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Glucocorticoid receptor mediated suppression of natural killer cell activity: Identification of associated deacetylase and corepressor molecules

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Abstract

Physical and psychological stressors reduce natural killer cell function. This reduction in cellular function results from stress-induced release of glucocorticoids. Glucocorticoids act upon natural killer cells to deacetylate and transrepress immune response genes through epigenetic processes. However, other than the glucocorticoid receptor, the proteins that participate in this process are not well described in natural killer cells. The purpose of this study was to identify the proteins associated with the glucocorticoid receptor that are likely epigenetic participants in this process. Treatment of natural killer cells with the synthetic glucocorticoid, dexamethasone, produced a significant time dependent reduction in natural killer cell activity as early as 8 hours post treatment. This reduction in natural killer cell activity was preceded by nuclear localization of the glucocorticoid receptor with histone deacetylase 1 and the corepressor, SMRT. Other class I histone deacetylases were not associated with the glucocorticoid receptor nor was the corepressor NCoR. These results demonstrate histone deacetylase 1 and SMRT to associate with the ligand activated 'glucocorticoid receptor within the nuclei of natural killer cells and to be the likely participants in the histone deacetylation and transrepression that accompanies glucocorticoid mediated reductions in natural killer cell function.

Keywords

Natural killer cell; Glucocorticoid receptor; Epigenetic; Histone deacetylase; Corepressor

1. Introduction

Stress negatively impacts immune function. For example, stress reduces natural killer (NK) cell activity [1-4] through the activation of the hypothalamic-pituitary-adrenocortical axis and the production of increased levels of circulating glucocorticoid [5]. Glucocorticoids

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(GCs) are known to reduce histone acetylation and to transrepress immune response genes, including those genes that mediate NK cell effector function [6-9]. GCs exert their effect by interaction with the glucocorticoid receptor (GR). GR is a ligand activated transcription factor, which is a member of the nuclear receptor super family of proteins. It predominantly exists within the cytoplasm, but when ligand activated, GR translocates to the nucleus [10]. GR's subcellular location is determined by the accessibility of GR's nuclear localization signals and nuclear retention signal [11]. GC:GR interactions in the nucleus affect histone acetylation status by both inhibiting the activity of histone acetyltransferases (HATs) and by recruiting histone deacetylases (HDACs) and corepressor complexes [12], resulting in decreased acetylation of histones, chromatin compaction, and reduced gene expression [6,9]. Chromatin compaction reduces the expression of molecules that mediate natural killer cell activity (NKCA), including perforin and granzyme B [13-15, 8, 9].

HDACs are enzymes that remove acetyl groups from the epsilon amino lysines of the N-terminal tails of histone proteins. In general, increased levels of histone acetylation are associated with increased transcription, while decreased acetylation is associated with transcriptional repression. HDACs are associated with large multiprotein complexes such as; mSin3A, nucleosome remodeling and histone deacetylation (NURD), corepressor for RE1 silencing transcription factor (CoREST), silencing mediator of retinoic acid and thyroid hormone (SMRT), and nuclear receptor corepressor (NCoR). HDACs are divided into four major classes, Class I – Class IV, based on their homology with yeast orthologs. Class I HDACs including; HDAC 1, 2, 3, and 8 associate with the four distinct multiprotein complexes identified above and are ubiquitously expressed with their functional activity optimized when associated with nuclear corepressor complexes. In contrast to HDAC 1, 2, 3, HDAC8's functional activity is limited to smooth muscle [15][16]. Class I HDACs are known to be involved in epigenetic regulation of lymphocyte function, as transcriptional repressors. The capacity of Class II-IV HDACs to repress transcription in lymphocytes is less well characterized and in many cases, the expression of Class II-IV is non-lymphoid. Both NCoR and SMRT are known to repress genes important for NK cell function and are also known to associate with GR [27]. Class I HDACs are known to associate with both SMRT and NCoR [17-21] and both HDAC1 and HDAC2 can be recruited by GR which is not necessarily the case for the other classes of HDACs [22,23].

The immunosuppressive effects of GCs are well documented [5, 24-26] and include the reduced production of immune effector molecules like perforin and granzyme B [9]. Diminished production is due to decreased transcription of the genes subsequent to decreased acetylation of lysine residues on histone tails [9]. Although decreased histone acetylation is observed during GC treatment, the molecular mechanism by which this occurs is unknown. It is known that histone deacetylase (HDAC) inhibitors restore histone acetylation status, transcript and protein levels of both effector molecules and cytokines, in GC treated NK cells [9]. Thus it is probable that GR recruits HDAC(s) that mediate histone deacetylation and associated with these HDAC(s) are corepressor(s) that mediate transrepression. Thus the aim of this study was to determine whether Class I HDAC(s) and/or corepressor complex(es) were associated with GR in the nuclei of NK cells.

