THE ROLE OF HORMONAL EFFECTS AND THE ENDOCANNABINOID SYSTEM IN CARDIOVASCULAR ADAPTATION PROCESSES

Ph.D. thesis

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1. Introduction

Humankind has been using *Cannabis Sativa* −a fitocannabinoid containing plant influencing the endocannabinoid system (ECS)− as a psychoactive agent for at least 3-4 millenia. The ECS is one of the most widespread endocrinal-homeostatic system in the human body with multifaceted physiological and pathophysiological importance, including metabolism and the cardiovascular system. Cannabinoid type-1 receptors (CB_1Rs) have significant effects on cardiovascular functions, as they can be found in the tissues of heart and vessels. ECS signaling was shown to affect the development of atherosclerosis (AS) and plaque stability.

Familial hypercholesterolemia (FH) is the leading cause of AS, consecutively cardiovascular diseases (CVDs). FH is characterized by elevated low-density lipoprotein (LDL) levels resulting in AS, a disease characterized by plaque formation in the circulatory system, elevated thickness and stiffness of the vessels, elevated blood pressure.

According to earlier studies, there is an involvement of the ECS in the control processes of the female reproductive system and is needed in the optimal function of the hypothalamic-pituitary-ovarian (HPO) axis.

Functional experiments of a specific receptor can be done by genetically bred animals that exist without that specific receptor, called receptor knockout (KO) animals, such as CB_1R-KO or LDLR-KO animals.

However, CVDs, such as ischaemic heart diseases caused by high cholesterol levels, has the highest mortality rate in western civilization, the relationship between CVDs and the ECS has not yet been fully defined. By understanding the potential interplay between estrogens and the ECS on the vascular functions and the connection between CB_1Rs and hypercholesterolemic AS, pharmaceutical researchers might be able to develop new medicines influencing the ECS. Novel

designed selective CB_1R inhibitors might have pharmatherapeutic benefits in the future in the treatment of hypercholesterolemia and AS, thus reducing the mortality rates and increase quality of life caused by ischemic heart diseases and hypertonia.

2. Objectives

2.1. In the first part of the experiments we aimed to understand the impact of the ECS and CB_1 receptor activation on vascular functions. We also examined the potential interplay between estrogens and the ECS on the vascular functions.

- We hypothesised, that there might be a connection between the endocannabinoid system and the female hormonal system in terms of vascular function, remodeling and that by knocking out CB_1Rs may enhance vascular function.
- Further hypothesis was, that knocking out CB_1Rs possibly alters female hormonal levels of estrogens and their metabolites.

2.2. In the second part we aimed to reveal the potential role of CB_1Rs and its signaling mechanisms in the functional vascular remodeling of an atherosclerosis-prone mouse model based on the absence of LDL receptors. By crossbreeding animals, we have developed a double knockout, LDLR-KO and CB_1R-KO mouse model, to be able to investigate the impact of the role of CB_1R in the functional and structural atherosclerotic remodeling of the aortic wall.

- We hypothesised, that knocking out the CB_1Rs might reduce high cholesterol levels in a novel atherosclerosis-prone LDLR-CB_1R double knockout mouse model kept on HFD.
- Further we hypothesised, that knocking out the CB_1Rs might moderate the deteriorating vascular functions and structural remodeling in LDLR-KO hypercholesterolemic, atherosclerosis-prone mouse model kept on HFD.

3. Methods

3.1. Experimental animals and alimentation

During experiments, we used genetically modified animals in terms of CB_1 receptor, LDL receptor, and we have grouped them by their genotype and their diet.

In the first project, female homozygous (4-6 months old, 20-23 g) CB_1R knockout mice ($CB_1R-/-$, n = 25) and their wild-type counterparts ($CB_1R+/+$; n = 35) were used, while in the second project, male animals (n=62) were used. These animals were divided into eight groups according to their genetics and their diet.

Our experiments were in line with the Guide for the Care and Use of Laboratory Animals (NIH, 8th edition, 2011). Institutional and National guidelines for animal care and breading were approved by the Animal Care Committee of the Semmelweis University, Budapest as well as by the Hungarian authorities (No. PE/EA/1428-7/2018 and PE/EA00670-6/2023).

Mice were either fed with Janvier standard laboratory chow (first project), or with special high-fat diet and its control diet (second project) from Ssniff Spezialdiäten GmbH.

3.2. Myography

Myography was performed in both parts of experiments according to our protocols. Animals were anaesthetized with intraperitonally administered pentobarbital-sodium (Euthasol), dosage up until 55 mg/kg. After perfusion with Krebs, aortas were dissected.

