New regulatory mechanisms in lipid metabolism: from polymorphisms of triglyceride uptake systems to mitochondrial stability

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Ph.D. Thesis

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Introduction

Cardiovascular diseases (CVD) are a major cause of death worldwide. The recent genome-wide association studies (GWAS) revealed genetic polymorphisms associated with blood lipid level changes. Nowadays, special attention gained on metabolic consequences, including triglyceride level increases, confirming risk for cardiovascular diseases, metabolic syndrome or for cerebrovascular diseases, especially stroke events.

While the exact reasons behind CVD are unknown several studies have established that elevated triglyceride (TG) levels affected TG metabolism, are independent risk factors for CVD. Thus, research of the TG level modifier factors, especially genetic susceptibility variants, may have clinical importance. These factors include, amongst others, ANGPTL3, CILP2, TRIB1, MLXIPL, GALNT2, GCKR and APOA5 genes. As a prominent example, the functional role of *APOA5* polymorphisms have already been widely investigated. Several of them are associated with elevated TG levels and higher risks for ischemic stroke and cardio- or cerebrovascular diseases or for metabolic syndrome. Recently, other TG modifying polymorphisms came into focus, which may also have role in development of different diseases. Some variants of these are mentioned in connection with increased, while others with decreased triglyceride levels. The elevated levels of certain TGs may have a higher risk for several vascular diseases, moreover significant associations between TG-elevating and polymorphisms were confirmed.

The intracellular fatty acids are catabolized predominantly in the mitochondria. Long chain fatty acids are transported to the mitochondrial matrix space by the carnitine acyl-transferase system, and on the end forming intramitochondrial long chain of fatty-acyl-CoA which is converted to acetyl-CoA by the mitochondrial beta-oxidation system. Free fatty acids besides contributing to ATP synthesis also cause serious stress to various tissues and can contribute to the development of cellular stress. Fatty acids contribute to intracellular reactive oxygen species (ROS) production in a significant extent in the mitochondria. Oxidative stress induced by palmitate can initiate Ca^{2+} release from the endoplasmic reticulum (ER) leading to ER stress and further ROS production. Elevated Ca^{2+} and ROS can initiate mitochondrial permeability transition causes superoxide production and the activation of mitochondrial apoptosis pathway. This vicious lipotoxicity pathway can lead to β-cell failure and insulin resistance and to diabetic complications.

Agents (UCP-1, uncouplers) lowering mitochondrial membrane potential ($\Delta\Psi$) and antioxidants (superoxide dismutase, N-acetylcysteine, lipoic acid) can prevent glucose-induced activation of PKC which leads to diabetic complications.

These observations among other data show the importance of mitochondrial reactive oxygen species production in the development of diabetes. Unfortunately antioxidants therapy fails in human studies for those compounds which are providing excellent protections in cell culture and animal studies. Therefore, it would be very advantageous to find molecules which are not antioxidant in the sense that they would not react with ROS, but to find molecules which bind to mitochondrial proteins, and so they can prevent, or highly reduces the mitochondrial ROS production at the respiratory complexes which are the major source of mitochondrial ROS.

BGP-15, a O-(3-pyperidino-2-hydroxy-1-propyl) pyridine-3-carboxylic acid amidoxime monohydrochloride has a wide range of cytoprotective effects. However, up to now has not been identify any clear intracellular targets for BGP-15. In diseases models BGP-15 prevented cell death, reduced oxidative stress (lipid peroxidation and protein oxidation), activated heat shock protein (HSP) expression. In addition, BGP-15 attenuated inflammatory reaction reduced DNA-breaks formation and poly ADP ribose polymerase (PARP) activation, facilitated mitochondrial energy production. Furthermore, BGP-15 decreased the nuclear translocation of apoptosis inducing factor (AIF) from mitochondria, reduced c-Jun N-terminal kinases (JNK) activation, and inhibited the activation of p38 mitogen-activated protein (MAP) kinase. Previous studies showed that BGP-15 is an insulin sensitizer in olanzapine-induced insulin resistance in human phase II studies, and in diabetic insulin resistant patients. Earlier, it was raised that this can be related to the co-inducer effect of BGP-15 on HSP, but no direct effect of BGP-15 on heat shock transcription factor (HSF1) has been shown.