2. Materials and methods

2.1 Cell culture

The human erythroleukemic-like cell line, K562, was obtained from the American Type Culture Collection. K562 cells were maintained in suspension culture in Corning 75 cm² tissue culture flasks (Corning Glass Works, Corning, NY) in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) low LPS (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin, 100 g/ml streptomycin

(Whittaker M.A. Bioproducts, Walkersville, MD), 0.1mM non-essential amino acids, 0.1mM 2-mercaptoethanol, and 2mM L-glutamine (Gibco Laboratories, Grand Island, NY).

The natural killer-like YT-Indy cell line (established from a child with acute lymphoma and thymoma [28]) was obtained from Christopher J. Froelich, M.D., Northshore University, Evanston, IL. YT-Indy cells were cultured in media containing RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 12% fetal bovine serum (FBS) low LPS (Gibco Laboratories, Grand Island, NY), 100 units/ml penicillin (Invitrogen, Carlsbad, CA), 100 g/ml streptomycin (Whittaker M.A. Bioproducts, Walkersville, MD), 0.1mM non-essential amino acids, 0.1mM 2-mercaptoethanol, and 2mM L-glutamine (Gibco Laboratories, Grand Island, NY).

2.2 Cellular treatment

YT-Indy cells, cultured at 2.5×10^5 cells/ml, were treated in 75 cm² tissue culture flasks with 100 nM (10^{-7} M) dexamethasone (Dex) (Sigma-Aldrich, St. Louis, MO) for 2, 4, 8, 12, and 24 hours. This concentration of Dex did not decrease cell viability and is a concentration demonstrated previously to differentially regulate Dex responsive genes [29]. This concentration of Dex approximates physiological concentrations [30, 31]. After treatment with Dex, YT-Indy cells were washed and resuspended to 5×10^6 cells/ml with media lacking all supplements. Cells treated with Dex for 2, 4, and 8 hours were used for subcellular localization assays. Cells treated with Dex for 4, 8, 12, and 24 hours were used for NKCA. Cells treated with Dex for 4 hours were used for co-immunoprecipitation analysis. Cells treated with Dex for 4, 8, and 24 hours were used for histone analysis. Cells treated with Dex for 4 hours were used for co-immunoprecipitation analysis. Cells were treated with RU-486 (Sigma-Aldrich, St. Louis, MO) for 24 hours. Cell number and viability were determined by vital dye exclusion using 0.1% Trypan Blue.

2.3 Natural Killer Cell Activity (NKCA)

YT-Indy cell lytic activity (NKCA) against tumor targets was assessed using a standard chromium release assay, as previously described [32]. K562 tumor target cells were radioactively labeled with 100 Ci of [⁵¹Cr] (New England Nuclear, Boston, MA). Radiolabeled K562 cells were incubated for 3 h with YT-Indy cells. Following incubation, the supernatants were removed using a Skatron harvesting press and the associated radioactivity was determined. Effector to target ratios for NKCA were 30, 20, and 10:1.

Results are expressed as percent cytotoxicity, calculated as:

$$\% \text{Cytotoxicity} = \frac{[(\text{experimental DPM}^*) - (\text{minimum DPM})]}{[(\text{maximum DPM}) - (\text{minimum DPM})]} \times 100.$$

All experimental means were calculated from triplicate values. Lytic units (LU) were calculated using a program written by David Coggins, FCRC, Frederick, MD. LU represents the number of cells per 10^7 effectors required to achieve 20% lysis of the target cells. *DPM = disintegrations per minute.

2.4 Immunofluorescent Flow Cytometric Analysis of Intracellular Perforin

After treatment as described above, YT-Indy cells (1×10^5 /assessment) were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen, San Jose, CA) for 20 min at 4°C. The cells were then washed twice with Perm/Wash Buffer (BD Biosciences, San Jose, CA) and then probed with antibodies specific for perforin (BD Biosciences, San Jose, CA) for 1 hr at 4°C. Following antibody staining the cells were washed twice with Perm/

Wash Buffer (BD Biosciences, San Jose, CA). After staining samples were analyzed by flow cytometry with a FACSCanto equipped with a 15mW argon-ion laser and a red diode laser using FACSDiva software for data acquisition [33-35]. 10,000-30,000 events were recorded and analyzed with FlowJo v8.4.1. Flow cytometric analysis was confirmed by fluorescence microscopy.

