Structure-function analysis of protein variants causing disturbances in glucocorticoid regulation

Ph.D. thesis

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Introduction

Glucocorticoids not only regulate glucose and fat metabolism, modulate behavior, the cardiovascular system and immune function, but also have additional effects on growth and development, calcium metabolism, connective tissue and bone regulation, thyroid and gonadal function. Therefore, both during treatment with exogenous glucocorticoids and under normal physiology, relatively small changes in glucocorticoid regulation may have important consequences for the organism. In addition, glucocorticoids have a great pharmacological importance as synthetic glucocorticoid derivatives exert a most significant effect on the inflammatory process.

The first part of our research has been focused on the glucocorticoid receptor. The effects of glucocorticoids are mainly mediated by glucocorticoid receptors, which belong to the group of nuclear receptors. The receptor, which is present within the cytoplasm before activation, is activated in the presence of hormone, and then translocated to the nucleus to act as a transcription factor. During our work we performed (1) a systemic review of reported mutation of the glucocorticoid receptor gene; (2) an *in silico* analysis of known and novel mutations of the glucocorticoid receptor gene using available

nucleotide sequence databases; (3) an analysis of protein structure and function contributed to known and novel mutations of the glucocorticoid receptor gene in order (4) to predict the effect of mutations on glucocorticoid receptor function.

The second part of the research included studies on protein variants causing disturbances in the cortisol biosynthetic pathway. Using hormone measurements in patients with hypertension and hypokalemia, steroid 17α-hydroxylase/17,20-lyase (cytochrome P450c17, P450c17) deficiency, a rare congenital defect in cortisol and androgen hormone biosynthesis was identified in two unrelated patients. Molecular biological analysis of peripheral blood samples in both patients and that of adrenal tumoral tissue obtained from one patient indicated a novel mutation of the gene coding for the P405c17 enzyme (Arg440Cys). Our additional studies confirmed that the mutation results in an almost complete loss of the enzyme activity.

A better understanding of the underlying causes of endocrine disorders may improve the chance for cure and the prognosis of many patients. It is possible that new knowledge on mutant proteins resulting in disturbances of glucocorticoid regulation, together with an earlier diagnosis, may help to

introduce more specific therapies for patients with these disorders.

Research Objectives

Our principal goals included the followings:

(1) To review all reported mutations of the glucocorticoid receptor gene that cause glucocorticoid resistance syndrome.

(2) To apply a novel method using *in silico* sequence analysis in an attempt to reveal previously undescribed sequence variants of the glucocorticoid receptor gene.

(3) To analyze the consequences of glucocorticoid receptor gene mutations causing glucocorticoid resistance on the three-dimensional protein structures and to predict the function of previously undescribed mutant receptor variants.

(4) To identify and clone the mutant CYP17 gene causing congenital 17α-hydroxylase/17,20-lyase deficiency in patients who were diagnosed by hormonal screening performed among patients with hypertension and hypokalemia.

(5) To determine the enzyme activity of mutant and wild type P450c17 enzymes expressed in eukaryotic cells.

(6) To analyze the structure-function relationship of the mutant P450c17 enzyme.

Materials and Methods

In silico search for novel sequence variants

We performed homology searches at the National Center for Biotechnology Information (NCBI) databases. Sequence comparison was performed by the Basic Local Alignment Search Tool (BLASTN)

Three-dimensional structural analyzes

The three-dimensional modeling was performed with Swiss-PDBViewer. The coordinates of the glucocorticoid receptor and the P450c17 enzyme were obtained from 1M2Z, 1P93, 1NHZ and 2C17 PDB structure files, respectively.

Patients with mutant P450c17

Patients were referred because of hypertension and low serum potassium level. The measurement of plasma steroids confirmed the suspicion of combined 17α-hydroxylase/17,20-lyase deficiency.

Measurements of hormone concentrations were performed in plasma samples by immunochemiluminometric and radioimmunoassay methods.