Previous works showed that ROS (including mitochondrial ROS production) produced in diabetes, and lead to the development of insulin resistance. Therefore, it was assumed that BGP-15 attenuates mitochondrial ROS production by binding to Complex I, or Complex III, and so prevent the development of the vicious cycle leading to mitochondrial ROS production and the abnormal activation kinase cascades characteristic to diabetic reprogramming and defective Glut4 translocation to cell surface.

Aims of the Investigations

During my PhD training I was involved in different research profiles performed on cells, in vitro models, animals, and on human samples. In my PhD I summarize two major focus points related to each other.

The first area includes human investigations targeted mainly on the possible functional roles of the triglyceride modifier genes in Roma population samples. This involved the examination of TG level modifier APOA5 polymorphisms in Roma samples as susceptibility factor because it is known, that the Roma population has high prevalence of increased TG levels [42]. Assuming that genetic background also plays a role in their susceptibility for cardiovascular diseases several gene polymorphisms were examined that literature has suggested to play a role in lipid metabolism and in the development of metabolic syndrome and cardio/cerebrovascular diseases. The following polymorphisms were examined: rs12130333 at the ANGPTL3, rs16996148 at the CILP2, rs17321515 at the TRIB1, rs17145738 and rs3812316 of the MLXIPL, rs4846914 at GALNT2, rs1260326, rs780094 residing at the GCKR loci and four APOA5 polymorphisms (rs662799, rs2266788, rs207560 and rs3135506) DNA samples. These samples were genotyped essentially using PCR-RFLP method. The targeted variants could be targets for therapeutic interventions. Often we used our biobank pooled samples as controls, in some experiments we performed also comparisons with huge public biobank and database results as well. In these experiments we often used internationally recognized biobanks; in all experiments we followed the Helsinki declaration for human experiments, and had the appropriate ethics committee approval.

The second part, the mitochondrial stability experiments were part of a long-term, quite complex series of mitochondrial investigations. Keeping in mind the importance of mitochondria in regulating cell death in ROS-related diseases, we investigated whether the protective effects of BGP-15 rely on the preservation of mitochondrial integrity and reduction of mitochondrial ROS production, using different biochemical approaches, as they are detailed under the section “Materials and methods”.

The link between the human and in vitro parts is the possible therapeutic significance of them.

Materials and methods

Study population: The Hungarian and Roma populations used for our study are part of the Biobank at the Department of Medical Genetics, which is part of the Hungarian National Biobank Network (www.biobanks.hu) as well as part of the European Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) (http://bbmri.eu/bbmri/). Sample size determination was based on the preliminary analyses of SNP prevalence. A total of 363 Roma and 404 Hungarian samples were used in the study.

Genetic approaches: For DNA extraction blood was drawn from peripheral vein into 10 ml EDTA containing tubes and DNA was extracted from the white blood cells with routine desalting method. DNA amplification took place with standard PCR methods. This was flowed by RFLP technique in order to detect variations in each specific DNA sequence.

Biochemical materials and methods:

WRL-68 (HeLa derivative), H9c2 (rat heart myoblast) and U-251 MG (human malignant glioblastoma) cells used for the study. The Wistar rats used were sacrificed, their livers were removed, and mitochondria were isolated from the liver homogenates by differential centrifugation, as described in a standard protocol. The membrane potential ($\Delta\Psi$) in isolated rat liver mitochondria was determined by measuring R123 fluorescence upon its release from the mitochondria. Alterations in $\Delta\Psi$ were induced by the addition of BGP-15. Uncoupling was induced using 50 μM 2,4-dinitrophenol Aliquots of the clear supernatant were freeze-dried, and taken up in aqueous formic acid solution (0.1%). Aliquots of the samples were injected into the HPLC-MS system. Liquid chromatographic separation was carried out using a Kinetex. Data analysis was performed using the Thermo Xcalibur software. Ion intensities were determined by matching them to a BGP-15 calibration curve.

