Introduction

1. Background: The metabolic syndrome is characterized by abnormalities in carbohydrate and lipid metabolism and affects one fourth of the population in developed and developing countries. Although symptoms (obesity, high triglyceride and serum cholesterol concentration, insulin resistance) and consequences (cardiovascular diseases, diabetes) of the syndrome are well-known, neither the pathomechanism, nor the proper treatment has been resolved. The candidate protein of the disease is the Stearoyl-CoA desaturase 1; the rate limiting enzyme in unsaturated fatty acid synthesis. Based on our hypothesis, the reducing force needed for desaturation is provided by *Ncb5or*, a recently described, functionally poorly characterized flavoheme reductase.

2. Preliminary results: Wild type and mutant *Ncb5or* proteins (p.E87G (rs28675051), p.E93G (re11539439), p.E118A (rs11539440), p.R140H (rs61762820) and p.N249S (rs13194584)) were successfully overexpressed in human cell cultures by transient transfections, mRNA and protein levels were followed by qPCR and immunoblots. Although the mRNA expression of the mutants were similar, the protein levels of two natural variants (p.E87G and p. E93G) were significantly reduced compared to wild type. The increased intracellular degradation of these Gly-to-Glu mutants was proved by using proteasome inhibitors, which abolished the difference in protein levels.

Results of first year

1. Analysis of naturally occurring Scd1 variants: In case of *Scd1* p.H125P (rs35602244), p.M224L (rs2234970), p.A333T (rs1054412), p.Tins/del (rs36110241) and p.Cins/del (rs36045940) mutations were subcloned into pcDNA3.1- vector. The intracellular expression of natural variants were tested in transiently transfected cell lines. Two mutants (p.H125P, p.A333T) showed significantly lower protein expression, since the level of p.M224L version was slightly elevated. As expected, the two frame shift mutations could not be detected. To find out the reason of the observed expressional pattern further research is needed.

2. Generation of Glu-Glu epitope tagged NCB5OR and SCD1 variants: The pcDNA3.1-based and Glu-Glu tagged forms of NCB5OR and SCD1 constructs were also prepared by overlap extension PCR. This glutamate rich tag on the C-terminal of the proteins was useful in easy detection with immunoblot and it was a powerful tool in the discrimination between the endogenously and transiently expressed proteins. In addition, the Glu-Glu tag provided identical immunoreactivity for the different proteins, which was highly required in the expressional comparison of the mutants. All vectorial constructs were tested in cell culture system. The Glu-Glu tagged vectors showed the same intracellular protein expressional pattern either in case of *Ncb5or* and its mutants or *Scd1* and its variants fully confirming the results described in chapter 1.

3. Further analysis of p.E87G and p.E93G Ncb5or natural variants: The remarkable decrease in p.E87G and p.E93G protein levels despite only a moderate difference between the wild type and mutant mRNA expressions suggested the role of accelerated protein elimination. Intracellular degradation of wild type and mutant *Ncb5or* molecules was compared by using Cycloheximide, a cell-permeable inhibitor of translation. Transiently transfected HEK293 cells were harvested for *Ncb5or* immunoblot analysis 0, 1, 2, or 4 h after translation arrest. The gradually decreasing band densities revealed a progressive degradation of all the three protein variants as expected; however, the time

courses were remarkably different. The amount of wild type *Ncb5or* protein decreased slowly during the 4 h long Cycloheximide treatment, and remained above 70% of the initial value. In contrast, both Glu-Gly mutant proteins were degraded rapidly with half-lives below 1 h; and the amounts of p.E87G and p.E93G decreased to the limit of detection after 4 h translation arrest. Elimination of either Glu side chain (i.e. replacement with Gly) proved to diminish the intracellular level of *Ncb5or*. Contribution of the loss of negative charge was studied by checking the effect of Glu to Gln mutations at positions 87 and 93 (p.E87Q and p.E93Q) on protein expression in transiently transfected HEK293 cells. Both mutations reduced the intracellular levels of *Ncb5or* significantly although less efficiently than the corresponding Glu-to-Gly mutations.