Molecular biology methods

Cloning and expression studies were performed by standard methods. Total RNA was isolated by RNeasy Mini Kit (Qiagen). cDNA was synthesized with Ready-To-Go You-Prime First-Strand Beads (Amersham Pharmacia Biotech). The CYP17 gene was amplified with Herculase Enhanced DNA Polymerase (Stratagene) in Mastercycler Gradient PCR (Eppendorf AG). The final PCR products were purified on 0.8% agarose gel with MinElute Gel Extraction Kit (Qiagen), and the fragment was ligated into the pcDNA3.1 V5/His vector (Invitrogen) The DNA sequences were determined by using Big Dye Terminator Cycle-Sequencing kit (Applied Biosystems), and run on an automated sequencer 310 Genetic Analyzer from Applied Biosystems. COS-1 cells (ATCC: CRL-1650) were transfected with vectors expressing the mutant or wild-type enzyme. The production of proteins was analyzed by Western blotting, immunocytochemistry and flow cytometry using a monoclonal anti-V5 antibody (Invitrogen). 17α-hydroxylase and 17,20-lyase activities of transfected cells were measured after incubating with progesterone or 17-hydroxypregnenolone. The concentrations of 17-hydroxyprogesterone and dehydroepiandrosterone were measured using radioimmunoassays.

Results

In silico search for novel glucocorticoid receptor gene mutations

In an attempt to reveal previously undescribed sequence variants of the glucocorticoid receptor gene, we applied a novel method using *in silico* sequence analysis. This method enables the rapid analysis of multiple sequences submitted to databases. It is intensively studied as a potentially efficient alternative of conventional time-consuming searches for sequence polymorphisms. As numerous sequences are being submitted recently to sequence databases, *in silico* approaches appear to gain expanding perspectives in the analysis of sequence variants.

We applied the following human DNA sequences as search sequences: exons 1-8, 9α, 9β with the surrounding introns (NCBI Accession Numbers: M60597.1, U78506.1-U78512.1, U80946.1, U80947.1) and mRNA for glucocorticoid receptor α (X03225.1), and mRNA for glucocorticoid receptor β (X03348.1). By this *in silico* approach, we managed to find five variants in the coding sequences that cause amino acid changes: Met646Thr, Leu647Pro, Ser651Tyr, Ile701Leu and Phe715Cys.

Structure-function analysis of glucocorticoid receptor mutations

We studied the mutations occurring in the ligand-binding domain (LBD) of the glucocorticoid receptor in detail. The mutations could be classified into several groups based on the location in the LBD structure. Some mutations affect residues in direct contact with the ligand (Met646Thr, Ile747Met and Leu753Phe). Other mutations affect residues of the hydrophobic core surrounding the LBD (Ile559Asp, Leu647Pro and Val729Ile). Two mutations may disturb the secondary structure and may affect overall folding and stability of LBD (Leu647Pro and Gly679Ser). The last group of mutations contains amino acids exposed to the surface of receptor (Asp641Val, Ser651Tyr, and Phe715Cys).

Mutations that affect residues in direct contact with ligand

Ligand binding is stabilized by extensive hydrophilic and hydrophobic interactions between the ligand and protein. Hydrophobic residue is in close vicinity to nearly every atom of the steroid core of dexamethasone. There are 23 residues in the van der Walls contact with ligand in the glucocorticoid receptor. These residues serve accurate definition of ligand recognition and specificity. In addition to hydrophobic

interactions, an extensive hydrogen bond network contributes to the ligand binding.

Met646Thr

This mutation was found by our *in silico* approach. The Met646 is in direct contact with the ligand. A smaller amino acid, such as threonine, would allow binding of the ligand, but very likely would not contribute to the tight binding of dexamethasone. The Met646Thr variant is noteworthy, since the corresponding amino acid in the rat glucocorticoid receptor gene (Met664) was previously studied by *in vitro* point mutagenesis in a yeast expression system. The receptor with Thr646 was strongly activated by dexamethasone, triamcinolone acetonide and deacylcortivazol, but in contrast to its wild-type counterpart it failed to respond to the glucocorticoid receptor agonist RU28362 and deoxycorticosterone.

Ile747Met

Vottero et al. described this mutation in a family with glucocorticoid resistance. Considering the van der Waals cutoff distance of 4.0 Angstrom, the side chain of Ile747 makes close contacts with dexamethasone as well as with seven residues in the protein chain (Trp557, Met560, Thr561, Asn564, Thr739, Met745 and Phe749). These residues are located in H3 and the

loop region between H11 and H12. In the case of the Met747, a change in the size of the side chain would have a large impact not only on the ligand binding but also would disturb the structural change during the activation. The mutated receptor shows a two-fold reduction of ligand binding and a 20 to 30-fold reduction in transcription activity on the glucocorticoid-responsive mouse mammary tumor virus promoter due to abnormal interactions with p160 steroid receptor coactivators.

