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Rapid immunosuppressive effects of glucocorticoids mediated through Lck and Fyn

Mark Löwenberg, Jurriaan Tuynman, Joyce Bilderbeek, Timo Gaber, Frank Buttgerit, Sander van Deventer, Maikel Peppelenbosch, and Daniel Hommes

Glucocorticoids (GCs) are effective immunosuppressive agents and mediate well-defined transcriptional effects via GC receptors. There is increasing evidence that GCs also initiate rapid nongenomic signaling events. Using activated human CD4⁺ lymphocytes and a peptide array containing 1176 different kinase consensus substrates, we generated a comprehensive profile of GC-induced rapid effects on signal transduction. The results show marked early differences in phosphorylation between GC-pretreated cells and control cells, including impaired phosphorylation of p56lck/p59fyn (Lck/Fyn) consensus substrates. Immunoprecipita-

tion and in vitro kinase assays reveal rapid GC-induced down-modulation of Lck and Fyn kinases using SAM68 (Src [pp60c-src]–associated in mitosis 68 kDa) as a substrate. Additionally, immunoprecipitation experiments revealed reduced Lck-CD4 and Fyn-CD3 associations, suggesting GC inhibited recruitment of these kinases to the T-cell receptor complex. Western blot analysis revealed reduced phosphorylation of a series of downstream signaling intermediates following GC treatment, including protein kinase B (PKB), protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs). Experiments with GC receptor–negative

Jurkat cells and a pharmacologic GC receptor ligand (RU486) indicated that rapid inhibition of Lck and Fyn kinases is GC receptor dependent. Parallel experiments conducted following the application of GCs in healthy individuals confirmed suppression of Lck/Fyn in T cells within 1 hour in vivo. These results identify the inhibition of Lck and Fyn kinases as rapid targets of GCs, mediated via a GC receptor–dependent pathway. (Blood. 2005; 106:1703-1710)

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Introduction

Glucocorticoids (GCs) are widely used therapeutically for immunosuppression. GC action is mediated through the intracellular GC receptor (GR), present in the cytosol of T cells. In the inactive state of the receptor, GRs are associated with heat shock proteins, which act as chaperones. Upon GC binding this complex dissociates, and the activated GR translocates into the nucleus where it binds to specific DNA motifs (glucocorticoid-responsive elements) and to transcription factors such as activator protein 1 (AP1) and nuclear factor κ B (NF κ B), thereby regulating the expression of a number of genes involved in the immunologic process.¹⁻³ Through regulation of gene expression, GCs reduce the production of proinflammatory mediators, including cytokines (interleukin 1 [IL-1], IL-2, tumor necrosis factor- α [TNF- α], interferon γ [IFN- γ], etc), prostaglandins, and nitric oxide. Moreover, GCs inhibit the expression of adhesion molecules and may induce death of T cells.⁴⁻⁶ However, the phenomenon of rapid GC-induced effects in cellular systems occurring within minutes is unlikely explained by the classic GR-mediated mechanism.⁷⁻¹⁸ The underlying mechanisms of rapid nongenomic GC-dependent immunosuppression remain to be elucidated.

Among the earliest recognizable events after T-cell receptor (TCR) stimulation are the activation of p56lck (Lck) and p59fyn (Fyn), resulting in TCR phosphorylation on tyrosine residues within immunoreceptor tyrosine-based activation motifs.¹⁹ Lck and Fyn, members of the Src (pp60c-src) family of tyrosine kinases, are expressed in T cells and are critically involved in TCR-mediated signal transduction.²⁰⁻²³ Lck and Fyn have been shown to associate with the TCR complex, and the cellular organization of these kinases is well coordinated and essential for efficient TCR signaling.²⁴⁻²⁸ Lck binds to the CD4 or CD8 coreceptor, whereas Fyn is known to associate with CD3.²⁹⁻³¹ Alternatively, Lck and Fyn can also physically interact with the CD3 and CD4 coreceptors, respectively, but these interactions are weak. The TCR-CD4 association largely determines the quality of TCR signaling, Lck being the critical mediator.^{32,33} As a subsequent step, ZAP70 (ζ -associated protein 70) tyrosine kinase is recruited to the TCR where it is activated by Lck through tyrosine phosphorylation.³⁴ Once activated, ZAP70 phosphorylates LAT (linker for activation of T cells), an adapter molecule, which ultimately promotes activation of essential downstream signaling pathways, including

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protein kinase C (PKC), protein kinase B (PKB), and the mitogen-activated protein kinases (MAPKs) (ie, p38 MAPK, extracellular signal-regulated kinase [ERK], and c-Jun N-terminal kinase [JNK]), resulting in T-cell activation.³⁵⁻⁴² The rapid effects of GCs on these signaling events remain to be established.

