Role of glucocorticoid receptor isoforms in the regulation of transcription

Ph.D. thesis

Zsolt Nagy, MD

Semmelweis University
Clinical Medicine Doctoral School

Supervisor: Attila Patócs, Ph.D., associate professor

Opponents: Pál Harjit Bhattoa, Ph.D., senior lecturer
András Masszi, Ph.D., assistant lecturer

Final Exam Committee
Head: Edit Buzás, Ph.D., D.Sc., professor
Members: Bálint Nagy,Ph.D., D.Sc., senior research follow
Hubina Erika, Ph.D., senior physician

Budapest
2017

Introduction

Glucocorticoids play an essential role in several physiological processes and are the most often prescribed anti-inflammatory agents. Despite their discovery in the 1940s the detailed mechanism of action of glucocorticoids are still not fully understood. It was long thought that glucocorticoids exert their diverse biological effects through only one receptor, the well known glucocorticoid receptor α (GRα). The ß isoform, discovered in the 1990's, is not able to induce gene transcription at glucocorticoid responsive elements (GRE), however it exerts a dominant negative activity on GRα function. Nowadays with the revolutionary development of molecular biology techniques several novel effects of the GR family have been described, and even a more complex understanding of the mechanism of diverse biological effects of glucocorticoid receptors have been suggested. In the present study I examined the role of GR α and ß isoforms in the regulation of gene transcription. Increased GRß expression was described in several autoimmune inflammatory diseases, including inflammatory bowel diseases (IBD) and its expression showed correlation with the development of glucocorticoid resistance. Therefore in the first part of my thesis an *in vitro* model developed from the Caco-2 intestinal cell line was used to study the role of GRß in the development of steroid resistance and IBD.

Circadian timing of gene expression is an important regulatory mechanism maintaining physiological processes and ensuring the physiological adaptation of the organism to the day/night cycles. Almost every human tissue preserves a peripheral circadian clock that consists of an approximately 24h oscillating feedback loop of circadian clock genes. The diurnally secreted glucocorticoids may play an important role in the regulation of clock genes. However the mechanism of glucocorticoid control of clock genes is poorly understood therefore in the second part of my thesis I investigated the effects of GR α and ß isoforms on the regulation of clock genes in H295R human adrenocortical cancer cell line.

1. Aims

Glucocorticoids regulate several physiological processes. The well known effects of glucocorticoids are transmitted through the GRα isoform, nevertheless the effects of other GR isoforms are less known. During my thesis I investigated the regulation of gene transcription by GRß in Caco-2 intestinal cell line, and I examined the role of GRα and GRß isoforms in the regulation of clock genes in human adrenocortical (H295R) cell line.

1. To investigate the effects of increased GRß expression on gene transcription and its role in the development of glucocorticoid resistance in an intestinal cell line (Caco-2).
2. To identify IBD specific gene expression alterations by the meta-analysis of microarray results in colonic mucosal biopsy samples obtained from healthy controls and patients with IBD.
3. To identify the biological function of gene common in GRß overexpressedcells and differentially expressed in IBD compared to healthy mucosa..
4. To verify the induction of peripheral circadian clock in human adrenocortical (H295R) cell line.
5. To investigate the role of GRα and GRß isoforms in the regulation of peripheral clock genes in H295R cell line.

2. Methods

2.1. Cell culture

Experiments were performed in Caco-2 and Caco-2GRß intestinal cell lines, as well as in human adrenocortical cancer (H295R) cell line. Maintenance of these cells were performed according to the instructions of the manufacturer.

2.2. Generation of a stably GRß expressing Caco-2GRß cell line

GRß was cloned from the GRα isoform using a sense oligonucleotide primer mapping to the shared α-β region and an antisense oligonucleotide specific for the GRß sequence. The PCR fragments were cloned into pcDNA3.1 vectors (Invitrogen). Sequences of plasmids were verified by direct DNA sequencing. Caco-2 cells were transfected either with the GRß containing plasmids or the empty pcDNA3.1 vector using FuGene Transfection Reagent (Promega) according to the manufacturer's instructions. Clonal selection was performed with neomycin treatment.