Abdominal aortic rings (3-4mm) segments were put into cold Krebs solution then were put onto wires attached to the holders of the multichamber isometric myograph system (610 M Multiwire Myograph System, Danish Myo Technology A/S, Aarhus, Denmark) to record isometric tensions. Powerlab data acquisition system registered all eight channels, in eight separate

chambers, simultaneously. Evaluation was done later with the LabChart version 8 evaluation software (ADInstruments Pty Ltd, Bella Vista, Australia. Introduced by Ballagi LTD, Budapest, Hungary). The myograph chambers were filled with exactly 37°C thermostated solution, bubbled with carbogen gas (95% O2 + 5% CO2), keeping the pH at 7.4. After pre-stretching to 10 mN, they were equilibrated for 30 min, then reference contraction was performed with high potassium level Krebs solution (K+ 124 mmol/L). Endothelium and smooth muscle function was tested and registered as dose response curves, with acetylcholine (after preconstriction, Ach), angiotensin II (AngII) and phenylephrine (Phe). Selective inhibitors were applied to test the mechanism of endothelial function. NOS inhibition by Nω-nitro-L-arginine and COX inhibition by indomethacin, while some parallel segments were treated with vehicle as controls. Inhibitors were administered to the chambers for 20 min before the repetition of the dose-response curves of Phe and Ach. In the first part of experiments also estradiol vasodilation was measured without and with the presence of inhibitors. Vasoconstriction responses were normalized to KCl contraction, vasodilation responses were calculated compared with the precontraction state. Presence and the effect of CB_1Rs were tested in CB_1R-KO and WT groups with WIN 55,212-2, a synthetic CB_1R agonist.

3.3. Estradiol determination

In the first project, free estradiol, conjugated estradiol, free and conjugated estrone, 2-hydroxyestrone and free 4-hydroxyestrone levels were determined from blood plasma samples by liquid chromatography-tandem mass spectrometry. Other estradiol metabolites were under threshold levels.

3.4. Body- and heart weight measurements

Body- and heart weights of the experimental animals' were measured with precision lab scale before and during preparation respectively (KERN EG2200-2NM, Kern&Sohn GmbH, Balingen, Germany).

3.5 Blood pressure measurements

External tail cuff method was used in order to measure the blood pressure (BP) of the mice. BP was measured under superficial anaesthesia with Euthasol, administered intraperitoneally in a reduced dose of 35 mg/kg. Blood pressure values were recorded using CODA tail-cuff blood pressure monitor (Kent Scientific Corporation, CT, US).

3.6. Cholesterol level determination

Determination of total cholesterol levels was carried out with EnzyChrom™ AF Cholesterol Assay Kit (BioAssay Systems, California, US). The Colesterol Assay Kist was used precisely according to the manufacturer's guideline to determine plasma cholesterol levels of the mice.

3.7 Immunohystological eNOS staining

To reveal endothelial nitric oxide synthase (eNOS) density we performed immunohystological staining. After paraformaldehyde fixation and embedding in paraffin, 2,5 μm abdominal aortic sections were cut and stained immunohistochemically for eNOS. Antigen retrieval was done by heating the slides in citrate puffer at a slightly acidic pH (pH = 6) after deparaffination. Endogenous peroxidase activity was blocked with 3 % H2O2. We used a 2.5 % normal horse serum blocking solution (Vector Biolabs, Burlingame, CA, USA), in order to eliminate the nonspecific labelling of the secondary antibody. Primary eNOS mouse monoclonal antibody 1:50

(Abcam, Cambridge, UK) was used with overnight application at 4 °C. For secondary labeling we used horseradish-peroxidase-(HRP) linked anti-mouse IgG polyclonal antibodies (Vector Biolabs, Burlingame, CA, USA). Visualization was performed with 3′3-diaminobenzidine (DAB, Vector Biolabs, Burlingame, CA, USA). Photos of slides were taken with Nikon eclipse Ni-U microscope with DS-Ri2 camera at 20× magnification (Nikon, Minato—Tokyo, Japan). The brown positivity and the background staining (DAB and hematoxylin) were separated and by noncalibrated optical density staining intensity was identified. We investigated the staining intensity in the endothelial layer using the FIJI® software (https://imagej.net/software/fiji/downloads, National Institutes of Health, Bethesda, MA, USA).

