Dexamethasone Facilitates NF- κ B Signal Pathway in TNF- α Stimulated Rotator Cuff Tenocytes

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Received: October 29, 2018
Revised: November 26, 2018
Accepted: November 27, 2018

First published online
November 28, 2018

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pISSN 1017-7825, eISSN 1738-8872

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Corticosteroids are commonly used for pain control in rotator cuff tear. Deregulated NF- κ B activation is a hallmark of chronic inflammatory diseases and has been responsible for the pathogenesis of rotator cuff tear. The Dexamethasone (DEXA) is a synthetic corticosteroid. The purpose of this study was to examine the exact effect of dexamethasone on NF- κ B signaling in rotator cuff tear. We measured NF- κ B expression in four groups: control, TNF- α -treated, DEXA-treated, and combined treatment with TNF- α and DEXA. Tenocytes were isolated from patients with rotator cuff tears and pre-incubated with TNF- α (10 ng/ml), DEXA (1 μ M), or both of them for 10 min, 1 h, and 2 h. Expression of p65, p50, and p52 in the nuclei and cytosol was analyzed by western blotting and immunofluorescence imaging using confocal microscopy. We also evaluated nucleus/cytosol (N/C) ratios of p65, p50, and p52. In our study, the combined treatment with DEXA and TNF- α showed increased N/C ratios of p65, p50, and p52 compared with those in the TNF- α group at all time points. Additionally, in the DEXA group, N/C ratios of p65, p50, and p52 gradually increased from 10 min to 2 h. In conclusion, DEXA promoted the nuclear localization of p65, p50, and p52, but was not effective in inhibiting the inflammatory response of TNF- α -stimulated rotator cuff tear.

Keywords: Rotator cuff, tenocytes, NF-kappa B, glucocorticoid, tumor necrosis factor-alpha

Introduction

A corticosteroid injection is one of the most commonly used procedures for reduction of chronic shoulder pain caused by rotator cuff tendinosis in clinical practice, although evidence and mechanisms that support the use of corticosteroid injection remain controversial [1]. Some studies have reported its short-term benefits for pain relief, without significant improvement in shoulder joint function over a long term [2–4]. However, other studies have reported worse long-term outcomes of corticosteroid injection compared with a placebo [5].

The cause of pain following rotator cuff tear has not been fully verified. In an unstabilized rotator cuff defect rat model, TNF- α , IL-1 β , and IL-6 levels were reported to be

significantly higher than those in the sham group at days 21 and 56 [6]. TNF- α and IL-1 β are upper stream inflammatory cytokines, which mediate an inflammatory reaction and ultimately induce composition of pain-related neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P [7]. The human antigen-presenting, cell-derived cytokines TNF- α , IL-1 β , and IL-6 induce IL-2 receptor (R) up-regulation and IL-2 production, and proliferation by both CD4+ and CD8+ T cells [8]. These inflammatory cytokines may serve as one source of pain during rotator cuff tear [6].

Deregulated nuclear factor-kappa B (NF- κ B) activation is a hallmark of chronic inflammatory diseases. NF- κ B plays an important role in gene regulation during inflammatory and immune reactions. The NF- κ B family consists of five

structurally related members: NF- κ B1 (also named p50), NF- κ B2 (also named p52), RelA (also named p65), RelB, and c-Rel. They mediate the transcription of target genes by binding to a specific DNA element, κ B enhancer, as various hetero- or homo-dimers [9]. Activation of NF- κ B involves two signaling pathways important for regulating immune and inflammatory responses: classic (canonical) and alternative (noncanonical) pathways [10]. TNF- α signaling regulates the transcriptional activity of NF- κ B by directly modifying p65 and p50. The noncanonical pathway of NF- κ B regulates the transcription of p52 [10].