The importance of GCs in clinical immunosuppression combined with the unknown basis of the nongenomic GC-dependent actions prompted us to investigate these rapid effects. Hence, we chose to screen rapid effects of the GC analog dexamethasone (DEX) on the CD4⁺ T-cell population kinome using a peptide array containing 1176 specific kinase pseudo-substrates.⁴³ The results of these experiments show marked differences in phosphorylation patterns between DEX-treated and non-DEX-treated cells, providing proof for rapid DEX-dependent effects on signal transduction. Among the most prominent effects observed upon DEX treatment was reduced phosphorylation of Lck/Fyn substrates. Subsequent *in vitro* kinase assays and Western blot analyses revealed that DEX impairs activation of both kinases as well as recruitment to the TCR complex. Lck/Fyn kinase activities were also found to be reduced *in vivo* at 1 hour following oral administration of prednisolone in human subjects. Experiments using GR-negative Jurkat cells and a pharmacologic GR ligand (RU486) indicate GR dependence of DEX-induced Lck and Fyn inhibition. Altogether, these results provide a molecular framework for understanding rapid GC-mediated immunosuppression in CD4⁺ T cells.

Patients, materials, and methods

Cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of healthy volunteers by Ficoll-Isopaque density gradient centrifugation (Amersham Biosciences, Roosendaal, the Netherlands). Approval was obtained from the Academic Medical Center institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki. The monocytes present in the PBMC pellet were removed by an adherence procedure: cells were plated out in 6-well plates (CellStar, Greiner Bio-One, Longwood, FL) at a final concentration of 5×10^6 cells/well for 1.5 hours at 37°C, and cells were harvested for subsequent magnetic cell sorting. CD4⁺ T cells were cultured in Iscoves modified Dulbecco medium (Gibco, Breda, the Netherlands), supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco), 2 mM L-glutamine (Gibco) and penicillin-streptomycin "complete" in a humidified 5% CO₂ environment. E6-1 Jurkat T lymphocytes (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 media with 10% FCS, 2 mM L-glutamine and penicillin-streptomycin complete.

CD4⁺ purification

CD4⁺ T cells were purified by negative selection using the magnetic-activated cell sorting (MACS) system. Briefly, non-CD4⁺ cells were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies bound to MicroBeads, as secondary labeling agent (Miltenyi Biotec, Auburn, CA). The magnetically labeled non-CD4⁺ T cells were depleted by retaining them on a MACS Column in the magnetic field of the autoMACS Separator (Miltenyi Biotec), and the unlabeled CD4⁺ T-helper cells were collected. The sample purity was assessed by fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA) with phycoerythrin (PE)-conjugated CD3 and fluorescein isothiocyanate (FITC)-conjugated CD4 monoclonal antibodies (mAbs) (purity, > 95% CD3⁺CD4⁺), according to routine procedures.

Reagents and antibodies

Antibodies (Abs) directed against p38^{Thr180/Tyr182}, Src^{Tyr416}, LAT^{Tyr171}, PKB^{Ser473}, PKC^{Ser660}, and ZAP70^{Tyr493} were purchased from Cell Signaling

Technology (Beverly, CA) as well as the Ab reacting with nonphosphorylated PKB. Phosphospecific Abs obtained from Santa Cruz Biotechnology (Heidelberg, Germany) were specific for the following targets: ERK1/2^{Thr202/Tyr204}, JNK^{Thr183/Tyr185}, Fyn^{Thr12}, and phosphotyrosine (PY20). Abs recognizing nonphosphorylated Lck, Fyn, Src, LAT, JNK, p38, ERK, PKC, ZAP70, CD3, CD4, actin, as well as SAM68 (Src [pp60c-src]-associated in mitosis 68 kDa) were obtained from Santa Cruz Biotechnology. The hypoxia-inducible factor-1 α (HIF-1 α) mAb was from Becton Dickinson (Heidelberg, Germany). Horseradish peroxidase (HRP)-conjugated goat-anti-rabbit, goat-anti-mouse, and rabbit-anti-goat were purchased from DakoCytomation (Heverlee, Belgium). Anti-human CD3 (CD3 ϵ ; mouse) was kindly provided by the group of Prof Dr H. Spits (Academic Medical Center, Amsterdam, the Netherlands),⁴⁴ and anti-CD28 mAb (mouse immunoglobulin G1) was from Sanquin (Amsterdam, the Netherlands). DEX, SU6656 (Src family kinase inhibitor), protein-A, and protein-G Sepharose, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were from Sigma-Aldrich (Zwijndrecht, the Netherlands). RU486 (mifepristone) was obtained from LKT Laboratories (Minnesota, MN). γ -³³P-ATP (adenosine triphosphate) was purchased from Amersham Biosciences. Lysis buffer and kinase buffer were from Cell Signaling Technology. Lysis buffer was supplemented with protease and phosphatase inhibitors, including 1 μ g/mL NaF, 1 μ g/mL leupeptin, 1 μ g/mL aprotinin, 10 mM Na₃VO₄, and 1 mM pefabloc "complete," obtained from Merck BV (Amsterdam, the Netherlands).

