SEX DIFFERENCES IN SPORT ADAPTATION OF THE RENAL AND FEMORAL ARTERIES

Ph.D. thesis
Márton Vezér MD

Doctoral School of Theoretical and Translational Medicine
Semmelweis University

Supervisor:	Szabolcs Várbíró MD, DSc Marianna Török, MD, PhD
Official reviewers:	Attila Keszthelyi MD, PhD István Sziller MD, PhD
Head of the Complex Examination Committee:	Gábor Varga MD, DSc
Members of the Final Examination Committee:	Barna Vásárhelyi MD, DSc Csaba Ambrus MD, PhD

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1. Introduction

Beneficial effects of regular exercise can be seen in several body functions of most mammalian species, humans included.
During aerobic exercise, hemodynamic alterations occur; while blood flow in skeletal muscle arteries increases, it decreases in visceral vessels due to mesenterial vasoconstriction. However, maintaining renal blood flow during intensive sport is also a priority. Our aim was to investigate the changes of vascular reactivity and histology of isolated renal and femoral artery of male and female rats in response to swim-training.

2. Objectives

Our hypothesis was that there are differences between the two sexes in the adaptation of renal and femoral vessels to long term-training. Isolated renal and femoral artery rings were examined by wire myography. We also performed histological and immunohistological measurements on the vascular rings.

Questions:

1. Does the renal artery adapt after long-term exercise, and are there sex differences in the ways of sport adaptation?
2. Does the femoral artery adapt after long-term exercise, and are there sex differences in the ways of sport adaptation?

3. Methods

3.1. Animals

Experimental protocols were approved by the Animal Care Committee of Semmelweis University (Permission Number: PEI/001/2374-4/2015) which met the regulations of the European Union (Directive 2010/63/EU) for the care and use of animals in research. Each animal received treatment in full compliance with the National Institutes of Health (NIH

Publication No. 86-23, revised 1996.) criteria for the 'Guide for the Care and Use of Laboratory Animals. All the animals were housed under the same conditions: constant climate-controlled temperature (22^{o}C-25^{o}C), relative humidity of 40%-to 70%, with a 12-hour light/dark cycle and provided with standard laboratory food and tap water. Following seven days acclimatization, animals were divided into four experimental groups: male sedentary (MSed), male trained (MTr, female sedentary (FSed,), and female trained (FTr). The animals were 8-week-old at the start of the training program.

3.2. Swim training protocol

Male and female trained rats (MTr and FTr) were participated in a long-term, 12-week swimming training program. The training program was built up gradually, starting with 15 minutes of swimming, increasing by an additional 15minute swim time every second day, until it reached the maximum of 200 minutes per day. The rats were trained for 12 weeks, 5 days a week. Sedentary rats were placed in water for 5 minutes each day (5 days a week) to reduce potential differences for swim load.

3.3. Echocardiography

Animals were anesthetized with isoflurane at week 12 (1-2% isoflurane in 100% oxygen). Transthoracic echocardiographic examination (Vivid I Echocardiac Image Analysis System, GE, Healthcare, Unites States) was performed. Left ventricular morphology was measered on standard two-dimensional short-axis images and M-mode images.

3.4. Myography

At the end of the swim training program, under pentobarbital anesthesia the hearts were removed and the renal and femoral artery were carefully dissected and removed under a microscope magnification. The experiments were performed on prepared vascular rings using a DMT 610 M Wire Myograph system (multi-chamber isometric myograph system, Danish Myo Technology, Aarhus, Denmark). The myograph system

contained four organ chambers, each filled with 6 ml of nKR. The temperature of the organ chambers was kept constant at 37 °C, pH=7.4 (O2 95%, CO2 5%). Data collection was performed using LabChart software. (ADInstruments, Oxford, UK-Ballagi LTD, Budapest, Hungary). A pretension of 10 mN was applied, which was achieved in 1 hour with continuous careful elevation of loading. The remaining segment was fixed in formalin and then embedded in paraffin for histological examinations.

3.4.1. Myography protocol for renal arteries

Animals were divided into four experimental groups: male sedentary (MSed, n=16), male trained (MTr, n=7), female sedentary (FSed, n=12), and female trained (FTr, n=12).

