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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.

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).