2.3. Cell proliferation assay

Proliferation rate of Caco-2, Caco-2 expressing the empty pcDNA3.1 vector (Caco-2pcDNA3.1) and Caco-2GRß cells were determined with AlamarBlue Proliferation Assay (Invitrogen) according to the manufacturer's instructions on at least seven biological replicates. Fluorescence was measured at the indicated time points on a Varioskan Multimode Reader (Thermo Scientific).

2.4. Immunocytochemistry

A Caco-2 and Caco-2GRß cells were treated either with 100 nmol DEX or vehicle for 4 hours. After fixation and washing procedures samples were incubated with PA3-514 (anti-GRß) (ThermoFisher Scientific)antibody and nuclear staining was carried out using Alexa Fluor 488 anti-rabbit IgG secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI). Microscopic images were quantified using the ImageJ software.

2.5. Luciferase reporter assay

Caco-2 and Caco-2GRß cells were co-transfected with pGRE-SEAP reporter vector (Clontech) and pGL3 control (Promega) plasmid using FuGene 6 transfection reagent. Measured SEAP activity was normalized to the pGL3 control firefly luciferase activity. Luminescence was detected on an Appliskan Microplate Reader on at least 3 biological replicates repeated 6 times.

2.1.6. Real-Time PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. 1µg RNA was reverse transcribed with Invitrogen Superscript VILO reverse transcriptase (Life Technologies, Carlsbad, California, USA) or High-Capacity RNA-to-cDNA Kit (Life Technologies). For quantitative Real-Time PCR (qRT-PCR) we used predesigned TaqMan Gene Expression Array Cards on a ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) or in some cases individual TaqMan gene expression assays were used on 7500 Fast Real-Time PCR system (Applied Biosystem) according to the manufacturer's instructions.

2.1.7. Microarray experiments

Caco-2 and Caco-2GRß cells were treated either with 100 nmol DEX or vehicle for 8 hours. After total RNA isolation whole genome mRNA expression analysis was carried out using Agilent44K cDNA microarrays. Array scanning and feature extraction was performed with Agilent DNA Microarray Scanner and Feature Extraction Software 9.5.3. Microarray data was processed using Genespring GX 12.5 with default parameters.

2.1.8. Meta-analysis of gene expression microarrays

For the meta-analysis of microarray gene expression studies raw data were downloaded from Gene Expression Omnibus (GEO) and ArrayExpress online databases. Overall 245 microarray samples (40 healthy control colon; 114 Crohn's disease (CD) and 91 ulcerative colitis (UC)) were re-analyzed by Genespring Gx 12.5.software.

2.1.9. Functional and pathway analysis

Genes differentially expressed by GRß overexpression in Caco-2 cells, genes differentially expressed in Crohn's disease compared to control colon and sets of overlapping GRß- and IBD related genes were analyzed with Ingenuity Pathway Analysis (IPA) (www.ingenuity.com) (Qiagen) software.

2. 1.10. Circadian experiments

H295R cells for serum shock experiments were serum starved for 24 h and incubated with 30% Nu Serum for 2h. To study the effects of GRα, cells were serum starved for 24h then treated with vehicle (0,01% ethanol), 100 nmol DEX, 1µmol RU486 alone or in combinations. In some experiments cells were treated with vehicle (0,01% ethanol), metyrapone (100µmol) or in combination with metyrapone (100µmol) and RU486 (1µmol).

2.1.11. Transient transfections

H295R cells were transfected overnight with 2500 ng empty pcDNA3.1 plasmid as control or GRß plasmid using Lipofectamine3000 reagent (LifeTechnologies). Then media was replaced with charcoal stripped Nu-serum containing medium and treated either with vehicle or DEX for 6 hours. Cells were harvested 48 hours post-transfection.

