOH. Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl [26]. Data were then normalized to the β1 integrin housekeeping gene. Level of β3 integrin mRNA (A) and FKBP51 mRNA (B) expression in the presence of DEX or EtOH for 6 days. Level of β3 integrin mRNA (C) in HTM cells treated with DEX or EtOH for 1 day only followed by media alone for 4 days. DEX was significantly different from EtOH $^{*}p < 0.05$. n = 4 biological replicates (A, B), n = 3 biological replicates (C).

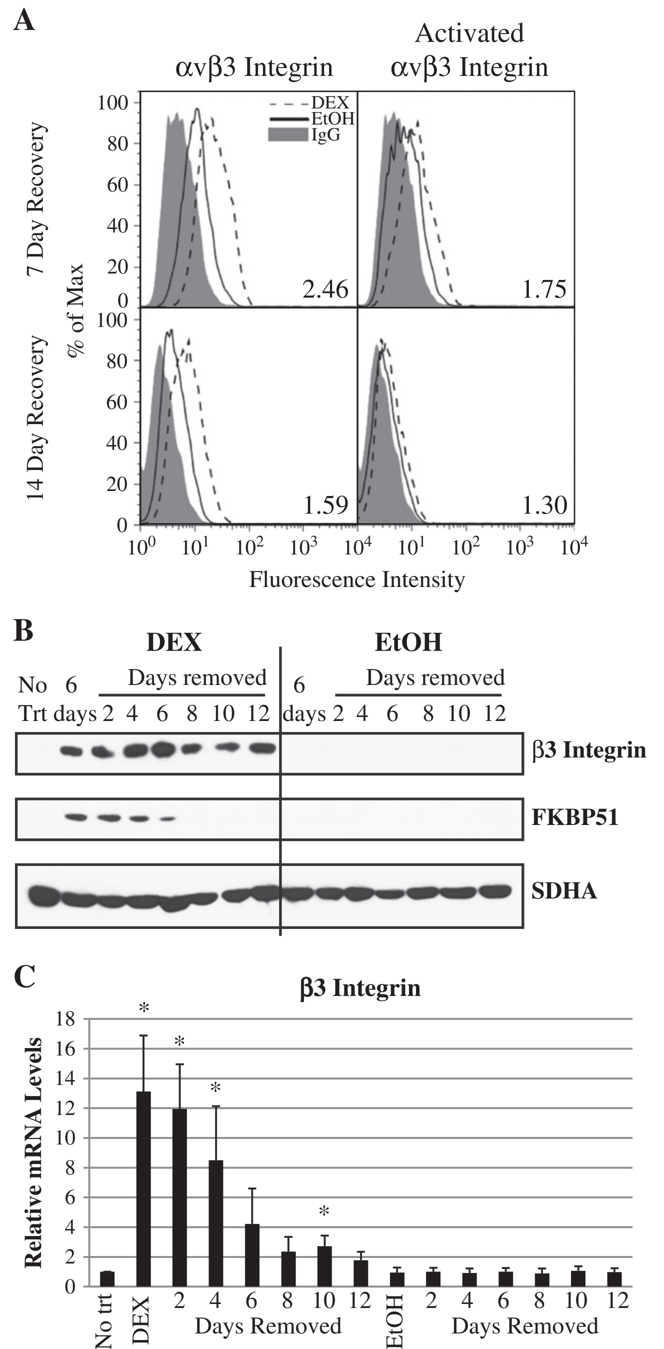

Fig. 4. Elevated levels of β3 integrin persists following the removal of DEX. (A) Cells were treated with DEX or EtOH for 6 days after which cells were incubated with media alone for 7 days (upper panels) or 14 days (lower panels). FACS analysis was done with the mAb LM609 against αvβ3 integrin (left) or mAb CRC54 which detects activated αvβ3 integrin (right). The fold change in geometric mean fold of DEX vs. EtOH is indicated in each of the panels for each antibody. Data representative of 2 biological replicates. (B and C) HTM cells were treated with DEX or EtOH for 6 days followed by media alone for 12 days. (B) Lysate was collected every other day after DEX or EtOH removal and analyzed for the expression of β3 integrin and FKBP51. SDHA was included as a loading control. Blots representative of 2 biological replicates. (C) Cells were collected every other day after DEX or EtOH removal and total RNA was analyzed by RT-qPCR. Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl [26]. Data was then normalized to the β1 integrin housekeeping gene. DEX significantly different from equivalent EtOH control, $^{*}p < 0.05$. n = 3 biological replicates.