The viability of WRL-68 cells after the different treatments were tested by sulforhodamine B (SRB) assay. Absorbance was determined using the GloMax Multi Detection System (Promega, USA). The optical density values were defined as the absorbance of each individual well extracted from the blank value. Intracellular ROS (peroxinitrite, •OH and iron + hydrogen peroxide (H_2O_2)) were determined by using two separate approaches, like fluorescence microscopy and quantitative determination of ROS-evoked fluorescence intensities by a plate reader. Mitochondrial production of reactive oxygen species production was determined by the oxidation of DHR123 (1 μM) to R123, as measured by a fluorescence spectrometer. The

antioxidant capacities of BGP-15 were determined by the chemical oxidation of DHR123 to R123. The mERFP-expressing plasmid was constructed by PCR amplification of the targeted mitochondrial sequence of cytochrome c oxidase subunit VIIIa (COX8A) gene (RZPD). The amplified sequence was then inserted into pDsRed-Monomer-N1 mammalian expression plasmid between the XhoI and HindIII restriction sites. ΔΨ was measured using the ΔΨ specific fluorescent probe. Nikon Eclipse Ti-U fluorescent microscope equipped with a Spot RT3 camera was zsed with a 40x objective lens with epifluorescent illumination. TMRM assay was used to assess aspecific adsorption of the dye, the fluorescence signal was remeasured after addition mitochondrial uncoupling agent carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). The ΔΨ value was calculated based on the difference of fluoresescence signal before and after FCCP-treatment. Cell death was detected by the GloMax Multi Detection System after annexin V-FITC/PI double staining procedure. Then green fluorescence signal (annexin V-FITC) was measured with the GloMax Multi Detection System (excitation wavelength was 490 nm and emission wavelength was 518 nm). The red fluorescence signal (PI) was excited at 525 nm and the evoked emission was measured at 617 nm.

Statistical analysis: Statistical analysis was performed with the program SPSS 18.0 (SPSS Inc, Chicago, IL, USA). All clinical data presented is shown as average ± SEM values.. For statistical analysis of the data PASW statistics 20 software package (SPSS Inc., Chicago IL) was used.

Results

GCKR, MLXIPL, ANGPTL3, CILP2, GALNT2, TRIB1 and APOA5: All allele distribution and allele frequencies of polymorphisms were in Hardy–Weinberg equilibrium both in Roma and in Hungarian individuals. Significant differences were found in allele frequencies for MLXIPL both variants, GALNT2 rs4846914 and ANGPTL3 rs1213033 polymorphisms comparing Roma participants to the Hungarians. The C alleles in rs17145738 and rs3812316 variants of MLXIPL occurred more frequently in Roma population than in Hungarians. The variants rs1213033 of ANGPTL3 and rs4846914 of GALNT2 genes showed lower allele frequency in Roma participants than in Hungarians. No association could be detected between serum triglyceride levels and carrying minor alleles compared with the non-carriers in Roma and Hungarian samples. For rs662799 APOA5 polymorphism we found that the frequency of the G allele was almost three times higher in the Roma population compared to Hungarian

samples (p=0.0001) and almost two times higher than in European population (1000Genomes; p=0.006. Results of rs207560 show the frequency of the T allele in Roma samples was almost double than in those of the Hungarian population (p=0.018). Data of rs3135506 show that the G allele frequency in Roma's was more than two times higher compared to the Hungarian population (p=0.001); however, it does not differ significantly from the European population (1000Genomes; p=0.066). We also analyzed the rs2266788 variant, where we did not find any difference between G allele frequencies of Hungarian and European populations (1000Genomes, HapMap) compared to Roma subjects (p=0.473; 0.062; 0.375).

The plasma triglyceride levels were significantly elevated in the carriers of the risk alleles when compared to non-carriers for all SNPs in both populations. Significantly higher TG levels were found in heterozygous carriers of rs207560, rs3135506 and rs2266788 variants compared to non-carriers in both study groups. Homozygous carriers of rs662799 variant have higher TG levels than non-carriers in Roma subjects. In Hungarians, we did not find any difference in TG levels between homo- or heterozygous carriers and non-carriers. Comparison of the cholesterol levels did not show any difference.

