The effect of endurance exercise and SIRT activation on physiological performance on rats artificially selected to high or low running capacity

Abstract of the PhD Thesis

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Introduction:

In 1996 Koch and Britton initiated a prospective test of the linkage between aerobic capacity and disease risk by applying large-scale artificial selective breeding in rats with widely varying genetic backgrounds to produce low and high strains that differ for intrinsic (i.e., untrained) aerobic endurance treadmill running capacity. The hypothesis was that rats selectively bred as Low Capacity Runners (LCR) would display disease risks and the rats bred as High Capacity Runners (HCR) would have positive health effects. HCR demonstrate greater maximal oxygen consumption, insulin sensitivity, lower level of oxidative damage, and longer life-span. There are a number of reports that exercise training and nutritional intervention have beneficial effects on groups suffering from metabolic disorders, those fed on high fat diet, the aged and those genetically selected for low running capacity. Much less information is available on how to upgrade endurance capacity in animals have genetically high VO_2max.

Other metabolic characteristics of skeletal muscle, such as the concentration or activities of the enzymes involved in oxidative metabolism, mitochondrial number, and respiratory capacity strongly affect aerobic endurance capacity. Therefore, impaired mitochondrial biogenesis could be a limiting factor of aerobic endurance. We were interested in how exercise training could further induce aerobic endurance capacity of rats. It was suggested that the activity of AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α), nuclear respiratory factor 1 (NRF1) mitochondrial transcription factor A (TFAM), and sirtuins could play an important role in the exercise-induced adaptive response. The mitochondria-dependent production of reactive oxygen species (ROS) is dependent on the density of mitochondria, since more mitochondria work at lower levels of respiration to produce the same amount of ATP. Therefore, mitochondrial biogenesis could be a part of the antioxidant system.

It is also not well known how mitochondrial fusion and fission would react to exercise-induced adaptation in animals with different intrinsic aerob capacity. Mitochondrial fusion

and fission are important mechanisms for maintenance of the mitochondrial network and for quality control, and thus impact mitochondrial function. The quality control of mitochondrial proteins is supervised by Lon protease and HSP78, which prevent the accumulation of oxidized and dysfunctional proteins in mitochondria. SIRT1 is an important regulator of metabolism by controlling the activity of key transcription factors such as PGC-1α, FOXO1, and p53, which play a key role in the training response. Therefore, activators of SIRT1, such as resveratrol could have potentially beneficial effects which enhance aerobic performance, even in rats having a high endurance capacity.

Therefore, in the present study, we investigated the mitochondrial adaptive response to exercise training and resveratrol supplementation on rats selectively bred for low or high running capacity.

Aims:

The aim of this study was to explore how mitochondrial biogenesis and aerobic capacity can be influenced by regular physical activity and resveratrol administration, in rats with different genetic background. How can affect these treatments on mitochondrial biogenesis in skeletal muscle and myocardium.

Experiment A: The effect of regular physical activity and resveratrol administration on mitochondrial biogenesis in skeletal muscle of rats have different genetic background:

The following hypothesis can be defined:

- Regular physical activity and resveratrol administration increases the running performance and aerobic capacity of animals.
- The treatments used in this study increased mitochondrial biogenesis in muscle tissue (PGC1α - SIRT1 pathway).
- The treatment may favorably influence of muscle mitochondrial enzymes to ensure quality control.
- Exercise and resveratrol treatments can compensate the mitochondrial deficits of LCR animals resulting from genetic setup. They can improving their quality of life, and reduces the risks of diseases.

Experiment B: The effect of regular physical activity and resveratrol administration on mitochondrial biogenesis in myocardium of rats have different genetic setup:

In this case, the following hypotheses were defined:

- The treatments used in this study were expected to improve mitochondrial functions in myocardium of LCR animals.
- Exercise and resveratrol treatments can enhance mitochondrial biogenesis in the myocardium through stimulation of AMPK - PGC1α (and other transcription factors) activity.
- The treatments can compensate the disadvantages of genetic setup in LCR's myocardium, and prevent cardio-vascular diseases.

Materials and methods:

Animals

Artificial selective breeding, starting with a founder population of 186 genetically heterogeneous rats (N:NIH stock), was used to develop rat strains differing in inherent aerobic capacity. The procedure has been described in detail by Koch and Britton (2001). Briefly, endurance running capacity was assessed on a treadmill and the total distance run during a speed-ramped exercise test was used as a measure of maximal aerobic capacity. Rats with the highest running capacity from each generation were bred to produce the HCR strain. A subgroup of 48 male rats from generation 22 was phenotyped for intrinsic treadmill running capacity when 11 weeks old, at the University of Michigan (Ann Arbor, USA) and then shipped via air freight to Semmelweis University (Budapest, Hungary) for further study. Investigations were carried out according to the requirements of The Guiding Principles for Care and Use of Animals, EU, and approved by the ethics committee of Semmelweis University.