Following equilibration, 124 mmol/L K+ was used for 3 minutes (100% contraction) to test the blood vessel contractility and to set the reference value. To check the viability of the endothelium, maximum concentration (10^{-6} mol/l) of phenylephrine and then the same concentration of acetylcholine was used. To measure contraction in response to alfa-agonist, phenylephrine was added to the bath in cumulative concentrations (10^{-8}–10^{-6} mol/L respectively). Acetylcholine (Ach) induced vascular relaxation was examined after phenylephrine (Phe) precontraction (10^{-6} mol/L) (without washing out phenylephrine) by applying acetylcholine in increasing concentrations (10^{-8} – 10^{-6} mol/L). Same procedure was repeated after 30 minutes pretreatment of the rings with only one of the following inhibitors: the cyclooxygenase (COX) inhibitor indomethacin (INDO 10^{-4} mol/L), the cyclooxygenase-2 (COX-2) specific inhibitor NS398 (10^{-5} mol/L), or the NO synthase blocker nitro-L-arginine methyl ester (L-NAME 10^{-5} mol/L). Control samples were treated with vehiculum (diluted dimethyl-sulfoxide, DMSO). Preteatment of a vascular ring was performed in each case with an inhibitor.

3.4.2. Myograph protocol for femoral arteries

Animals were divided into four experimental groups: male sedentary (MSed, n = 20), male trained (MTr, n = 19), female sedentary (FSed, n = 21), and female trained (FTr, n = 21). Following equilibration, 124 mmol/L K+ was used for 3 minutes (100% contraction) to test the blood vessel contractility and to set the reference value. To check the viability of the endothelium, maximum concentration (10^{-5} mol/l) of phenylephrine and then the same concentration of acetylcholine was used. To measure the contraction ability, the alpha receptor agonist phenylephrine (Phe) was administered in cumulative increasing concentrations (10^{-9}-10^{-5} mol/l). Between the use of different types of vasoactive agents, the organ chambers were thoroughly washed, 3 times. During examintation of femoral arteries the degree of contraction was also measured in the presence of thromboxane agonist U46619 (10^{-9}-10^{-7} mol/l). The vasorelaxation resulting from acetylcholine (Ach) was measured following phenylephrine precontraction (10^{-6} mol/L) (without washing the organ chambers) with increasing concentrations of Ach (10^{-9}-10^{-6} mol/l). Protocol was repeated after 30 minutes pretreatment of the rings with the NO synthase blocker nitro-L-arginine methyl ester (L-NAME 10^{-5} mol/L), the cyclooxygenase (COX) inhibitor indomethacin (INDO 10^{-4} mol/L), as well as the cyclooxygenase-2 (COX-2) specific inhibitor NS398 (10^{-5} mol/L). Control vascular rings were treated parallel with the vehicle only (diluted dimethyl-sulfoxide, DMSO).

3.5. Hystology and immunohistochemystry

Formalin fixed paraffin embedded tissue samples were cut in 5 micrometer sections. Resorcin-fuchsin (RF) was used to examine the density of elastic fibers.

Fixed sections were deparaffinized for immunohistochemical staining. The density of nitro-tyrosine (NT), α-smooth muscle actin (α-SMA), cyclooxygenase 2 enzyme protein (COX2), and

endothelial nitric oxide synthase protein (eNOS) were investigated using immunohistochemistry followed by colorimetry.

Histological sections were photographed with Nikon Eclipse Ni-U microscope with DS-Ri2 camera (Nikon Minato - Tokyo Japan) at 10x magnification in case of eNOS & α-SMA, and at 20x magnification in case of NT & COX2 stains. To evaluate the results, immunohistochemistry, and background staining (DAB + Hematoxylin) were separated on the images using ImageJ software (National Institutes of Health (NIH), Bethesda, MA, U.S.A.). After converting the separated images to black and white, the degree of staining was determined by non-calibrated optical density (OD) in the intima (in case of eNOS and COX2 evaluation) and in the media (in case of NT and α-SMA evaluation).

3.6. Statistical analysis

GraphPad Prism software (ver. 8. GraphPad Software, Inc., San Diego, CA, USA) was used for data analysis and graphical representation. Data are expressed as mean ± SEM. Normal distribution was checked using the Shapiro-Wilk test. In the case of a normal distribution, two-way repeated measures analysis of variance (ANOVA) was performed for the statistical analysis. As a post hoc test, Dunnett's or Tukey's post hoc test was used. Histological and immunohistochemical evaluations were compared using two-way ANOVA with Tukey's post hoc test and Kruskal-Wallis test with Dunn's multiple comparison test. $P < 0.05$ was accepted throughout the work as a level of significance.