Cytokine bead array

The effect of DEX on cytokine synthesis was evaluated in supernatants of stimulated CD4⁺ T cells by cytokine bead array (BD Biosciences, Alphen a/d Rijn, the Netherlands). Cells were plated out in a 96-well plate (CellStar) at a cell density of 1×10^5 cells/well, activated with anti-CD3 and anti-CD28 Abs, and incubated overnight at 37°C in the presence or absence of 10^{-6} M DEX. This initial experiment aimed at showing that activated human CD4⁺ cells indeed represent a valid model for studying GC effects.

GC stimulation of T cells (in vitro and in vivo)

CD4⁺ T cells and Jurkat cells were seeded in 6-well plates at a concentration of 5 to 10×10^6 cells per well and incubated at 37°C for 2 hours in complete media. Subsequently, cells were pretreated for 10 minutes with 10^{-6} M DEX dissolved in DMSO (dimethyl sulfoxide), or DMSO-supplemented media (control). Cells were then incubated for 15 minutes with immobilized anti-CD3 Abs on plastic and soluble anti-CD28 mAbs (3 μ g/mL) and subsequently ice-cold PBS (phosphate-buffered saline) was added. After centrifugation complete ice-cold lysis buffer was added, and cell lysates were subjected to *in vitro* kinase assay or diluted in sample buffer for Western blotting. In addition, to study the effects of GCs on Lck/Fyn kinase activities *in vivo*, whole blood was collected from 2 healthy volunteers before and 1 hour after oral administration of 20 mg prednisolone. PBMCs activated *ex vivo* for 15 minutes with anti-CD3 and anti-CD28 Abs were lysed in complete lysis buffer and subjected to immunoprecipitation for Lck and Fyn. *In vitro* kinase assays were performed, and phospho-SAM68 expression was analyzed on Western blot with PY20.

Kinome array analysis

The protocol of the kinome array is described in detail on the website (<http://www.pepscan.nl/pdf/Manual%20PepChip%20Kinase%200203.pdf>). After a 10-minute DEX treatment and incubation with anti-CD3 and anti-CD28 Abs for 15 minutes, CD4⁺ T cells were washed in PBS and lysed in complete lysis buffer. Cell lysates were corrected for protein concentrations using Bradford analysis (Biorad, Veenendaal, the Netherlands). To study kinase activity, 50 μ L cell lysate was added to 10 μ L activation mix, containing 50% glycerol, 50 μ M ATP, 60 mM MgCl₂, 0.05% vol/vol Brij-35, 0.25 mg/mL bovine serum albumin (BSA) and 2000 μ Ci/mL (74 MBq) γ -³³P-ATP. The peptide arrays (Pepscan, Lelystad, the Netherlands),

containing 1176 different kinase pseudo-substrates in duplo,⁴³ were incubated with cell lysates for 2 hours in a humidified stove at 37°C. Subsequently, the arrays were washed in 2 M NaCl, 1% Triton X-100, 0.1% Tween in H₂O, and slides were exposed to a phosphoimaging screen for 24 to 72 hours and scanned on a phosphoimager (Fuji, Stamford, CT).

Data acquisition and statistical analysis of PepChip array

Acquisition of the peptide array was performed with a phosphoimager (Fuji) and quantified using ArrayVision 6.0 software (Molecular Dynamics, Sunnyvale, CA). Subsequently, the data were exported to a spreadsheet program (Microsoft Excel 2002; Microsoft, Redmond, WA). We corrected the spot density for the individual background to diminish interarray variances. In addition, the variation between arrays and individual experiments was reduced by normalization to the 75% percentile of the intensity of each array. Differential kinase activities in lysates from activated cells incubated in the presence or absence of DEX were determined by the comparison of the median density of the spots of each condition using the algorithm originally developed for microarray analysis (<http://www.stat.stanford.edu/~tibs/SAM/>) and fold change ratios. In short, inconsistent data (ie, SD between the data points > 1.96 of the mean value) were excluded from further analysis. Second, spots were averaged and included for dissimilarity measurement to extract kinases of which activity was either significantly induced or reduced. Alternatively, differential kinase activities were analyzed using a ranking method. The full list of peptides spotted on the peptide array can be found online (<http://www.pepscan.nl/pdf/Content%20PepChip%20Kinase%20%200303.pdf>).

