REPORT

On the Project NKFI K106060

„Role of fatty acid desaturating enzymes in the pathomechanism of diabetes mellitus"

Background

Increased level of plasma free fatty acids (FFA) plays an important role in the development of type 2 diabetes (T2D). Excessive amounts of FFA cause lipotoxicity or even lipoapoptosis with the underlying inflammatory signaling, oxidative and endoplasmic reticulum (ER) stress, ceramide accumulation and triglyceride deposition in various cells. Lipotoxicity causes insulin resistance, β-cell dysfunction and β-cell death, hallmarks of T2D. Saturated fatty acids, such as palmitate are more toxic than the unsaturated ones (e.g. oleate). Moreover, the unsaturated ones often reduce the toxicity of saturated fatty acids. It became evident that acyl-CoA desaturase enzymes play a protective role against lipotoxicity by maintaining a balanced supply of saturated and unsaturated fatty acids. Δ9 Stearoyl-CoA desaturases (SCD1 or SCD5 in human) are integral proteins of the ER membrane, and they receive electrons from cytosolic NAD(P)H via cytochrome b5 (b5) and b5 reductase (b5R), another two ER membrane proteins.

NADH cytochrome b5 oxidoreductase (Ncb5or) was discovered on the basis of homology with both b5R and b5 redox proteins. The assumption that Ncb5or also contributes to fatty acid desaturation is strengthened by the progressive loss of white adipose tissue observed in Ncb5or-null mice. Ncb5or-deficient β-cells are more sensitive to palmitate-induced lipotoxicity, to oxidative stress in general and more susceptible to streptozotocin-induced death, which suggests that the novel enzyme bears additional as yet unresolved functions in the maintenance and control of cellular redox homeostasis.

Our earlier studies proved the central role of ER redox homeostasis in nutrient sensing and consequently in obesity-related human diseases, such as T2D. Conversion of the prohormone, cortisone to cortisol is the concerted action of 11β-hydroxysteroid dehydrogenase type 1 (11βHSD1) and hexose 6-phosphate dehydrogenase (H6PD), and it requires the reduced state of ER luminal NADPH. Inhibition of luminal cortisol production or even enhancement of cortisol oxidation is considered as a promising novel strategy for the treatment of metabolic syndrome and T2D.

Acyl-CoA desaturation and ER redox homeostasis – subcellular localization of Ncb5or

Ncb5or is a particulate protein, which had been reported to be localized in the ER. However, it remained to be elucidated whether it is loosely associated to the cytosolic surface of the ER membrane or it is emplaced within the ER lumen. The NADPH pool of the organelle is separated from the cytosol and serves mostly the prereceptor cortisol synthesis. Therefore, the possible luminal topology of Ncb5or would create an intriguing functional link between acyl-CoA desaturation and the control of glucocorticoid action in various hormone target cells.

In order to establish an experimental tool, which allows us to induce an oxidative shift in the ER lumen, we investigated the effect of EGCG a known anti-obesity and anti-diabetic agent of unclarified mechanisms of action on the pre-receptor glucocorticoid production in liver microsomes [Szelényi P et al. BIOFACTORS 39(5): 534-541, 2013]. Our results proved that EGCG is suitable for oxidizing the ER

luminal NADPH pool to $NADP^+$ without causing lipid peroxidation or directly affecting the activity of proteins involved in cortisol production. These findings do not only help us to understand the beneficial health effects of tea flavanols but also highlights the ER luminal pyridine redox system as a promising new drug target in metabolic disorders. Modulation of NADPH:$NADP^+$ ratio by glucose 6-phosphate, fructose 6-phosphate, cortisol, cortisone, methyrapone, or EGCG provides us with the opportunity to investigate the potential relationship between acyl-CoA desaturation and pre-receptor cortisol synthesis through Ncb5or activity.

Then we aimed to clarify the ambiguous compartmentation of Ncb5or and test the possible effect of stearoyl-CoA on microsomal NADPH level [Zámbó V et al. FEBS Lett, 590(5): 661-671, 2016]. Elucidated amino acid sequence of human Ncb5or was analyzed by computational tools to identify any ER-targeting signals and to predict the subcellular localization of the protein. None of the applied tools revealed signal peptide or ER-retention signal in the polypeptide, and the most likely topology of Ncb5or protein was unequivocally predicted to be cytoplasmic.

HEK293T cells were transiently transfected to express Ncb5or-EGFP fusion protein, and these cells were examined by flurescence microscopy. Green autofluorescence signal was diffuse in the cytoplasm, and it did not co-localize with fluorescent staining of either the nuclei or the ER. Intracellular localization of Ncb5or was further analyzed by detecting the endogenously expressed protein in subcellular fractions prepared by differential centrifugation from HEK293 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. Ncb5or protein could be detected only in the cytosolic fractions of both of the cultured cells and of rat livers. 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.