2.5 Subcellular localization

Nuclear and cytoplasmic fractions of YT-Indy cells were separated from 5×10^6 cells via the Fermentas ProteoJET Cytoplasmic and Nuclear Protein protocol (Fermentas, Burlington, ON). Nuclei were lysed using non-denaturing lysis buffer (20mM Tris HCl pH=8, 137 mM NaCl, 10% glycerol, 1 % Nonidet P-40, 2mM EDTA). Both lysed nuclear and cytoplasmic fractions were resuspended in Laemmli SDS-sample buffer (4x) (Boston Bioproducts, Boston, MA). Samples were boiled for 5 min and proteins were separated by electrophoresis with a 10% polyacrylamide gel and transferred to nitrocellulose membrane for immunoblotting. Proteins were visualized with anti-GR alpha (AbCam, Cambridge, MA), anti-HDAC1 (Millipore, Temecula, CA), anti-HDAC2, anti-HDAC3 or anti-acetylated histone 4 antibodies (AbCam, Cambridge, MA), horseradish peroxidase (HRP) conjugated anti-IgG secondary antibody (Millipore, Temecula, CA), and chemiluminescence reagent (ThermoScientific, Rockford, IL). Nuclear and cytoplasmic separation efficiency was determined with anti-LaminB1 (AbCam, Cambridge, MA) anti-GAPDH (Cell Signaling, Danvers, MA) antibodies, respectively. LaminB1 served as the control for nuclear contents, as it is localized to the nucleus only; GAPDH is only found within the cytoplasm, and was used as a control for cytoplasmic contents (See Supplemental Figure 1 for an example of the efficiency of separation of nuclear and cytoplasmic constituents.) Blot density was quantified using ImageJ software.

2.6 Co-immunoprecipitation

Nuclear and whole cell lysates were pre-cleared using Protein G magnetic beads (New England BioLabs, City, ST) and added to tubes containing antibody cross-linked to magnetic beads; anti-GR, anti-HDAC2, anti-HDAC3, anti-NCoR, anti-SMRT (AbCam, Cambridge, MA), and anti-HDAC1 (Millipore, Temecula, CA) supernatants were immunoprecipitated overnight at 4°. Immune complexes were collected by magnetic separation, washed and eluted. Immune complexes were resuspended in Laemmli SDS-sample buffer (4x) (Boston Bioproducts, Boston, MA). Samples were boiled for 5 min and proteins were separated by electrophoresis with a 10% polyacrylamide gel and transferred to nitrocellulose membrane for immunoblotting. Proteins were visualized with anti-GR, anti-HDAC2, anti-HDAC3, anti-NCoR, anti-SMRT (AbCam, Cambridge, MA), anti-HDAC1, and normal mouse IgG (Millipore, Temecula, CA) antibodies, horseradish peroxidase (HRP) conjugated anti-IgG secondary antibody (Millipore, Temecula, CA), and chemiluminescence reagent (ThermoScientific, Rockford, IL).

2.7 Statistical Analysis

Data are presented as mean with standard error of the mean (SEM). Quantitative data were analyzed by two-tailed, two sample, homoscedastic Student's T-test. Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of dexamethasone on natural killer cell activity

The effect of dexamethasone (Dex) on YT-Indy cell lysis of tumor cell targets was evaluated. Treatment of YT-Indy cells with varying amounts of Dex for 24 hr produced a

dose-dependent reduction in lytic activity, Figure 1 A. Treatment of YT-Indy cells for 24 hours with (10^{-7} M) Dex produced a greater than 50% reduction in NKCA and had no effect on the viability of the natural killer cells. Viability data are not shown. Concomitant treatment of the YT-Indy cells with the glucocorticoid receptor (GR) antagonist RU-486, ablated the effect of Dex in a dose dependent manner. See Figure 1 B. Human NKCA is primarily mediated by perforin dependent lysis of tumor cell targets and as demonstrated in Figure 2, Dex at a concentration of (10^{-7} M) reduced YT-Indy cytoplasmic perforin levels, which were restored with RU-486. The effect Dex was dose dependent in that Dex at (10^{-9} M) was indistinguishable from No Treatment. Data are not shown. In Figure 3 a time course for the effect of Dex on NKCA is presented. YT-Indy cells were treated with Dex (10^{-7} M) for 4, 8, 12, or 24 hours and then assessed for NKCA. At the 4 hour time point, lytic activity of YT-Indy cells was numerically reduced but significant reductions in activity were at 8, 12 and 24 hours. These data demonstrate the effect of Dex on YT-Indy cells to be both time and GR dependent.

3.2 Comparative analysis of the subcellular localization of the glucocorticoid receptor during dexamethasone treatment

Subcellular localization of GR was assessed in nuclear and cytoplasmic fractions extracted from Dex-treated and non-treated YT-Indy cells over an 8 hour period. The presence of GR within nuclear and cytoplasmic fractions was determined by Western blot analysis, with an antibody specific for GR alpha. An example of a western blot from an SDS-PAGE gel is shown in Figure 4 A. The density of the bands was quantified using ImageJ software. Figure 4 B shows the percent of total cellular GR present in either fraction throughout the time course averaged from multiple independent experiments.