Preparation of cell lysates and Western blot analysis

CD4⁺ T cells and Jurkat cells were pretreated with 10⁻⁶ M DEX for 10 minutes and stimulated with anti-CD3/anti-CD28 Abs for 15 minutes, after which ice-cold PBS was added. Cells were harvested in sample buffer (150 mM Tris [tris(hydroxymethyl)aminomethane]-HCl pH 6.8, 30% glycerol, 6% sodium dodecyl sulfate [SDS], 3% β-mercaptoethanol, and broom phenol-blue), sonicated, and heated to 90°C for 5 minutes. Whole-cell extracts were loaded on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Amsterdam, the Netherlands). The membranes were blocked with 1% Protifar (Nutricia, Zoetermeer, the Netherlands) in TBS/T (0.05 M Tris, 150 mM NaCl, 0.05% Tween-20). Primary antibodies and secondary HRP-conjugated antibodies were diluted in 1% Protifar in TBS/T. Proteins were visualized using the Lumi-Light^{PLUS} substrate (Roche, Woerden, the Netherlands). Blots were subsequently stripped with strip buffer (62.5 mM Tris-HCl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS) and reprobed with adequate antibodies for evaluation of equal protein loading.

Immunoprecipitation and in vitro kinase assay

CD4⁺ T cells were lysed in complete nondenaturing lysis buffer after a 10-minute DEX treatment (10⁻⁶ M) and stimulation with anti-CD3, anti-CD28 Abs for 15 minutes. Lysates were subjected to immunoprecipitation with the indicated Abs for Lck and Fyn. First, a preclearance step was performed by incubating the samples with protein-A or protein-G Sepharose for 2 hours at 4°C. Lysates were then centrifuged at 20 000g (13 000 rpm) for 10 minutes, and supernatants were incubated overnight with Lck- or Fyn-specific Abs at 4°C. Samples were subsequently incubated for 2 to 3 hours with a Sepharose-conjugated polyclonal antibody at 4°C. Next, kinase buffer supplemented with 200 μM ATP and 2 μg/mL SAM68 was added to the immunoprecipitates, and in vitro kinase reactions were performed at 30°C for 30 minutes. Immunoprecipitates were dissolved in sample buffer, loaded on 10% SDS-PAGE, blocked with 2.5% BSA in TBS/T (0.05 M Tris, 100 mM NaCl, 0.05% Tween-20), and immunoblotted using PY20 and a secondary HRP-conjugated Ab. Cell lysates were also incubated with 2 × 10⁻⁶ M SU6656 (a selective Src family kinase inhibitor) for 45 minutes on ice prior to the in vitro kinase assay.

Experiments with RU486, a pharmacologic GR ligand

CD4⁺ lymphocytes were pretreated for 10 minutes with increasing concentrations of DEX (10⁻¹¹, 10⁻⁸, 10⁻⁵ M) or RU486 (10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴ M) in complete RPMI 1680 (4 × 10⁶ cells/mL) at 37°C. Subsequently, cells were incubated for 6 hours in closed microtubes to induce hypoxia and compared with noncapped incubations (ie, hypoxia versus control). After incubation, cells were centrifuged, and whole-cell extracts were analyzed on Western blot using Abs against HIF-1α and actin.

In addition, CD4⁺ cells were pretreated (1 hour, 37°C) with increasing RU486 concentrations (50 × 10⁻⁹, 50 × 10⁻⁸, 50 × 10⁻⁷, 50 × 10⁻⁶ M), subsequently incubated with 10⁻⁶ M DEX (10 minutes) and activated using anti-CD3, anti-CD28 Abs (15 minutes). Lysates, subjected to immunoprecipitation using anti-Fyn mAbs, were then used for in vitro kinase assay and Western blotting.

Finally, the effects of RU486 and DEX alone, as well as RU486 and DEX in combination, were compared in preincubations of CD4⁺ T cells (50 μM RU486, 1 hour, 37°C),⁴⁵ followed by a 10-minute DEX treatment (10⁻⁶ M) and subsequent incubation with anti-CD3, anti-CD28 Abs for 15 minutes. After immunoprecipitation in vitro kinase assays and Western blotting were performed using the PY20 mAb.

Supplemental material

Supplemental Figure S1 discloses the results obtained with the PepChip experiment (available at the *Blood* website; see the Supplemental Figure link at the top of the online article).