A rat study has reported gene expression of NF- κ B in mesangial cells in the kidney of rats after treatment with corticosteroid. The Dexamethasone (DEXA) is a synthetic corticosteroid. Pretreatment with dexamethasone failed to inhibit degradation of I κ B- α or - β in response to stimulation or prevent the increase in NF- κ B binding activity [11]. However, corticosteroid injections can quickly alleviate pain and inflammation by suppressing resistance symptoms, without long-term effects on NF- κ B suppression [1, 2, 12]. Moreover, there are limited studies regarding the effect of corticosteroid treatment on NF- κ B in tenocytes of human rotator cuff tears [13]. To our knowledge, this is the first study of increased nuclear localization of the NF- κ B family in TNF- α -treated rotator cuff tenocytes after glucocorticoid treatment. The purpose of this study was to investigate whether DEXA would inhibit or increase expression levels of NF- κ B family proteins in TNF- α -stimulated tenocytes from torn rotator cuff tendons, because deregulated NF- κ B activation is a hallmark of chronic inflammatory diseases.

Materials and Methods

Isolation and Culture of Tenocytes

Human rotator cuff tendon was obtained during elective surgery of rotator cuff repair from a 63-year-old woman. Informed written consent was taken from the patient for this study.

The sample, 5 × 5 mm out of a 30 × 20 mm-full thickness of rotator cuff tear, was washed with phosphate buffered saline (PBS: HyClone, USA) three times, and then cut into 1–2 mm³ with a No. 11 knife. Pieces of torn rotator cuff tissue were placed on cover slides and incubated at 37°C in a CO₂ incubator for two weeks. Cell suspension was collected, filtered through a 100- μ m cell strainer (BD Biosciences, USA), and centrifuged at 300 g for 10 min at room temperature. Suspension was incubated with 1 ml of red blood cell lysing buffer (Sigma R7757, USA) at room temperature for 1 min, pipetted for 1 min to mix, added to 9 ml of PBS, and then centrifuged at 300 ×g for 10 min at room temperature.

Isolated cells were seeded into a 100-mm culture dish (Corning, USA) and incubated in Dulbecco's modified Eagle medium (DMEM: HyClone, USA) containing 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco). The cultures were maintained at 37°C CO₂ incubator. Culture medium was replaced three times per week. All experiments on tenocytes were conducted at three passages.

Stimulation of Tenocytes

To verify their effects on intracellular NF- κ B expression, tenocytes (1 × 10⁶ cells) were incubated with TNF- α (10 ng/ml, R&D Systems Inc., USA), DEXA (1 μ M, Sigma D-4902, USA), or both of them for 10 min, 1 h, and 2 h. Control group did not receive any treatment. Thus, there were four groups in our study: control group, TNF- α group, DEXA group, and a TNF- α and DEXA-treated group (combined treatment). This procedure was repeated three times to obtain statistical significance.

Western Blot Analyses of NF- κ B Family

After washing cells twice with ice-cold PBS to extract proteins, cells were scraped and centrifuged at 1,600 ×g for 5 min at 4°C. Nuclear and cytosolic proteins were extracted from the four groups using a kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents 78833, Thermo Scientific, USA), according to the manufacturer's instructions. These separated proteins were quantitated by Bradford assay and boiled with sample buffer for 5 min. These samples were then subjected to 10% SDS-PAGE at 100 V for 1 h and 30 min, transferred to PVDF membrane, and incubated with anti-p65 (diluted 1: 500), anti-p50 (diluted 1: 500), and anti-p52 (diluted 1: 500) antibodies at 4°C overnight. After washing with PBS, the membrane was incubated with secondary antibody (anti-rabbit IgG HRP-linked antibody, diluted 1: 1000, Cell Signaling Technology Inc., USA) at room temperature for 2 h, washed with PBS, developed, and imaged with ECL. We evaluated the nuclear and cytosolic expression of NF- κ B proteins and the nucleus to cytosol ratio (N/C ratio) of p65, p50, and p52 expression levels.

