Studies on intracellular localization and some human mutations of Ncb5or flavoheme reductase

PhD thesis outline

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Introduction

The endoplasmic reticulum (ER) as a real metabolic compartment responding to changes of extra- and intracellular environment and modulating functions of the whole cell has an outstanding significance in the maintenance of intracellular homeostasis. Due to the selective permeability of the ER membrane, the lumen has a special milieu differing from the cytoplasm, characterised by distinct state and connections of its redox systems and a several orders of magnitude higher calcium concentration. Luminal redox systems in the ER have major importance in the metabolic sensor function of the organelle. Besides the thiol oxidizing environment of the lumen, the locally maintained reduced pyridine nucleotide pool is indispensable for intraluminal reactions. The high [NADPH]:[$NADP^+$] ratio is ensured by the cooperation of H6PD and G6PT. The produced NADPH pool is used by the 11βHSD1, which catalyzes reduction of cortisone to active cortisol in glucocorticoid sensitive organs.

Our laboratory contributed to the understanding of the physiological and pathological functions of the G6PT-H6PD-11βHSD1 triad by several findings. Long-term nutritional surplus increases the amount of G6P and F6P, which enhances luminal NADPH production and hence it can stimulate prereceptor cortisol activation, which takes part in development of the metabolic syndrome and diabetes. However, lipotoxic effect of saturated fatty acids also plays an important role in the pathomechanism of metabolic disorders in obesity. Endogen pathways, which are able to decrease fatty-acyl-CoA oversupply are currently in focus of research. The first step of unsaturated fatty acid synthesis is catalysed by the ER-membrane-bound stearoyl-CoA desaturase 1 (SCD1), which contributes to the synthesis of complex lipids by maintaining a balanced saturated/unsaturated fatty acyl-CoA ratio. Fatty acyl-CoA desaturases of human and animal cells function as terminal members of an electron transfer chain, which receives electrons from cytosolic NAD(P)H through the flavoprotein cytochrome b_5 reductase (b5R) and the hemoprotein cytochrome b_5 (b5).

The recently discovered NAD(P)H cytochrome b_5 oxidoreductase (Ncb5or) flavohemoprotein got to the focus of our interest because it was reported as a

soluble microsomal oxidoreductase, and it raised the possibility of new connections in ER redox homeostasis. The natural fusion protein contains both microsomal cytochrome b5 and cytochrome b5 reductase homology domains, which indicates its participation in fatty acid desaturation. Both physiological and pathological roles of Ncb5or are highlighted by the knock-out phenotype, which includes a progressive loss of white adipose tissue, an increased fatty acid sensitivity, and diabetes due to a progressive loss of pancreatic β-cells.

Ncb5or was described as an ER localised, soluble oxidoreductase, thus first of all we wanted to examine its possible connections with luminal redox homeostasis. If the enzyme is located on the cytosolic side of ER membrane it would supply fatty acyl-CoA desaturation from the same redox system as b5R and b5 do. However, luminal localization of Ncb5or would raise the possibility of utilizing ER luminal NADPH for acyl-CoA desaturation, thus it would create a direct link between cellular lipid metabolism and prereceptor hormone activation. This finding would reveal a new mechanism for the nutrient sensor function of the ER. However, controversial data have been published regarding the intracellular localization of the protein.

The phenotype of knock-out mice implies that mutations leading to loss of function or decreased activity of Ncb5or protein can be linked to human diabetes as well. There are limited available data about the relationship between diabetes and mutations in *NCB5OR*. A minor correlation has been found in a human genetic analysis between polymorphism of *NCB5OR* gene and type 2 diabetes. One of the studied variants showed significant association with type 2 diabetes in a case-control study. In this report, only intronic variations were analysed with two exceptions, and they were not involved in the pathogenesis of the disease. However examination of further, particularly exonic mutations can lead to important findings.

Objectives

Regarding the biological function of the recently discovered (Ncb5or) natural fusion protein, several questions are still unanswered. First of all, it is essential to elucidate the intracellular localization of the flavohem reductase enzyme.

It is of great importance to clarify which side of the ER membrane the likely microsomal oxidoreductase is located on. The ER localization of the protein raises the possibility that fatty acyl-CoA desaturation might consume luminal NAD(P)H, which would imply that it potentially interacts with the prereceptor cortisol production as well.

Both the observed β-cell death and diabetes in Ncb5or-null mouse suggests the putative role of human *NCB5OR* mutations in diabetes. Despite the obvious relationship between the lack of Ncb5or protein and the development of diabetes in animal models, very limited information is available on the possible role of human gene variants – especially exonic mutations – in the disease.