4. Results

4.1. Cardiac changes

After 12 weeks of swimming, left ventricular myocardial mass increased significantly in FTr and MTr as well ($p<0.001$),

suggesting the effective induction of cardiac adaptation in our experimental model. The sex difference found in the sedentary groups, according to which males had higher absolute left ventricular heart mass, persisted after training ($p<0.001$) (LV mass (g): MSed, 1.18±0.029; MTr, 1.31±0.031; FSed, 0.89±0.008 and FEx, 1.05±0.023).

4.2. Renal arteries

4.2.1. Contraction of renal arteries

Male sedentary rats showed significantly higher contraction at Phe concentration of 10-7 mol/L and 10-6 mol/L as compared to female sedentary animals. Effect of exercise training, the Phe-induced contraction decreased at Phe concentration of 10-6 mol/L in trained male animals **(Figure 1)**.

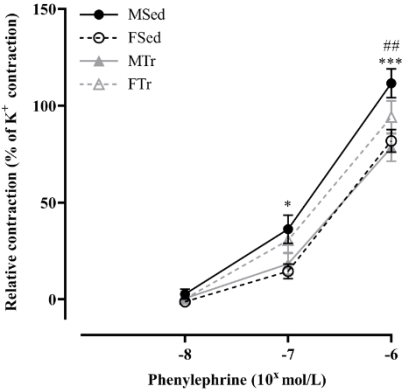

Figure 1. Phenylephrine (Phe) induced contraction. Data are shown as mean ± SEM. Two-way RM ANOVA, with Tukey post hoc test. N = 5-13 in each group; Pint=0.0285; Pconcentration<0.0001; Pgroup=0.0114; Panimal<0.0001. *, $p<0.05$ MSed vs. FSed; ***, $p<0.001$ MSed vs. FSed; ##, $p<0.01$ MSed vs. MTr, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary and FTr—female trained

Non-selective and selective COX inhibition led to decreased contraction in male sedentary animals at 10^{-7} mol/L and non-selective COX inhibition led to decreased contraction at 10^{-7} mol/L and 10^{-6} mol/L in female sedentary ones. The L-NAME

caused increased contraction at 10^{-7} mol/L and 10^{-6} mol/L in male and at 10^{-7} mol/L in female trained rats. General COX inhibition led to decreased contraction at 10^{-6} mol/L in male trained animals as well **(Figure 2)**.

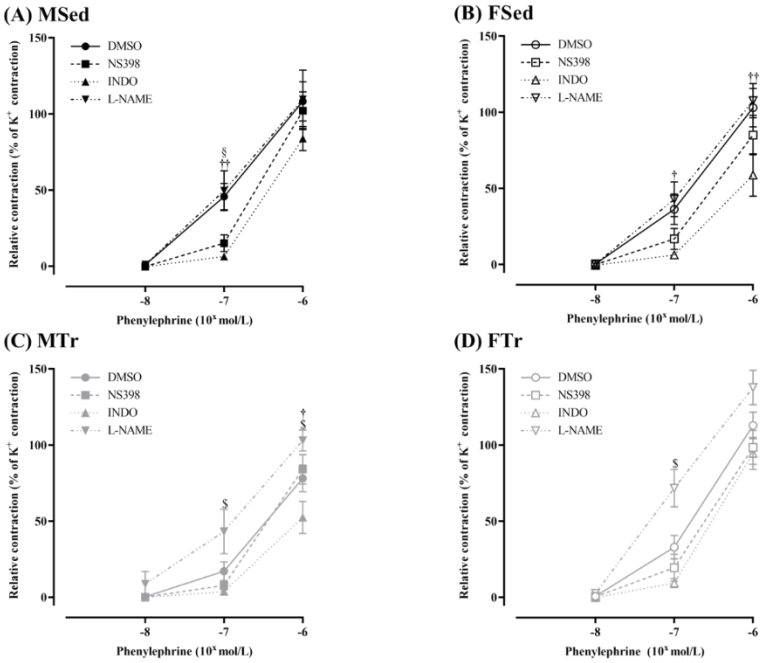

Figure 2. Phe-induced contraction in the presence of the selective COX-2 inhibitor (NS398) or non-selective COX inhibition (indomethacin; INDO), or nitric oxide synthase inhibitor (L-NAME), or their vehicle DMSO (a) in male sedentary rats, (b) in female sedentary rats, (c) in male trained rats and (d) in female trained rats. Data are shown as mean ± SEM. Two-way RM ANOVA, with Dunnett's post hoc test. N = 5-13 in each group; †, $p<0.05$ DMSO vs. INDO ††, $p<0.01$ DMSO vs. INDO; §, $p<0.05$ DMSO vs. NS398; $,$p<0.05$ DMSO vs. L-NAME, Abbreviations: MSed— male sedentary; MTr— male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl-sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin.