Immunofluorescence Analyses of NF- κ B Family

Cells grown on glass coverslips were treated with TNF- α (10 ng/ml, R&D systems Inc.), DEXA (1 μ M, Sigma, USA), or both of them for 10 min, 1 h, and 2 h. Cells were fixed in 4% paraformaldehyde for 15 min and washed with ice-cold PBS containing 0.2% triton X-100 for 2 min. After washing with PBS twice for 5 min, cells were reacted with 5% bovine serum albumin (BSA) for 1 h. They were then incubated with primary antibodies (anti-p65, diluted 1:50; anti-p50, diluted 1:50; and anti-p52, diluted 1: 50) at 4°C overnight, washed with ice-cold PBS containing 0.2% triton X-100 three times. These cells were then incubated with secondary antibody (cy3-conjugated goat anti-rabbit IgG (H&L), diluted 1: 500) for 1 h. After washing with PBS three times for 2 min each, cells were counterstained with nuclear probe DAPI

and then mounted. Image acquisition was performed using a LSM5 live configuration VarioTwo VRGB laser scanning microscope (Zeiss, Germany).

Statistical Analysis

SPSS software (version 15; SPSS, USA) was used for all statistical analyses. Comparisons between the four groups were performed using Student's *t*-test. Statistical significance represented by asterisks is marked correspondingly in the figure (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Nuclear Expression of NF- κ B Family Was Higher After Combined Treatment of TNF- α and Dexamethasone than After the Single Treatments at All Exposure Times

The nuclear expression of p65 and p50 proteins was higher after a combined treatment of TNF- α and DEXA than after the single treatments, at all exposure times. For p52, the nuclear expression was higher after combined treatment of TNF- α and DEXA at 10 min and 2 h of exposure than after TNF- α treatment alone (Fig. 1). DEXA-treated samples showed an increase in the nuclear expression of p65 and p52 from 10 min to 1 h of exposure, while the nuclear expression levels were similar between the 1 h- and 2 h-treated samples. However, the levels of p50 expression increased nuclear expression as exposure time increased (Fig. 1). NF- κ B proteins showing an increase in nuclear expression accompanied by a concomitant decrease in the cytosolic expression were p65 (treated with the combination of TNF- α and DEXA for 1 h compared to treatment for 10 min), p50 (treated with DEXA for 2 h compared to treatment for 1 h), p50 (treated with the combination of TNF- α and DEXA for 2 h compared to treatment for 1 h), and p52 (treated with DEXA for 1 h compared to treatment for 10 min) (Fig. 1). Combination treatment with TNF- α and DEXA showed a greater increase in the nuclear localization of NF- κ B proteins than that induced by TNF- α treatment. Therefore, DEXA could not be assumed to suppress the nuclear expression of NF- κ B.

Dexamethasone Does Not Inhibit the Nuclear Expression of NF- κ B Family

In TNF- α -, DEXA-, and combined TNF- α and DEXA-treated groups, the nucleus/cytosol ratio of p65 and p50 expression showed a similar pattern for increase and decrease in the nuclear expression levels of p65 and p50, respectively (Figs. 1 and 2). In contrast to the single treatments, combined treatment with TNF- α and DEXA resulted in a greater N/C ratio for three NF- κ B proteins at

Fig. 1. The relative expression of p65 (A), p50 (B), and p52 (C) measured by western blotting following treatment with TNF- α , dexamethasone, or both TNF- α and dexamethasone.

The nuclear expression of p65 and p50 proteins was higher after a combined treatment of TNF- α and DEXA than after the single treatments alone, at all exposure times (A, B). For p52, the nuclear expression was higher after combined treatment of TNF- α and DEXA at 10 min and 2 h of exposure than after TNF- α treatment alone (C). This was normalized to actin expression. Data are represented as mean $\pm$ standard error.

all exposure times (Fig. 2). Combination treatment with TNF- α and DEXA significantly increased the N/C ratio for p65 after 1 or 2 h of exposure compared to single treatments.

Fig. 2. Nucleus/cytosol ratios of p65, p50, and p52 measured from the western blot. Combined treatment with both TNF- α and DEXA resulted in increased N/C ratios of p65, p50, and p52 compared with that seen after TNF- α treatment alone (A–C). In DEXA groups, N/C ratios of p65, p50, and p52 gradually increased as exposure time increased (A–C). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Moreover, in case of p50, the N/C ratio was significantly increased at all exposure times, compared with the single treatments. In case of p52, the N/C ratio was significantly increased at 10 min and 2 h, compared to TNF- α treatment,

Table 1. Statistical analyses to compare nucleus/cytosol ratio of NF- κ B between different exposure times of 10 min and 1 or 2 h within groups treated with TNF- α , dexamethasone, or both TNF- α and dexamethasone.