The aims of our study were the followings:

- Clarification of the intracellular localization of Ncb5or protein.
- Investigation on the effect of natural occurring human Ncb5or missense mutations in an *in vitro* cellular system.

Methods

The *in silico* search for naturally occurring missense mutations of the human *NCB5OR* gene was performed in NCBI SNP and 1000 Genomes databases. The 3D structure of Ncb5or mutants were modelled by I-TASSER online prediction program. The subcellular localization of the protein was analyzed by six different protein prediction programs based on amino acid sequence.

For the expression of Ncb5or and Ncb5or-GFP fusion protein, the enzyme encoding sequence was cloned into pcDNA3.1- and pEGFP-N1 plasmids. The studied missense mutations were generated by overlap extension PCR mutagenesis based on wild type expression plasmids. Wild type and p.E87G and p.E93G variants with Glu-Glu epitope tag were also prepared by the same method. HEK293T and HepG2 cells were transfected to express Ncb5or variants. mRNA levels were assessed by RT-PCR and qPCR using GAPDH as an intrinsic control housekeeping gene.

Proteins of cell lysates were separated by SDS-PAGE and then blotted onto PVDF membranes. Primary and secondary antibodies were applied overnight at 4 °C and for 1 h at room temperature, respectively. Detection was carried out with chemiluminescence. Specific band intensities were evaluated by densitometry using ImageQuant 5.2 software and normalized to β-actin bands.

Homogenates obtained from HEK293T and HepG2 cells by Dounce homogenizer or rat liver tissue using Potter-Elvehjem were separated by differencial centrifugation. Nuclei were sedimented from the homogenates (1,000× *g*, 10 min, 4 °C), and the supernatant was subjected to repeated centrifugations of increasing force. The pellet was removed each time and yielded the fractions of mitochondria (11,000× *g*, 20 min, 4 °C) and microsomes (100,000× *g*, 1 h, 4 °C), while the cytosolic fraction was gained as the remaining supernatant. Pellets of subcellular fractions obtained at the centrifuging steps were suspended in PBS. The origin and purity of each subcellular fraction was confirmed by immunoblot using antibodies to organelle-specific marker proteins.

Endogenous reduced pyridine dinucleotide content of intact rat liver microsomes was monitored by fluorimetry. Stearoyl-CoA was administered at concentrations below 100 μM (10 és 50 μM) to avoid membrane permeabilization in these experiments.

Intracellular degradation of wild-type and p.E87G mutant Ncb5or protein was investigated by pulse-chase method. Cells were incubated in methionine- and cysteine-free media and pulse-labeled with 100 μCi ^{35}S-TransLabel. After washing with media containing excess unlabeled methionine and cysteine, the cells were lysed at different times (0, 1, 3 and 22 h). The radioactively labeled wild-type and p.E87G Ncb5or proteins were immunoprecipitated and the immune-complexes were recovered with Protein-A Sepharose beads. Immunoprecipitates were separated by SDS-PAGE and then dried gels were exposed to phosphoscreen.

The intracellular expression of Ncb5or-EGFP fusion protein was studied by labeling the nucleus (DAPI) and the ER (BODIPY^{TM} TR-X thapsigargin) using fluorescence microscope. For the investigation ont he subcellular localization of endogenous Ncb5or by immuncytochemistry, HepG2 cells were grown on microscope slides. Then, cells were fixed and incubated with mouse monoclonal antibody against human Ncb5or and rabbit polyclonal antibody against PDI. Alexa Fluor 488 donkey anti-mouse IgG and Alexa Fluor 568 goat anti-rabbit IgG fluorescent secondary antibodies were used.

Data were compared using the Tukey-Kramer multiple comparison test and GraphPad InStat program. Differences with a P value below 0.01 or 0.001 were considered to be statistically significant.

Results

Subcellular localization of Ncb5or protein

In the first part of our study, we wished to analyze the intracellular localization of the protein. *In silico* peptide analysis using various tools unequivocally predicted cytosolic placement of Ncb5or as it possesses neither ER targeting nor retention sequence. This presiction was verified experimentally by different ways:

- The localization of endogenously expressed Ncb5or was assessed in two cell lines (HEK293T and HepG2) and in rat liver tissue. Cells and the tissue samples were homogenized to separate the subcellular fractions by differential centrifugation. Ncb5or and specific marker proteins of various cellular organelles were detected by Western blot. **Ncb5or protein could be detected only in the cytosolic fractions of both of the cultured cells and of rat livers.**