In non-treated YT-Indy cells, GR is found in both the cytoplasm (Figure 4 A, lane 2) and the nucleus (lane 1), with the majority present in the cytoplasm (74%), indicated in Figure 4 B. Dex (10^{-7} M) for 2 hours induced a small increase in the percentage of GR within the nucleus (26% in non-treated cells to 34% in treated cells). Four hour Dex (10^{-7} M) treatment resulted in a significant increase in the percentage of GR in the nucleus (69%, $p < 0.05$), when compared with GR in the nucleus of non-treated cells. After 8 hours GR localization returned to levels similar to that of untreated cells, with only 30% of total GR present in the nucleus. GR was found in the cytoplasm and nucleus in approximately equal levels following a 24 hour dex (10^{-7} M) treatment (data are not shown).

3.3 Comparative analysis of the subcellular localization of Histone Deacetylases (HDACs) 1, 2, and 3 following dexamethasone treatment

Dex treatment has been shown to alter both the global and promoter specific epigenetic patterns of Histone (H) 4 acetylation in the IL-2 dependent NK cells line, NK92 [9]. To determine whether Dex (10^{-7} M) had a similar effect on the IL-2 independent YT-Indy cell line, a time course analysis of total H4 acetylation was performed. Total H4 acetylation was reduced at 8 hours by Dex treatment to 70.9%, at 12 hours to 64.1% and at 24 hours to 21.6%, when compared to untreated YT-Indy cells (data are not shown). No change in H4 acetylation was observed prior to 4 hours of Dex treatment. Deacetylation of H4 is achieved by HDACs; therefore, the subcellular localization of HDACs 1, 2, and 3 was assessed using nuclear and cytoplasmic extracts from YT-Indy cells treated for 0, 2, 4, and 8 hours with Dex. The location of HDACs in nuclear or cytoplasmic fractions was determined by western blot with antibodies specific for HDAC1, HDAC2, and HDAC3. The density of protein bands was quantified using ImageJ software. The total cellular level of each HDAC was calculated as the sum of the nuclear and cytoplasmic levels.

HDAC1—Figure 5 A is an example of a western blot for HDAC1 quantification. Lanes 1 and 2 demonstrate HDAC1 to be a 65 kDa protein found in both the nucleus and cytoplasm, respectively, of untreated cells. Lanes 3 – 8 show the effect of Dex treatment on HDAC-1 in these cells. Figure 5 B shows the percent of total cellular HDAC1 present in either fraction throughout the time course averaged from multiple independent experiments. In untreated cells, 56% of total cellular HDAC1 is nuclear. At 2 hours a significant ($p < 0.01$) increase in the nuclear localization of HDAC1 was observed, with 76% of HDAC1 in the nuclear compartment, at 4 hours 70% of HDAC1 is in the nucleus. By 8 hours of treatment, HDAC1 was found in equal proportions in either compartment, a distribution similar to untreated cells.

HDAC2—An example of HDAC2 localization is shown in Figure 6 A. HDAC2 is a 55 kDa protein that is present in both nuclear (lane 1) and cytoplasmic (lane 2) fractions of untreated YT-Indy cells, as well as the nucleus (lanes 3, 5, and 7) and cytoplasm (lanes 4, 6, and 8) of Dex-treated cells. Figure 6 B shows the percent of total cellular HDAC2 present in either fraction throughout the time course averaged from multiple independent experiments. On average, HDAC2 is divided almost equally between the nucleus and cytoplasm in YT-Indy cells; 52% of all HDAC2 in the cell is nuclear. Dex initially induces a statistically significant ($p < 0.05$) increase in the proportion of HDAC2 in the nucleus at 2 hours, with 71% of HDAC2 found in the nucleus. However, continued Dex treatment results in HDAC2 almost equally distributed between the nucleus and cytoplasm at 4 and 8 hours.

HDAC3—Figure 7 A is an example of a western blot for HDAC3 localization. HDAC3 is a 49 kDa protein and like HDACs 1 and 2 is present in both the nucleus and cytoplasm compartments. Lanes 1 and 2 display HDAC3 in both the nuclear and cytoplasmic fractions, respectively, of untreated cells, while lanes 3 – 8 are from Dex-treated cells. Figure 7 B shows the percent of total cellular HDAC3 present in either fraction throughout the time course averaged from multiple independent experiments. In general, Dex treatment had little effect on subcellular localization of HDAC3. In untreated cells, 64% of total cellular HDAC3 is in the nucleus. Similarly, 57% to 66% of total HDAC3 is within the nucleus of cells treated with Dex. This trend is displayed in the western blot (Figure 7 A) as well. Therefore, Dex treatment does not impact the subcellular localization of HDAC3 under these treatment conditions.