Results

DEX inhibits cytokine production in activated CD4⁺ T cells

The CD4 isolation procedure yielded cells that were greater than 95% CD4⁺, CD3⁺ (data not shown). CD4⁺ cells were activated with anti-CD3 and anti-CD28 Abs, incubated with 10⁻⁶ M DEX or control media, and supernatants were collected after overnight incubation. DEX treatment reduced the secretion of IL-2, IFN-γ, and TNF-α (Figure 1), and this was not a consequence of reduced cell viability (as assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide [MTT] colorimetric assay; data not shown). Thus, incubation of activated human CD4⁺ cells with DEX has a potent effect on CD4⁺ cell function.

DEX rapidly alters kinomic profiles in activated CD4⁺ T cells

To investigate the early effects of GCs on the kinome, CD4⁺ T cells were incubated for 10 minutes with DEX and then stimulated for 15 minutes with anti-CD3 and anti-CD28 Abs and subsequently

Figure 1. DEX blocks IL-2, IFN-γ, and TNF-α cytokine production in activated human CD4⁺ T cells. Cells were stimulated with anti-CD3 and anti-CD28 Abs and incubated in the presence or absence of 10⁻⁶ M DEX. Supernatants were collected after overnight culturing, and cytokine protein levels were measured using a cytokine bead array. Results are expressed as the mean ± SD of triplicate determinations. DEX indicates dexamethasone.

analyzed using the peptide array (PepChip) with 1176 different kinase consensus substrates spotted in duplo. Analysis of kinomic profiles revealed 116 differential kinase substrates with either significantly increased or decreased phosphorylation upon DEX treatment. The dot plot depicted in Figure 2B shows the median density of the spots for DEX versus control conditions. Figure 2C shows the same data set analyzed using ranking. Among the most prominent effects of DEX treatment was significant decreased phosphorylation of Lck/Fyn consensus substrates. A complete listing of the peptide substrates with significantly altered phosphorylation upon DEX treatment can be found in Figure S1. Thus, the results show that a short treatment with DEX significantly influences kinase activities upon TCR ligation and reveal that the rapid immunosuppressive effect of GC treatment correlates with profound alterations in cellular signaling.

DEX inhibits Lck and Fyn kinase activity in vitro

To verify the data of the PepChip analysis, enzymatic activities of Lck/Fyn kinases were assessed using in vitro phosphorylation of the Src-family substrate SAM68.^{46,47} Prior to DEX treatment and TCR stimulation (anti-CD3/anti-CD28 Abs), cells were pretreated for 45 minutes with a Src family kinase inhibitor (SU6656). As shown in Figure 3A, TCR stimulation leads to SAM68 phosphorylation. SAM68 phosphorylation depended on Src family kinase activity, as treatment with SU6656 resulted in a complete abrogation of SAM68 phosphorylation. DEX-treated cells were neither capable of sustaining SAM68 phosphorylation. These experiments provide direct evidence that a short exposure to DEX interferes with Src family tyrosine kinase activation. Immunoprecipitation of Lck and Fyn and subsequent testing for in vitro enzymatic activities (Figure 3B) demonstrated the specific involvement of Lck and Fyn, 2 Src family members. SAM68 phosphorylation in Lck and Fyn immunoprecipitates from DEX-treated cells was impaired. Thus, DEX rapidly inhibits Lck and Fyn kinases in activated CD4⁺ T cells, corroborating the PepChip data.

Figure 2. Kinome analysis of rapid effects of DEX in activated CD4⁺ T cells. (A) Peptide micro arrays (PepChip) incubated with lysates of activated CD4⁺ T cells treated with or without DEX. (B) Dot plot representing the phosphorylation status of specific peptide substrates spotted on the PepChip array. Differential kinase activities in lysates from activated cells incubated in the presence or absence of DEX using median densities of the spots are shown. Each spot represents the amount of phosphorylation of a specific peptide substrate. Lck/Fyn kinase consensus substrates demonstrated significantly decreased phosphorylation (0.32- and 0.51-fold changes) because of DEX. A full description of the substrates of which phosphorylation was either significantly increased or decreased because of DEX treatment is provided in Figure S1. (C) The same data set was analyzed using a ranking method; each spot representing the phosphorylation status of a specific kinase pseudo-substrate. Because of the ranking method, a bisymmetric distribution is generated of peptides of which phosphorylation was either significantly increased or decreased because of DEX treatment. Peptide substrates demonstrating increased phosphorylation (corresponding with higher ranks of peptides) are reflected by an equivalent number of peptides of which phosphorylation was decreased because of DEX (lower ranks of peptides). Lck/Fyn kinase consensus substrates are marked by an arrow. Spots representing peptides of which phosphorylation is significant decreased (A), increased (C), or unaltered (B) as a result of DEX treatment are shown. DEX indicates dexamethasone.