FKBP51 mRNA levels also increased by 7.3 fold ($p < 0.04$) and remained elevated throughout the treatment (Fig. 3B). The increase in FKBP51 mRNA levels appeared after only 1 day of DEX treatment compared to 2 days for β3 integrin mRNA.

To determine if 2 days of DEX treatment was needed to increase β3 integrin mRNA levels, cultures were treated with DEX for 1 day only. As shown in Fig. 3C, β3 integrin mRNA levels still increased on day 2 even though DEX was not present on the second day. However, in the absence of any additional DEX, the mRNA levels started to decrease and by day 5 the levels of β3 integrin mRNA returned to baseline.

Since some individuals treated with glucocorticoids go on to develop glaucoma even after glucocorticoid treatment has stopped, we asked what happens to αvβ3 integrin expression once DEX is removed. FACS analysis (Fig. 4A, left panels) showed that β3 integrin expression remained 1.59 fold higher 14 days after removal of DEX compared to EtOH. Interestingly, there was still an elevation (~1.75 fold) in the level of activated αvβ3 integrin in the DEX-treated cells compared to the EtOH treated cells 7 days after the removal of the DEX (Fig. 4A, right panels). By day 14, little of the αvβ3 integrin in the DEX-treated cells was activated, although protein expression was still upregulated. Western blot analysis of lysate from cells treated with DEX followed by DEX removal verified the FACS analysis and showed that total β3

integrin expression was still elevated 12 days after DEX removal (Fig. 4B). The increase in β3 integrin levels coincided with its mRNA levels which remained elevated for up to 10 days after removal of DEX from the medium (Fig. 4C). In contrast, FKBP51 protein levels returned to EtOH control levels 8 days after removing DEX. Similar results were seen at the mRNA level for FKBP51.

3.2. β3 integrin mRNA half-life and synthesis rate increase with DEX

We next used actinomycin D to determine if the accumulation of β3 integrin mRNA could also be attributed to an increase in mRNA half-life. Cells were treated for two days with DEX or EtOH after which a final concentration of 5 μg/ml actinomycin D was added to fresh media containing DEX and EtOH for 4, 8, 12 or 24 h. Fig. 5A shows that in the presence of actinomycin D there was no significant decrease in the β3 integrin mRNA compared to the zero time at 4, 8 or 12 h. However, β3 integrin mRNA levels were significantly different from time zero at 24 h ($p < 0.025$). In contrast, there was a significant decrease in the level of β3 integrin mRNA in the EtOH treated controls over the entire 24 h. The half-life in DEX and EtOH treated cells was 60.7 and 22.5 h respectively ($p < 0.05$ overall). This suggests that the half-life of the β3 integrin mRNA was increased in the presence of DEX. In addition to mRNA half-life, DEX also increased β3 integrin mRNA synthesis. Cells treated with DEX or EtOH for 2 days were labeled with 5-ethynyl uridine (EU) for 1 h to detect nascent RNA. Fig. 5B shows a 5.9 fold increase in nascent β3 integrin mRNA compared to EtOH treated cells ($p < 0.025$).