3.4 Interactions among GR, HDACs, and corepressor complexes

Class I HDACs associate with nuclear corepressor complexes (e.g NCoR and SMRT); furthermore, GR is known to interact with these corepressors. In order to investigate interactions among these proteins, co-immunoprecipitation experiments were performed following 4 hours of treatment with Dex. At this time period GR was maximally located within the nucleus and HDACs 1, 2, and 3 were present in the nuclear compartment. Additionally, a significant reduction in NKCA was observed within 8 hours of treatment; thus, the time period prior to the 8 hour point was of particular interest. Whole cells were lysed using non-denaturing lysis buffer to maintain protein-protein interactions. Antibodies specific for GR, HDACs 1, 2, and 3, as well as the corepressors NCoR and SMRT were used for co-immunoprecipitation from Dex treated and untreated whole cell lysates. Figure 8 A demonstrates GR to be immunoprecipitated with SMRT and HDAC1, but not NCoR, HDAC2, or HDAC3. Immunoprecipitation with antibody specific for SMRT also precipitated GR and HDAC1 (lane 3) while immunoprecipitation with antibodies specific for HDAC1 only precipitated GR (lane 5). NCoR, HDAC2, and HDAC3 were not found to be associated with any of the proteins/complexes investigated (lanes 4, 6, and 7). Immunoprecipitation with IgG antibody alone (lane 1) did not immunoprecipitate any of the protein complexes.

GR and HDAC1 are present in both the nucleus and cytoplasm of YT-Indy cells. To determine whether such interactions occur within nuclei, nuclear fractions were isolated and immunoprecipitated using antibodies specific for GR, SMRT, and HDAC1, as those proteins were shown to interact in whole cell lysates. Figure 8 B demonstrates that similar to whole cell lysates, the GR-specific antibody precipitated GR, SMRT, and HDAC1 (lane 2). Both GR and HDAC1 co-immunoprecipitated with antibody specific for SMRT (lane 3). Antibody specific for HDAC1 immunoprecipitated both GR and SMRT (lane 4). Co-immunoprecipitates of proteins found in nuclear extracts confirmed that GR interacts with SMRT and HDAC1.

4. Discussion

The results presented demonstrate GR to interact with HDAC1 and the corepressor SMRT in the nucleus of YT-Indy cells during dexamethasone (Dex) treatment. Prior studies have demonstrated that GR and HDAC1 co-immunoprecipitated in Dex-treated lysates, that GR recruits SMRT to glucocorticoid response elements during Dex-treatment, and that SMRT binds HDAC1 [17-21] [29]. While such associations have been documented, we believe this work to be the first to demonstrate interactions among all three proteins, GR, SMRT, and HDAC1, and also the first to demonstrate such in natural killer cells. Others have shown that GR interacts with HDAC2 [36] and HDAC3 [37], as well as NCoR [37]. Contrary to those studies, these data demonstrate that GR does not appear to interact with NCoR, HDACs 2 or 3 in YT-Indy cells during Dex treatment.

These interactions among GR, corepressors, and HDACs have been found in a variety of cell types, suggesting that recruitment of specific complexes and HDACs by GR may be context dependent. As GR has been shown to interact with both NCoR and SMRT [37], the preference for one corepressor over the other may be due to differences in the subcellular environment among different cell types. Interaction between hormone nuclear receptors like GR and corepressors depend on a number of factors, including the direct binding between GR and the corepressor's interacting domains, as well as the composition of the corepressor complex. It is feasible that different proteins are part of the SMRT or NCoR complexes in different cell types; perhaps in NK cells, another protein blocks the sites where GR is capable of binding NCoR, but those sites are available on SMRT. This may also explain SMRT's recruitment of HDAC1 instead of HDACs 2 or 3. The site at which HDAC1 binds in the SMRT complex may be exposed, while the sites for HDACs 2 or 3 binding may not.

In the nuclei of Dex treated YT-Indy cells, GR co-precipitates with SMRT and HDAC1 and each reciprocal immunoprecipitation confirms the association. These results demonstrate GR to bind both HDAC1 and SMRT, but the order of this interaction is unknown. In the whole cell lysates of Dex treated cells, GR only co-precipitated with HDAC1 or GR only coprecipitated with SMRT with no apparent interaction of all three. Interaction between GR and HDAC1 has been reported previously [29] and it may be that the absence of SMRT interaction with the two proteins in whole cell lysates results from a preponderance of GR:HDAC1 or GR:SMRT interactions within the cytoplasm. Interaction of GR:HDAC1:SMRT was only observed within the nucleus. It could be that the environment of the nucleus may be such that the stoichiometry of SMRT interaction with GR and HDAC1 is optimal and such an optimal situation is only revealed within the nucleus. Alternatively, the stability of the interaction may be greater due to the presence of other cofactors within the nucleus. Further, it could be that the preponderance of GR:HDAC1 and GR:SMRT interactions within the cytoplasm may compete out the interaction of all three when whole cells are lysed and analyzed. The reason for this distinction between whole cell lysates and the nucleus is unknown but is under investigation.