We analyzed associations among the four APOA5 variants in both study groups. Strong correlations were found among rs662799, rs207560 and rs2266788 variants. However, rs3135506 variant did not show significant correlation with other APOA5 variants in Roma samples as well as in Hungarians. Significant correlations were found between all of the APOA5 variants and TGs in both populations. We did not observe any significant correlation between allelic variants and cholesterol levels in both populations. Linkage disequilibrium examination yielded moderate association between the rs2266788 and the rs3135506 variants, likewise between rs207560 and rs3135506 in Hungarian population. In Roma population we found strong association between the rs207560 and the rs3135506 variants. Seven haplotypes were identified in each population. Six of these haplotypes, APOA5*1, APOA5*2, APOA5*3, APOA5*4, APOA5*5 and ht7 were found to occur most frequently in both populations. Significant differences were found in the presence of APOA5*2, APOA5*4, APOA5*5 and ht7 haplotypes between the Roma and Hungarian populations. However, we did not identify differences in the presence of APOA5*1 and APOA5*3 haplotypes between these populations. Ht5 haplotype in Roma and ht4 haplotype in Hungarian population could not be detected.

BGP15:

We found that the BGP-15 possesses a delocalized positive charge, therefore, it is suitable for determining membrane potential-dependent uptake. We measured BGP-15 uptake in both energized and uncoupled mitochondria. The void volume was determined using glucose-6-phosphate, which substance not able to permeate the mitochondrial inner membrane. When incubated in the presence of 50 μM BGP-15 for 10 minutes, the energized mitochondria took up more than 85% of the drug, suggesting that the majority of BGP-15 was taken up in a membrane potential-dependent manner. Complete uncoupling by dinitrophenol significantly decreased BGP-15 uptake. However, even the uncoupled mitochondria were found to bind more BGP-15 than the amount corresponding to the void volume, indicating that BGP-15 interacted with the mitochondrial proteins and/or lipids. Extrapolating this finding to physiological conditions, it is likely that more than 90% of BGP-15 had accumulated in the mitochondria, which raises the possibility that BGP-15 may protect cells via mitochondrial mechanisms.

As mild uncoupling of the mitochondria could be beneficial in insulin resistance, we tested the effect of BGP-15 on $\Delta\psi$ by using a $\Delta\psi$-sensitive dye (R123) in isolated rat liver mitochondria. Treatment by BGP-15 alone resulted in a concentration-dependent decrease in $\Delta\psi$ at millimolar concentrations. The effect on $\Delta\psi$ of submillimolar concentrations of BGP-15 was below the detection limit suggesting that at the 50 μM concentration we have used throughout the study the drug could hardly cause any mitochondrial depolarization.

Under cell culture conditions we analyzed the effect of BGP-15 on $\Delta\psi$ using JC-1, a cell-permeable voltage-sensitive fluorescent mitochondrial dye. JC-1 emits red fluorescence in highly energized mitochondria, while depolarized mitochondria emit green fluorescence (monomer dye). WRL-68 cells were treated with JC-1 dye, after which fluorescent microscopy was performed. In the control and BGP-15-treated cells, fluorescence microscopy showed strong red fluorescence and weak green fluorescence, which indicates a high $\Delta\Psi$ in mitochondria. The addition of H_2O_2 to cells facilitates the depolarization of mitochondria, resulting in weaker red fluorescence and stronger green fluorescence. When H_2O_2 was added to cells in addition to BGP-15, the depolarization of mitochondria was found to be weaker.

Destabilization of the mitochondrial membrane systems has previously been reported to contribute to mitochondrial ROS production, and BGP-15 was shown to protect the mitochondrial membrane system under oxidative stress, we assumed that it could also affect mitochondrial ROS production. To investigate this possibility, WRL-68 cells were transfected with mERFP, green R123 fluorescence was added and as a result of oxidation from non-

fluorescent DHR123, was measured by fluorescence microscopy. The addition of BGP-15 reduced the substantial increase in green fluorescence induced by the H_2O_2 treatment.

We analyzed the effect of BGP-15 on ROS-induced ROS production at concentrations ranging between 1–50 μM under cell culture conditions using a quantitative, plate-reader based method instead of microscopy. BGP-15 had a concentration-dependent inhibitory effect on the ROS-induced ROS production in WRL-68 cells, which was significant even at the 1 μM concentration. In order to show that these observations apply to other cell lines too, we analyzed the effect of BGP-15 on H_2O_2-induced ROS production in H9c2 cardiomyocytes using the same system. BGP-15 decreased the ROS-induced ROS production in H9c2 cardiomyocytes in a concentration-dependent manner.