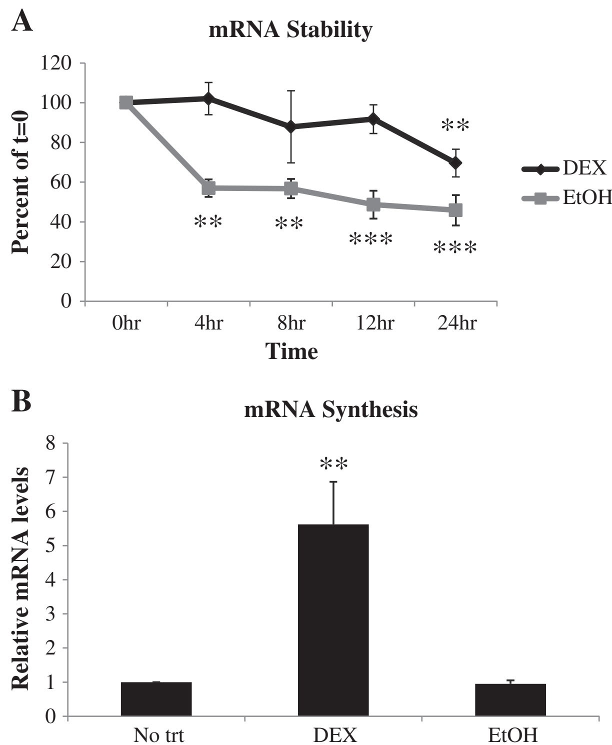

Fig. 5. DEX treatment increased β3 integrin mRNA stability and synthesis. (A) HTM cells were treated with DEX or EtOH for 2 days after which 5 μg/ml actinomycin D was added to cells in the presence of DEX or EtOH. RNA was isolated from cells after 4, 8, 12 or 24 h of actinomycin D treatment. RT-qPCR was performed and the average mRNA half-life was determined from the slopes of the linear regression lines from each experiment. Data from cells treated with DEX or EtOH with actinomycin D were normalized to αvβ3 integrin mRNA levels after 2 days of DEX or EtOH alone respectively ($t = 0$). Significantly different from 0 h, $^{**}p < 0.025$, $^{***}p < 0.02$. DEX was significantly different from EtOH overall $p < 0.05$. (B) HTM cells were treated with DEX or EtOH for 2 days after which nascent RNA was labeled with 5-ethynyl uridine (EU). After 1 h, EU labeled RNA was isolated using the Click-iT® Nascent RNA Capture Kit from Invitrogen (see Materials and methods section). Relative mRNA levels compared to no trt were determined. DEX significantly different from EtOH, $^{**}p < 0.025$. n = 4 biological replicates (A) or 3 biological replicates (B).

3.3. The increase in β3 integrin mRNA requires glucocorticoid receptor signaling

The delay in β3 integrin mRNA induction by DEX suggests that this is a secondary glucocorticoid response and that another factor induced by DEX is needed for the increase seen in β3 integrin mRNA levels. To test if the DEX-induced increase in αvβ3 integrin is mediated through glucocorticoid receptor (GR) signaling, cultures were treated with the GR antagonist RU486. The RU486-GR complex interacts with DNA at a much lower affinity than that of the dexamethasone–GR complex and can act as an agonist when other transcriptional coactivators are activated or in specific cells lines [31–33]. For this experiment, RU486 was added 1 h prior to the addition of DEX or EtOH, to determine if a DEX-induced factor was responsible for the increase in β3 integrin mRNA. In the absence of RU486, DEX increased β3 integrin mRNA by 5.4 fold on the second day of treatment ($p < 0.001$; Fig. 6A). Preincubation with RU486 eliminated this increase in β3 integrin mRNA, suggesting that the increase was due to a glucocorticoid signaling event. RU486 by itself was able to increase β3 integrin mRNA in the EtOH controls, but not to the same extent as DEX alone.

3.4. DEX induces β3 integrin through a secondary glucocorticoid response mechanism

To demonstrate that this is a secondary glucocorticoid response and required de novo protein synthesis expression for induction [34], cultures were pretreated with cycloheximide. As shown in Fig. 6B, treatment with cycloheximide caused a significant decrease in the level of β3 integrin mRNA induction observed at day 2 ($p < 0.05$). In contrast, pre-treatment with cycloheximide had no inhibitory effect on the DEX-induced increase in FKBP51 mRNA levels observed after one day of DEX treatment (Fig. 6C). These results indicate that transcription of the FKBP51 mRNA was directly induced by DEX whereas transcription of the β3 integrin mRNA required translation of a DEX-induced protein.