- To study the protein location *in situ* by fluorescent microscopy, the vector construct for eukaryotic expression of green fluorescent Ncb5or-EGFP fusion protein was prepared and then GFP-tagged Ncb5or was expressed in transiently transfected human HEK293T cells. The fusion protein was efficiently expressed in transfected cells and the translated Ncb5or-EGFP polypeptide retained its stability. The green fluorescence signal was diffuse in the cytoplasm, and it did not co-localize with fluorescent staining of either the nuclei or the ER on the microscopic images. **Distribution of the exogenous EGFP-labeled Ncb5or protein also demonstrated a cytosolic localization.**

- **Immunocytochemistry revealed the cytosolic localization of endogenous and native Ncb5or.** Specific fluorescent signals for Ncb5or were seen to be disseminated in the cytosol. Images obtained by using the Nomarski DIC technique clearly showed that the fluorescent signal is homogenously distributed within the cell borders, revealing the cell processes, and it did not co-localize with the ER labeling.

- The possible effect of stearoyl-CoA on the luminal pyridine dinucleotide redox state was checked in rat liver microsomal vesicles. We found that fluorescence was not altered by repeated addition of stearoyl-CoA, i.e. **the luminal NAD(P)H level remained unaffected by the substrate of microsomal stearoyl-CoA desaturase.**

Investigation on natural human NCB5OR missense mutations by molecular biology methods

We have studied the effect of natural exonic missense mutations on the expression of human Ncb5or:

- Five non-synonymous coding variants of Ncb5or (p.E87G, p.E93G, p.E118A, p.R140H, p.N249S) were selected by *in silico* search in NCBI SNP and 1000 Genomes databases. Comparison of the expression of wild-type and mutant proteins revealed alteration in the protein levels in two cases that could not be explained by differences in mRNA levels. **Amounts of p.E87G and p.E93G variants were 10 and 5 times lower than the wild-type Ncb5or, respectively.** Ncb5or variants were also expressed with Glu-Glu epitope tag in both HEK293T and HepG2 cells. The amount of tagged proteins showed the same pattern as the corresponding tag-free ones.

- Effect of altered size and/or charge of the side chain on the expression of Ncb5or protein was further studied by generating and investigating E/Q mutants, which contain Glu to Gln mutations at the same positions, i.e. replecement of Glu^{87} or Glu^{93} residue with uncharged Gln of similar size. Both mutations reduced the intracellular levels of Ncb5or significantly, although less efficiently than the corresponding Glu to Gly mutations. These data indicate that **loss of negative charge on its own is not fully responsible for the remarkably decreased protein levels of the two natural Ncb5or mutants.**

- The intracellular degradation of wild-type, p.E87G and p.E93G Ncb5or proteins was monitored by inhibition of translation and determination of the half-life of proteins. The amount of wild-type Ncb5or protein decreased slowly, and remained above 70% of the initial value by the end of a 4 h long cycloheximide treatment. In contrast, only 10 and 20% of Glu-Gly mutant proteins remained detectable. **We concluded that the two natural mutations in human *NCB5OR* gene resulted in enhanced intracellular degradation. The protein half-life of the p.E87G mutant was indeed 10 times lower (1,5 h) than that of the wild-type Ncb5or protein (14,2 h).**

- **Proteasome inhibitors prevented the rapid intracellular degradation of p.E87G and p.E93G Ncb5or mutants**, which suggests the role of accelerated proteasomal degradation of these natural variants.

- The generated (artificial) double mutant (p.E87G_p.E93G) Ncb5or protein was expressed at even lower level than the single mutants, its detectable amount was only 7% of the wild-type. Intracellular degradation of the protein containing two amino acid replacements was more rapid than that of the natural mutants containing only a single aminoacid replacement, i.e. **the two mutations were synergistic in decreasing protein expression**. While inhibition of proteasomal degradation by lactacystin completely prevented the reduction of double mutant Ncb5or protein levels, the other proteasome inhibitor MG132 only partially protected against enhanced proteasomal degradation.

Conclusions

In this study, we aimed to clarify the subcellular localization of human Ncb5or protein and also wanted to investigate whether known exonic missense mutations in human *NCB5OR* gene would cause decreased protein levels, which are likely associated with the development of diabetes.

Our results led to the following conclusions:

1. Ncb5or protein does not contain any ER targeting or retention signals in its amino acid sequence. Both endogenous and exogenous proteins were detected in the cytosol tested by various experimental methods, and neither of them showed co-localization with ER marker proteins. We concluded that Ncb5or is a cytosolic protein and it does not have a strong and permanent connection with the ER network.