4.2.2. Relaxation of renal arteries

The Ach induced relaxation did not show significant differences between thefour groups **(Figure 3)**.

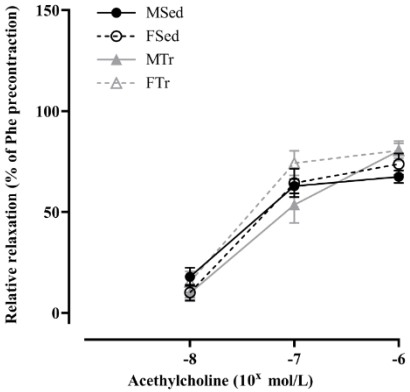

Figure 3. Acetylcholine (Ach) induced relaxation. Data are shown as mean ± SEM. Two-way RM ANOVA, with Tukey post hoc test. N = 7-15 in each group. Abbreviations: MSed—male sedentary; MTr— male trained; FSed—female sedentary and FTr—female trained.

The L-NAME caused decreased relaxation at 10^{-7} mol/L and 10^{-6} mol/L in all experiment groups. As expected, the relaxation caused by Ach is predominantly realized by the NO pathway, which is not influenced by sex, or training.

4.2.3. Histological alterations of renal arteries

The optical density (OD) of elastic fiber on resorcin-fuchsin-stained sections was significantly lower in male sedentary animals than in female sedentary rats. The OD was significantly reduced in trained female animals compared to sedentary female animals. The optical density was significantly lower in FTr animals than in MTr rats **(Figure 4).** The OD of smooth muscle

actin (SMA staining) was significantly reduced in trained female animals compared to sedentary female animals **(Figure 5).** The optical density of nitrotyrosine and COX-2 did not differ between groups.

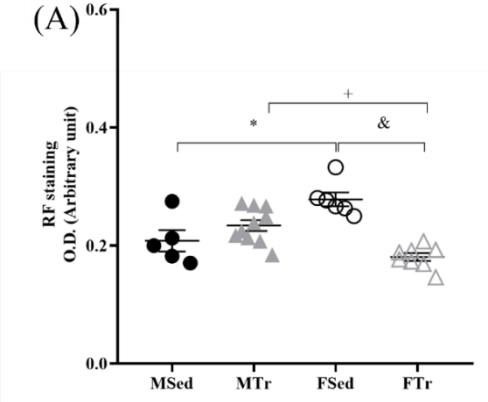

Figure 4. Optical density of elastica on resorcin-fuchsin-stained segments. Data are presented as individual data points, lines represent mean ± SEM. Two-way ANOVA with Tukey post hoc test. N = 5-10 in each group. *, $p<0.05$ MSed vs. FSed; &, $p<0.05$ FSed vs. FTr; +, $p<0.05$ MTr vs. FT, Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained.

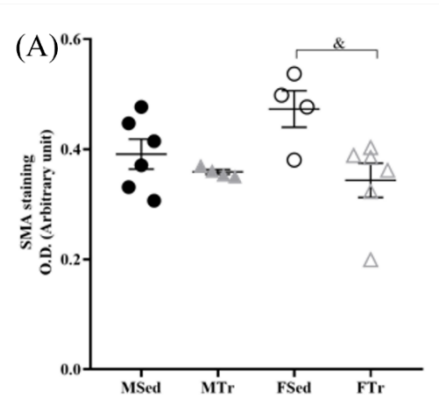

Figure 5. Optical density on smooth muscle actin-stained segments. Data are presented as individual data points, lines represent mean ± SEM. Two-way ANOVA with Tukey post hoc test. N = 4-6 in each group. &, $p<0.05$ FSed

vs. FTr, Abbreviations MSed— male sedentary; MTr— male trained; FSed—female sedentary; FTr—female trained.