Although these data did not support the assumption that Ncb5or might create a link between ER-luminal pyridine nucleotides and acyl-CoA desaturation in the membrane, the possible effect of stearoyl-CoA on the luminal redox state was checked in rat liver microsomal vesicles. The well-established method based on continuous fluorimetric monitoring of intrinsic luminal NADH and NADPH is suitable to reveal redox shifting inside the vesicles. Oxidation was clearly detected upon the addition of metyrapone or cortisone (substrates of 11βHSD1) and their effect could be antagonized by glucose-6-phosphate (a substrate of H6PD). However, 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. Our results unequivocally demonstrated the cytosolic rather than ER luminal localization of exogenous or endogenous Ncb5or in cultured cells and liver tissue. Utilization of ER luminal reducing equivalents in acyl-CoA desaturation was also contradicted. It remains to be elucidated, how and in which conditions Ncb5or participates in fatty acid desaturation as an alternative cytosolic electron supplier. This activity requires the protein to directly interact with

integral membrane proteins of the ER. It is, therefore, likely that Ncb5or can be associated transiently to the cytosolic surface of the organelle. Further study is needed to reveal the contribution of this enzyme to the protection of pancreatic β-cells against lipotoxicity.

The applied method to monitor ER luminal NAD(P)H levels is widely used and accepted. However, the research on ER redox homeostasis would clearly benefit from the development of more accurate and reliable analytical approaches to quantitate the individual pyridine dinucleotides (NAD^+, $NADP^+$, NADH and NADPH) in the organelle. Such an innovation is hindered mostly by the particularly small volume:surface ratio of the ER, and thus the appropriate methodology is still to be established [Somogyi A et al., PERIOD POLYTECH CHEM ENG 60(4): 218-230, 2016].

Studies on natural missense mutations of human Ncb5or

Ncb5or-null mice develop insulin-dependent diabetes at age 4-6 weeks. It is, therefore, intriguing whether polymorphisms of NCB5OR gene have any relationship with human pathology. We investigated the effect of natural missense mutations on the expression of human NCB5OR gene [Kalman SF et al., BIOCHIMIE 95(7): 1403-1410, 2013].

In silico search in NCBI SNP and in 1000 Genomes databases yielded five non-synonymous coding variants of Ncb5or: p.E87G (rs28675051), p.E93G (rs11539439), p.E118A (rs11539440), p.R140H (rs61762820) and p.N249S (rs13194584). The two Glu to Gly mutations in positions 87 and 93 are located in the third exon while p.E118A, p.R140H and p.N249S variants can be found in the fourth, fifth and tenth exons, respectively.

HEK293 cells were transiently transfected to express wild type and mutant human Ncb5or variants. Real-time Quantitative PCR showed a 170-300 fold elevation in NCB5OR mRNA level in all transfected cells compared to the endogenous expression. Endogenous Ncb5or protein was barely detectable in the control cells, and a massive overexpression of the wild type protein was revealed by immunoblot. Three of the mutants were present at essentially the same level as the wild type, while the amounts of p.E87G and p.E93G variants were 10 and 5 times lower, respectively. The phenomenon was further verified by using a C terminal Glu-Glu epitope tag and in HepG2 cells. Artificial Glu to Gln mutations at positions 87 and 93 (p.E87Q and p.E93Q) reduced the intracellular levels of Ncb5or significantly although less efficiently than the corresponding Glu to Gly mutations. The artificial p.E87G_p.E93G double mutant protein was barely detectable in the cell lysates; its amount was only 7% of that of the wild type, which indicates that the two mutations act synergistically.

Intracellular degradation of wild type and mutant Ncb5or molecules was compared by using Cycloheximide, a cell-permeable inhibitor of translation as well as by pulse-labeling and immunoprecipitation in transfected HEK293 cells. The results indicated a largely accelerated degradation of mutant Ncb5or. Analysis of wild type and p.E87G mutant protein samples revealed half-lives of 14.2 h and 1.5 h, respectively. Treatment of transfected HEK293 cells with cell-permeable proteasome inhibitors MG132 or Lactacystin abolished the difference in protein levels, i.e. wild-type and mutant Ncb5or variants were expressed at similar extent.

Our results support the importance of Glu87 and Glu93 in the stabilization of the native structure of human Ncb5or. The investigated natural or artificial replacements of these residues promote proteasomal degradation presumably through distortion of polypeptide conformation in the vicinity

of heme-ligating residues of the b5 domain. Our findings provide insight into the factors determining the spherical arrangement in the unique heme-binding environment of this protein. Most importantly, this study provides first in vitro evidence for natural mutations, which lead to decreased human Ncb5or protein levels. Since Ncb5or deficiency causes insulin-dependent diabetes in animal models, and human Ncb5or mutations are linked with diabetes and breast cancer, the possible role of these mutations in human pathology deserves further investigation.

