The role of resveratrol treatment and regular physical activity on sirtuins in brain of rats artificially selected for intrinsic aerobic running capacity

PhD thesis

Linda Sárga
(Linda Szűcs)

University of Physical Education
Doctor School of Sport Sciences

Supervisor: Dr. Zsolt Radák, professor, DSc

Official reviewers: Dr. László Virág, professor, DSc
Dr. Anikó Pósa, associate professor, PhD

Chairman of Committee: Dr. Kornél Sipos, professor emeritus, CSc
Members of Committee: Dr. Gábor Pavlik, professor emeritus, DSc
Dr. Péter Osváth, associate professor, PhD
Dr. József Pucsok, professor, DSc

Budapest
2015

Introduction

One of the biggest wishes of mankind is that of eternal life. Although it is not possible, humanity is still seeking new ways to elongate life. We know more and more about cell differentiation, cell proliferation, apoptosis …etc. every day. These processes are built up like a symphony of molecular mechanisms. In the orchestra one of the most important members is DNA, surrounded by histones. Among many functions histones protect the genetic information by making it untouchable for harmful molecules. In these reactions the key regulators are the histone deacetylases. There are four classes of deacetylases in mammals, among them, enzymes in class III are unique ones. They are referred to as silent information regulator two proteins (sirtuins), named after their yeast homologue (sir2). Since their first debut this family of molecules has been quoted many times as promises of a longer life.

Sirtuins are NAD^+-dependent enzymes that are phylogenetically conserved from archeobacteria to humans. Sirtuins have either deacetylase or mono-ADP-ribosyl transferase activity or both. Mammals possess seven types of sirtuins. SIRT1 is the best known member among the sirtuin family. This was the first sirtuin in which the deacetylator function was proved at first on histone targets, later on several protein targets in the cell. SIRT1 is expressed in the adult brain, at high levels in the cortex, hippocampus, cerebellum, and hypothalamus, and at low level in white matter. Among the various cell types of brain, SIRT1 is predominantly, if not exclusively, expressed in neurons. More than 2000 publications are dedicated to the therapeutic potential of sirtuins. A main trend is to activate sirtuins via a natural, dietary way. To start a diet like this, it is suggested to consume several types of fruits, vegetables and nuts. These foods contain high amounts of antioxidants. A huge section of antioxidants are polyphenols.

Polyphenols are naturally occurring phytochemicals which are present within fruits, vegetables and natural products. They can be divided into two main categories - flavonoids and non-flavonoids. Within the non-flavonoids' group a huge category is stilbenes. Resveratrol (3,5,4'-trihydroxy-stilbene), which is the main stilbene, can be found in grapes, wine and peanuts. Although resveratrol accumulates in a low level in the brain there are already publications where its beneficial effects are discussed. It has been shown that SIRT1 is also activated by resveratrol resulting in cell survival, but it is still under investigation if this activation happens in a direct or an indirect way. To sum up the previous facts: sirtuins may have the potential to elongate life. To obtain this it seems to be useful to elevate the level of consumed polyphenols. Although eating antioxidants have several well-documented

advantages, for a healthy body everyone should do one more thing: to have some regular exercise!

Originally we were built up to walk and run tens of miles a day seeking for fruits to collect or animals to hunt. Nowadays in developed countries people take up a huge protein and sugar surplus day by day. On the other hand we forgot about our in-built locomotion needs. Of course there are honorable exceptions for those who practice some kind of regular exercise. Statistically a cleft is about to open up between these two types of people, so in biology these was a need of a new model which can illustrate the aforementioned differences. Lauren G. Koch and Steven L. Britton generated a rat model which is close enough to characterize the biological differences between the two extremities. They undertook a large-scale selective breeding program to develop rat lines that would diverge widely for intrinsic aerobic capacity. Four years later Wisløff et al. characterized the 11th generation of the same model. By this time the low capacity runners (LCR) and high capacity runners (HCR) differed in running capacity by 347%. LCR animals on the other hand started to represent the average person with metabolic syndrome. In 2008 I had the chance to work with male rats from the 22nd generation. I tried to map the differences between the central nervous system of these animals, especially the brain and hippocampus region. Several studies reflect that there are huge benefits of regular exercise in the central nervous system. According to these regular physical activity increases the endurance of cells and tissues to oxidative stress, vascularization, energy metabolism, and neurotrophin synthesis, all important in neurogenesis, memory improvement, and brain plasticity. On the other hand it is also well-known that exercise generates a huge population of reactive oxygen species due to the increased oxygen consumption. It is still under investigation how training has such beneficial effects despite the ROS.

Reactive oxygen species is a general term for molecular oxygen-derived molecules that are reactive species or that are converted easily to reactive species. Many of them are free radicals. Oxygen-derived free radicals are highly reactive chemical species involved in a variety of disorders. They are mostly produced in the mitochondria during the reduction of molecular oxygen to water. Large amount of evidence has shown the important roles of ROS in cell proliferation, homeostasis, intracellular signaling, angiogenesis, and modifications of the extracellular matrix… etc. On the other hand ROS are described as harmful products and capable of DNA mutations, lipid peroxidation and protein oxidation. All these can lead to inflammation and cell death. Neurons are particularly sensitive to oxidative stress; therefore the brain is more vulnerable to reactive oxygen species-induced damage due to its high rate of oxygen consumption and high polyunsaturated lipid content. Prevention is very important

because regular training increases endurance of cells to oxidative stress, vascularization, energy metabolism and neurotrophin synthesis which can be seen via improved memory and brain plasticity. Drugs which have antioxidant property do and will have attention as therapy. Including but not limited to: statins, alkaloids, catapol and the big family of polyphenols. These molecules have the capacity to chelate metal ions and to directly quench free radical species. Unfortunately antioxidants are not enough to avoid every danger which threats our cells. Sometimes the amount of free radicals is too high to deal with and molecules of the cells' get damaged. There have to be mechanisms to repair these damages.