4.3. Femoral arteries

4.3.1. Contraction of femoral arteries

In femoral arteries, contraction ability was tested with phenylephrine at increasing concentrations. There was no significant difference between the four groups.

The functional vascular effects associated with cyclooxygenases and endothelial oxide synthase (eNOS) were explored as follows: Phe-induced contraction, repeated in the presence of the L-NAME (10^{-5} mol/L), INDO (10^{-4} mol/L), and NS398 (10^{-5} mol/L). In the male and female sedentary groups, the presence of INDO decreased Phe-induced contraction significantly. Following the swimming training, an increased Phe-induced contraction in the presence of L-NAME was observed in both the trained groups such new phenomena. As a gender difference, we found that there was a specific COX-2 vasoconstriction effect only in the FSed group. After exercise training, this specific COX-2 inhibition (NS398) effect disappeared in FTr rats. Moreover, the INDO effect mentioned in the sedentary groups remained in trained female animals, but not in males. L-NAME effects did not demonstrate significant differences when comparing the trained groups **(Figure 6)**.

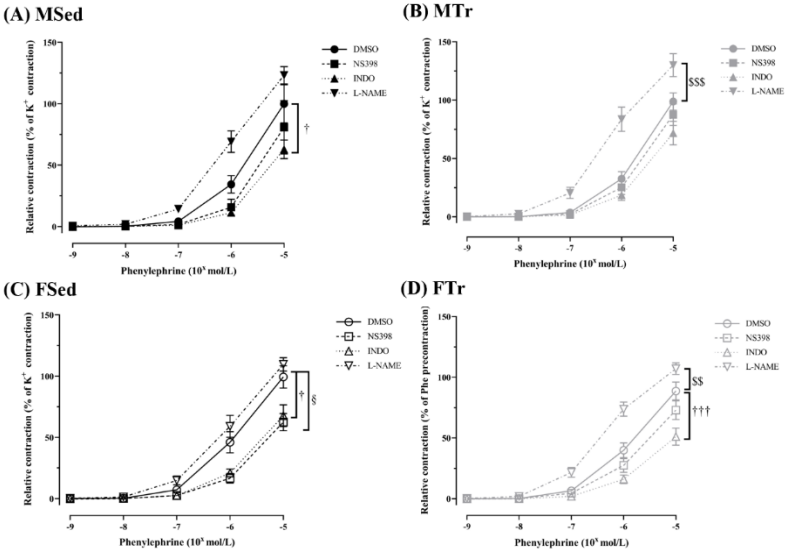

Figure 6. Phenylephrine induced contraction in the presence of NS398, INDO, L-NAME, or DMSO in sedentary male rats (A), in trained male rats (B), in sedentary female rats (C), and in trained female rats (D). Data are shown as means ± SEM; n = 5–17 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. † $p < 0.05$, ††† $p < 0.001$: DMSO vs. INDO; $$ $p < 0.01$, $$$ $p < 0.001$ DMSO vs. L-NAME; § $p < 0.05$ DMSO vs. NS398. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl-sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin

4.3.2. Relaxation of femoral arteries

The Ach induced relaxation itself did not show significant differences between the four groups studied. As expected, when L-NAME was administered, Ach-induced relaxation decreased significantly in all animal groups **(Figure 7).** In addition, when L-NAME was administered in the trained male rats group, Ach-dependent relaxation was markedly decreased compared to in the female rats **(Figure 7).**