Exercise protocol and resveratrol treatment

Twenty four HCR male rats, aged 13 months, were assigned to control HCR (HCR-C), trained HCR (HCR-Tr), resveratrol treated control HCR (HCR-Rsv), trained resveratrol treated HCR (HCR-TrRsv) groups (n=6 rats per group). Control rats had access to the treadmill three times a week for ten min with an electrical stimulator in place. Trained rats were introduced to treadmill running for three days, then for the next two weeks the running speed was set to 10 m/min, on a 5% incline for 30 min. The treadmill was equipped with a high pressure air pipe and electric grid to stimulate running.

In the following week, maximal oxygen uptake (VO_2max) was measured on a motor driven treadmill (Columbus Inst. Columbus, Ohio) with a gradually increasing intensity. VO_2max was measured for each animal, using three criteria: (i) no change in VO_2 when speed was increased, (ii) rats no longer kept their position on the treadmill, and (iii) respiratory quotient (RQ = VCO_2/VO_2) > 1. Based on the level of VO_2max, a treadmill speed corresponding to 60% VO_2max was determined and used for daily training for one hr, five times per week. VO_2max was measured every second week and running speed was adjusted

accordingly. The total training period lasted 12 weeks. In addition, the forelimb strength of the animals was assessed weekly by using a gripping test as described by Marton et al. Resveratrol supplementation (100 mg/kg, oral dosing) was started two weeks before habitual treadmill running was introduced to the animals, and four weeks before the actual training started, therefore lasting 16 weeks. A dose response study on the toxicity of resveratrol revealed no toxic effects up to 100 mg/kg in rats.

The animals were sacrificed two days after the last exercise session to avoid the acute metabolic effects of the final run. The skeletal muscle gastrocnemius was dissected and homogenized in buffer (HB) containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 2% NP 40, 10% glycerol and protease inhibitors.

ROS, protein carbonyl and antioxidant enzyme activities

Intracellular oxidant and redox-active iron levels were estimated using modifications of the dichlorodihydrofluorescein diacetate (H_2DCFDA) staining method. The oxidative conversion of stable, nonfluorometric, DCF-DA, to highly fluorescent 2'7'-dichlorofluoorescein (DCF) was measured in the presence of esterases, as previously reported. This assay approximates levels of reactive species, such as superoxide radical, hydroxyl radical, and hydrogen peroxide. The method has been widely used in the literature but does have the problem of not being particularly specific, and results can be strongly affected by release of labile iron or copper. Briefly, the H_2DCFDA (Invitrogen-Molecular Probes #D399) was dissolved to a concentration of 12.5 mM in ethanol and kept at -80^{O}C in the dark. The solution was freshly diluted with potassium phosphate buffer to 125 μM before use. For fluorescence reactions, 96-well, black microplates were loaded with potassium phosphate buffer (pH 7.4) to a final concentration of 152 μM/well. Then 8 μl diluted tissue homogenate and 40 μl 125 μM dye were added to achieve a final dye concentration of 25 μM. The change in fluorescence intensity was monitored every five minutes for 30 minutes with excitation and emission wavelengths set at 485 nm and 538 nm (Fluoroskan Ascent FL) respectively. Data obtained after 15 min were used. The fluorescence intensity unit was normalized with the protein content and expressed in relative unit production per minute.

The protein carbonyl measurement was done as described earlier.

Western blots

Ten to fifty micrograms of protein were electrophoresed on 8-12% v/v polyacrylamide SDS-PAGE gels. Proteins were electrotransfered onto PVDF membranes. The membranes were subsequently blocked and incubated at room temperature with antibodies (1:500 #sc-13067 Santa Cruz PGC-1 (H-300), 1:1,400 #2532 Cell Signaling AMPKα, 1:500 #2535 Cell Signaling p-AMPKα (Thr172) (40H9), 1:1,000 #sc-33771 Santa Cruz NRF-1 (H-300), 1:500 #sc-30963 Santa Cruz mtTFA (E-16) /TFAM/, 1:500 #sc-98900 Santa Cruz Fis1 (Fl-152), 1:10,000 #sc-50330 Santa Cruz Mfn1 (H-65), 1:1,000 #sc-99006 Santa Cruz PNPase (H-124), 1:200 #U7757 Sigma-Aldrich UCP3, 1:5000 #ab87253 Abcam CLPB /HSP78/, 1:15,000, #T6199 Sigma alpha-tubulin). The antibody for Lon protease was generated in our laboratory, as described previously. After incubation with primary antibodies, membranes were washed in TBS-Tween-20 and incubated with HRP-conjugated secondary antibodies. After incubation with the secondary antibody, membranes were repeatedly washed. Membranes were incubated with an ECL Plus reagent (RPN 2132, Amersham) and protein bands were visualized on X-ray films. The bands were quantified by ImageJ software, and normalized to tubulin, which served as an internal control.