DNA damage occurs in daily life and is aggravated following metabolic and oxidative stresses. Accordingly, DNA repair is essential to maintenance of genomic integrity and cellular viability. Among the four bases of DNA guanine has the lowest redox potential, thus it is prone to oxidation resulting 7,8-dihydro-8-oxoguanine (8-oxoG) formation. This lesion is particularly mutagenic because in addition to its ability to form a Watson-Crick pairing with cytosine, 8-oxoG has the ability to form a stable Hoogsteen pair with adenine. Because of the high mutagenic potential during evolution arose a special enzyme to cut out 8-oxoG. It is 8-oxoguanine DNA glycosylase-1 (OGG1) which catalyzes the first step of base excision repair in the case of an oxidated guanine. OGG1's activity can be modified during post-translational changes: OGG1 is phosphorylated in vitro by CDK4 (Cyclin-dependent kinase 4), resulting in a 2.5-fold increase in the activity of OGG1. On the other hand OGG1 is acetylated by p300 which also increased its activity. The excision activity of OGG1 is quite important because accumulation of 8-oxoG in brain has been implicated in neurodegeneration. It was recently reported that aging results in increased levels of 8-oxoG in the hippocampus, which was associated with decreased level of acetylation of the most powerful repair enzyme of 8-oxoG, OGG1. In contrast to this Stuart et al. proved that OGG1 null mice do not exhibit abnormal phenotype. In 2012 Boldogh et al. published for the first time several lines of evidence that OGG1 is able to bound free 8-oxoG, thus interacting with Ras family GTPases that initiates a signaling cascade.

In this context it might be possible that OGG1 is needed to be deactivated sometimes. To decrease its activity one option is deacetylation, so there might be a connection between OGG1 and SIRT1 deacetylase.

Objectives of the study

The aim of the study was to test how regular exercise can overcome the health risks which occur at metabolic syndrome. An animal model from the Michigan University was ideal for this purpose. As I mentioned in the "Introduction", there is a model in which rat lines were developed that diverge widely for their intrinsic aerobic capacity. This is not the first time when artificial selection was used to investigate such question in exercise. But as we know this is the first time when researchers selected animals for a very long time (more than ten years passed, which is quite long compare to the life-span of rats) for the final goal to determine the genetic components of aerobic capacity. Of course the 22nd generation, which I worked with, is not enough yet to reach that goal, but ideal to get conclusions about the extremities the two rat types typify. From this point of view observations on this model may reveal mechanisms, which can mean new information after all about us.

The laboratory, where most of the experiments were conducted, has a special interest in addition to sport sciences. Since their discovery, sirtuins have stood in a main focus in many of the investigations. No doubt that sirtuins are a very old and conservative protein family which on the other hand is barely known by modern biology. This experiment was the first in this laboratory when we attempted to get information about sirtuins not only in a descriptive way, but we tried to enlarge their effects by administering a well-known activator: resveratrol.

During my PhD years I had the opportunity to spend some time at the University of Texas. In those days I learnt how to work with cell cultures and I could test my hypothesis on cell culture. According to the observations on rat brain we presumed a connection between sirtuins and OGG1 repair enzyme, so my last hypothesis arose from this topic.

Hypotheses:

1. Regular physical activity and resveratrol treatment will enhance the cognitive function of both rat strains.
2. Our aim was to illustrate that the cognitive enhancement was caused via sirtuins and neurotrophic factors in the brain which overall can be seen in neurogenesis.
3. Training and/or resveratrol will compensate the differences which come from the genetic origin of the animals.
4. Sirtuins can deacetylate OGG1 protein and this might moderate its activity.

Materials and methods

Protocols of the animal house

Most of my results are based on the testing of a special type of rats. These Sprague-Dawley rats are artificially selected for intrinsic aerobic endurance running capacity. This selection is based on a large scale breeding program. The protocol for estimation aerobic running capacity required 2 weeks. The first week consisted of introducing each rat to the treadmill. During the second week, each rat was evaluated for maximal endurance running capacity on five consecutive days. Using the criterion of single best day, the 13 lowest and 13 highest capacity rats of each sex were selected from the founder population and randomly paired for mating. At 10 weeks of age the offsprings were introduced to the treadmill and subsequently tested for running capacities as described above. I had the chance to work with 24 low capacity of running (LCR) and 24 high capacity of running (HCR) male rats from the 22^{nd} generation. The rats were arrived in September 2008 and were housed 2 per cage. The first week was taken up with adaptation. All the investigations took 15 weeks and were carried out according to the requirements of the Guiding Principles for Care and Use of Animals in the European Union, approved by the local ethics committee. The first two weeks consisted of teaching the rats how to run on the treadmill. The goal was to run for 10 minutes at a speed 10 m/min on a 5^{o} slope. This amount of exposure to treadmill running is likely below that required to produce a significant change in their aerob capacity.