4. Analysis of double mutant of NCB5OR: Effect of the two Glu-to-Gly mutations on Ncb5or protein expression was further studied by generating and investigating the p.E87G_p.E93G double mutant. Nearly equal NCB5OR mRNA levels were detected in the wild type and mutant transfected HEK293 cells by RT-PCR or qPCR further supporting that these mutations do not affect the expression at this level. However, the double mutant protein was barely detectable in the cell lysates, which indicates that the two mutations act synergistically. The combined mutation remarkably shortened the half-life of Ncb5or protein beyond the effect of single mutations as revealed by the assessment of intracellular degradation after Cycloheximide treatment. Inhibition of proteasomal degradation by Lactacystin completely prevented the reduction of double mutant *Ncb5or* protein levels in accordance with our findings with each single mutation.

5. Generation of pEU based vectorial constructs of NCB5OR and SCD1: The wild type and mutant *Ncb5or* and *Scd1* proteins were also subcloned into pEU3 vectorial background with a ligase independent cloning method. This vectorial background can be used in a cell free *in vitro* translational system producing pure protein solutions. The glutation S-transferase and histidine tagged versions of both target proteins were generated and confirmed by sequencing.

6. In vitro translation of Ncb5or and Scd1 proteins: The *in vitro* translation of His and GST tagged versions of *Ncb5or* was easily elaborated because of the soluble character of this protein. In contrast, *Scd1* is a membrane anchored protein, hence its production needed more optimization. However, we were able to produce *Scd1 in vitro*, the protein was precipitated and formed aggregates in a membrane free translational system. To solve this problem an artificial, soybean based liposome generating method was elaborated and optimized. In the presence of liposomes, the *Scd1* was already translated and folded in a native manner. All together, at this point we possess both *Ncb5or* and *Scd1* protein preparations in a pure and native form, which are ready for further *in vitro* protein-protein interaction analysis.

Results of second year

The results of second year are in agreement with the workplan of OTKA Grant (104113).

1. In silico prediction of Ncb5or subcellular localization: The amino acid sequence of human Ncb5or was analyzed by six online localization predictors. None of the applied tools revealed signal peptide or ER-retention signal in the polypeptide. Although the possibility of mitochondrial or nuclear localization was also speculated as a second option, the most likely topology of *Ncb5or* protein was unequivocally predicted to be cytoplasmic.

2. Generation and characterization of Ncb5or-EGFP fusion protein expression vector: Ncb5or-EGFP fusion protein expressing vector was generating using pEGFP-N1 as a host plasmid for further *in vitro* and *in vivo* localization analysis. Prior to fluorescence microscopy, the expression, stability and intactness of Ncb5or-EGFP was tested using gel electrophoresis and autofluorescence detection as well as immunoblot with anti-Ncb5or primary antibody. HEK293T cells were transiently transfected with pcDNA3.1-_NCB5OR, pEGFP_NCB5OR or empty pEGFP-N1 vectors to express endogenous human cb5or, Ncb5or-EGFP fusion protein or EGFP, respectively. Fluorescent signals of EGFP and Ncb5or-EGFP were clearly visible at the appropriate molecular weights in polyacrylamide gels after separation. Most importantly, the 27 kDa band corresponding to EGFP alone was not detectable in the samples prepared from Ncb5or-EGFP-expressing cells, which indicates the lack of significant intracellular breakage of the tagged protein.

3. Analysis of intracellular localization of Ncb5or in cell lines and rat liver tissue: After having verified that pEGFP-N1_NCB5OR-transfected cells are free of interfering protein degradation products, and EGFP-specific fluorescence corresponds to Ncb5or-EGFP, these cells were examined by fluorescence microscopy. Green autofluorescence was observed in more than 80 out of 100 investigated cells, i.e. the transfection efficiency was above 80%. The signal was diffuse in the cytoplasm, and it did not co-localize with fluorescent staining of either the nuclei (DAPI) or the ER (BODIPY TR-X thapsigargin).