Of particular note are the kinetics of GR subcellular localization during the first 8 hours of Dex treatment. As anticipated, the majority of GR was within the cytoplasm in non-Dex treated cells. However, GR is known to shuttle between the cytoplasm and the nucleus, depending on the accessibility of its nuclear localization sequence and nuclear retention signals [38-40] and the YT-Indy cell line was cultured in fetal bovine serum supplemented media, which may contain factors that reveal GR's nuclear localization signal, allowing for translocation into the nucleus. Exogenous Dex treatment induced a shift of GR into the nucleus, as early as 2 hours with maximal effect 4 hours post treatment. By 8 hours, the majority of GR returned to the cytoplasm and by 24 hours of treatment, GR was distributed equally between the nucleus and cytoplasm, indicating that GR continues to shuttle between compartments throughout treatment. GR's optimal presence within the nucleus at 4 hours has been demonstrated previously [41, 42], as has GR's localization in both the nucleus and the cytoplasm during Dex treatment [36]. It is worth noting that for the western blot analysis, several molecular sizes for GR were detected, ranging from approximately 95 kDa to 100 kDa. GR is reported to be a 97 kDa protein that undergoes various post-transcriptional modifications, including phosphorylation, acetylation, and ubiquitination [43], and is known to have up to eight isoforms of varying size [44]. It is probable that the observed variation in molecular size is a consequence of these various post translational modifications and less likely a consequence of the various isoforms. Further, the apparent visualized quantities of GR at various time periods showed degrees of inconsistency. For example, levels found in the untreated cells appear to be much greater than the levels in Dex-treated cells. Dex is known to decrease the level of cellular GR by both inhibiting transcription of the GR locus [43] as well as by increasing proteosomal degradation of GR [45] and these explanations may contribute to the observed differences.

With regard to western blot analysis of the subcellular location of HDACs, HDAC1 is present in both the nucleus (56%) and cytoplasm (45%) of untreated cells, which is consistent with previously published observations [46]. HDAC1 is typically found within both the nucleus and the cytoplasm and requires a shuttle protein for nuclear export to the cytoplasm [47]. Dex initially increased the proportion of HDAC1 in the nucleus at 2 and 4 hours of treatment. At 8 hours of treatment, HDAC1's subcellular localization returned to the pattern found in untreated cells. HDAC1 is known to interact with many multi-protein complexes including ligand bound GR. It is conceivable that interactions between HDAC1 and GR or other complexes may prevent association with its shuttle protein. As GR dissociates from HDAC1 to exit the nucleus by 8 hours, HDAC1's shuttle protein may bind and escort HDAC1 to the cytoplasm, localizing the protein in a manner similar to untreated cells.

HDAC2 is also found in both the nucleus (52%) and cytoplasm (48%) of untreated YT-Indy cells and this is consistent with published observations [46]. Dex significantly increased the proportion of HDAC2 in the nucleus at 2 hours (71%), followed by a return to a more equal distribution between both compartments at 4 and 8 hours of treatment. Nuclear HDAC2 peaked at 2 hours and subsequently decreased. The initial increase coincided with the increase in HDAC1. HDACs 1 and 2 are known to associate with each other [37] in multiple protein complexes; thus, the initial retention of HDAC1 may also result in the initial increase in HDAC2 in the nucleus. Then, as increasing levels of ligand bound GR enter the nucleus (peaking at 4 hours of treatment), GR recruitment of HDAC1 may cause HDAC1 to dissociate from HDAC2. Upon interacting with GR, HDAC1 is acetylated, which decreases interaction with HDAC2 [15]. As previously demonstrated [46], HDAC3 is found in both the nucleus and cytoplasm. HDAC3 favors nuclear localization (57% - 66%) in both untreated and Dex-treated cells at all time points, indicating that Dex does not impact the subcellular localization of HDAC3, unlike HDACs 1 and 2. HDACs 1, 2, and 3 are all present in the nucleus when GR's nuclear localization peaks at 4 hours. Therefore, all three

HDACs are available to interact with GR during Dex treatment, and the impact of these HDACs on GR cannot be ruled out by changes in localization alone. However, the retention of HDAC1 in the nucleus overlaps the time period for peak levels of nuclear GR, perhaps creating optimal conditions for interaction between these two proteins. These results do identify an HDAC and corepressor that associate with GR in the nucleus after Dex treatment. These results do not preclude the possibility that other proteins are involved in the process. In that sense, this study is limited by the number of molecules evaluated and also by analysis in a single cell line. However, the purpose of this study was to determine whether such associations did occur and to identify participants that may contribute to histone deacetylation and gene corepression.