P65 (Nucleus/Cytosol)		p-value		
Group		10 min vs 1 h	10 min vs 2 h	1 h vs 2 h
TNF- α		1.2×10^{-2}	1.6×10^{-2}	N.S.
DEXA		N.S.	4.2×10^{-3}	N.S.
TNF- α + DEXA		1.4×10^{-2}	2.4×10^{-3}	N.S.

P50 (Nucleus/Cytosol)		p-value		
Group		10 min vs 1 h	10 min vs 2 h	1 h vs 2 h
TNF- α		1.0×10^{-4}	3.0×10^{-2}	N.S.
DEXA		N.S.	1.0×10^{-5}	1.2×10^{-5}
TNF- α + DEXA		4.6×10^{-4}	1.2×10^{-3}	5.0×10^{-2}

P52 (Nucleus/Cytosol)		p-value		
Group		10 min vs 1 h	10 min vs 2 h	1 h vs 2 h
TNF- α		2.3×10^{-2}	6.9×10^{-3}	N.S.
DEXA		2.9×10^{-2}	3.6×10^{-2}	N.S.
TNF- α + DEXA		4.3×10^{-2}	3.6×10^{-2}	N.S.

DEXA: dexamethasone, min: minutes, h: hour, vs: versus, N.S.: not significant

but at 10 min and 1 h compared to DEXA treatment (Fig. 2). In DEXA groups, the N/C ratio of p65 significantly increased between 10 min and 2 h exposure time. In the case of p50, the nuclear expression increased as the exposure time increased, and the N/C ratio significantly increased between 10 min or 1 h and 2 h of exposure. In the case of p52, the N/C ratio significantly increased between 10 min and 1 h or 2 h (Table 1 and Fig. 2). Combination treatment with TNF- α and DEXA resulted in a greater increase in the levels of NK- κ B proteins than with the single treatments. Hence, it could be assumed that DEXA does not inhibit the nuclear expression of NK- κ B proteins.

Nuclear Expression of NK- κ B Family Was Clearly Increased upon TNF- α Treatment and Simultaneous Treatment with TNF- α and Dexamethasone after 1 H of Exposure

In a confocal image analysis for immunofluorescence staining, p65, p50, and p52 levels were not increased in the nucleus at 10 min in the TNF- α alone, DEXA alone, and combination treatment groups (Fig. 3A). In contrast, after 1 h of exposure, the nuclear expression of p65, p50, and p52 was clearly increased upon TNF- α treatment and simultaneous treatment with TNF- α and DEXA, while the nuclear expression did not increase after DEXA treatment (Fig. 3B).

Fig. 3. Immunofluorescence analysis of NF- κ B at 10 min (A) and 1 h (B).

In the TNF- α , DEXA, and combination treatment groups, p65, p50, and p52 were not increased in the nucleus at 10 min (A). In contrast, after 1 h of exposure, the nuclear expression of p65, p50, and p52 was clearly increased upon TNF- α treatment and simultaneous treatment with TNF- α and DEXA, while the nuclear expression did not increase after DEXA treatment (B).

Discussion

In our study, the group simultaneously treated with TNF- α and DEXA showed increased nuclear localization of p65, p50, and p52 compared with samples treated with TNF- α alone.