4. Results

4.1 Results of the first project

4.1.1. Results of myography in CB_1R knockout and wild-type female mice

Dose-dependent vasodilatory responses to acetylcholine (Ach) and E2 were significantly higher in case of CB_1R-KO mice, compared to their WT counterpart in the aortas of mice (Ach: 10^{-8} and 10^{-7} mol/L, p = 0.002 and 0.044, respectively, E2: 10^{-5} mol/L, p = 0.008, 2-way ANOVA with Bonferroni post-hoc test). Vasodilation to CB_1R agonist WIN 55,212-2 was only observed in WT female mice (12.1 ± 3.4%, n=5), not in CB_1R-KO animals (2.3 ± 3.3%, n=6). In order to test potential different mechanism in vascular tone control, vasoconstriction to phenylephrine, vasodilations to Ach and estradiol were obtained in the presence of the NO synthesis inhibitor LNA and COX inhibitor INDO in CB_1R-WT and -KO female mice. LNA did not significantly influence the contraction dose–response to Phe in either genetic group. In the presence of INDO, Phe contractions were significantly attenuated both in the CB_1R-WT (at 10^{-6} to 10^{-5} mol/L, $p<0.001$, interaction: p=0.006, 2-way ANOVA) and in the CB_1R-KO (at 10^{-6} to 10^{-5} mol/L, $p<0.001$, interaction: $p<0.001$, 2-way ANOVA). Direct comparison of the two strains showed that Ach relaxation was statistically different in the presence of INDO (p = 0.026 with two-way ANOVA, Bonferroni post hoc test, $p<0.05$ at 10^{-7} and 10^{-6} mol/L Ach). NOS inhibitor LNA significantly reduced E2-relaxation ($p<0.001$ at 10^{-5} mol/L) in both groups.

4.1.2. Estrogen metabolite levels

Free estradiol (fE2) levels did not show a significant difference between $CB_1R+/+$ (n=24) and $CB_1R-/-$ (n=17) female mice. In spite of this, conjugated estradiol levels were significantly higher (p=0.039) in the $CB_1R-/-$ (n=11) female mice than in

CB_1R+/+ (n=16). Free 4-hydroxyestrone levels showed higher values in CB_1R−/− mice (n=13), but they didn't reach significant difference compared to CB_1R+/+ female mice (n=14)

4.2 Results of the second project

4.2.1. Results of myography in double knockout atherosclerotic male mouse model

Acetylcholine-induced dose-dependent vasorelaxation showed a significant difference between CD and HFD groups (p=0.026). which was significantly attenuated in HFD groups. Endothelium dependent Ach relaxed the vessels the most in LDLR+/+, CD groups, compared to LDLR–/–, CD groups (p=0.047). When CB_1R receptor was absent, significantly less relaxation was recorded for LDLR-KO, CD compared to LDLR+/+, CD (p=0.016, two-way ANOVA, Holm Sidak test). In case of HFD groups no significant statistical difference could be obtained between LDLR+/+ vs. LDLR–/–, in addition, there was no statistical difference in case of CB_1Rs either. With a curve fitting method, there was a significant difference in EC50 values between CD and HFD in the genotype of CB_1R+/+, LDLR−/− (p=0.043), demonstrating that these 5 months of HF dieting deteriorated Ach-induced vasorelaxation, by elevating EC50 values (from 13.4±2.9 to 26.4±5.3 nmol/L, Table 3). We have found that values significantly decreased by knocking out the CB_1Rs in LDLR−/−, HFD group (from CB_1R-WT 26.4±5.3 to CB_1R-KO 14.5±3.0 nmol/L, $p<0.05$) showing an enhancement in vasorelaxation in CB_1R-KO mice. ENOS inhibition with LNA significantly attenuated the Ach-induced vasodilation in all groups in concentrations of 10^{-8}–10^{-6} mol/L. COX-inhibition with INDO slightly modulated vasodilation in just a some of the groups, in 10^{-8} mol/L concentration. Vasodilation induced by Ach was attenuated by NOS inhibition in all groups, which effect was elevated in all CD groups compared to HFD groups. There was a less pronounced vasodilation response between CD

and HFD fed mice regarding Ach relaxation attenuated with LNA in CB_1R-KO groups, a significance was found only between groups in CB_1R-LDLR double knockout group at Ach 10^{-6} mol/L, compared to CB_1R+/+ groups.

4.2.2. Body- and heart weight values

HFD effectively and significantly increased body weight of mice, but with a CB_1R-KO genotype, mice developed lower body weight ($p<0.001$ HFD vs. CD and $p<0.001$ CB_1R+/+ vs. CB_1R−/−). The heart weight of the mice didn't change significantly.

