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Promoter-specific relevance of histone modifications induced by dexamethasone during the regulation of pro-inflammatory mediators

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ABSTRACT

Glucocorticosteroids (GCs) are widely used to treat different kinds of chronic inflammatory and immune diseases through transcriptional regulation of inflammatory genes.
Modulation of gene expression by GCs is known to occur through diverse mechanisms of varying relevance to specific classes of genes. Epigenetic modifications are indeed a pivotal regulatory feature of glucocorticoid receptor and other transcription factors.
In this study, histone post-translational modifications were investigated for their involvement in the regulation of selected pro-inflammatory genes – expressed in human monocyte-derived macrophages – in response to treatment with synthetic GC dexamethasone (DEX). We show that histone tail acetylation status is modified following DEX administration, through distinct and alternative mechanisms at the promoters of interleukin-8 and interleukin-23. In addition to histone H3 acetylation, our results demonstrate that H3 lysine 4 trimethylation is affected following drug treatment.

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1. Introduction

It is well known that glucocorticosteroids (GCs) suppress the inflammation process through several mechanisms of action. Chromatin remodeling, which is at the basis of several of these mechanisms, has been extensively explored. Inflammation requires the expression of multiple genes – cytokines, chemokines, adhesion molecules, and inflammatory enzymes – regulated by pro-inflammatory transcription factors, such as nuclear factor (NF)-κB and activator protein (AP)-1. This increase in gene expression is facilitated by several co-activators, which accomplish acetylation and other modifications of the histones located in promoter regions of target genes, thus promoting the open state of chromatin [1–4].

Activated glucocorticoid receptors (GRs) counteract inflammation through *trans-activation* of anti-inflammatory target genes such as annexin-1, interleukin-10, inhibitor of -κB α (IκBα), MAPK-phosphatase-1, tristetraprolin, and glucocorticoid-induced leucine zipper. GRs bound to glucocorticoid response elements (GREs) in target promoters recruit co-activator proteins to increase histone acetylation, producing a DNA–protein structure that allows enhanced gene transcription [5–8]. On the other hand, GCs down-modulate pro-inflammatory genes by *trans-repression*, reversing histone acetylation through: i) binding of ligand-engaged GRs to co-activator molecules thus inhibiting their histone acetyltransferase (HAT) activity and ii) recruitment of histone deacetylases (HDACs) to the transcription complex, thus favoring compaction of DNA around core histones, exclusion of RNA polymerase, and suppression of gene transcription [9]. Inflammatory genes are specifically recognized by GRs because of the presence of NF-κB and its cognate pro-inflammatory co-factors [10–12]. Nonetheless, the precise mechanism mediating the inhibition of each gene depends on the cell type, availability of specific co-factors, chromatin status, promoter environment, and other aspects. Therefore, much remains to be uncovered regarding the role of specific histone modifications at the promoter level during the modulation of gene expression by GC treatment.

This work focuses on a model of therapeutic intervention on an ongoing inflammatory state, obtained by administering a GC drug to cells several hours after the pro-inflammatory stimulus. We speculated that DEX would target pre-existing histone post-translation modifications in, or near, the same DNA regions modified by the presence of active NF-κB. Interleukin (IL)-23 and IL-8/CXCL8 were investigated, as they represent well known pro-inflammatory mediators involved in the

Abbreviations: ChIP, chromatin immunoprecipitation; DEX, dexamethasone; DMSO, dimethyl sulfoxide; GC, glucocorticosteroid; GR, glucocorticoid receptor; GRE, glucocorticoid response element; H3Ac, acetylated histone H3; H3K4me3, trimethylated lysine 4 of histone H3; HAT, histone acetyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; IκBα, inhibitor of -κB α; IL-23, interleukin-23; IL-8, interleukin-8; LPS, lipopolysaccharide; Mφ, macrophage; MKP-1, MAPK phosphatase 1; NF-κB, nuclear factor -κB; PBS, phosphate buffered saline; TSA, trichostatin A; TSS, transcription start site

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development and maintenance of autoimmune inflammation, chronic inflammatory disorders, premature cellular senescence and cancer progression [13–16]. Both cytokines are produced in macrophages upon transcriptional induction by NF-κB [17–22], and are efficiently repressed by glucocorticoid treatment [23–26]. We show here that transcriptional repression in each of the two promoters is associated with a distinct pattern of modulation of HAT and HDAC activities, suggesting that the classical scheme of epigenetic modifications could be of varying relevance during the repression of distinct pro-inflammatory transcripts by the GC analog DEX.