Figure 3. DEX inhibits Lck and Fyn kinase activity in vitro. (A) CD4⁺ cells were pretreated with a specific Src family kinase inhibitor (SU6656) for 45 minutes and subsequently incubated with DEX or DMSO supplemented media (control) for 10 minutes and activated with anti-CD3 and anti-CD28 Abs for 15 minutes. In vitro kinase assays were performed using SAM68 as a substrate, and Src-like kinase activity was analyzed on Western blot using PY20. Equal loading was evaluated with an Ab against actin. Phospho-SAM68 expression is observed upon TCR ligation. Incubation with SU6656 resulted in abrogation of SAM68 phosphorylation, and a similar effect was demonstrated in activated cells treated with DEX. (B) Lysates of CD4⁺ cells, pretreated with DEX (10 minutes) and subsequently activated (15 minutes), were subjected to Lck and Fyn immunoprecipitation, followed by in vitro kinase assay. To test for equal loading, Western blots were analyzed for total Lck and Fyn expression. Reduced phospho-SAM68 expression was seen in activated cells incubated with DEX, compared with cells incubated in the absence of DEX. Similar results were obtained in 3 independent experiments. DEX indicates dexamethasone; IB, immunoblotting; IP, immunoprecipitation.

DEX suppresses phosphorylation of signaling molecules downstream of the TCR

To further investigate the effects of DEX on early steps of TCR signal transduction, the activation status of signaling molecules downstream of the TCR was analyzed by immunoblotting using phosphospecific Abs. DEX rapidly suppresses phosphorylation of Fyn, Src, LAT, PKB, PKC, ERK1/2, JNK, and p38 MAPK in activated CD4⁺ cells (Figure 4). These observations demonstrate that DEX affects Lck/Fyn-induced TCR-dependent signaling cascades.

Figure 4. DEX inhibits signaling pathways downstream of the TCR. Cells were preincubated for 10 minutes with or without DEX and subsequently stimulated for 15 minutes using anti-CD3 and anti-CD28 Abs. The phosphorylation status of Fyn, Src, LAT, PKB, PKC, p38, ERK1/2, and JNK in whole-cell lysates was assayed using phosphospecific mAbs on Western blot. An Ab against phosphorylated Lck associated with increased activity (not available) was not included.⁴⁸ The Ab we used to detect phosphorylated Src may cross-react with other Src kinase family members, including Fyn, Lck, Lyn, pp62c-yes (Yes), hematopoietic cellular kinase (Hck), and Src itself when phosphorylated at equivalent sites. Therefore, we cannot exclude the possibility that suppressed Src phosphorylation represents reduced phosphorylation of Src itself or other Src family members. To test for equal loading Western blots were stripped and reprobed using the appropriate Abs. The Western blots represent 3 independent experiments. LAT indicates linker for activation of T cells; PKC, protein kinase C; PKB, protein kinase B; DEX, dexamethasone.

DEX affects Lck-CD4 and Fyn-CD3 interactions

Next, it was assessed whether DEX affects spatial distribution of Lck and Fyn within the cell membrane, as translocation of these kinases to the TCR complex is crucial for Lck and Fyn activation and efficient TCR signaling.^{19,25,49} Lysates from DEX-treated cells (CD3/CD28 activated) were subjected to Lck and Fyn immunoprecipitation and subsequent Western blot analysis using anti-CD4 and anti-CD3 mAbs. Activated cells displayed strong CD4 and CD3 protein expression in Lck and Fyn immunoprecipitates respectively (Figure 5). In contrast, CD4 protein expression was reduced in Lck immunoprecipitates prepared from activated cells treated with DEX. Similarly, CD3 expression was suppressed in Fyn immunoprecipitates following DEX exposure. Effects of DEX on Lck-CD3 and Fyn-CD4 associations were not evident (Figure 5). Apparently, DEX treatment rapidly affects the recruitment of Lck and Fyn to the CD4 and the CD3 coreceptors, respectively.

DEX-induced inhibition of Lck/Fyn kinase activity is GR dependent

To address the question as to whether DEX inhibition of Lck/Fyn kinases is GR dependent or independent, GR-negative Jurkat cells were pretreated with DEX for 10 minutes and subsequently incubated with anti-CD3/anti-CD28 Abs for 15 minutes. Western

Figure 5. DEX affects Lck-CD4 and Fyn-CD3 associations. Cells were preincubated with DEX for 10 minutes and subsequently stimulated with anti-CD3/anti-CD28 Abs for 15 minutes. Lck and Fyn were then immunoprecipitated, and samples were analyzed for CD4 and CD3 expression on Western blot. To test for equal loading, blots were analyzed for total Lck and Fyn expression. Upon TCR ligation, CD4 and CD3 expression is increased in Lck and Fyn immunoprecipitates, respectively. Reduced CD4 expression was observed in Lck immunoprecipitates prepared from activated cells treated with DEX. Similarly, Fyn-immunoprecipitated lysates demonstrated decreased CD3 protein levels upon DEX treatment. CD3 expression was analyzed in Lck immunoprecipitates, and CD4 protein levels were studied in Fyn immunoprecipitates to ensure specificity in this experiment. Three experiments were performed and comparable results were obtained. DEX indicates dexamethasone; IB, immunoblotting; IP, immunoprecipitation.