Assessment of SIRT1 activity

To measure SIRT1 deacetylase activity, a Cyclex SIRT1/Sir2 Deacetylsase Fluorometric Assay Kit (Cyclex, CY-1151) was used according to the established protocol including the separation of nuclear extract (Koltai et al., 2012). To prepare nuclear fractions, the homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C, and the pellet was suspended in HB and re-centrifuged. The pellet was then re-suspended in HB with 0.5% NP40 and again centrifuged. Next, the pellet was washed twice in HB. After centrifugation, the final nuclear pellet was rocked for 30 min after the addition of a 1/10 (vol/vol) of 2.5 M KCl and centrifuged at 14,000 rpm for 30 min. For the measurement of SIRT1, five microliters of nuclear extracts of rat gastrocnemius muscle were mixed with a reaction mixture (40 µl) containing 50 mM Tris-HCl pH 8.8, 4 mM $MgCl_2$, 0.5 mM DTT, 0.25 mAU/ml Lysyl endpeptidase, 1 µM Trichostatin A, 20 µM Fluoro-Substrate Peptide, and 200 µM NAD^+

on a microplate. The samples were mixed and incubated for ten min at room temperature and the fluorescence intensity (ex. 355nm, em. 460 nm) was read every ten min for two hours and normalized by the protein content.

Measurement of mtDNA by PCR

The mtDNA content was quantified as the mtDNA to nuclear DNA (nDNA) ratio (mtDNA/nDNA). Total DNA was extracted (Fast DNA kit #6540-400 BIO 101 Systems Qbiogene) and quantified spectrophotometrically. The mtDNA content was measured by PCR (Rotor-Gene 6000, Corbett Research, Australia) using the following conditions: 94 °C for 2 min (initial denaturation), 94°C for 30 s, 60°C for 30 s, 72°C for 45 s (25 cycles), 72°C for 10 min (final extension) and corrected by the simultaneous measurement of a single copy nuclear BDNF gene. Primers used for the analysis of mtDNA were R-CYTB-F (5′-CCC CAG AGG ATT AAA CTC CAA CGC A-3′), and R-CYTB-R (5′-GGG TGG GGT CAG GGG GT-3′). Primers used for the analysis of nDNA were R-BDNF-genom-exon-IV-F (5′- TTG GGA TGG GAA AGA TGG G -3′), and R-BDNF-genom-exon-IV-R (5′- CAG AGT AGG AGG GAA CAA GTG TGA C -3′). The mtDNA content was normalised to nDNA. Data are expressed as the mean of three measurements.

Statistical analyses

Because of the limited sample size and for the purpose of finding the appropriate statistical procedure, normality was tested on all dependent variables (Shapiro Wilk's W-test). It was found that few of the dependent variables demonstrated a normal distribution, namely: NRF1, Fis1, NAMPT, LonP, Mfn1, and VO_2max. Therefore, non-parametric Kruskal-Wallis ANOVA analysis was used to test for differences among the dependent variables. The Mann-Whitney U-test was applied for post-hoc analyses. Significance level was set at $p < 0.05$.

Results:

The effects of exercise training:

Regular exercise training has significantly increased the level of VO2max in low running capacity rats which achieved the levels of CH. VO2max is not completely covers the endurance capacity, so we measured the running distance during the measurements of VO2max, in which indeed we have found larger differences among the experimental groups.

The level of ROS was measured by the fluorescent activity if H2DCFDA which increased by exercise load in LCR rats.