We wanted to investigate whether BGP-15 could reduce the production of mitochondrial superoxide. We determined H_2O_2-induced ROS production in WRL-68 and H9c2 cells using the mitochondria-targeted redox fluorescent dye MitoSOX instead of DHR123. Essentially, the results were comparable to those we obtained with DHR123. We repeated these experiments in the presence of the mitochondria-targeted antioxidant MitoTEMPO. MitoTEMPO abolished H_2O_2-induced ROS production in all groups indicating mitochondrial localization of the BGP-sensitive ROS production. These data provide evidence that BGP-15 reduced mitochondrial superoxide production in both WRL-68 cells and H9c2 cardiomyocytes.

In order to provide unequivocal evidence for the mitochondrial mechanism underlying the inhibitory effect of BGP-15 on ROS-induced ROS production, we used Percoll gradient-purified mitochondria. We determined the effect of BGP-15 on the oxidation of DHR123 in the presence of glutamate and malate as substrates, with antimycin A for complex III inhibition, and showed the production of ROS in complex I and the complex III cytochrome b region of the respiratory chain. We found that the addition of BGP-15 at concentrations of 10 to 50 μM had a ~50% inhibitory effect on mitochondrial ROS production. These results suggest that the target of BGP-15 was between complex I and the complex III cytochrome b region of the respiratory chain.

BGP-15 was also shown to reduce mitochondrial ROS production in the presence of succinate as a substrate and CN^- as the cytochrome oxidase inhibitor, however, to a much smaller extent. This suggests that BGP-15 affected ROS production mainly via complex I and the cytochrome b part of complex III. These data show that BGP-15 has a specific inhibitory effect on mitochondrial ROS production at the complex I and complex III cytochrome b region of the respiratory chain, which is not an antioxidant effect.

As BGP-15 was shown to protect against H_2O_2-induced mitochondrial damage in addition to reducing mitochondrial ROS production, we analyzed its effects on H_2O_2-induced cell death. We found that, consistent with its aforementioned protective effects, BGP-15 increased cell survival in a concentration-dependent manner. To further investigate the underlying mechanism behind the cytoprotective effect of BGP-15, we determined the proportion of apoptosis and necrosis using annexin V-conjugated fluorescein-isothiocyanate and PI staining. We found that under these conditions, H_2O_2-induced cell death was predominantly necrotic and only approximately 10% of the cells died by apoptosis. BGP-15 significantly reduced both apoptotic and necrotic cell death, which was likely to be a result of mitochondrial protection.

Mitochondria have been reported to play an important role in LPS signaling. We analyzed the effect of BGP-15 on LPS-induced mitochondrial depolarization in the U-251 MG cell line. Mitochondrial depolarization was found to be induced by the addition of 1 μg/mL LPS for 1 hour, as determined by JC-1 staining and fluorescent microscopy. The addition of BGP-15 alone did not affect $\Delta\psi$, but significantly attenuated LPS-induced mitochondrial depolarization in the U-251 MG human malignant glioblastoma cells. We obtained identical results when we assessed $\Delta\Psi$ by using TMRM, another membrane potential sensitive fluorescent dye and a quantitative, plate reader-based method. The data suggest that BGP-15 may play a role in inflammatory processes by a novel mitochondrial mechanism.

Due to the association between mitochondrial depolarization and ROS production, we measured LPS-induced ROS production in the U-251 MG cells. Very low fluorescence intensities were detected in the untreated and BGP-15-treated cells, however, the addition of LPS was found to greatly induce ROS production. BGP-15 reduced the LPS-induced ROS production almost to control levels. Similarly to the H_2O_2-induced ROS production, we wanted to determine the intracellular localization of the LPS-induced ROS. To this end, we repeated the previous experiment using MitoSOX instead of DHR123 and a plate-reader instead of microscopy. The results were comparable to those we obtained with DHR123. We repeated the experiment in the presence of MitoTEMPO. MitoTEMPO abolished LPS-induced ROS production indicating mitochondrial localization of the BGP-sensitive ROS production. These data provide evidence that BGP-15 reduced LPS-induced mitochondrial superoxide production.