Protection of β-cells against lipotoxicity and lipoapoptosis

Lipotoxicity can lead to β-cell dysfunction and even to reduced β-cell mass due to enhanced apoptosis. Reduction of lipotoxicity induced ER stress, with special emphasis on JNK activation and serine phosphorylation of IRS-1, in pancreatic β-cells is a promising antidiabetic strategy. One of the aims of the ongoing research is to identify potential β-cell-protective agents that efficiently prevent lipotoxicity and/or lipoapoptosis in these cells.

Metformin, a widely used insulin sensitizer has been reported recently to prevent ER stress induced apoptosis in a mouse β-cell line. We examined whether attenuation of the ER stress response might play a role in the β-cell protection by metformin in lipotoxicity [Simon-Szabo L et al. PLOS ONE 9(6): e97868, 2014]. To this end, palmitate-induced lipotoxic ER stress and lipoapoptosis were assessed in RINm5F rat insulinoma cells in the presence or absence of metformin. Treatment of RINm5F cells with 500 μM palmitate for 6 h nearly tripled the apoptotic index compared to control untreated cells. Metformin (10 μM or 100 μM) alone did not affect the apoptotic index; nevertheless it completely abolished the pro-apoptotic activity of palmitate when administered simultaneously, i.e. the apoptotic index was reduced to the level of untreated control cells.

ER chaperones (GRP78 and PDI) and the pro-apoptotic transcription factor CHOP were largely induced, caspase-3 cleavage and the phosphorylation eIF2α were enhanced in the palmitate-treated cells indicative of a massive ER stress. Activation of the most intriguing IRE1 pathway was marked by XBP-1 mRNA splicing and phosphorylation of JNK, IRE-1 and c-Jun. All these effects were efficiently, although to various extents counteracted by simultaneous addition of metformin. Therefore, our findings revealed a significant reduction in several palmitate-induced UPR events by metformin. Most importantly, the observed decrease in lipoapoptosis can be, at least partly, due to the interference of metformin with lipotoxic JNK activation, IRS-1 serine phosphorylation and CHOP induction.

The established a cellular model of β-cell lipotoxicity has been applied to test novel kinase inhibitors capable to reduce IRS-1 Ser307 phosphorylation for the therapy of T2D [Simon-Szabo L et al. Bioorg Med Chem Lett 26(2):424-428, 2015]. Two compound families of JNK inhibition potential were identified in Vichem's EVL library to be druglike and suitable for optimization. In addition to six reference compounds, 46 novel derivatives were also synthesized and investigated. They were pre-filtered in HEK293 cells treated with the direct JNK-activator, anisomycin and using BI-78D3, a known inhibitor of JNK activation. There was a correlation between the phosphorylation of JNK, c-Jun and IRS-1 Ser307, and 12 inhibitors were selected for further investigation on the basis of inclusion criteria. Palmitate-induced JNK, c-Jun and IRS-1 Ser307 phosphorylations were also assessed in RINm5F cells in the presence of the selected compounds. Although the signaling mechanism that leads to IRS-1 Ser307 phosphorylation in palmitate-treated insulinoma cells is more complex, the effects observed in this model are much more relevant with respect to diabetes pathology and treatment. A remarkable reduction in lipotoxicity-induced IRS-1 Ser307 phosphorylation was observed in case of 5 compounds,

4 of which also decreased JNK phosphorylation at statistically significant, yet smaller extent. It seems likely that these molecules act by inhibiting other protein kinases involved in IRS-1 phosphorylation beside JNK. The development process yielded a narrow group of novel structures with excellent inhibitory effect on IRS-1 Ser307 phosphorylation, opening the opportunity for further drug development.

Functional relationship between Ncb5or and acyl-CoA desaturases

We also tried to collect experimental evidence for the involvement of Ncb5or in the activity of acyl-CoA desaturases. To this end, HEK293T cells were transfected with SCD1 or Ncb5or or both, and the fatty acid profile of the cells was assessed by GC-FID or GC-MS analysis with special regard on the alterations of the ratio of saturated:unsaturated fatty acids. In accordance with our expectation, overexpression of SCD1 caused a marked decrease in the ratio, which indicated an increase in the desaturation of fatty acids. However, overexpression of Ncb5or, either on its own or in combination with SCD1 did not alter the ratio significantly. It is a possible interpretation of these findings that Ncb5or does not cooperate with SCD1. However, it is also plausible that the electron supply of SCD1 is abundant and it is not rate limiting, so the presence or absence of Ncb5or does not make a difference. Therefore, we are continuing these experiments by testing the effect of b5 and b5R overexpression, and also by assessing the effect of Ncb5or when the expression of b5 or b5R is silenced.