2. Material and methods

2.1. Cell cultures and reagents

Human monocyte-derived Mφs were obtained from healthy blood. Adult volunteers signed an informed consent form before making an anonymous donation at the blood collection center of S. Maria della Misericordia Hospital in Urbino (Italy). Mφs were prepared by density gradient separation using Histopaque-1077 solution (specific density 1.077; Sigma-Aldrich, St. Louis, MO, USA). Cells were resuspended in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. All cell culture reagents were from Lonza (Basel, Switzerland). Alternatively, Mφs were cultured in a medium supplemented with charcoal/dextran-stripped FBS (Hyclone, South Logan, UT, USA) to verify that cortisol levels in the media did not interfere with the performance of dexamethasone treatment. Since no interference was observed, all the subsequent experiments were performed in heat-inactivated FBS. Monocytes were separated by plastic adherence to tissue culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany), overnight at 37 °C in a humidified 5% CO_2 atmosphere. Non-adherent cells were removed by repeated washes. Cells were cultured for 12 days, after which >95% of adherent cells were differentiated Mφs, as revealed by immunostaining and surface marker analyses.

Monocyte-derived Mφs (10^6–10^7) were either left untreated or stimulated with 1 μg/mL of LPS (serotype 0111:B4, Sigma-Aldrich), for 8 h. DEX (Sigma-Aldrich) was resuspended in PBS to 1 mM and working dilutions were dosed spectrophotometrically at 239 nm and administered to cells for 2 h. Trichostatin A (TSA) (Sigma-Aldrich) was resuspended in dimethyl-sulfoxide (DMSO) at a stock concentration 20 mM and used at a final concentration of 10 nM (DMSO 0.05% v/v). An equal volume of DMSO was administered to cells as a negative control. Sodium orthovanadate (Na_3VO_4) (Sigma-Aldrich) was activated according to the standard procedure prior to use at a final concentration of 250 μM.

2.2. ChIP

For each experimental condition, 3×10^6 cells were crosslinked by adding 1/10 (v/v) of the cross-linking solution (11% formaldehyde, 50 mM HEPES pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA) for 10 min at 37 °C. The crosslinking reaction was stopped by the addition of 0.125 M glycine and incubation on ice for 5 min. Cells were then washed twice with ice-cold PBS, and chromatin extraction was performed as described elsewhere [27,28], with minor modifications. The isolated chromatin was fluorimetrically quantified by Qubit (Invitrogen, Life Technologies, Carlsbad, CA, USA) and the correct chromatin average size (300–500 bp) verified. Chromatin immunoprecipitation assays were performed on 750 ng of chromatin using antibodies directed against histone H3 trimethylated at lysine 4 (ab8580, Lot 533389; Abcam, Cambridge, UK), acetylated histone H3 (06-599, Lot DAM1422332; Millipore, Billerica, MA, USA), or with normal rabbit IgG (not commercial) as negative control. Immunoprecipitated chromatin (bound fractions) and an aliquot of input chromatin (1%) were subjected to de-crosslinking and DNA purification.

Primer pairs specific to the genes studied were designed in the TSS proximal-regions of interest, within 300–500 bp from reported transcription factor binding sites and histone post-translation modifications. Primer sequences are reported in Table 1. Real-time PCR assays were then performed with the Fast Start Sybr Green Master Mix (Roche, Mannheim, Germany) in a Corbett Rotor-Gene 6000™ (Qiagen, Hilden, Germany). Cycling conditions were as follows: one step of 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 59 °C, 30 s at 72 °C, and a melt from 70 to 95 °C, rising at 0.5 °C/s. The experiments were first validated by comparing the enrichment levels of a housekeeping gene, represented by vinculin (Vcl), and of the constitutively silent gene cartilage-link protein 1 (Crtl1) (data not shown). Raw data (threshold cycle, Ct) of promoter-specific amplifications from immunoprecipitated chromatin (IP sample) were expressed as % of chromatin input controls, calculated as $2^{-(Ct_{IPsample} - Ct_{input})}$.