3.5. Calcineurin regulates β3 integrin

To understand the mechanisms by which DEX induced β3 integrin mRNA expression we treated the cells with CsA and FK506. CsA and FK506 are immunosuppressants that inhibit calcineurin, a phosphatase that activates the NFAT family of transcription factors [23]. NFATc1 had previously been shown to directly induce β3 integrin expression in osteoclasts [21]. FK506 has also been shown to suppress endogenous expression of NFATc1 [35]. Figs. 7A and B show that in cells pretreated with either CsA or FK506, the DEX-induced increase in β3 integrin mRNA was significantly inhibited, suggesting that calcineurin may play a role in regulating β3 integrin mRNA levels. Only CsA, however, completely inhibited the increase in β3 integrin mRNA back to the control level. This was not completely unexpected since several studies have shown that these compounds exhibit different efficiencies at inhibiting calcineurin [36,37] which may be due to the levels of their respective target immunophilins.

4. Discussion

In this paper we demonstrate that the upregulation of β3 integrin mRNA and protein levels by DEX was a secondary glucocorticoid response that was regulated by calcineurin. This novel induction of β3 integrin expression appeared to be triggered by a classic GR-mediated pathway since it could be inhibited by RU486. The increase in β3 integrin expression was due both to an increase in the half-life of the β3 integrin mRNA as well as an increase in transcription. It resulted in an expression of the β3 integrin subunit that persisted for days even after the removal of DEX. Thus, DEX appears to specifically trigger a long-term dysregulation of αvβ3 integrin signaling in trabecular