2.1.12. Statistical analysis

Statistical analysis of immuncytochemistry, proliferation assay and qRT-PCR was performed with Student's t-test using SPSS Statistics 20.0 (IBM). Microarray data was analyzed with Genespring GX 12.5 software. Identification of gene sets differentially expressed between the groups was carried out by two-way ANOVA, followed by Tukey's post hoc test for all pairwise multiple comparisons and Benjamini-Hochberg multiple correction. Fold change filter was set to 2-fold in each comparison. To identify rhythmic expression within each group in circadian experiments gene expression was first analysed with ANOVA to exclude random oscillation, then rhythmic gene expression was further calculated by the cosinor method with an online available program (http://www.circadian.org). A value of $p<0,05$ was considered to be significant.

3. Results

3.1 Investigation of GRß isoform in inflammatory bowel diseases (IBD)

3.1.1. Characterization of stably GRß expressing Caco-2 cell line (Caco-2GRß*)*

The expression GRß isoform was barely detected in Caco-2 cells by qRT-PCR. After transfection with GRß overexpressing plasmid GRß was more abundant both at the mRNA and protein levels in Caco-2GRß cells. Immunocytochemistry showed that GRß was present in both the cytoplasm and nucleus with a strong nuclear distribution. Microscopic analysis revealed that Caco-2GRß cells show markedly different cell shape. GRß overexpressing cells had a significantly lower proliferation rate compared to either to the normal Caco-2 cells or to the Caco-2pcDNA3.1 cells.

3.1.2. Role of GRß isoform in the regulation of gene transcription

Whole genome gene expression microarray experiments revealed that DEX treatment affected the expression of 116 genes (47 under- and 69 overexpressed) in Caco-2 cells, but the expression of only 12 genes (5 under- and 7 over-expressed) was altered in Caco-2GRß cells. To test the effect of GRß overexpression on glucocorticoid sensitivity, both cell lines were transfected with a glucocorticoid responsive element (GRE) driven SEAP promoter reporter vector. Upon DEX treatment luciferase activity in Caco-2GRß cells was significantly lower compared to Caco-2 cells. Our results suggest that overexpression of GRß rendered the cell line glucocorticoid insensitive.

Microarray data analysis revealed that without DEX treatment 852 genes were differentially expressed (196 under- and 656 overexpressed) in Caco-2GRß cells compared to the Caco-2 cells. Interestingly there was only a small overlap between GRß and DEX regulated genes. Of the significantly induced genes we measured the expression of genes which are involved in the regulation of apoptosis (*BCL2*, *CASP1*), metabolism (*CPE*, *NNMT*, *LARGE*, *PAH*, *SLC26A9*), immune and inflammatory response (*IL1RAP*, *SAMD9*, *DEFB1*), signal transduction (*RHOBTB1*, *RICH2*, *TGFB2*), gene transcription and/or RNA processing regulators (*RBMS3*, *SATB1*) and genes encoding matrix proteins (*VIM*, *CDH6*, *SPP1*, *SPARC*, *COL4A6*). Among genes showing reduced expression in Caco-2GRß cells compared to Caco-

2 cells, genes involved in immune response (*CXCL1*, *CXCL2*, *CXCL3*), gene transcription (*NFIA*, *RPL39L*), metabolism (*PDE4A*) and cell signaling (*SAMD1*, *S100P*, *SSTR1*) were identified.

3.1.3. Identification and functional analysis of GRß regulated genes in inflammatory bowel disease

A meta-analysis of 245 microarray data from 8 different studies which examined the gene expression in colon mucosa from patients with IBD compared to healthy subjects was performed. We found 737 differentially expressed genes in CD compared to healthy control samples, and 838 differently expressed genes between ulcerative colitis and healthy control. When the profile of differentially expressed genes in Caco-2GRß vs Caco-2 cells were compared to that found in colonic mucosa samples of patients with CD vs healthy controls, we detected overlaps in the expression of 64 genes (55 were up-regulated and 9 down-regulated). Interestingly 28 additional genes showed opposite direction of expression in the comparison.