2.3. RT-PCR

Total RNA from Mφs was extracted, reverse-transcribed and analyzed in Real-Time PCR as described in ref. [23]. Primer oligonucleotides were purchased from Sigma-Aldrich and sequences are: IL-23p19 Fwd 5′-CGT CTC CTT CTC CGC TTC A-3′, IL-23p19 Rev 5′-GTG CCT GGG GTG GTA GAT TT-3′ (annealing at 65 °C); IL-8 Fwd 5′-ATG ACT TCC AAG CTG GCC GT-3′, IL-8 Rev 5′-CAG CCC TCT TCA AAA ACT TCT CC-3′ (annealing at 61 °C); MKP-1 Fwd 5′-AGC AGA GGC GAA GCA TCA TC-3′, MKP-1 Rev 5′-CCC AGC CTC TGC CGA AC-3′ (annealing at 61 °C); and β2m Fwd 5′-GGC ATT CCT GAA GCT GAC-3′, β2m Rev 5′-ATC TTT GGA GTA CGC TGG ATA-3′ (annealing at 61 °C). Thermal cycling was performed as follows: 10 min at 95 °C; 45 cycles of denaturation at 95 °C for 15 s, annealing for 30 s, and extension at 72 °C for 30 s. At the end

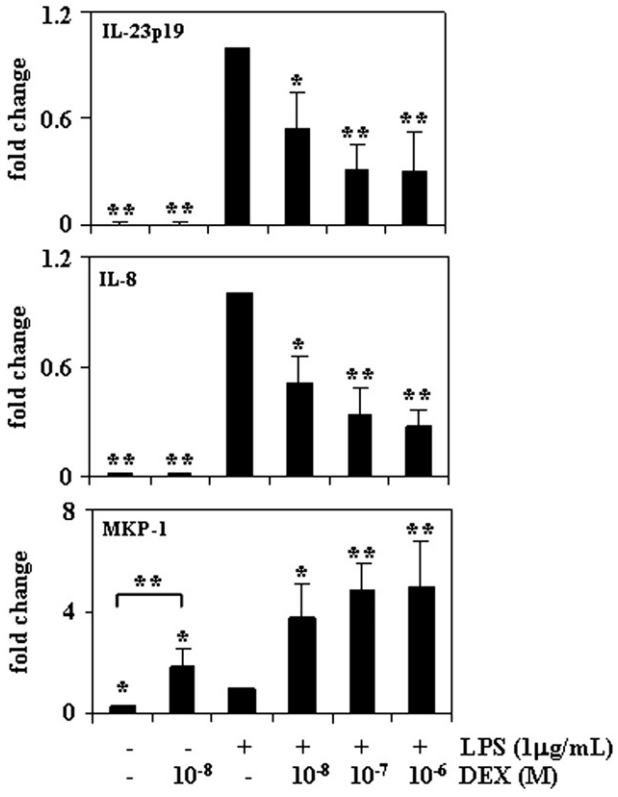

Fig. 1. Interleukin expression following LPS and DEX treatments. H3 acetylation levels of IL-23p19 (upper bar graph), IL-8 (middle bar graph) and MKP-1 (lower bar graph), in basal Mφs and Mφs stimulated with 1 μg/mL LPS for 8 h, treated or not treated with the indicated concentrations of DEX. β2microglobulin was used as internal reference gene. Relative quantification was performed by Real Time RT-PCR according to the ΔΔCt comparative method and data are expressed as mean fold changes compared to the calibrator sample (+LPS) ± s.d. from three independent experiments. *$p < 0.05$, **$p < 0.01$.

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