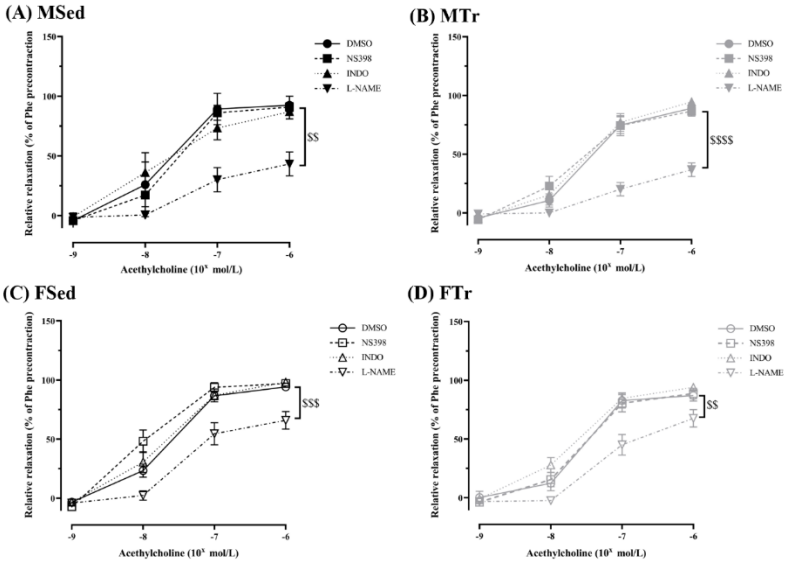

Figure 7. Acetylcholine induced relaxation in the presence of NS398, INDO, L-NAME, or DMSO in sedentary male rats (**A**), in trained male rats (**B**), in sedentary female rats (**C**), and in trained female rats (**D**). Data are shown as means ± SEM; n = 5–19 in each group; analysis: two-way repeated measures ANOVA; test: the Tukey's post hoc test. $$ $p < 0.01$, $$$ $p < 0.001$, $$$$ $p < 0.0001$ DMSO vs. L-NAME. Abbreviations: MSed— male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; DMSO—diluted dimethyl sulfoxide; NS398—the cyclooxygenase-2 specific inhibitor; L-NAME—nitro-L-arginine methyl ester; INDO—indomethacin.

4.3.3. Histological alterations of femoral arteries

The optical density (OD) of eNOS protein expressed was increased in MTr groups after the 12-week training. The OD in the MTr group was higher compared with in the FTr group (**Figure 8)**. Examining COX-2 staining after exercise training, no significant differences were found (26.35 ± 5.299, 77.16 ± 9.001, 38.77 ± 8.580, and 32.93 ± 12.130 arbitrary units for the MSed, MTr, FSed, and FTr groups, respectively (n.s.)). The OD measured with NT staining also did not show differences (0.08 ± 0.003, 0.07 ± 0.007, 0.08 ± 0.004, and 0.07 ± 0.003 arbitrary

units for the MSed, MTr, FSed, and FTr groups, respectively (n.s.)).

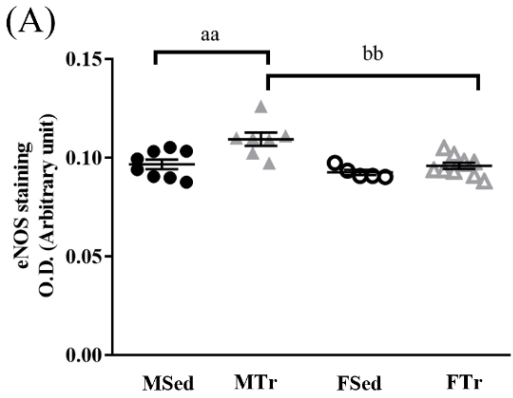

Figure 8. Optical density of eNOS labeling in the intimal layer of femoral arteries. Data are presented as individual data points, and lines represent means ± SEM; *n*= 5–10 in each group; analysis: two-way ANOVA; test: the Tukey's post hoc test. aa, $p < 0.01$ MSed vs. MTr; bb, $p < 0.01$ MTr vs. FTr. Abbreviations: MSed—male sedentary; MTr—male trained; FSed—female sedentary; FTr—female trained; OD—optical density.

5. Conclusions

Our experiments focused on the following questions:

1. Does the renal artery adapt after long-term exercise, and are there sex differences in the ways of sport adaptation?

Our results suggest that aerobic physical activity induces sex specific renal arterial adaptation in Wistar rats. In male rats, phenylephrine-induced contraction is reduced, in which decreased COX-2 dependent contraction and increased NO dependent compensation may play role. In females, no significant functional change was observed; however, decreased COX dependent contraction and NO dependent compensation was still present together with reduced elastin and SMA density.

2. Does the femoral artery adapt after long-term exercise, and are there sex differences in the ways of sport adaptation?

As a result of swim training the balance between endothelium-derived vasocon-strictor and vasodilator substances shifted towards vasodilation in both male and female animals. In swim trained males, NO-dependent relaxation and relaxation reserve increases. We found a greater eNOS expression to be the underlying cause. Sex hor-mones can have a beneficial effect on eNOS, COX and COX-2 signaling. In conclusion, NO release/bioavailability increased as a result of training counteracts vasoconstriction and improved relaxation in femoral arteries. NO release/bioavailability is likely to be more beneficial in males than in females after training

6. Bibliography of the candidate's publications

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∑IF: 12,154