Intracellular localization of *Ncb5or* was further analyzed by detecting the endogenously expressed protein in subcellular fractions prepared by differential centrifugation from HEK293T and HepG2 cells as well as from rat livers. Proteins of well-defined localization and regarded as characteristic to different cellular compartments or organelles were employed as markers specific to each fraction in our experiments. The markers of nuclei (CREB-1 and Lamin A/C), ER (PDI) and cytosol (Procaspase-3 and GAPDH) were detected only in the appropriate samples, while some contamination of nuclear fractions with mitochondria was indicated by the presence of mitochondrial markers (Bcl-XL or Cyclophilin D) in both lanes. This, however, did not disturb the evaluation of our assay as Ncb5or protein could be detected only in the cytosolic fractions of both of the cultured cells and of rat livers.

4. Immuncytochemistry of endogeous Ncb5or in HepG2 cells: To avoid the drawbacks of exogenous over-expression and peptide tagging that might affect intracellular protein targeting, while taking advantage of *in situ* analysis, endogenously expressed naive Ncb5or was immunolabeled and localized by fluorescence microscopy in untreated and untransfected HepG2 cells.The specific signal for Ncb5or was seen to be disseminated in the cytosol, showing a clearly different pattern compared to the ER labeling. In certain cells, Ncb5or seemed to be localized in the processes of the cytoplasm as well, while others lacked the Ncb5or protein in their cytoplasmic extensions. Accumulation of Ncb5or at the nuclear border was also observed in a few cells. Nevertheless, the protein did not show obvious co-localization with either the nuclear staining or the ER labeling.

5. Analysis of heme binding capacity of rapidly degrading Ncb5or variants: Based on 3D structural prediction, E87G and E93G natural variants can have a negative effect on the heme binding pocket of *Ncb5or* protein. The peroxidase based protocol, generally used in literature, was not proved specific for heme binding proteins. We elaborated and optimized a new fluorescence quenching based method highly specific for heme bindig

proteins using *in vitro* translated positive controls (such as globin and cytocrome c). For analyzing *Ncb5or*, wild type, E87G, E93G and the double mutant were subcloned into pEU3 vector proper for *in vitro* translation. To obtain reliable negative control, heme binding cysteins were mutated and H89A, H112A and H89A_H112A variants were generated and *in vitro* translated. As a preliminary result, the double His mutant was not able to bind heme as we expected, furthermore, the natural variants showed similar poor heme binding capacity. It suggests, that the rapid intracellular degradation of *Ncb5or* natural variants is caused by their insufficient heme binding related loss of function.

6. Elaboration of enzyme activity measurement protocol for Scd1 desaturase enzyme: HEK293T cells were transiently transfected by pcDNA3.1-_NCB5OR or pcDNA3.1-_SCD1 vectors. After 24 hour of transfection 50 μM BSA conjugated palmitic acid was add. 15 hour after palmitic acid treatment, cells were harvested, measured, suspended in MeOH. The fatty acid profile of samples was analyzed by GC-FID (Gas Chromatography-Flame Ionization Detector). As a preliminary result, the ratio of saturated acids to unsaturated ones was significantly shifted in SCD1 transfected cells compatred to un- or mock transfected samples. *Ncb5or* overexpression, however, had no effect on fatty acid profile under these experimental conditions.