Leu753Phe

The Leu753Phe mutation has been described in a human leukemic cell line isolated from a specimen which showed reduced responses to attempts of corticosteroid therapy. This mutation has been confirmed to occur in the patient from whom the cell line was isolated. The Leu753 is a member of the H12 helix. Agonist binding induces a conformational change of the AF-2 region, stabilizing the receptor in an active conformation to facilitate its association with coactivator proteins, such as steroid receptor coactivator-1 (SRC-1) and transcriptional intermediary factor 2 (TIF2). During activation, Leu753 is buried inside a small hydrophobic cavity. This hydrophobic cavity is made up of the side chains of Val571, Trp600 and Pro750 and the backbone carbonyl of Asn564 and Gly567. Interestingly, Leu753 also makes a direct contact with

the ligand. These interactions result in the stabilization of the AF-2 region in the active conformation. In the case of mutant Phe753, the side chain of phenylalanine would not fit correctly into the cavity. The larger side chain of Phe753 physically prevents H12 helix from adopting the characteristic agonistic position over the ligand-binding pocket. Since the receptor cannot form a stable and active conformation during ligand binding, it results in an increased dissociation rate of the hormone and reduced transactivation capability.

Mutations that affect the amino acids of the hydrophobic core which surround the ligand binding pocket

The ligand binding is stabilized by extensive hydrophilic and hydrophobic interactions between the ligand and the protein. The ligand-binding cavity is buried within the hydrophobic core of the LBD. The hydrophobic binding pocket is formed not only by the residues with direct contact with the ligand but also by the hydrophobic core surrounding these residues. These residues are very important in the correct formation of the binding pocket. In this group we studied the following mutations: Ile559Asp, Val729Ile and Leu647Pro. These mutations have different effect in receptor structure and function. The consequences of the Val729Ile mutation cannot

be easily explained by the structure, isoleucine is larger than valine only by a methyl group. This extra methyl group may disturb the tightly packed structure by locally rearranging the binding pocket and by reducing the affinity of ligand binding. In the case of Ile559Asp mutation the tight hydrophobic packing are disrupted with the charged side chain of Asp559. This mutation induces a conformational change of the ligand binding pocket, causing receptor inactivation and glucocorticoid resistance syndrome in the patients. The Leu647Pro mutation was found by our *in silico* approach. The Leu647 is a member of helix 7 and the hydrophobic core, which surrounds the ligand-binding pocket. In the mutant structure proline cannot form a regular α-helix since the proline residue breaks the helix structure. Accordingly this mutation would cause complete inactivation of the receptor.

Mutations in other parts of the receptor

The analysis of the role of these mutations is more difficult. The Gly679Ser mutation disturbs the turn region between the β strand 2 and helix 9. In the Asp641Val mutation, valine would be unfavorable in intensive hydrophilic environment of the receptor surface and would, therefore, disturb the local structure of the receptor. The Ser651Tyr mutation leads to a large, lipophilic residue exposed on the surface of the receptor.

However there are additional 12 Tyr residues in the receptor structure that are almost all exposed to the surface. The Ile701Leu and Phe715Cys variants were found by our *in silico* approach in the same germline nucleotide sequence. The side chains of these amino acids seem to face each other and they are involved in the stabilization of the tightly packed H9 and H10. Although the Leu701 and Cys715 may fit into this surround, the modified environment is less tightly packed, and the undistributed space would reduce the local and overall stability of the LBD. We cannot predict the structural and functional effects of these mutations exactly, since they are far away from the ligand binding pocket and other functional structural elements.

Glucocorticoid receptor β isoform

In earlier studies characterization of the genomic structure of the human glucocorticoid receptor gene suggested that alternative splicing of exons 9a and 9b was the mechanism responsible for generating the two receptor isoforms, named glucocorticoid receptor α and β, containing different carboxyl termini. Both isoforms share the same amino acid sequence through amino acid 727 but diverge beyond this position with glucocorticoid receptor α having an additional 50 amino acids and with glucocorticoid receptor β harboring an additional 15

non-homologous amino acids. In the case of the β isoform, the missing part of the receptor would make important interactions with the ligand, with the coactivator and with other part of the receptor. Residues in the helix 11 (Leu732, Tyr735, Cys736 and Thr739), helix 12 (AF-2 helix) (Leu753) and in the loop between them (Ile747 and Phe749) are in direct contact with the ligand. The Glu755 clamps the C-terminal ends of the coactivator. Following the H12 helix there is an extended strand that forms a conserved sheet which has an important role in receptor activation by stabilizing the H12 helix in the active conformation. The β isoform misses these important amino acids (727-777) whose functions include ligand binding, coactivator binding and receptor stabilization. Surprisingly, the β isoform does not lack the ability to fold into a native protein. As a result, the β isoform keeps the dimerization, DNA binding, and nuclear translocation functions. DeRijk et al. described an association between the β variant and rheumatoid arthritis. As the β isoform is supposed to repress the glucocorticoid receptor α activity, the increased expression of the β isoform may result in glucocorticoid resistance.