Figure 6. DEX-induced inhibition of Lck and Fyn kinase activities is GR dependent. (A) GR-negative Jurkat cells (confirmed by Western blot analysis; not shown) were pretreated with DEX for 10 minutes and incubated with anti-CD3, anti-CD28 mAbs for 15 minutes. Whole-cell lysates were used for Western blot analysis. Because there are no Abs commercially available targeting phospho-Lck associated with increased activity, phospho-ZAP70 expression (a downstream target of Lck) was analyzed. DEX treatment did not affect phospho-Fyn expression, nor did it affect phospho-ZAP70 protein levels in GR-negative Jurkat cells. Three experiments were performed, and similar outcomes were obtained. (B) CD4⁺ T cells were incubated for 1 hour with a GR mimetic (RU486; 50 μM), which acts as a GR agonist at this concentration. Subsequently, cells were treated with DEX (10 minutes) and activated with anti-CD3, anti-CD28 Abs (15 minutes). Lck and Fyn immunoprecipitates were subjected to in vitro kinase assay, and phospho-SAM68 expression was studied on Western blot using PY20. Decreased phospho-SAM68 expression was seen in Lck and Fyn immunoprecipitates prepared from activated cells treated with DEX, and cells treated with RU486 together with DEX. Incubation with RU486 alone (at this high/agonistic concentration) also resulted in impaired SAM68 phosphorylation. DEX indicates dexamethasone; IB, immunoblotting; IP, immunoprecipitation.

blot analysis was performed using Abs against phospho-Fyn and phospho-ZAP70, a downstream target of Lck. DEX treatment did not affect phospho-Fyn or phospho-ZAP70 protein expression in Jurkat cells (Figure 6A), suggesting that GC effects on Lck/Fyn require a functional GR.

Further evidence that the inhibitory effects of GCs on Lck/Fyn activities are GR mediated was obtained in experiments with a GR mimetic (RU486). Depending on the concentration, RU486 may either stimulate or inhibit this receptor (dualistic agonistic or antagonistic actions). The effects of RU486 on GR activation were studied in CD4⁺ T cells activated with hypoxia. Hypoxia strongly induces HIF-1α (hypoxia inducible factor 1α) expression (not shown). DEX treatment (at 10⁻⁸, 10⁻⁵ M) reduces HIF-1α expression. At 10⁻⁷, 10⁻⁶, and 10⁻⁵ M RU486 concentrations the effects of RU486 were neutral. Of note, cells incubated with the highest concentrations of RU486 (10⁻⁴ M) showed the opposite effect (ie, reduced HIF-1α protein levels). DEX and RU486

synergized in suppressing hypoxia-induced HIF-1 α expression at 10^{-11} M DEX and 10^{-5} M RU486.

Further evidence that RU486 has a dualistic nature was obtained in experiments in which activated CD4⁺ cells were pretreated with increasing RU486 concentrations together with DEX (10^{-6} M) and subsequently used for in vitro kinase assay. Pretreatment with the highest RU486 concentration (50 μ M) showed an agonistic effect on Fyn kinase activity (ie, strongly reduced phospho-SAM68 phosphorylation on Western blot; not shown). The lowest RU486 concentration (10^{-9} M) was not able to block the inhibitory effects of DEX on Fyn kinase activity (ie, reduced phospho-SAM68 expression). Cells pretreated with intermediate RU486 concentrations (10^{-8} M) together with DEX demonstrated clear phospho-SAM68 protein levels, suggesting an inhibitory effect of RU486 on DEX-dependent Fyn inhibition (ie, antagonistic effect).

Finally, we studied the effects of the highest RU486 concentration (50 μ M, 1 hour) on Lck and Fyn kinases in activated CD4⁺ T cells in the absence or presence of DEX (10^{-6} M). Cells incubated with RU486 (in the absence of DEX) exhibited strongly reduced phospho-SAM68 expression (Figure 6B) in both Lck and Fyn immunoprecipitates (ie, agonistic effect). Altogether, these experiments support a rapid GR mediated mechanism of Lck/Fyn inhibition.