4.2.3. Results of blood pressure measurements

Significantly elevated systolic and diastolic BP values were registered in CB_1R+/+, LDLR−/−, HFD mice compared to CB_1R+/+, LDLR+/+, HFD mice ($p<0.001$), while this elevation wasn't registered in CB_1R−/−, LDLR−/−, HFD group. There is also a statistical difference ($p<0.001$) between CB_1R+/+, LDLR−/− and CB_1R−/−, LDLR−/−, HFD animals.

4.2.4. Results of cholesterol level measurements

Plasma total cholesterol levels did not differ significantly in the LDLR+/+ groups. In LDLR knockout groups the HFD significantly elevated the plasma cholesterol levels pushing it into pathophysiological ranges compared to CD groups ($p=0.001$ between CB_1R−/−, LDLR−/−, HFD and CB_1R−/−, LDLR−/−, CD and $p=0.006$ between CB_1R+/+, LDLR−/−, HFD and CB_1R+/+, LDLR−/−, CD group). LDLR−/− animals kept on control diet showed a significantly increased plasma cholesterol concentration compared to LDLR+/+ groups with CD. This effect was independent from the presence of the CB_1 receptor. Presence of CB_1Rs did not change the plasma cholesterol levels significantly.

4.2.5. Immunohistochemistry results of endothelial NOS

Endothelial nitric oxide synthase didn't change in $CB_1R+/+$, HFD group, but was significantly elevated in $CB_1R-/-$, HFD animals. Thus, the missing CB_1Rs resulted in higher levels of eNOS in the HFD group compared to CD animals ($p=0.016$).

5. Conclusions

5.1. Conclusions of the impact of CB_1R on the vascular function and on estrogen status in female mice (first project)

In the first project, we have found functional changes of the aortic wall of $CB_1R-/-$ female mice, indicating augmented vasorelaxant responses partially regulated by NO and the alteration of vasoactive endoprostanoid function. In CB_1R knockout mice, the vasodilatory responses are lack of the dominance of vasoconstrictor prostanoids, which are present in WT. Our team also found structural changes in accordance with the functional results. A life-long functional role of CB_1Rs had been revealed according to our results. We suggest that by administering selective CB_1 receptor inhibitors chronically, a significant part of the registered effects may be realized. Our results are in line with the previous experiments, which suggest that a suppression of the ECS through selectively blocking CB_1Rs may enhance favorable vasodilatory effects, and vascular remodeling (Figure 1A-B).

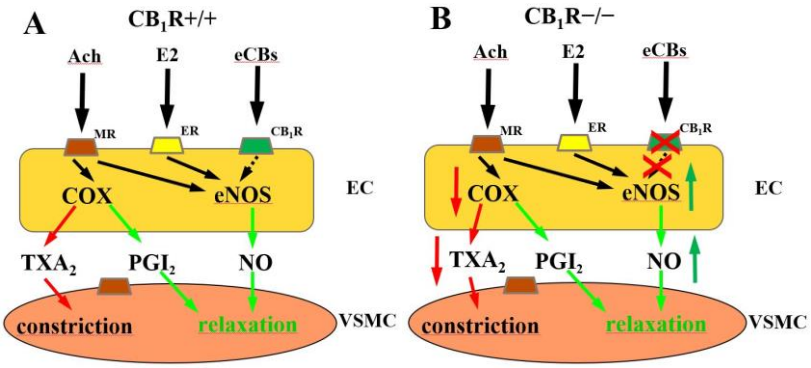

Figure 1. Schematic representation of the functional changes in the aortic wall of CB_1R-WT and CB_1R-KO female mice.

Knocking out the CB_1Rs induces elevation of eNOS expression and NO availability, parellel to a decreased constrictor prostanoid production, resulting in an enhanced relaxation of the vascular smooth muscle cells. (Own figure based on Bányai and Vass et al., 2023.) *Abbreviations: CB_1R+/+, cannabinoid type-1 receptor wild-type; CB_1R−/−, cannabinoid type-1 receptor knockout; Ach, acetylcholine; E2, estradiol; eCBs, endocannabinoids; MR, muscarinic receptors; CB_1R, cannabinoid type-1 receptor; COX, cyclooxygenase; eNOS, endothelial nitric oxide synthase; TXA_2, thromboxane A_2; PGI_2, prostacyclin; NO, nitric oxide; EC, endothelial cell; VSMC, vascular smooth muscle cell; CB_1R-WT, cannabinoid type-1 receptor wild-type; CB_1R-KO, cannabinoid type-1 receptor knockout.*

5.2. Conclusions of the investigation of the functional and remodeling effect of CB_1R in double CB_1R-LDLR knockout atherosclerotic male mouse model (second project)