After the learning period each animal's maximal oxygen uptake was measured with the use of a special rat ergospirometer system (Piston Medical Ltd. Hungary). After 10 minutes of calm and 5 minutes of warm up we increased the speed of the treadmill by 5m/min every 3^{rd} minutes. This measurement was kept until: 1: the rat's VO_2 did not change when speed was increased, 2: the rat could not keep the position on the belt of the treadmill, 3: the respiratory quotient ($RQ= VCO_2/VO_2$) >1. The VO_2 measurement was repeated on every 2^{nd} week and the training was set up according to the VO_2 values. The initial parameters at the training were 10 m/min, 30 minutes, on a 5% slope. Then based on the level of VO_2 max, the speed corresponding to the 60% VO_2 max was determined and used for daily training for 1 hour five times a week.

During the 15 weeks of the procedure the animals treated with resveratrol got 100 mg/body mass kg resveratrol solution per os on every 2^{nd} day. The body weight of the animals was measured every week. The blood sugar of the animals was defined once in every month from a drop of blood which was collected from the tail vein.

The balance and coordination of the rats was also determined using a rotarod test, in which the rodent is placed on a horizontally oriented, rotating cylinder (rod) suspended above a cage floor. The length of time that a given animal stays on this faster and faster rotating rod is a measure of their balance, coordination, physical condition, and motor-planning.

Behavioral tests are meant to measure cognitive ability of rodents. The Novel Object Recognition (NOR) task was used to evaluate cognition, particularly recognition memory, in rodent models. The choice to explore the novel object reflects the use of learning and recognition memory. During habituation, the animals are allowed to explore an empty arena. (It is also called Open field test.) Twenty-four hours after habituation, the animals are exposed to the familiar arena with two identical objects placed at an equal distance. The next day, the rats are allowed to explore the open field in the presence of the familiar object and a novel object to test long-term recognition memory. The time spent exploring each object as well as their discrimination index percentage is recorded.

The Passive Avoidance task is a fear-aggravated test used to evaluate learning and memory. In this test, subjects learn to avoid an environment in which an aversive stimulus (such as a foot-shock) was previously delivered. The animals can freely explore the light and dark compartments of the chamber and a mild foot shock is delivered in one side of the compartment. Animals eventually learn to associate certain properties of the chamber with the foot shock. The latency to pass the gate in order to avoid the stimulus is used as an indicator of learning and memory. We measured short time (after 24 hours) and longtime memory (after 10 days).

In order to detect new cell formation, BrdU was injected into each animal for the last four weeks of the program. At the end of the experiments the animals were sacrificed two days after the last exercise session to avoid the metabolic effects of the final run. Half of the brain was used for histochemistry. From the other half the hippocampus and the frontal lobe was excised and frozen in liquid nitrogen.

Protocols in the laboratory

For protein analysis a piece of frontal lobe tissue was separated according to the followings:

The mass of every tissue piece before thawing was measured, and cold lysis buffer (containing NP40) was added. The protein concentration of the samples was measured according to the Bradford method with a kit (Bio-Rad DC #500-0002).

For western blot assays the resolving gels were between 6-15% and the stacking gel was 10%. Usually 20-50μg protein/well was loaded plus the protein bench mark. For the electrophoresis a Bio-Rad electrophoresis system was used. The blotting to the membrane (PVDF) was made

by Bio-Rad Mini blotting system. After blotting the membranes were blocked between 1-12 hours with 5% nonfat dry milk in 1x TBST. Then the primary antibody was dissolved according to the manufacturers' protocol into 5% milky TBST or 1% bovine serum albumin (BSA) TBST. The membranes were soaked in the primary antibody solutions between 1-12 hours. After the incubation with the primary antibody the membranes were washed and soaked into the secondary antibody solution. After incubation with the secondary antibody the labelled protein bands were revealed with the use of Pierce ECL Western Blotting Substrate. For detection, membranes were exposed to x-ray films. Finally the x-ray films were scanned and the protein densities were quantified using ImageJ. On every membrane β-actin was used as internal control.

SIRT1 activity assay was performed with a CycLex kit (#CY-1151). All the recipes can be found in the kit's manual. For the assay solutions were mixed into the microplate wells: Assay buffer, Fluoro-Deacetylated Peptide, NAD, TSA, my sample or recombinant SIRT1 and finally LEP which initiates the reaction. "No enzyme control", "no NAD control" and positive control (recombinant protein) was used as well. Deacetylase enzyme activity is measured by measuring this fluorescence intensity by a fluorometer. The machine measured the excitation at 340 nm and emission at 440 nm in every 5^{th} minute for 3 hours.