Based on results from the TNF- α and combined treatment groups, it was expected that DEXA might have a modulating effect on the pain caused by rotator cuff tear within a short time. However, it failed to inhibit the NF- κ B pathway

induced during TNF- α -simulated rotator cuff tear. This might be associated with the negative effects of DEXA on tendon homeostasis. Clinically, steroids are most commonly used for chronic shoulder pain caused by degenerative rotator cuff tendinosis. However, the precise mechanism for this DEXA treatment remains unknown. Effects of corticosteroids on inflammation, matrix synthesis, and cytoprotective potential of tenocytes in conditions simulating tendinopathy have not been investigated yet [14]. Meta-analysis of randomized controlled trials of subacromial corticosteroid injection versus placebo injection for rotator cuff tendinosis showed a slight, transient pain relief at assessment between 4 and 8 weeks. However, the injection failed to reduce pain intensity at 3-month assessment, indicating that corticosteroid injection could not modify the natural course of the inflammatory disease [15].

Concerns about a local corticosteroid injection for tendinopathy arise from its potential side effects, including tendon degeneration, the weakening of the injected tendon, decreasing biomechanical strength at the injected site, and ruptures [16–23]. Intra-articular treatment with corticosteroids also increases apoptosis in human rotator cuff tears and the tendency was similar in all rupture sites [24].

Upstream regulation of NF- κ B activation was reported to occur in skeletal muscle atrophy via increasing in vivo expression of NF- κ B inducing kinase following glucocorticoid administration [25]. Early and intermediate stage supraspinatus tendon disease increased expression of genes and proteins regulated by NF- κ B activation pathways [26].

Oh *et al.*'s study concluded that glucocorticoid may not inhibit the transcription of NF- κ B stimulated by TNF- α in intervertebral disc cells [27].

In this study, DEXA treatment alone gradually increased nuclear localization of p65, p50, and p52 from 10 min to 2 h. The group simultaneously treated with TNF- α and DEXA showed increased nuclear localization of p65, p50, and p52 compared with samples treated with TNF- α alone. DEXA did not inhibit migration of NF- κ B to the nucleus, but increased its induction by TNF- α .

Therefore, improvement of symptoms after DEXA treatment might be due to induction of short-term inflammatory factor mobilization or immune response rather than a decrease of NF- κ B in TNF- α -stimulated rotator cuff tenocytes.

Moreno *et al.* reported that the kinetic behavior of p65 nuclear import and export showed an oscillatory behavior with most p65 molecules in the nucleus 30–60 min after TNF- α treatment, followed by nuclear export and a cytosolic localization of p65 at later time points in HeLa

cells [28]. In our study, TNF- α treatment increased the nuclear expression of all three proteins, p65, p50, and p52, until 1 h and then decreased slightly at 2 h compared with the expression at 1 h. This could be the natural course of NF- κ B proteins in TNF- α -stimulated rotator cuff tenocytes. The canonical NF- κ B pathway might have responded to diverse stimuli, including ligands of various cytokine receptors including proinflammatory cytokine TNF- α in torn rotator cuff tenocytes.

This study has a few limitations. First, the number of samples was small. Nonetheless, the results presented here are important, as limited studies have reported the increased nuclear expression of p65 by DEXA in TNF- α stimulated tenocytes obtained from rotator cuff tendon tear. Further preclinical experiments on NF- κ B with specimens should be performed to confirm the results and to understand the role of canonical and non-canonical NF- κ B pathway in DEXA-mediated effects on torn rotator cuff tendon. Second, we did not perform identification of the cultured tenocytes. However, an expert and experienced orthopedic surgeon had obtained the sample from a torn rotator cuff. Third, as shown in the in vitro experiment that DEXA treatment alone did not inhibit the inflammatory response after TNF- α stimulation, an additional in vivo experiment should be performed to confirm this result.

In conclusion, the simultaneous treatment with TNF- α and DEXA showed stronger nuclear localization of NF- κ B family proteins including p65, p50, and p52 than with TNF- α treatment alone. Additionally, DEXA treatment alone led to a gradual increase in the nuclear localization of p65, p50, and p52 with increase in exposure time. Thus, DEXA promoted movement of NF- κ B into the nucleus in the presence of TNF- α and was not effective in inhibiting the inflammatory response in rotator cuff tear. This ex vivo evidence shows that DEXA has negative effects on tendon homeostasis with increase in the nuclear localization of NF- κ B as previously reported [12, 23, 29–31].

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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