Discussion

GCKR, MLXIPL, ANGPTL3, CILP2, GALNT2, TRIB1 and APOA5:

The possible role of serum TGs and total cholesterol in relation to development of several diseases, including cardio-and cerebrovascular diseases, metabolic syndrome and diabetes mellitus are extensively investigated worldwide. In the past decade a spectrum of studies described genetic polymorphisms which have an effect on triglyceride levels, like GCKR and more intensively investigated, the APOA5 variants.

In genome-wide association studies the possible effect of functional variants in GCKR gene in association with hypertriglyceridemia was also investigated.

Variants rs780094 and rs1260326 are the most widely investigated, the last one indirectly affects triglyceride levels, has a role in impaired fasting glycemia, and is a possible risk for type II diabetes mellitus. An association with hepatic fat accumulation along with large VLDL and triglyceride levels was observed.

Several studies found that ANGPTL3 has an effect on lipid metabolism, the protein indirectly inhibits the activity of lipoprotein and other endothelial lipases. The loss-of-function mutations of ANGPTL3 gene causing total ANGPTL3 absence, which shows a high association rate with recessive hypolipidemia.

GWAS studies between the plasma TG-level alterations and MLXIPL locus correlations were found. Moreover, the influence of TG level increase of the major alleles of the rs17145738 and rs3812316 variants in MLXIPL locus were observed.

In recent GWAS studies, the minor G-allele of the rs4846914 intronic variant of the GALNT2 gene was found to associate with increased TG concentrations of the plasma. In a study the association between elevated TG levels and genotypes for MLXIPL rs17145738 variant and for GCKR rs780094 was confirmed, but not for GALNT2 rs4846914 polymorphism.

An association has been found between dyslipidemia and the rs16996148 (near CILP2), rs17321515 (near TRIB1), rs12130333 (near ANGPTL3) variants. In addition, these loci were correlated with the manifestation of cardiovascular diseases.

The CILP2 gene the proteins' relation to lipid metabolism is not well understood. However, in a GWAS study, a TG level reducing role of the rs16996148 variant was confirmed analyzing Caucasian individuals.

The human TRIB1 facilitates the proteosome-dependent protein degradation. In an Asian Malay population, the variant adjacent to the TRIB1 locus (rs17321515) showed a significant

correlation with increased total cholesterol and LDL-cholesterol, and also a higher risk for coronary heart disease and CVD was documented.

Our present findings support the earlier results, the allele frequencies showed significant differences in both MLXIPL variants, GALNT2 rs4846914 and ANGPTL3 rs1213033 polymorphisms comparing Roma individuals to the Hungarians. Analyzing Roma and Hungarian population samples we could not confirm any significant associations between triglycerides levels and minor allele carriers compared with the non-carriers.

We examined the effect of major APOA5 polymorphisms on lipids, especially in TG levels. The result of this study showed that heterozygous carriers of rs2266788; rs3135506; rs207560 variants had higher TG levels than non-carriers. Homozygous carriers of rs662799 variant have higher TG levels than non-carriers in Roma subjects. Combining the homozygous and heterozygous samples we found significantly elevated plasma TG levels in carriers of risk alleles of the APOA5 variants when compared to the non-carriers in both Hungarian and Roma populations. All four APOA5 variants showed correlation to TG levels with or without adjustment factors like age and cholesterol levels.

Earlier studies of susceptibility genetic variants revealed that Roma are a genetically unique population and their genetic constitution can differ from other populations. For rs662799 significantly different risk allele frequencies were detected between Roma and other studied populations. We found, that the risk allele frequencies were significantly higher in Roma than in Hungarian population for rs662799; rs3135506 and rs207560 variants.

The most common APOA5 variants, like rs662799, rs207560, rs2266788 and rs3135506 are in strong linkage disequilibrium and create two major haplotype variants (APOA5*2 and *3). These two haplotypes together with wild type haplotype (APOA5*1-3) constitute approximately 98% of the average population. Five common haplotypes were identified so far (APOA5*1-5). Additional possible haplotypes are also known (ht4, 5, 7) composing only a small portion of the population. Different linkage was found between the rs207560 and rs3135506 in Roma and Hungarian populations. In Roma the linkage between the variants was strong, while in Hungarians moderate, possibly because of the distinct origin of the two populations.