Functional analysis of differentially expressed genes in Caco-2GRß cells and CD samples revealed that the overlapping set of genes are annotated as “cell-to cell signaling”, “cellular movement” and “proliferation of cells” categories that represent “adhesion of tumor cell lines”, “migration of cells” and “proliferation of cells” cellular functions. Among GRß regulated genes that play role in cell adhesion, cell migration, and cell proliferation the expression of *SPP1*, *CHI3L1*, *VIM*, *CASP1* and *S100P* was significantly increased, meanwhile expression of *SSTR1* was decreased in Caco-2GRß cells compared to Caco-2 cells.

3.2. Role of GR isoforms in the regulation of peripheral circadian clock

3.2.1. Investigation of synchronizing effect of serum shock in H295R cell line

After synchronization of H295R cells with serum shock we detected the rhythmic expression of 4 clock genes: *PER1, PER2, REV-ERBα and ARNTL* by cosinor analysis.

3.2.2. Identification of primary glucocorticoid targets and GRα mediated changes in peripheral clock of H295R cells

I confirmed the expression of total GR in H295R cell line. Clock genes that respond early to GRα activation may be considered as direct targets of GRα. DEX treatment caused an immediate induction of *PER1*, *PER2* and *CRY1* levels in a GRα dependent manner after 2 hours, however. *PER1* and *PER2* levels remained constantly significantly elevated (except for *PER2* at time point 6) compared to control until 12 hours of stimulation by DEX, meanwhile *PER1* and *PER2* induction was blunted by the simultaneous administration of RU486. For *CRY1* significantly higher levels were measured after 4 and 12 hours of DEX treatment compared to control group that was again abolished by simultaneous administration of RU486. *REV-ERBα* became suppressed from 6 to 12 hours in response to DEX that was prevented by addition of concomitant RU486.

3.2.3. Rhythm of expression of peripheral clock genes in H295R cells

In the control group cosinor analysis revealed the rhythmic oscillation of *PER1, PER2, CRY1* and *ARNTL*. DEX treatment abolished the rhythmic oscillation of *PER1* and *CRY1* and provoked the rhythmic expression of *REV-ERBα* accompanied with a phase-shift in *PER2* levels. Treatment of cells with the non-specific GR antagonist RU486 totally abolished the oscillation of expression of these genes but, a rhythmic expression of *REV-ERBα* was revealed.

3.2.4. Effect of GRß overexpression on clock gene transcription

H295R cells were transiently transfected with GRß expressing or empty plasmids and a treatment with vehicle or DEX for 6 hours was carried out. Overexpression of GRß at basal conditions did not influence the expression of the examined clock genes. DEX treatment as expected significantly increased the expression of *PER1, PER2* and suppressed the *REV-ERBα* levels in empty vector transfected cells. Overexpression of GRß did not influence the induction of *PER1* and *PER2*, however, prevented the down-regulation of *REV-ERBα* after stimulation of GRα.

3.2.5. Endogenous steroid synthesis is not involved in the induction of PER1

We hypothesized that endogenous steroid production present in the media of H295R cells might be sufficient for induction of rhythmic of expression of clock genes. After serum starvation and administration of 100μM metyrapone, concentration of cortisol and testosterone in the media determined using liquid chromatography tandem mass spectrometry decreased under the detection limit, while progesterone level was just above the detection limit. However, surprisingly, the expression of *PER1* still oscillated suggesting that other factors may drive its expression. We repeated experiments by adding RU486 to metyrapone and the induction of *PER1* was successfully prevented. Nevertheless if serum starvation was not applied before experiments, we could not detect the induction of *PER1*. These results suggest that serum starvation might have induced the rhythmic expression of *PER1* gene in the control group.