We have previously demonstrated the epigenetic effect of GC on the NK cell line, NK92 [9]. For continuous culture, NK92 requires the exogenous addition of recombinant IL-2. In order to circumvent this potential confound, we have evaluated the distinct cell line, YT-Indy, which is a continuous cell line requiring no such exogenous growth factor. The findings demonstrate that Dex inhibits the NKCA of the YT-Indy cell line as has been reported previously for many cell populations [5,9,24,48,49]. Further, this effect is ablated by RU-486. In our previous study Dex not only reduced NKCA but also reduced the constitutive and the stimulated production of cytokines and perforin. Further, Dex treatment also reduced global histone acetylation, the acetylation of histone 4 lysine position 8, and the accessibility of the proximal promoters of perforin, interferon gamma and granzyme B. Histone acetylation was recovered by treatment of the NK cells with a histone deacetylase inhibitor, which also restored NKCA and IFN gamma production. Those results demonstrated Dex to dysregulate NK cell function at least in part through an epigenetic mechanism, which reduces promoter accessibility through modification of histone acetylation status and decreased the expression of effector proteins necessary to the full functional activity of NK cells [9]. In this study, we have extended those observations to include an analysis of the likely mediators of the GR mediated reduction in NKCA. Taken together, these data suggest that glucocorticoids, like Dex, enter the cell and induce translocation of the GR into the nucleus. Within the nucleus, HDAC1 recruited by GR, deacetylates H4 residues and with SMRT reduces access for the promoter regions of immune effector genes like perforin. The decreased acetylation and the recruitment of SMRT result in the observed reduction in NKCA. Therefore it appears that the glucocorticoid receptor mediated suppression of natural killer cell activity in this cell line is a result of the recruitment of this histone deacetylase and this corepressor, which decrease histone acetylation, increase chromatin compaction, reduce gene expression and immune effector molecule production. These findings are the next important step in understanding the molecular mechanism(s) by which epigenetic processes contribute to the immune dysregulation that accompanies psychological and physical stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Glucocorticoids inhibit NK cell function through an epigenetic mechanism.
- This epigenetic mechanism is mediated by histone deacetylation.
- The deacetylation is mediated by histone deacetylase 1 and the corepressor SMRT.

Figure 1.

Effect of dexamethasone on Natural Killer Cell Activity (NKCA). (A) YT-Indy cells were treated with varying amounts of dexamethasone for 24 hrs. Percent (%) inhibition was calculated by: $[(\text{Lytic Units No Treatment}) - (\text{Lytic Units Dexamethasone Treatment})] / (\text{Lytic Units No Treatment}) \times 100$. Values are presented as the mean and standard error of the mean (SEM). $N =$ at least three independent experiments. (B) YT-Indy cells were treated with 10^{-7}M dexamethasone (Dex) for 24 hours with and without varying concentrations of RU-486 and assessed for NKCA. Values are presented as mean Lytic Units (LU) and standard error of the mean (SEM). ($n =$ at least three independent experiments). Comparisons are among treated (Dex 10^{-7}M or Dex 10^{-7}M plus RU-486 -log, M) and non-treated cells (no treatment with Dex and no treatment with RU-486) *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

Figure 2.

Effect of dexamethasone on constitutive production of perforin. YT-Indy cells were treated with 10^{-7} M Dex for 24 hours with and without 10^{-7} M RU-486. Intracellular perforin staining was performed using anti-human perforin antibodies and assessed by flow cytometry. Data are expressed as mean fluorescent intensity and are expressed as mean $\pm$ SEM (n = at least three independent experiments). Comparisons are among Dex treated and non-treated cells (No treatment) *, $P < 0.05$

Figure 3.

Time course for the effect of dexamethasone on Natural Killer Cell Activity. YT-Indy cells were treated with 10^{-7} M Dex for varying periods of time and assessed for NKCA. Values are presented as mean Lytic Units (LU) $\pm$ SEM. (n = at least three independent experiments). Comparisons are between treated (open bars) and non-treated cells (closed bars) at the individual time periods, ***, $P < 0.001$.

Figure 4.

Subcellular localization of the glucocorticoid receptor following dexamethasone treatment. (A) An example of a western blot with non-treated nuclear fraction (lane 1), non-treated cytoplasmic fraction (lane 2), nuclear fraction from 2-hour 10^{-7} M Dex treatment (lane 3), cytoplasmic fraction from 2-hour Dex treatment (lane 4), nuclear fraction from 4-hour Dex treatment (lane 5), cytoplasmic fraction from 4-hour Dex treatment (lane 6), nuclear fraction from 8-hour Dex treatment (lane 7), and cytoplasmic fraction from 8-hour Dex treatment (lane 8) probed for the glucocorticoid receptor (GR). GR is 97kDa; and blot is marked at 95kDa. (B) Blot density quantified using ImageJ software. Each bar represents the percent of total GR within the cell that is present in either the nucleus or cytoplasm. Nuclear fractions are represented by closed bars; cytoplasmic fractions are represented by open bars. Values are presented as the mean percentage of total cellular GR $\pm$ SEM. (n = three independent experiments). Comparisons are between nuclear fractions of non-treated cells and nuclear fractions of Dex treated cells, *, $P < 0.05$.

Figure 5.