Five haplotypes (APOA5*1, APOA5*2, APOA5*3, APOA5*4 and APOA5*5) are the most extensively studied haplotypes of the gene. Comparison of the prevalence's in Roma and Hungarian populations revealed APOA5*2, APOA5*4 and ht7 haplotypes were significantly prevalent in Roma population, whereas APOA5*5 haplotype was more frequent in Hungarians. Our results suggest that Roma people have higher risk for hypertriglyceridemia and for vascular

events because of increased prevalence of the APOA5 susceptibility alleles. Our findings on APOA5*5 also provide indirect support for APOA5 variant's having a role in the Roma susceptibility to CVD.

BGP15:

As previously mentioned BGP-15 has been shown to have a protective effect in several disease models, including ischemic heart disease, Duchenne muscular dystrophy, neuropathy, cisplatin-induced kidney disease, glivec-induced cardiac disease and paracetamol-induced liver disease, in addition to insulin resistance. Here, oxidative stress and inflammatory processes play a key role in disease progression, and in several cases, mitochondrial damage is essential. This is why we studied the effect of BGP-15 on ROS- or inflammatory response-induced mitochondrial damage in cell culture models, with focus on the effects of BGP-15 on mitochondrial membrane stability and ROS production, which are critical for mitochondrial-induced signaling, energy metabolism and mitochondrial cell death pathways.

Recently, it has been shown that mild mitochondrial uncoupling can be protective in several disease models, including insulin resistance, hypertriglyceridemia and fatty liver disease, and can have a regulatory role in endocrine cross-talk via the induction of fibroblast growth factor 21 and the growth hormone/insulin-like growth factor I axis. However, we saw that BGP-15 exerted only mild uncoupling effect in millimolar concentrations. Since we previously used BGP-15 at a 50 μM concentration, it is unlikely that its uncoupling effect played a significant role in its mitochondria- and cytoprotective effects. But as we saw using two membrane potential sensitive dyes BGP-15 reduced the oxidative stress-induced mitochondrial depolarization by ROS-induced mitochondrial depolarization could result in decreased ATP synthesis, increased superoxide production by the electron-transport chain, release of proapoptotic proteins from the intermembrane space, decreased mitochondrial fusion and increased fission. All of these processes disturb cellular energy metabolism and a lead toward proapoptotic signaling eventually resulting in cell death. Thus, the membrane potential stabilizing effect of BGP-15 likely contributed to its protective effect in the aforementioned diseases.

BGP-15 was also found to be critical in the reduction of ROS- and LPS-induced ROS production, which can play a significant role in the progression of several diseases. Unfortunately, it is difficult to localize the site of ROS production by using conventional mitochondria-targeted redox dyes since oxidation of the dye by extramitochondrial or mitochondrial ROS results in identical fluorescence localized to the mitochondria. For this

reason we used MitoSOX. Under these conditions, the resulting fluorescence is believed to result from oxidation of the dye by mitochondrially produced superoxide. Furthermore, we quenched mitochondrial ROS by the mitochondria-targeted antioxidant MitoTEMPO. All the results supported that BGP-15 reduced mitochondrial ROS production. This effect could not result from antioxidant property of the molecule as it was revealed by our experiments on various cell-free ROS-generating systems. We believe that we localized the most important target of BGP-15, complex I-III, which is critical for ROS production by the respiratory chain. It can generate substantial amounts of ROS under different conditions including hypoxia, mitochondrial hyperpolarization, inhibition of respiratory complexes. The significant ROS-reducing effect of BGP-15 may be important in regulating ROS-dependent processes including cell death, MAPK and PARP pathways, in addition to transcription factor. The high enrichment of BGP-15 in the mitochondria, combined with the significant reduction in mitochondrial ROS production at complex I by BGP-15, suggests that the protective effect of BGP-15 observed in different disease models is likely to be mediated by the previously mentioned mitochondrial mechanisms. Our results also suggest that the effect of BGP-15 on signaling pathways, such as BGP-15-induced reduction in JNK and p38 MAPK activation or BGP-15-induced Akt activation, could also potentially be related to reduced ROS production. LPS induces a complex stress pattern in sensitive cells, including ROS production by NADPH oxidases, an increase in cytoplasmic free calcium level and activation of mitochondria damaging signaling pathways.