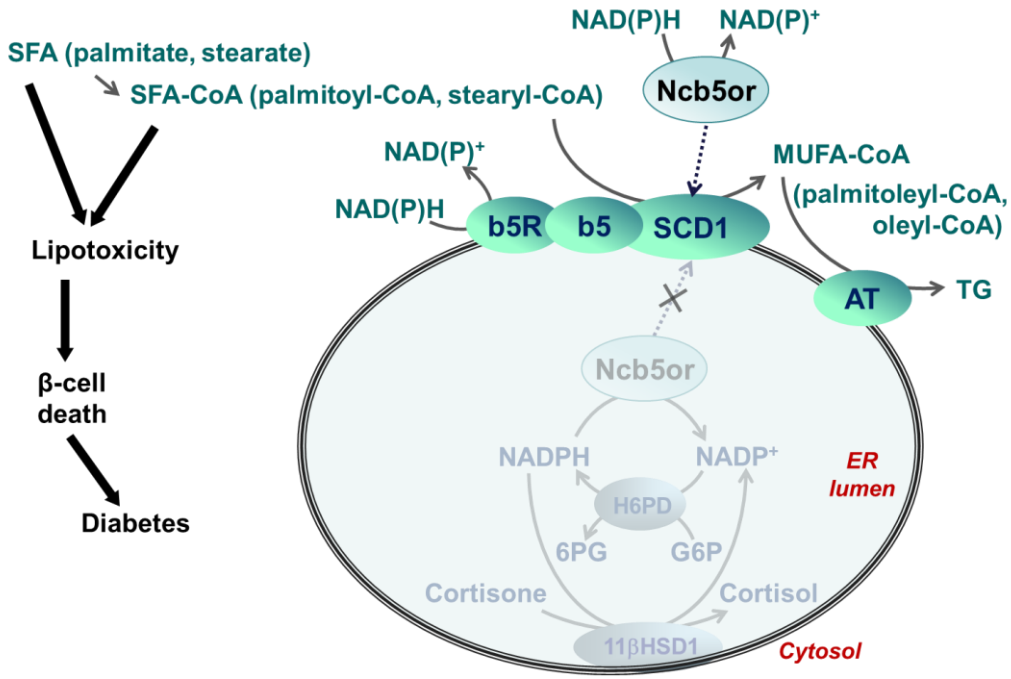

Figure 1. Ncb5or protein might serve as an alternative electron supplier of SCD1 from the cytosol

2. Stearyl-CoA addition to liver microsomes did not affect the intraluminal NAD(P)H pool, thus in accordance with the subcellular localization of Ncb5or, the SCD enzyme functioning on the cytosolic side of ER membrane is not able to utilize ER luminal pyridine dinucleotides as

electron sources. This data suggests that Ncb5or does not create a functional link between fatty acid desaturation and prereceptorial cortisol activation (Figure 1).

3. Naturally occurring missense mutations in *NCB5OR* such as p.E87G and p.E93G caused a significant decrease in the protein levels in the examined human cell lines. Based on the phenotype of knock-out mice, this finding can imply the potential role of these two mutations in development of human diabetes.

4. p.E87G and p.E93G amino acid replacements in human Ncb5or remarkably and synergistically shortened the intracellular half-life of the expressed proteins, which could be effectively prevented by proteasome inhibitors. This result indicates that these naturally occurring mutations enhance proteasomal degradation of the mutant proteins due to possible polipeptide destabilization (Figure 2.).