In the second project we established a LDLR-CB_1R double knockout mouse model, which served as a tool to explore the possible involvement of CB_1Rs in a development of hypercholesterolemia and atherosclerosis in male mice. According to our results, LDLR knockout mice fed with HFD develop functional vascular deterioration which manifests in a depressed NO-dependent vasodilation. The key discovery of our experiments that this deteriorated function can be partially improved in the absence of CB_1Rs, which result is partially supported by an augmentation in the NO availability in CB_1R−/−. LDLR−/−, HFD mice. In parallel, the systolic and diastolic BP values developed significantly higher in HFD, which was attenuated in the absence of CB_1Rs. Patophysiological alterations caused by HFD indicates towards

the development of atherosclerosis, hypertension and other cardiovascular diseases. These mechanisms can be partially prevented in the absence of CB_1Rs, or also by a selective inhibition of the CB_1 receptors. Our results may open new therapeutic strategies to prevent or improve the deteriorated vascular functions in atherosclerosis (Figure 2A-C).

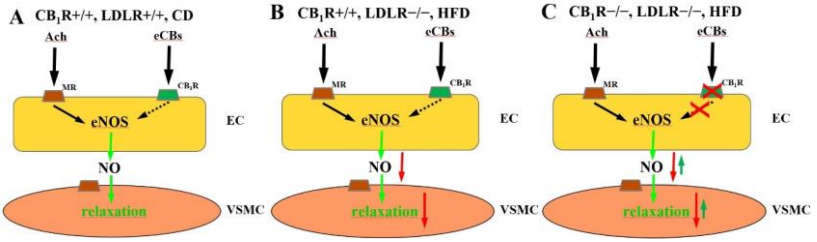

Figure 2. Schematic representation of the functional changes in the aortic wall of CB_1R-LDLR double knockout high-fat diet male mice compared to CB_1R-LDLR-WT, control diet male mice. Knocking out LDLR with HFD results in a decreased NO production and a deteriorated vasodilatory function, which can be partially prevented by knocking out the CB_1Rs. (Own figure based on Vass et al., 2024.) *Abbreviations: CB_1R+/+, cannabinoid type-1 receptor wild-type; CB_1R−/−, cannabinoid type-1 receptor knockout; LDLR+/+, low density lipoprotein receptor wild-type; LDLR−/−, low density lipoprotein knockout; Ach, acetylcholine; eCBs, endocannabinoids; MR, muscarinic receptors; CB_1R, cannabinoid type-1 receptor; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; EC, endothelial cell; VSMC, vascular smooth muscle cell; CB_1R-WT, cannabinoid type-1 receptor wild-type; CB_1R-KO, cannabinoid type-1 receptor knockout, CD, control diet; HFD, high-fat diet.*

6. Bibliography of the candidate's publications

Publications related to the thesis:

I. Bálint Bányai†, **Zsolt Vass†**, Stella Kiss, Anikó Balogh, Dóra Brandhuber, Gellért Karvaly, Krisztián Kovács, György L Nádasy, László Hunyady, Gabriella Dörnyei, Eszter Mária Horváth, Mária Szekeres. Role of CB1 Cannabinoid Receptors in Vascular Responses and Vascular Remodeling of the Aorta in Female Mice. Int. J. Mol. Sci. 2023 Nov 17;24(22):16429. doi: 10.3390/ijms242216429. **†Shared first co-author. IF: 4.9**

II. **Zsolt Vass**, Kinga Shenker-Horváth, Bálint Bányai, Kinga Nóra Vető, Viktória Török, Janka Borbála Gém, György L. Nádasy, Kinga Bernadett Kovács, Eszter Mária Horváth, Zoltán Jakus, László Hunyady, Mária Szekeres and Gabriella Dörnyei. Investigating the Role of Cannabinoid Type 1 Receptors in Vascular Function and Remodeling in a Hypercholesterolemic Mouse Model with Low-Density Lipoprotein–Cannabinoid Type 1 Receptor Double Knockout Animals. Int. J. Mol. Sci. 2024, 25, 9537. https://doi.org/10.3390/ijms25179537. **First author. IF 4.9**

Publications not related to the thesis:

I. Gabriella Dörnyei, Zsolt Vass, Csilla Berta Juhász, György L. Nádasy, László Hunyady, Mária Szekeres. Role of the Endocannabinoid System in Metabolic Control Processes and in the Pathogenesis of Metabolic Syndrome: An Update. Biomedicines. 2023 Jan 21;11(2):306. doi: 10.3390/biomedicines11020306. Review, **IF: 3.9**

∑IF: 13,7