SIRT1 can activate PGC-1α and hence, alter mitochondrial biogenesis however exercise training failed to increase the activity of SIRT1 in LCR and HCR rats. Since the activity of SIRT1 is dependent on NAD^+ levels we have measured the level of NAMPT, which is involved in the biosynthesis of NAD, and interestingly exercise training tend to decrease the NAMPT levels in LCR and tend to increase it in HCR rats. AMPK activity is a known activator of PGC-1α activity, and our data revealed that exercise significantly decreased the ratio of pAMPK/AMPK in LCR rats, while exercise tend to increase the level in HCR rats. The PGC-1α level did not change after the exercise program in LCR rats, while it tend to increase in the HCR animals. The concentration of NRF-1 was not significantly affected by exercise load. On the other hand, the TFAM protein level decreased significantly by exercise training in LCR rats, and unchanged in HCR group. Fis 1 level was not significantly affected in either groups by exercise regime. However, the Mfn1 concentration of control LCR rats was significantly lower than that of HCR control group and exercise increased the level of Mfn1 in LCR animals, while significant alteration was not seen in HCR groups. Significant effects of exercise on Lon protease level was not observed. However, the mitochondrial HSP78 concentration was higher in HCR rats and exercise increased the level of this protein in LCR rats. The level of PNPase was induced by the joint effects of exercise and resveratrol. The level of FOXO1 was significantly higher at LCR control rats than that of HCR group, moreover exercise decreased the level of this transcription factor in LCR rats, while the FOXO1 levels remained unaffected in HCR groups. Exercise very similarly impacted the SIRT4 levels in both running capacity groups.

The effects of resveratrol treatment:

Resveratrol increased the VO2max in high running capacity rats, and the increase was even more significant in the running distance, but wasn't effective in case of LCR animals. The effects of resveratrol were also different in SIRT1 activation in LCR and HCR rats, since no effects was present in LCR rats while resveratrol stimulated the activity of SIRT1 in HCR rats.

Interestingly, resveratrol treatment decreased the activity of AMPK in LCR rats, but significantly supported the enhancing effects of exercise on the ratio of pAMPK/AMPK in HCR rats. However, the level of PGC-1α was not altered by resveratrol intervention and this was true for the NRF-1 levels too. Resveratrol treatment appears to differently act on TFAM in LCR and HCR rats, since it decreased the concentration of TFAM in LCR animals and increased it in HCR groups. The mitochondrial fission controlling protein, Fis1 concentration was not altered by resveratrol treatments. On the other hand, resveratrol administration increased the protein concentration of LCR rats but not change it in HCR groups. Lon protease was not altered by resveratrol. Resveratol intervention significantly upregulated the level of HSP78 in LCR rats. The effects of resveratrol appears to be different in LCR and HCR groups in terms of the level of PNPase, resveratrol decreased the levels of FOXO1 and SIRT4 in the LCR groups.

Conclusion:

Here we test if 12 weeks of treadmill exercise training and/or resveratrol can retrieve the low running performance of the LCR and enhance performance in HCR. Training increased running performance in both strains but resveratrol alone did not change running performance in either strain. Resveratrol degraded running performance in trained LCR and increased running performance in trained HCR. Resveratrol increased performance on a test of forearm gripping strength in HCR but had no effect in LCR. Activities of 15 factors that regulate mitochondrial biogenesis and differentiation from gastrocnemius muscle were interrogated for explanation. AMPK, SIRT1, and TFAM increased in HCR and decreased in LCR by resveratrol. Mitochondrial fission and fusion levels were significantly lower in LCR rats and increased to levels not different from HCR by exercise and resveratrol. mtDNA was lower in LCR compared to HCR, and was increased by resveratrol given alone or in combination with training HCR group. In case of myocardium- factors of mitochondrial biogenesis shows less, but similar changes then in skeletal muscle. Fis1 and Mfn1 levels altered in both group-, and SDHA level in HCR group by exercise. But in case of LCR group exercise decreased the activity of AMPK. The effect of resveratrol wasn't so pronounced, although it increased SDHA content in HCR rats, but decreased TFAM level in LCR rats. The two treatment together had positive effect on PGC1α level, and AMPK activity in case of HCR animals, but not in LCR.

Thus, it appears that although exercise could be an effective treatment for both group, ergogenic responses to resveratrol can be influenced differentially by heritable determinants of exercise performance.

Publications:

Publications closely related to the thesis:

Hart N, Sarga L, Csende Z, Koltai E, Koch LG, Britton SL, Davies KJ, Kouretas D, Wessner B, Radak Z. (2013) Resveratrol enhances exercise training responses in rats selectively bred for high running performance. Food Chem Toxicol. [Epub ahead of print]

IF: 2.999

Koltai E, **Hart N**, Taylor AW, Goto S, Ngo JK, Davies KJ, Radak Z. (2012) Age-associated declines in mitochondrial biogenesis and protein quality control factors are minimized by exercise training. Am J Physiol Regul Integr Comp Physiol. 303: R127-34.

IF: 3.336

Publications not related to thesis:

Radak Z, **Hart N**, Sarga L, Koltai E, Atalay M, Ohno H, Boldogh I. (2010) Exercise plays a preventive role against Alzheimer's disease. J Alzheimers Dis. 20(3):777-83.

IF: 4.261

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IF: 3.380

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Muscle Plasticity: Advances in Biochemical and Physiological Research

Karela: Research Signpost Karela, 2009. pp. 37-46.