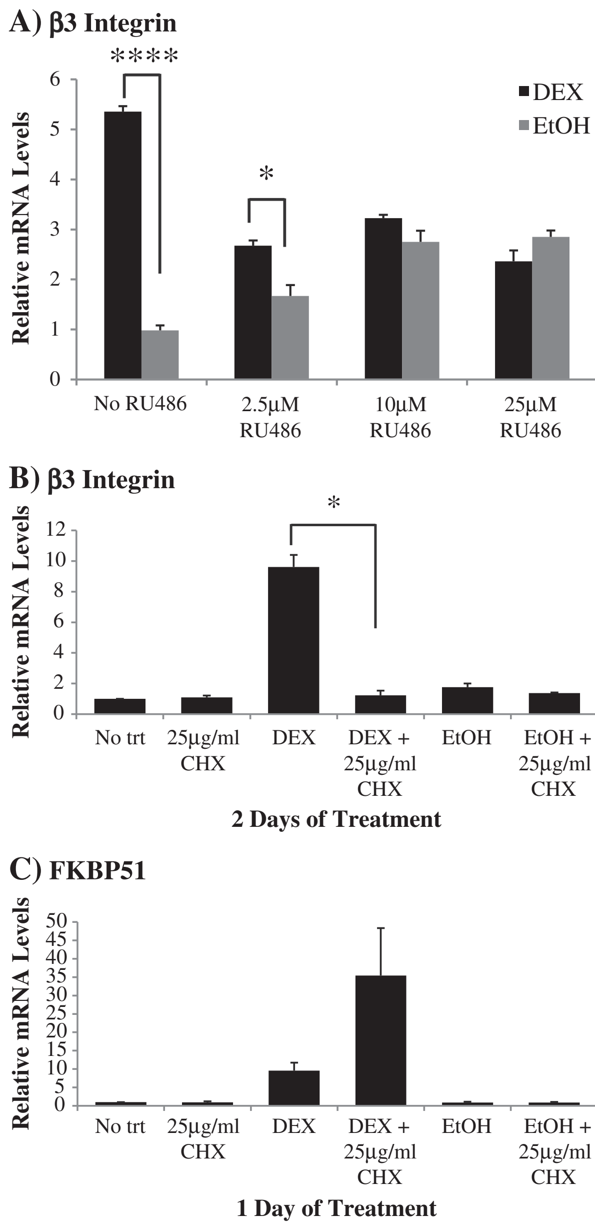

Fig. 6. RU486 and CHX prevented the induction of β3 integrin mRNA by DEX. RT-qPCR was performed on HTM cells pretreated for 1 h with 2.5, 10 or 25 μM RU486 (A) or 25 μg/ml CHX (B, C) prior to the addition of DEX or EtOH for 2 days (A, B) or 1 day (C). Fresh media containing inhibitor and DEX or EtOH were added after 1 day (A, B). Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl [26]. Data were normalized to the β1 integrin housekeeping gene. Significantly different as indicated with brackets, *$p < 0.05$, ****$p < 0.001$. n = 3 biological replicates.

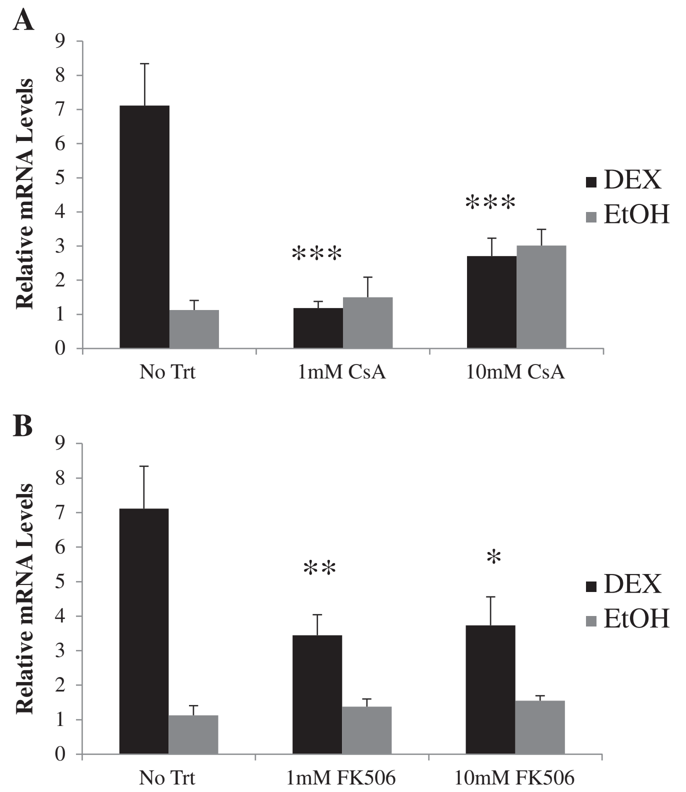

Fig. 7. CsA and FK506 prevented the induction of β3 integrin mRNA by DEX. RT-qPCR was performed on HTM cells pretreated for 1 h with 1 or 10 μM CsA (A) or FK506 (B) prior to the addition of DEX or EtOH for 2 days. Fresh media containing inhibitor and DEX or EtOH were added after 1 day. Ct values were corrected for primer efficiency and compared to cells with no treatment as described by Pfaffl [26]. Data were normalized to the β1 integrin housekeeping gene. Significantly different from DEX alone (no trt), *$p < 0.05$, **$p < 0.025$, ***$p < 0.02$. n = 4 biological replicates (A, B) or 3 biological replicates (C).

meshwork cells which could explain the cytoskeleton changes associated with glaucoma.

The DEX-induced upregulation of β3 integrin in HTM cells differed from that previously reported in osteoblasts [20]. In those studies, β3 integrin was transiently expressed. In comparison, the upregulation of β3 integrin persisted in HTM cells for 2 weeks even after the removal of DEX. The most likely explanation for this difference is that the upregulation of β3 integrin mRNA in osteoblasts involved specific palindromic sequences called glucocorticoid response elements (GREs) in the 5′ region of the β3 integrin promoter, suggesting a direct interaction between the GR and the GREs in the promoter. In contrast, this interaction with the GREs did not appear to function in HTM cells, since the DEX-induced upregulation of the β3 integrin subunit could be inhibited with cycloheximide, indicating the need for factor(s) to be synthesized prior to the upregulation of the β3 subunit.