Results of third year

1. The protective effect of Ncb5or in lipotoxicity. *i. Effect of free fatty acids on Ncb5or and Scd1 expression:* HEK293T cells were treated by high dosage (500 μM) of saturated fatty acids (palmitic acid). 12 hours after treatment cells were harvested and endogenous Ncb5or and Scd1 gene and protein expressions were analyzed. We demonstrated by qPCR that palmitic acid caused significant mRNA expression increment in both genes. This effect was confirmed on protein level by immunoblot. Saturated fatty acid exposure triggered Ncb5or and Scd1 expression both in gene an protein expression levels. *ii. Role of Ncb5or in saturated- and trans-lipotoxicity:* Ncb5or overexpressing HEK293T cells were treated with high dose (500 μM) of saturated (palmitic- and stearic acid) and unsaturated (palmitoleic- and oleic acid) fatty acid pairs. The viability of HEK293T cells was quantified by MTT test. While, the viability of saturated fatty acid treated samples was merely one fifth compared to controls, the cell death was completely abolished by Ncb5or overexpression in case of unsaturated fatty acid treatment. Furthermore, in palmitic- and stearic acid treated cells the endoplasmic reticulum stress, measured by eiF2α phosphorilation, also was significantly diminished.

2. Analysis of physical or functional connection between Ncb5or and Scd1: *i. Co-immunopecipitation of Ncb5or and Scd1:* The GST-tagged version of Ncb5or was subcloned and transfected into HEK293T cell line. Despite the suitable Ncb5or-GST fusion protein expression, the detection of direct connection between the two proteins was unsuccessful. *ii. Functional connection of Ncb5or and Scd1 in fatty acid desaturation:* Ncb5or is a soluble natural fusion protein of a cytochrome b5-like and cytochrome b5 reductase-like domain in the cytosol (as we demonstrated previously). Because of its nature, this protein can be a good candidate for supplementary reducing force for fatty acid desaturation. To investigate this hypothesis, we used GC-FID method described above. The fatty acid profile were measured in control and Scd1 or Ncb5or transfected cells. The ratio of unsaturated (UFA) and saturated fatty acids (SFA) was two times higher in Scd1 overexpressing samples comparing control cells, indicating that the

transfected desaturase is functional. This increment in UFA/SFA ration was doubled when the experiment was supplemented with palmitic acid treatment. However, Ncb5or – alone or Scd1 co-trasfected manner – had no effect on fatty acid profile in this experimental setup. Nevertheless, the possibility of adequate UFA/SFA measurement by GC-FID enabled us further Scd1 characterization.

3. The effect of endoplasmic reticulum stress on Ncb5or and Scd1 expression*: i. The effect of ER stressors:* The high amount of fatty acids in cells can trigger endoplasmic reticulum stress. To analyze Ncb5or and Scd1 expression under stressful environment in HEK293T cells, beside palmitic acid, stress was induced by chemical and oxidative stressors as well. While the majority of stressors (palmitic acid, tunicamycin, menadione) caused Ncb5or protein increment, there was no change detected in mRNS levels. In case of Scd1, the same expressional pattern was observed. However, one ER stressor (thapsigargin) caused no protein elevation (Ncb5or), what is more, caused protein level decrement (Scd1) still without mRNA level change. This notable finding raised the possibility of ER-stress inducing microRNA based modulation of protein translation or mRNA degradation. *ii. The role of microRNAs in modulation of Ncb5or and Scd1 expression:* miRanda and miRWalk databases were used for microRNA selection. Eight microRNSs were selected, two of them were predicted to modify Ncb5or level and six of them were predicted to regulate Scd1 expression. These miRNSs are expressed ER-stress dependent manner and contained solid Ncb5or or Scd1 complementer binding seed sequences, respectively. To analyze the effect of lipotoxicity and ER-stress on the expression of these microRNAs, HEK293T cells were treated by palmitic acid or thapsigargin. RNAs were detected by qPCR. As a result of palmitic acid treatment, one (125b-5p) out of the two Ncb5or related microRNAs showed remarkably diminished expression. The same effect was detected when thapsigargin was used. In case of Scd1 related microRNAs, three (24-3p, 346, 708-5p) out of six microRNAs showed slight but significant decrease in their expression.

Presentation of scientific results

The results of this project were presented in six posters at international and Hungarian conferences. The results were also published in four international scientific papers (Biochimie IF: 3.142; Biofactors IF: 3.088; World J Hepatology IF: 0; FEBS Lett. IF: 3.169).