Structure-function analysis of a novel mutation of the CYP17

We present the clinical history, hormonal findings, and mutational analysis of two patients from unrelated families, who were diagnosed as having congenital 17α-hydroxylase/17,20-lyase deficiency by hormonal screening studies. Nucleotide sequence analysis of peripheral blood DNA obtained from the two patients showed a novel homozygous mutation (Arg440Cys) of the CYP17. To demonstrate that the Arg440Cys mutation causes 17α-hydroxylase/17,20-lyase deficiency, RNA samples were isolated from adrenocortical tissue obtained from one patient during operation of an adrenal tumor, and these RNA samples were used to generate cDNA clones for expression studies. DNA sequencing of the recombinant clone demonstrated the presence of a change from C to T at nucleotide position 1359, which corresponded to the novel mutation at amino acid position 440 documented in our two patients. The presence of wild-type CYP17 sequence was also confirmed in the mutagenized recombinant clone, which was used as control in subsequent expression experiments.

COS-1 cells transfected with vectors expressing either the wild-type or mutant P450c17 enzyme showed a clear immunostaining with a monoclonal anti-V5 antibody. Using

the same monoclonal antibody the proportion of transfected cells was determined quantitatively by flow cytometric analysis, and these studies indicated that 39.5% of the cells were transfected with the V5 protein. In addition, Western immunoblotting indicated that COS-1 cells transfected with vectors expressing the wild-type or mutant P450c17 enzyme produced comparable amounts of V5 protein, whereas cells not transfected had no V5 protein.

To determine 17α-hydroxylase and 17,20-lyase activities of COS-1 cells transfected with vectors expressing the wild-type or mutant P450c17 enzyme, progesterone or 17-hydroxypregnenolone was added to the incubation medium of the cells. Cells expressing the mutant P450c17 enzyme had negligible 17α-hydroxylase and 17,20-lyase activities, as the conversion of added progesterone to 17-progesterone and the conversion of added 17-hydroxypregnenolone to dehydroepiandrosterone was less than 1 % compared to that found in COS-1 cells expressing the wild-type p450c17.

Three-dimensional computerized modeling of the heme-binding site of the P450c17 enzyme indicated that replacement of Arg by Cys at amino acid position 440 predicts a loss of the catalytic activity of the enzyme. The mutant enzyme containing Cys at position 440 fails to form a hydrogen

bond with the propionate group of heme, which renders the mutant enzyme unable to stabilize the proper position of heme.

Conclusions

An extensive review of reported clinical cases of the glucocorticoid resistance syndrome indicated, that mutations in the glucocorticoid receptor are causative factors in most cases of this inherited disorder. The majority of the reported mutations are located in the ligand-binding domain of the glucocorticoid receptor gene.

By our *in silico* approach, we managed to find novel variants in the coding sequences of the glucocorticoid receptor gene. These variants may be relevant in further clinical studies.

Comparative protein modeling of mutations located in the LBD domain of the glucocorticoid receptor offers a useful means to analyze the functional consequences of these mutant receptor variants. This approach may have a great value in predicting functional abnormalities of novel sequence variants discovered by the *in silico* method.

With the use of hormone measurements we showed that previously undiagnosed congenital 17α-hydroxylase/17,20-lyase deficiency may occur in adult patients with hypertension and hypokalemia. In these patients, both hyperplasia and tumor of the adrenal glands may be present.

We showed that congenital 17α-hydroxylase/17,20-lyase deficiency in two unrelated patients was due to a novel mutation of the CYP17 gene, which resulted in a change of arginine to cysteine at amino acid position 440.

We demonstrated that eukaryotic cells expressing the Arg440Cys mutant P450c17 enzyme have negligible 17α-hydroxylase and 17,20-lyase activities.

We showed that three-dimensional computerized modeling of the heme-binding site of the P450c17 enzyme provides a useful tool for the evaluation of the role of arginine at position 440, and that replacement of arginine by cystein predicts a loss of the catalytic activity of the enzyme. Arginine at position 440 is a member of the electron-transfer pathway from FAD to heme and it participates in proper positioning of both the heme and the substrate.

List of publications closely related to the Ph.D. thesis

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