This DEX induced increase in β3 integrin mRNA levels may be unique to HTM cells and is similar to that observed for the glucocorticoid response protein called myocilin [38,39]. Myocilin is a secreted glycoprotein that is mutated in some juvenile open-angle glaucomas [40–42]. Like the β3 integrin gene, myocilin also contains GREs and the upregulation of its expression is also due to a secondary glucocorticoid response [38,39]. Why the GREs in myocilin and the β3 integrin subunit are not utilized by the GR is not clear. There are a number of factors which are responsible for mediating the interactions between the GREs and the GR [43] including the multi-subunit complexes that remodel chromatin, target the initiation sites and stabilize the RNA polymerase II machinery.

It is presently unclear which factor(s) are involved in the upregulation of β3 integrin. One possible mechanism may be that the GR is exerting its effect through an indirect non-DNA binding mechanism termed transrepression [44]. In this mechanism, transcriptional modulation of the β3 integrin subunit could be achieved by cross-talk between the GR and other transcription factors such as NF-κB, AP-1 and members of the STAT family. However, because it took 2 days to see the upregulation and it required de novo protein synthesis, we tend to favor the idea that DEX induced the expression of a soluble factor which in turn activated calcineurin. In support of this idea, recent proteomic studies have shown that phosphatidylinositide 3-kinase (PI-3K) and protein kinase C (PKC), both of which modulate calcineurin signaling [23], are upregulated in HTM cells in response to DEX [25]. It was not surprising that calcineurin was involved. Previous studies have shown that the regulation of β3 integrin subunit involves NFATc1 [21,45] which is a well established target of calcineurin [23]. Interestingly, in

bone marrow cells NFATc1 expression is upregulated in the cytoplasm after one day of treatment with RANKL, a factor that induces osteoclast formation, and detected in the nucleus after 2 days of treatment [46,47]. Whether NFATc1 is upregulated by DEX in TM cells is unknown.

Calcineurin, has been shown to regulate the organization of the cytoskeleton. The involvement of calcineurin fits with the observations that in HTM cell cultures and in anterior segments, DEX induces the reorganization of the actin cytoskeleton into CLANs [7,16]. This unique actin structure is thought to make the cells more rigid so they cannot change shape in response to pressure changes. This, in turn, causes the TM to become more rigid which could restrict the outflow of aqueous humor from the eye [14,48].

Interestingly, the DEX treatment also increased the stability of the β3 integrin mRNA. The regulation of mRNA stability is a powerful mechanism to alter long term gene expression and has been shown to be controlled by hypoxia, inflammation, cancer and aging [49]. In terms of steroid induced glaucoma or primary open angle glaucoma, this enhanced mRNA stability could have large implications especially since the elevated levels of β3 integrin subunit at the cell surface also appeared to be more stable and more of the αvβ3 integrin was in the activated state. At the present time, however, it is not possible to say whether the increased mRNA expression was due to an increase in mRNA stability or in transcription. This supports previous work from our laboratory which showed that in the presence of DEX, CLAN formation is associated with a change in the conformation of αvβ3 integrin that results in integrin activation. In the absence of DEX, CLANs can be formed simply by activating αvβ3 integrin using either the AP-5 antibody or the thrombospondin peptide 4N1K [8]. Thus, a DEX induced increase in β3 integrin expression and activation could explain the increased frequency of CLAN formation observed in glaucomatous and DEX-treated anterior segments.

In summary, this study suggests that some of the long term effects normally attributed to SIG could be due to the indirect activation of calcineurin that results in a prolonged dysregulation of αvβ3 integrin signaling. Clearly additional studies looking at the other transcriptional factors responsible for the secondary glucocorticoid response and their role in the expression of proteins such as myocilin are warranted, since it may provide insight on how to prevent undesirable steroid induced side effects in the treatment of eye diseases.

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