Subcellular localization of HDAC1 following dexamethasone treatment. (A) An example of a western blot with non-treated nuclear fraction (lane 1), non-treated cytoplasmic fraction (lane 2), nuclear fraction from 2-hour 10^{-7} M Dex treatment (lane 3), cytoplasmic fraction from 2-hour Dex treatment (lane 4), nuclear fraction from 4-hour Dex treatment (lane 5), cytoplasmic fraction from 4-hour Dex treatment (lane 6), nuclear fraction from 8-hour Dex treatment (lane 7), and cytoplasmic fraction from 8-hour Dex treatment probed for HDAC1. HDAC1 is 65kDa; blot is marked at 72kDa. (B) Blot density quantified using ImageJ software. Each bar represents the percent of total HDAC1 within the cell that is present in either the nucleus or cytoplasm. Nuclear fractions are represented by closed bars; cytoplasmic fractions are represented by open bars. Values are presented as the mean percentage of total cellular HDAC1 $\pm$ SEM. ($n =$ three independent experiments). Comparisons are between nuclear fractions of non-treated cells and nuclear fractions of Dex treated cells, *, $P < 0.05$.

Figure 6.

Subcellular localization of HDAC2 following dexamethasone treatment. (A) An example of a western blot with non-treated nuclear fraction (lane 1), non-treated cytoplasmic fraction (lane 2), nuclear fraction from 2-hour Dex treatment (lane 3), cytoplasmic fraction from 2-hour 10^{-7} M Dex treatment (lane 4), nuclear fraction from 4-hour Dex treatment (lane 5), cytoplasmic fraction from 4-hour Dex treatment (lane 6), nuclear fraction from 8-hour Dex treatment (lane 7), and cytoplasmic fraction from 8-hour Dex treatment probed for HDAC2. HDAC2 is 55kDa; blot is marked at 52kDa. (B) Blot density quantified using ImageJ software. Each bar represents the percent of total HDAC2 within the cell that is present in either the nucleus or cytoplasm. Nuclear fractions are represented by closed bars; cytoplasmic fractions are represented by open bars. Values are presented as the mean percentage of total cellular HDAC2 $\pm$ SEM. ($n =$ three independent experiments). Comparisons are between nuclear fractions of non-treated cells and nuclear fractions of Dex treated cells, *, $P < 0.05$.

Figure 7.

Subcellular localization of HDAC3 following dexamethasone treatment. (A) An example of a western blot with non-treated nuclear fraction (lane 1), non-treated cytoplasmic fraction (lane 2), nuclear fraction from 2-hour Dex treatment (lane 3), cytoplasmic fraction from 2-hour 10^{-7} M Dex treatment (lane 4), nuclear fraction from 4-hour Dex treatment (lane 5), cytoplasmic fraction from 4-hour Dex treatment (lane 6), nuclear fraction from 8-hour Dex treatment (lane 7), and cytoplasmic fraction from 8-hour Dex treatment probed for HDAC3. HDAC3 is 49 kDa; blot is marked at 50 kDa. (B) Blot density quantified using ImageJ software. Each bar represents the percent of total HDAC3 within the cell that is present in either the nucleus or cytoplasm. Nuclear fractions are represented by closed bars; cytoplasmic fractions are represented by open bars. Values are presented as the mean percentage of total cellular HDAC3 $\pm$ SEM. (n = three independent experiments).

Figure 8.

Interaction between GR, corepressors and HDACs. (A) YT-Indy cells treated with 10^{-7} M Dex for 4 hours. Cell lysates were immunoprecipitated with antibodies specific for GR (lane 2), SMRT (lane 3), NCoR (lane 4), HDAC1 (lane 5), HDAC2 (lane 6), and HDAC3 (lane 7); as a non specific control, lysates were immunoprecipitated with IgG antibody (lane 1). This is an example of western blot results ($n = 3$). No precipitate appears in lysates immunoprecipitated with IgG (lane 1). Antibodies specific for GR immunoprecipitated SMRT and HDAC1. Antibodies specific for SMRT immunoprecipitated GR and HDAC1 (lane 3). Antibodies specific for HDAC1 immunoprecipitated GR (lane 5). NCoR only immunoprecipitated NCoR (lane 4), antibodies specific for HDAC2 immunoprecipitated only HDAC2 (lane 6), and antibodies specific for HDAC3 only immunoprecipitated HDAC3 (lane 7). (B) Interaction among GR, SMRT, and HDAC1. YT-Indy cells were treated with 10^{-7} M Dex for 4 hours. Nuclear and cytoplasmic fractions were separated, and nuclear fractions immunoprecipitated with antibodies specific for GR, SMRT, and HDAC1. This is an example of western blot results ($n = 2$). As a nonspecific control, nuclear lysates were immunoprecipitated with IgG (lane 1). Antibodies specific for GR immunoprecipitated both SMRT and HDAC1 (lane 2). Antibodies specific for SMRT immunoprecipitated GR, as well as HDAC1 (lane 3). Antibodies specific for HDAC1 immunoprecipitated GR and SMRT (lane 4).