We assumed that similar to the oxidative stress situation, BGP-15 would protect against LPS-induced mitochondrial damage and we found that BGP-15 prevented LPS-induced mitochondrial depolarization and ROS production, demonstrating that BGP-15 can protect mitochondria against complex inflammatory damage as well as against ROS-induced damage. These results suggest the potential of BGP-15 as an experimental drug, not only in ROS-related diseases, but also in inflammatory diseases.

The critical mechanism underlying the protective effect of BGP-15 on the mitochondria appear to be due to reduced ROS production, predominantly at the first and third respiratory complexes. By this mechanism, it may regulate several pathways that play critical roles in the progression of ROS-related and inflammatory diseases.

Summary

1. Significant differences were observed in both variants of *MLXIPL*, *GALNT2* rs4846914 and *ANGPTL3* rs1213033 polymorphisms comparing Roma individuals to Hungarians. However, there were no associations between levels of triglycerides and minor allele carriers in Roma and Hungarian population samples.

2. GCKR, CILP2, GALNT2 and TRIB1 loci were not associated with increased triglyceride levels in any population investigated.

3. In APOA5 rs662799, rs2266788, rs207560 and rs3135506 we found elevated plasma triglyceride levels in the risk allele carriers compared to non-carriers in both populations. At least a two-fold significant increase was detected in minor allele frequencies in Roma when compared to Hungarians, except the rs2266788 variant.

4. Haplotype analysis revealed significant increase of APOA5*2, APOA5*4 in Roma, as opposed to the higher levels of APOA5*5 found in Hungarians.

5. Different linkage disequilibrium was found between rs207560 and rs3135506 variants in Roma compared to Hungarians.

6. BGP-15 accumulates in mitochondria and attenuates ROS-induced mitochondrial ROS production.

7. BGP-15 has a protective effect against ROS-induced mitochondrial depolarization and preserves mitochondrial integrity.

8. BGP-15 attenuates bacterial lipopolysaccharide (LPS)-induced collapse of mitochondrial membrane potential and ROS production in LPS-sensitive cells.

9. BGP-15 did not have any antioxidant effects and reduces ROS production mainly at Complex I, and so a novel mitochondrial drug candidate for the prevention of ROS-related and inflammatory disease progression.

Publications

Publications related to thesis:

1. Marked Differences of Haplotype Tagging SNP Distribution, Linkage, and Haplotype Profile of APOA5 Gene in Roma Population Samples.
Sumegi K, Duga B, Melegh BI, Banfai Z, Kovesdi E, Maasz A, Melegh B
PATHOLOGY AND ONCOLOGY RESEARCH &: p. &. (2017)
Impact factor: 1.94

2. BGP-15 Protects against Oxidative Stress- or Lipopolysaccharide-Induced Mitochondrial Destabilization and Reduces Mitochondrial Production of Reactive Oxygen Species.
Sumegi K, Fekete K, Antus C, Debreceni B, Hocsak E, Gallyas F Jr, Sumegi B, Szabo A
PLOS ONE 12:(1) Paper e0169372. 19 p. (2017)
Impact factor: 3.057

3. Functional Variants of Lipid Level Modifier MLXIPL, GCKR, GALNT2, CILP2, ANGPTL3 and TRIB1 Genes in Healthy Roma and Hungarian Populations
Katalin Sumegi, Luca Jaromi, Lili Magyari, Erzsebet Kovesdi, Balazs Duga, Renata Szalai, Anita Maasz, Petra Matyas, Ingrid Janicsek, Bela Melegh
PATHOLOGY AND ONCOLOGY RESEARCH 21:(3) pp. 743-749. (2015)
Impact factor: 1.94

Total impact factors related to thesis: 6.937

Publications non-related to thesis:

1. Activation of mitochondrial fusion provides a new treatment for mitochondria-related diseases
Szabo A, **Sumegi K**, Fekete K, Hocsak E, Debreceni B, Setalo Gy, Kovacs K, Deres L, Kengyel A, Kovacs D, Mandl J, Nyitrai M, Febbraio M, Gallyas F, Sumegi B
BIOCHEMICAL PHARMACOLOGY 150, 86-96 (2018)
Impact factor: 4.235

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