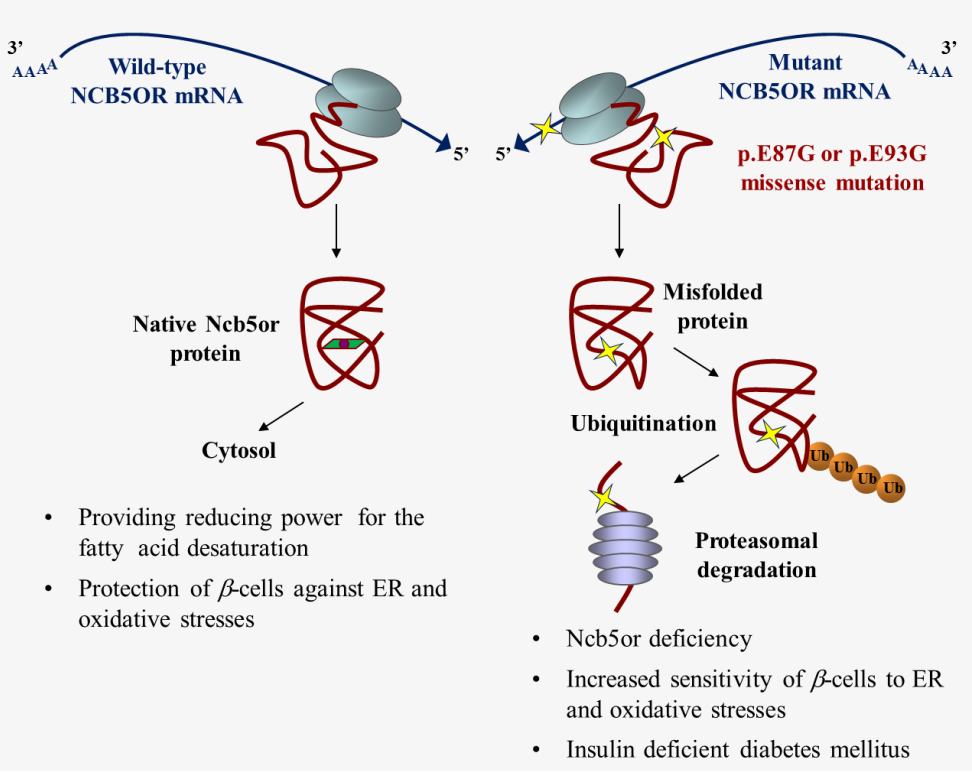

Figure 2. Assumed *in vivo* consequences of missense mutations, which shorten the half-life of Ncb5or protein

5. Examining stability of natural p.E87G and p.E93G mutants and artificially introduced p.E87Q and p.E93Q amino acid replacements suggested that the negative charge and hydrophilic nature of the two conserved Glu residues are indispensable factors in maintaining stable protein structure with long half-life. We found correlation between the predicted alteration in the b5-like domain of the protein and the extent of decreased protein levels. These data indicate that the conformation destabilizing effect of missense mutations in E87 and E93 residues are due to structural distortion at the hem-binding region of the b5-like domain.

Our observations concerning the newly discovered ER oxidoreductase answered the original questions in our aims; however they also raise new problems in the field. Further investigations have to clarify why the cell needs an alternative electron transfer pathway of fatty acyl-CoA desaturation once it uses the same cellular NAD(P)H pool as electron source as the already known classic components. We have to examine the nature of factors that induce or increase the participation of Ncb5or in fatty acid desaturation and to investigate which other redox processes the enzyme might be involved. It is also to be analysed how certain mutations influence the structure of the protein's unique hem-binding region, and how the possible changes in the affinity to the prosthetic group contributes to the accelerated degradation of the polypeptide.

Abbreviations:

11βHSD1: type 1 isoenzyme of 11β-hydroxysteroid dehydrogenase, **G6PD**: glucose-6-phosphate dehydrogenase, **G6PT**: glucose-6-phosphate-transporter, **H6PD**: hexose-6-phosphate dehydrogenase, **GAPDH**: glyceraldehyde 3-phosphate dehydrogenase, **DAPI**: 4,6-diamidino-2-phenylindole, **SDS-PAGE**: sodium dodecyl sulfate polyacrylamide gel electropforesis, **GFP**: green fluorescent protein, **Gln/Q**: glutamine, **Glu/E**: glutamate, **Gly/G**: glycine, **DIC**: differential interference contrast

Publications

Publications related to doctoral thesis:

1. Kalman FS, Lizak B, Nagy SK, Meszaros T, **Zambo V**, Mandl J, Csala M, Kereszturi E. (2013) Natural mutations lead to enhanced proteasomal degradation of human Ncb5or, a novel flavoheme reductase. Biochimie, 95(7):1403-1410. IF: 3.123

2. **Zambo V**, Toth M, Schlachter K, Szelenyi P, Sarnyai F, Lotz G, Csala M, Kereszturi E. (2016) Cytosolic localization of NADH cytochrome b(5) oxidoreductase (Ncb5or). FEBS Lett, 590(5):661-671. IF: 3.623

Publications not related to doctoral thesis:

1. **Zambo V**, Simon-Szabo L, Szelenyi P, Kereszturi E, Banhegyi G, Csala M. (2013) Lipotoxicity in the liver. World J Hepatol, 5(10):550-557.

2. Polgar N, Csongei V, Szabo M, **Zambo V**, Melegh BI, Sumegi K, Nagy G, Tulassay Z, Melegh B. (2012) Investigation of JAK2, STAT3 and CCR6 polymorphisms and their gene-gene interactions in inflammatory bowel disease. Int J Immunogenet, 39(3):247-252. IF: 1.355