For the PCR measurements half of the hippocampus was used. RNA separation was made with RNA NucleoSpin kit (Macherey-Nagel #740955.50) according to the kit's manual. The cDNA synthesis was made with cDNA Synthesis kit (BIOLINE #BIO-65026) according to the kit's manual. The geNorm Housekeeping Gene Selection kit (Primerdesign #ge-SY-6 rat) was used to determine the appropriate housekeeping gene. According to the guideline reactions were elicited with βAKT, YWHAZ, UBC, ATP5B, CYC1, GAPDH primers. Finally the geNorm analysis was shown that βAKT is a suitable housekeeping gene. Afterwards cDNAs were diluted {20 µl cDNA + 180 µl DEPC-water} and RT-PCR was made with βAKT primer. The cDNAs were diluted to the same concentration, and this was checked via agarose gel electrophoresis. For this and every following RT-PCR reactions 5 µl cDNA, 10 µl ImmoMix (BIOLINE #BIO-25020), 1 µl of the reverse-forward primer mix, 1 µl SYBR green (QIAGEN) and 3 µl DEPC-treated water was used. RT-PCR measurement was performed on Rotor-Gene 6000 real-time system (Corbett Life Sciences) and gene expression levels were determined by delta CT method with the help of the Rotor-Gene software. The cDNAs were diluted to the same concentration, and this was checked via agarose gel electrophoresis. The thermocycling profile conditions used were: 95 °C for 10 minutes, 95 °C for 10 seconds, 60 °C for 15 seconds, 72 °C for 20 seconds. 35 cycles were used in case of each primer. Each run was finished with a melt phase (50-95 °C).

For histochemistry and immunofluorescent measurements half of the brain was fixed with paraformaldehyde (Sigma-Aldrich #47608) embedded into paraffin and cut with microtome into 5 μm slides.

For the detection of neurogenesis the sections were de-paraffinated with xylol, rehydrated with ethanol solutions and washed 3 times with PBS. The sections were needed to be digested in DNase I and for antigen retrieval citrate buffer pH: 6.0 was used. The slides were blocked in normal goat serum and washed 3 times in PBS. Then BrdU primary antibody was added after solved in the blocking solution and incubated overnight at 4^{o}C. Next morning the slides were washed and the secondary Alexa Fluor 546 antibody was applied after solving it in the blocking solution and incubated the slides at room temperature for 30 minutes. After the washing steps I added the anti-Neuronal Nuclei (NeuN) Alexa 488 conjugated antibody and incubated the slides overnight again. Next morning after the washing steps Hoechst 33342 stain was used. Slides were washed twice in DW and mounted with Gel Mount. Microscopy was performed on Zeiss ELYRA Superresolution Microscopy. Colocalization was visualized by superimposition of green, red and blue images using Zeiss LSM Image Browser Version 4.2.0.121 All measurements were done on coded slides, so during the evaluation I was blind to the animal groups.

For the acetylated OGG1 detection Mouse specific HRP/DAB detection kit (Abcam #ab64264) was used. So according to the Abcam protocol the sections were de-paraffinated, rehydrated and washed 3 times with PBS. After the hydrogen peroxide block for antigen retrieval citrate buffer (pH: 6.0) treatment was used then slides were washed 3 times in PBS. Protein block was applied and incubated for 10 minutes at room temperature. Acetylated OGG1 antibody or the OGG1 antibody (affinity purified mouse anti-OGG1 antibody generated against a synthetic peptide, C-DLRQSRHAQEPPAK, representing the C-terminus of OGG1, acquired from Antibodies-Online GmbH) was added and incubated overnight. Next morning after the required washing steps the biotinylated goat anti-mouse IgG was applied. In the next phase the Streptavidin Peroxidase was also added. Finally the DAB chromogen and the substrate were added and the results were improved with a 1 minute Hematoxylin staining. After the tap water washing the slides were mounted with 1:1 glycerol: DW. The density of the AcOGG1 was determined with ImageJ software.

Protocols of the cell culture

For the cell culture experiments I chose HCT116. This is a human colorectal carcinoma cell line, which feeds on McCoy's 5a Modified Medium + 10% fetal bovine serum + 1%

penicillin-streptomycin. The cells were lysed for western blot using 1x RIPA buffer + 1% protein inhibitor cocktail + 2% NaF solution + 10% SDS. The western blot was made as mentioned above. The cells were lysed for PCR studies using RLT buffer (Qiagen RNeasy kit #74104) and the RNA was separated according to the kit's handbook. The cDNA synthesis was similar to the BIOLINE kit, but Invitrogen's SuperScript III (#18080-300) was used this time. The housekeeping gene was GAPDH.

To monitor the SIRT1's deacetylation activity on OGG1 SIRT1 was silenced via siRNA with the help of siSMART pool (Dharmacon #M-094699-01-0005). The cells were transfected using INTERFERin system (Polyplus transfection #409-10), reverse transfection, and 4 groups were made (Without siRNA, Control siRNA, siSIRT1, siSIRT3). The plates were harvested after 48 hours or after 72 hours of the treatment.

I also wanted to check whether the acetylation status of OGG1 changes after treatment of the cells with known SIRT1 activator/inhibitor. So resveratrol (100 μM), nicotinamide (10 mM) and Trichostatin A (100 nM, as HDACI-II inhibitor) was applied on the cells. Then the plates were harvested for western blot analysis.

Statistics

At the beginning the rats were randomly assigned to groups as follows: Control LCR (CL), Trained LCR (TrL), Resveratrol treated LCR (RsvL), Trained and resveratrol treated LCR (TrRsvL), Control HCR (CH), Trained HCR (TrH), Resveratrol treated HCR (RsvH), Trained and resveratrol treated HCR (TrRsvH).