4. Conclusions

Investigating the role of GRα and GRß isoforms in the regulation of gene transcription the following conclusions were drawn:

1. The Caco-2GRß cell line is an important tool for studying glucocorticoid resistance *in vitro* related to increased GRß expression. In accordance with previous studies our results showed that increased GRß expression leads to glucocorticoid insensitivity through the inhibition of GRα function.

2. GRß regulates gene transcription in a gene specific manner the same and opposite direction compared to GRα. We also showed that GRß has a GRα independent transcriptional activity.

3. Comparison of gene expression profiles observed in Caco-2GRß cells compared to Caco-2 cells and IBD samples showed that several genes found in intestinal mucosa samples of IBD patients were also regulated by GRß overexpression. We found that the overlapping genes play a role in cell adhesion, cell migration and cell proliferation. These cellular processes play an important role maintaining intact barrier function.

4. Overexpression of GRß isoform altered the morphology of Caco-2 cells accompanied by a diminished proliferation rate. Functional analysis revealed that a set of genes differentially expressed by GRß overexpression influence cell growth and cell death and may explain decreased proliferation rate. This particular feature may suggest that overexpression of GRβ may have a tumorsuppressor role, however this hypothesis needs further studies.

5. A functional peripheral clock is present in H295R human adrenocortical cell line. Rhythmic oscillation of clock genes was related to serum shock/starvation and did not show correlation with endogenous cortisol, testosteron and progesteron production of H295R cells.

6. A GRα isoform influenced simultaneously the expression of multiple genes encoding members of the peripheral clock. Stimulation of GRα increased the expression of *PER1*, *PER2* and *CRY1* genes while inhibited the expression of *REV-ERBα*. Overexpression of GRß at basal conditions did not influence the expression of the examined clock genes, however GRß isoform may modify the entrainment of peripheral clocks by glucocorticoids through *REV-ERBα.*

5. List of publications

Publications related to the doctoral thesis:

Nagy Z, Acs B, Butz H, Feldman K, Marta A, Szabo PM, Baghy K, Pazmany T, Racz, K, Liko I, Patocs A. Overexpression of GRß in colonic mucosal cell line partly reflects altered gene expression in colonic mucosa of patients with inflammatory bowel disease. (2016) ***J. Steroid Biochem. Mol. Biol***. 155:76–84 **IF: 3,98**

Nagy Z, Marta A, Butz H, Liko I, Racz K, Patocs A. Modulation of the circadian clock by glucocorticoid receptor isoforms in the H295R cell line. (2016)
Steroids 116:20-27 **IF:2,51**

Nagy Zs, Rácz K, Patócs A. A perifériás cirkadián órák jelentősége az anyagcserezavarok kialakulásában. (2014)
Magyar Belorvosi Archívum 67(6): 374-380

Szappanos A, **Nagy Z**, Kovács B, Poór G, Tóth M, Rácz K, Kiss E, Patócs A. Tissue-Specific Glucocorticoid Signaling May Determine The Resistance Against Glucocorticoids In Autoimmune Diseases. (2015)
Curr Med Chem. 22(9): 1126-1135 **IF:3,45**

Other publications:

Igaz I, Nyírő G, Nagy Z, Butz H, **Nagy Z**, Perge P, Sahin P, Tóth M, Rácz K, Igaz P, Patócs A. Analysis of Circulating MicroRNAs In Vivo following Administration of Dexamethasone and Adrenocorticotropin. (2015)
Int J Endocrinol. 2015:589230 **IF:2,37**

Kacso G, Ravasz D, Doczi J, Nemeth B, Madgar O, Saada A, Ilin P, Miller C, Ostergaard E, Iordanov I, Adams D, Vargedo Z, Araki M, Araki K, Nakahara M, Ito H, Gal A, Molnar MJ, **Nagy Z**, Patocs A, Adam-Vizi V, Chinopoulos C: Two transgenic mouse models for beta subunit components of succinate-CoA ligase yielding pleiotropic metabolic alterations. (2016)
Biochem J 473(20):3463-3485 **IF:3,56**