Glucocorticoid-induced suppression of Lck and Fyn activity in vivo

GC effects on Lck and Fyn kinase activities were studied in PBMCs isolated from 2 individuals before and at 1 hour following oral administration of a single dose of 20 mg prednisolone. In isolated cells stimulated ex vivo for 15 minutes (with anti-CD3/anti-CD28 Abs) Western blot analysis revealed suppressed SAM68 phosphorylation in Lck and Fyn immunoprecipitates at 1 hour following prednisolone treatment in both experiments (Figure 7).

Figure 7. Glucocorticoid-induced inhibition of Lck/Fyn enzymatic activity in vivo. Human PBMCs were isolated from whole blood obtained from 2 (no. 1, no. 2) healthy volunteers before and 1 hour after oral administration of 20 mg prednisolone. Next, cells were stimulated ex vivo for 15 minutes with anti-CD3 and anti-CD28 Abs and subjected to immunoprecipitation for Lck and Fyn. After performing in vitro kinase assays, lysates were analyzed on Western blot for SAM68 phosphorylation. Suppressed phospho-SAM68 expression in both Lck and Fyn immunoprecipitates was observed at 1 hour after prednisolone administration in both individuals. IB indicates immunoblotting; IP, immunoprecipitation.

Discussion

GCs form the basis of current immunosuppressive therapy. The classic mechanism of GC action involves the GR and modulation of transcriptional and translational events. Over the past years increasing evidence for rapid nongenomic GC action has accumulated that cannot be explained by the traditional mechanistic model.⁷⁻¹⁸ The molecular mechanisms underlying the early effects of GCs are poorly characterized. The present study was undertaken to study rapid effects of GC action in activated human CD4⁺ T cells. Using an array of kinase pseudo-substrates we demonstrated that the activities of multiple kinases are rapidly altered in activated T cells following short-term treatment with DEX, a synthetic GC analog. Further analysis revealed that DEX inhibits Lck and Fyn kinases within minutes in these immune cells. This was demonstrated in vitro as well as in vivo in healthy individuals who had received GCs. Phosphorylation of Lck and Fyn are proximal events in T-cell activation. Lck and Fyn are subsequently known to positively regulate the signaling initiated upon TCR stimulation through a variety of downstream pathways. Accordingly, reduced Lck and Fyn kinase activities may have an important role in the fast immunosuppressive effects of GCs in immune cells. In line with this notion, decreased activation of several signaling pathways downstream of the TCR upon DEX treatment was observed, including suppression of PKB, PKC, ERK, JNK, and p38 MAPKs. It is known that the biochemical and functional responses to TCR ligands are largely determined by Fyn-CD3 and Lck-CD4 associations.^{24,28,49-52} The data reported here show that DEX treatment rapidly alters the cellular distribution of Lck and Fyn which would likely result in decreased Lck/Fyn kinase activities and suppressed TCR signaling.

Others have previously reported that DEX disturbs the submembrane localization of Lck and Fyn in a murine T-cell hybridoma, because lipid rafts purified from DEX-treated cells displayed a decrease in Lck and Fyn protein concentration.⁵³ However, in those experiments DEX treatment did not affect cellular expression levels of Lck and Fyn kinases, as measured by Western blot performed on whole-cell extracts. Furthermore, in this particular study no effect of DEX on Lck/Fyn kinase activities was noted. We assume that these discrepant findings could be due to differences in experimental set-up. Murine T cells were used in their study, and it is known that murine cells markedly differ from human T cells in their GC activation responses.⁵⁴⁻⁵⁸ Also the DEX incubation time of T cells was considerably different: they used a 16-hour DEX preincubation time in contrast to the 10 minutes in our study. It is well possible that feedback mechanisms upon GC treatment were activated within the 16-hour time frame, counteracting the negative effects on Lck/Fyn activation and the spatial distribution of these kinases.^{59,60} Finally, it is well possible that the observed early effects are short lived and as a result come and go rapidly.

Recent advances in nuclear hormone receptor biology provide evidence for novel types of receptors binding steroids and mediating rapid nongenomic signaling events.⁶¹ Are the early GC effects that we observed in activated T cells, GR dependent or GR independent? Phospho-ZAP70 (an Lck downstream substrate) and phospho-Fyn protein expression in Jurkat cells, which do not express the GR, did not respond to DEX. These results are supported by experiments with CD4⁺ T cells treated with agonistic concentrations of RU486, a pharmacologic GR ligand. Again, a

strict correlation between GR activation and inhibition of Lck and Fyn kinase activities was observed.

In conclusion, we have identified Lck and Fyn, key players in TCR activation, as rapid molecular targets of GC action in activated human CD4⁺ T lymphocytes via a GR-dependent mechanism. These observations open the possibility that compounds selectively targeting Lck and Fyn may constitute a potent anti-inflammatory therapy.

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