In results: At those data which fit a Gaussian curve (according to a Shapiro Wilk's W-test) the statistical significance was assessed by ANOVA, followed by Tukey's posthoc test. The significance level was set at $p < 0.05$. If the values don't fit the "bell curve" shape the statistical significance was assessed by Mann-Whitney U test. The significance level was also set at $p < 0.05$ everywhere. For statistics Statistica 9.1 software was used. The differences between groups were shown with the help of " ┌──────┐ " sign. The statistically significant differences were marked with the help of " ┌──────┐ " plus a "*" sign.

Results

Results from the animal experiments

At the beginning a main goal was to measure the maximal oxygen uptake capacity (VO_2max) of the rats. These data were used to follow the progression of exercise and of course to adjust the running speed. If anyone compares the results of the first and the last measurements it is easy to recognize that every group which was trained shows development when compared to the untrained ones. As an example TrL reached ~40% better result than CL by the last week. These differences are significant in LCR groups, but only tendencies in HCR groups. It is normal to see the bigger differences in LCR animals, because they had worse fitness, than the HCR ones so they could show greater progression as a result of training.

We checked the body mass of the animals weekly, to track the changes which can come up because of the exercise or the resveratrol treatment. Finally the body mass of TrL group was significantly lower (470 ± 47 g) compared to CL (595 ± 38 g), while the difference was much smaller between CH and TrH (433 ± 21 g vs 403 ± 39 g). LCR groups had significantly higher body mass than corresponding HCR groups. The resveratrol treatment did not change significantly the body weight during the experimental period.

We also measured the blood sugar levels of the animals. Previous studies referred to the fact that low aerobic running capacity is associated with high blood glucose values and insulin resistance among other signs of metabolic syndrome. Animals with low running capacity had higher blood glucose levels than animals with high running capacity. It is also proven that exercise had a meliorating effect on blood sugar especially at the LCR groups. The trained low capacity of running groups had almost as low blood glucose levels as the high capacity of running animals.

Rotarod test was used to map the balance of the animals and check if training or resveratrol has a result on it or not. According to the results resveratrol raised the time the animals could spend on the barrel of the machine. Exercise had the same result but it was not statistically significant. The highest values could be observed at groups which had both of the treatments.

The nervous system is responsible for behavior, and so behavioral analysis is the ultimate assay of neural function. Sensory tests may also be performed on an animal that is removed from its home cage to a new environment, for example an open field cage. Normally an animal in a novel environment ignores food in favor of making exploratory movements.

These movements are quantifiable by counting latency time, line crossing, rearings…etc. The most remarkable difference in latency time was that LCR animals spent more time still when placed into the unknown cage. HCR animals started the exploration of the new environment faster. The exploration rate in the open field test can be calculated from line crossings and rearings which were made by the animals in the test field. HCR groups also reached greater values in exploration of the new cage. This difference is significant in the groups which were treated with resveratrol.

During object recognition in a natural environment the time spent sniffing and examining objects placed in the animal's home or familiar environment is used as the measure of recognition. Rats recognize the old object as familiar so they spend more time discovering the new one. Exercised HCR groups spent the greater time periods at the new object compare to the LCR groups.

Memory is described as being either short-term, to be used only for the moment, or long-term, to be used for long durations. Passive avoidance has been found to be a very sensitive measure of both types of memory. After the learning period (when they had the mild foot shock in the dark chamber), each group spent longer time at the light chamber before entering the dark one. In short term memory test control HCR group had slightly better results than control LCR. It is also remarkable that only resveratrol treated groups reached the highest latency values. By the 10^{th} day of the test the differences became greater between the LCR and HCR animals. In control, exercised and resveratrol treated groups genetic differences seem to stand out. LCR animals had worse long term memory than HCR groups. Only TrRsv gangs had the similar results.

Results from investigation of brain tissue

Animal behavior tests, especially the long term memory results suggested that we should look for cellular and molecular differences between the groups of LCR and HCR animals.

For a start we used immunofluorescence to test the number of the newly produced neurons in the hippocampus' gyrus dentatus region. The new cells were labeled with BrdU staining and NeuN staining helped to count the neurons among the new cells. Resveratrol enhanced the new cell production in the hippocampal region. Training had the same effect but only at the HCR animals. The results show new neurons in the hippocampus, especially after the resveratrol administration. Exercise only caused increasing at HCR rats.

Since the immunofluorescent results showed a difference between the groups we checked the mRNA levels of most common sirtuin molecules.

SIRT1 is a nucleus located protein with deacetylase activity. Resveratrol is a well-known activator of sirtuins. Here resveratrol made no difference compared to the control groups in the level of Sirt1 mRNA. But there were significant differences between RsvL-RsvH.

SIRT3 is a mitochondria located protein with proven deacetylase activity. As far as we know from literature, resveratrol in not a direct activator of SIRT3 enzyme. But we wanted to check this statement and we found no differences between the groups at the Sirt3 mRNA levels.

SIRT4 protein is also located in the mitochondria. Compared to the previous sirtuins it has mainly just ADP-ribosyltransferase activity. We saw no difference in the Sirt4 mRNA levels among the groups. Neither training nor resveratrol changed the Sirt4 mRNA amounts.

SIRT6 is nucleus located and it has mainly just ADP-ribosyltransferase activity as the SIRT4 in the mitochondria. Because SIRT6 is a nuclear protein like SIRT1, it seemed reasonable to check if resveratrol has any effect on the amount of SIRT6 or not. Sirt6 mRNA level showed decrease at the exercised animals compared to the controls. There was also difference among the control groups which could be caused by the different genetic background. Resveratrol did not seem to modify the mRNA levels of Sirt6 in the treated groups.

Of course mRNA results do not show exactly the amount of active proteins in the tissues, so I measured the SIRT1 protein level via western blot. The most remarkable difference was between TrL-TrH. On the other hand resveratrol seemed to elevate slightly the SIRT1 amount in RsvL group but did not show the same elevation in RsvH animals. There can be a big difference between a protein's level and activity, so I measured its activity in an artificial deacetylation reaction with a kit. The activity showed the same result as the western blot, so the activity of SIRT1 in TrH is significantly higher than TrL. Resveratrol also did not seem to change the activity of SIRT1 (neither in low capacity of running nor in high capacity of running animals) according to this measurement.

Sirtuins are NAD^+ dependent deacetylases. Inside the cell there are several targets which can be deacetylated. For a start I measured an overall acetylation with the help of the acetylated lysine western blot. Both treatments significantly decreased the lysine molecules' acetylation levels. It shows that exercise and resveratrol had the same effect in deacetylation.

Deacetylation can change a protein's activity through changing its conformation. But carbonylation can change a protein's turn-over. The carbonyl level of proteins is used as a marker for oxidative stress. It has been shown that exercise elevates the level of carbonylated proteins because of the elevated ROS levels in the cells. This effect is clearly seen at HCR animals. However, resveratrol seems to ameliorate this disadvantage both in LCR and HCR animals.

While sirtuins deacetylate other molecules they use a NAD^+ which will hydrolyze and transfer the acetyl-group. Finally nicotinamide will be produced until it will be converted back into NAD^+. For this conversion NAMPT (also known as PBEF) is required. According to the western blot results, the level of NAMPT protein is significantly higher in the resveratrol-treated groups. It suggests that resveratrol might elevate the NAMPT level through SIRT1. Training did not inflict the same results.

CREB (cAMP response element-binding protein) is a cellular transcription factor. It binds to certain DNA sequences called cAMP response elements (CRE), thereby increasing or decreasing the transcription of the downstream genes (e.g.: BDNF). CREB has been shown to be integral in the formation of spatial memory, in neuronal plasticity and the formation of long-term memory in the brain. It turned out that exercise increased the mRNA level of Creb spectacularly.

Brain-derived neurotrophic factor, also known as BDNF acts on certain neurons of the central nervous system and the peripheral nervous system, helping to support the survival of existing neurons, and encouraging the growth and differentiation of new neurons and synapses. It is active in the hippocampus, the cortex, and the basal forebrain areas vital to learning, memory, and higher thinking. At low capacity of running type rats both resveratrol and training enhanced the BDNF protein's level. These effects were also detectable at high capacity of running type rats, but only significant at the TrH group.

Poly(ADP)-ribose (PAR) is synthesized by the nuclear DNA repair enzyme poly(ADP)-ribose polymerase (PARP). PARP is selectively activated when DNA strands break and catalyze the addition of long branched chains of PAR to a variety of nuclear proteins. The amount of PAR formed in living cells with DNA damage is directly related to the extent of the damage (e.g. at oxidative stress). Interestingly, both in low and high running capacity animals, resveratrol decreased the level of PAR. On the other hand training with resveratrol supplementation enhanced the PAR level to the control animal's value.

Decreased PAR levels at resveratrol treatment suggest repair mechanisms at the background. As written earlier, OGG1 is a base excision repair enzyme, which is responsible for the excision of 7,8-dihydro-8-oxoguanine (8-oxoG), a mutagenic base byproduct that occurs as a result of exposure to reactive oxygen species (ROS). OGG1 is a bifunctional glycosylase, as it is able to both cleave the glycosidic bond of the mutagenic lesion and cause a strand break in the DNA backbone. Like PAR, OGG1's density is decreased at resveratrol treatment. Training had different results: at low running capacity animals training made no significant change compare to the controls, but there was huge difference between the LCR and HCR control groups. At high running capacity animals training increased the OGG1 level. OGG1's activity can be fine-tuned through posttranslational modification. One type of these potential modifications is acetylation-deacetylation. It seemed interesting to test if sirtuins are potent deacetylators of OGG1. Therefore I applied histochemistry staining on the half-brain slides. Acetylated OGG1 had higher concentration in almost every LCR groups than the parallel HCR ones. It correlated inversely with the passive avoidance's long term memory data.

Results from experiments on cell cultures

Data from the animal experiments foreshadowed some kind of connection between the memory results, resveratrol and the DNA repair via OGG1. To evaluate if there is a molecular link behind these findings I made tests on a simpler model system, the HCT116 cell culture.
I checked whether the acetylation status of OGG1 changes after treatment of known SIRT1 activator/inhibitor. So I applied resveratrol, nicotinamide and Trichostatin A as HDACI-II inhibitor. Resveratrol treatment decreased the amount of AcOGG1, but nicotinamide increased it. If the deacetylator is SIRT1 it makes sense that a SIRT1 activator (resveratrol) will decrease the acetylation state and a known inhibitor (NAM) will increase it. TSA does not have any effect because it only blocks HDAC I-II proteins.
To prove the connection between the SIRT1 and OGG1 molecules I chose to silence the SIRT1 protein with siRNA technology. After the silencing I measured the relative density of acetylated OGG1. It was significantly higher in the group where SIRT1 was silenced with siRNA. siSIRT3 which was used as a "deacetylator control" had no effect on AcOGG1.

Conclusions

Although we measured VO_2max only for practical reasons (to adjust the training speed regularly), the results are quite interesting. We perceived 152% difference between the control groups. This result is in accordance with Koch and Britton's results, because high running capacity animals have better capabilities in genetic compare to low running capacity animals. The effectiveness of the training is also clearly visible at the end of the exercise program. Each exercised group produced higher VO_2max than the corresponding control. These differences came up significant only at LCR animals, at HCR these are mainly tendencies. It is plausible that training's effectiveness is higher in LCR animals, because HCR animals were already at a higher level of fitness.

We likely can use the same idea at explanation of the body weight and blood sugar data. Koch, Britton and Wisløff presented that the signs of metabolic syndrome is evincible on LCR animals. Low running capacity animals had a greater body weight, and their body composition was also far from ideal. We did not measure any fat : muscle ratio, but there was a pronounced difference between low and high running capacity animals in the amount of the abdominal fat (just observation during the autopsy). It was also remarkable that TrL and TrRsvL groups had significantly less abdominal fat compare to CL. So, regular exercise already resulted in a leaner body shape after these few months. In metabolic syndrome the blood glucose level is higher due to imbalanced glucose homeostasis. The glucose depots do not function well at skeletal muscles, which is in accordance with insulin resistance. Exercise could meliorate the blood glucose levels too. We could not detect the same effect at resveratrol supplementation.

We certainly could see the effects of resveratrol in rotarod performance test. Rotarod test measures the balance and coordination of rodents. Both resveratrol and exercise enhanced the balance of the animals and the enhancement was cumulative. In balance and coordination the main characters are the vestibular nuclei and cerebellum of course. In this study there was no space to go after this, but in 2011. Steiner and colleagues have previously demonstrated that exercise training increases brain mitochondrial biogenesis (via SIRT1 and PGC-1α) in various regions (i.e. cerebellum) and it results increased neuronal functions. It is also interesting to watch the results of cognitive tests all together. HCR animals performed better in every task. They had the shorter latency and higher exploration in open field, they spent more time investigating a new object and they could remember the learnt things for longer time. Unfortunately in most cases neither resveratrol nor training could meliorate the disadvantages of low animals in these tasks. According to the literature, exercise should increase cognitive

function, but in this case improved function is seems to be genetically inherited, not acquired with this few month of training.

The better cognitive function might be a result of some kind of cellular alteration. In most studies this alteration is neurogenesis. In our results high animal groups reacted to each type of treatment with neurogenesis, in contrast at low animals only resveratrol affected the show up of new neurons. The neuronal changes do not follow correctly the cognitive data. NeuN staining can label the majority of neurons, but maybe the neurons which we detected were immature and could not affect cognitive function yet. As the literature reflects, BDNF is a sensitive marker, widely used to measure exercise's effect on cognitive function, especially in barely-invasive human studies. So we measured the amount of BDNF as well. It turned out that training elevated the BDNF levels in both animal types and there was a beneficial tendency at resveratrol too. It is still under investigation, how SIRT1 is capable to change the levels of BDNF. In 2011 Jeong et al. provided a possible interaction. They proved that SIRT1 can deacetylate and also activate TORC-1 which will increase BDNF expression through CREB. We measured the expression levels of Creb and data was in accordance with the BDNF levels. CREB also can verify exercise's beneficial effects through BDNF.

We checked if SIRT1 will also underpin these assumptions. Two signs were referring to this. The acetylated lysine levels were decreased in both high and low running capacity animals to every treatment, particularly to resveratrol treatment. Resveratrol is a well-known activator of SIRT1, so we hoped this polyphenol enlarged the deacetylase activity of SIRT1. On the other hand the level of PBEF (or NAMPT or visfatin) was markedly higher in resveratrol treated groups. NAMPT is the key enzyme of NAD^+ production from nicotinamide. As we thought an increased NAD^+ production is the sign and protector of the increased deacetylase activity. Controversially the results did not prove this theory fully. After measuring Sirt1 mRNA, protein level and relative activity it is sure that SIRT1 was more active at exercised groups but only at high capacity of running type animals. Resveratrol also did not raise the activity of SIRT1 spectacularly. Some publications say that resveratrol is not only a SIRT1 activator (or not a SIRT1 activator at all) but it has its own function probably as an antioxidant. The answer for this question is not in focus at this study, but we think that resveratrol both can increase SIRT1 activity and work as an antioxidant what we can see at results in the level of carbonylated proteins.

The carbonyl level of proteins is used as a marker for oxidative stress. According to our data at resveratrol supplemented groups we measured lower carbonyl levels. It's a well-known fact that exercise increases protein's carbonyl amount through the enhanced level of ROS. This found to be true, especially at HCR animals. It might seem to be confusing because how can

animals do better cognitive performance with higher level of damaged proteins? Radak et al. publicized an explanation in 2011, where they suggested that certain types of carbonyl groups could be important to stimulate protein turnover.

Reactive species also produce multiple oxidative DNA damage such as oxidized DNA bases, oxidized sugar fragments, abasic (AP) sites, and single-strand breaks (ssbs). Training increased BrdU incorporation into hippocampal cells in high performing animals. On the other hand, we did not observe any indication for the S-phase and, thus, we considered that BrdU incorporation may represent DNA synthesis due to repair processes of the oxidative base and strand lesions. (Elevated PAR results might mean the same, particularly because the PAR results show the same pattern as OGG1.) 8-oxoG is repaired via the base excision repair pathway that is initiated by the OGG1. Unexpectedly, in HCR rats there was a significantly lower Ogg1 expression in the hippocampus compared to LCR at both protein and RNA levels. Intriguingly the activity-related post-translational modification of OGG1 (acetylation), was lower in high performing rats, when compared to LCR rats. These results appear to contradict previously published observations showing the imperative role of DNA damage repair in the hippocampal cells.

We used a cell culture model to test if SIRT1 is the deacetylator of OGG1 or not. Nicotinamide, a SIRT1-specific inhibitor, caused the greatest increase in the acetylation of OGG1. Resveratrol an activator of SIRT1 decreased AcOGG1 levels and TSA (histone deacetylase inhibitor) had no significant effect on AcOGG1. Also silencing SIRT1 via siRNA increased the level of AcOGG1. Exercise in high running capacity groups increases the activity of SIRT1, leading to a decreased acetylation of OGG1, which implies a decreased enzymatic OGG1 activity and lower efficiency of 8-oxoG repair in the brain. It also has been reported earlier that, exercise increases DNA repair activity of OGG1 in human skeletal muscle from young individuals. It seems possible that OGG1's activity is differentially regulated in response to exercise, and that specifically its activity is transiently downregulated in the brain, while upregulated in muscle. These observations raise the possibility that a delay in the repair of 8-oxoG lesions could be beneficial for brain function. As summarized before despite a genomic accumulation of 8-oxoG, Ogg1–/– mice appeared to have a normal phenotype and showed an increased resistance to inflammation. Moreover, no organ defects were observed, and these Ogg1–/– mice showed an increased tolerance to chronic oxidative stress. These observations imply that the 8-oxoG base released from the genome of the brain cells (and not the transient 8-oxoG accumulation in DNA) could have a higher physiological/patho-physiological relevance compared to skeletal muscle. Indeed, in 2012. Boldogh et al. shown that OGG1 binds its excision product, the 8-oxoG base. In complex

with the 8-oxoG base, OGG1 interacts with the canonical Ras family members and induces guanine nucleotide exchange. Activated Ras then initiates signal transduction via Raf1-MEK1,2/ERK1,2, leading to the transcriptional activation of genes. Activation of Ras and the MAPK pathway has been shown to cause apoptosis in neurons. Therefore deactivation of OGG1 by SIRT1-mediated deacetylation could favor its control of the OGG1-initiated repair of DNA, but also imply an anti-apoptotic role of SIRT1.

At the Objectives chapter I made up the aims of the study. Now it is time to review the four hypotheses I set up:

1. Regular physical activity and resveratrol treatment will enhance the cognitive function of both rat strains. FALSE Exercise had positive effects on the animals, but it was only seen in cognitive function at HCR animals. Resveratrol did not elevate the performance in cognitive tests.
2. Our aim was to illustrate that the cognitive enhancement was caused via sirtuins and neurotrophic factors in the brain which overall can be seen in neurogenesis. PARTLY TRUE At high running capacity animals the activity of SIRT1 was elevated, and we also could detect the elevated BDNF levels which enhanced the expression of Creb.
3. Training and/or resveratrol will compensate the differences which come from the genetic origin of the animals. PARTLY TRUE Training could overcome the signs of metabolic syndrome at LCR animals but it did not enhance the cognitive function compare to HCR animals. The meliorating results of resveratrol could not be seen uniformly too.
4. Sirtuins can deacetylate OGG1 protein and this might moderate its activity. TRUE SIRT1 certainly can deacetylate OGG1, because at SIRT1-silenced cell cultures the levels of AcOGG1 was elevated. On the other hand AcOGG1 was also increased when nicotinamide was applied as a SIRT1 inhibitor, and AcOGG1 was decreased when SIRT1 activator (resveratrol) was applied.

According to the results it would be useful to do further investigations on the connection between SIRT1 and OGG1 protein through special circumstances. (For example, performing experiments on cell cultures from SIRT1 knockout mice.)

Bibliography of own publications

In connection with the thesis:

- **Sárga** L, Hart N, Koch LG, Britton SL, Hajas G, Boldogh I, Ba X, Radák Z (2013) Aerobic endurance capacity affects spatial memory and SIRT1 is a potent modulator of 8-oxoguanine repair. Neuroscience 252:326-336.
  IF: 3.122
- Radák Z, Hart N, **Sárga** L, Koltai E, Atalay M, Ohno H, Boldogh I (2010) Exercise plays a preventive role against Alzheimer's disease. J Alzheimers Dis. 20(3):777-783.
  IF: 4.174

Not in connection with the thesis:

- Hart N, **Sárga L**, Csende Z, Koltai E, Koch LG, Britton SL, Davies KJ, Kouretas D, Wessner B, Radák Z (2013) Resveratrol enhances exercise training responses in rats selectively bred for high running performance. Food Chem Toxicol. 61:53-59.
  IF: 3.010
- Marosi K, Bori Z, Hart N, **Sárga L**, Koltai E, Radák Z, Nyakas C Long-term exercise treatment reduces oxidative stress in the hippocampus of aging rats. Neuroscience. 226:21-28.
  IF: 3.122