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

PhD thesis

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List of abbreviations

2-AG	2-arachidonoylglycerol
2OH-E1	2-hydroxyestrone
7-TM	7-transmembrane receptors
Ach	acetylcholine
AEA	arachidonoylethanolamide, anandamide
AMI	acute myocardial infarction
Ang II	angiotensin II
ApoER2	apolipoprotein E receptor 2
AS	atherosclerosis
AT1	angiotensin type 1
CB_1R	cannabinoid type 1 receptor
CB_2R	cannabinoid type-2 receptor
CBD	cannabidiol
CD	control diet
cE1	conjugated estrone
cE2	conjugated estradiol
COX	cyclooxigenase
CV	cardiovascular
CVRFs	cardiovascular risk factors
DAGL	diacylglycerol lipase
DM	diabetes mellitus
E1	estrone
E2	17β-estradiol
E3	estriol
E4	estetrol
EC	endothelial cell
EC50	effective concentration at 50% of maximum response
eCBome	endocannabinoidome
eCBRs	endocannabinoid receptors

eCBs	endocannabinoids
ECS	endocannabinoid system
Emax	maximum effect
eNOS	endothelial nitric oxide synthase
ERα	estrogen receptor α
ERβ	estrogen receptor β
f4OH-E1	free 4-hydroxyestrone
fE1	free estrone
fE2	free estradiol
FH	familial hypercholesterolemia
GnRH	gonadotropin releasing hormone
GPCRs	G protein-coupled receptors
GPR55	G protein-coupled receptor 55
HDL	high density lipoprotein
HFD	high-fat diet
HPO	hypothalamo–pituitary–ovarian
HT	hypertension
INDO	indomethacin
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LDLR-KO	low density lipoprotein receptor knockout
LH	luteinizing hormone
LNA	Nω-nitro-L-arginine
LRP	lipoprotein receptor-related protein
MAGL	monoacylglycerol lipase
NAPE	N-arachidonoyl phosphatidylethanolamine
NO	nitric oxide
OD	optical density
OEA	N-oleoylethanolamine

PEA	N-palmitoylethanolamine
pEC50	negative log of effective concentration at 50%
PG	prostaglandin
PGI_2	prostacyclin
Phe	phenylephrine
PPARα	peroxisome proliferator-activated receptor α
ROS	reactive oxygen species
THC	Δ^9 -tetrahydrocannabinol
TXA_2	thromboxane A_2
VLDL	very low density lipoprotein
VSMC	vascular smooth muscle cell

1. Introduction

1.1. Historical overview of the endocannabinoid system and cannabis consumption

The presence of *Cannabis sativa* plant appeared around 11,700 years ago and had been used for ropes, nets, food etc. in central and South-East Asia, while people had been using cannabis as a psychoactive drug for at least the past 3-4 millennia. (Crocq, 2020; Pisanti & Bifulco, 2019) Chinese archeological excavations found cultivated cannabis containing high levels of Δ^9 -tetrahydrocannabinol (THC), dating back to 750 B.C.. (Crocq, 2020; Russo et al., 2008) Medical use of cannabis might date back to circa 2000 bc., and might be used to create numbness, general anaesthesia, later to treat inflammation topically, diminish depression and pain. Graeco-Romans, Greeks used cannabis for its antalgic and anti-inflammatory properties in arthritis and gout. (Crocq, 2020) Later the cannabis plant spread the world. (Crocq, 2020; O'Shaughnessy, 1840) Before the full therapeutic range of cannabis could be exploited, it was subjected to strict regulation (such as the Marihuana Tax Act of 1937), replaced with other medications, and later classified as a Schedule 1 drug in 1970, hindering research efforts. (Crocq, 2020) Cannabidiol (CBD) in 1940, which is a non-psychoactive agent, 4 years later THC, the main psychoactive agent in *Cannabis sativa* was also isolated (Burstein, 2015; Crocq, 2020) Mechoulam and Gaon determined the structure of THC (Crocq, 2020; Mechoulam & Gaoni, 1965; Pertwee, 2006), while Devan and his team described cannabinoid type 1 receptor (CB_1R) and endocannabinoid arachidonoylethanolamide called anandamide (AEA). (Devane et al., 1988; Devane et al., 1992) Endocannabinoid receptors (eCBRs) were initially identified due to their binding affinity to THC, the main psychoactive agent of *Cannabis sativa*. Endocannabinoids (eCBs) act as endogenous ligands for cannabinoid receptors and are involved in tissue-specific paracrine regulatory pathways initially described in the nervous system. (Di Marzo et al., 1998; Dörnyei et al., 2023; Freund et al., 2003) In 2016 CB_1R, in 2019 CB_2R was crystallized for better understanding and to create new routes for drug developments. (Hua et al., 2016; Huang et al., 2020; Shao et al., 2016; Xing et al., 2020) CB_1R is encoded by the *cnr1* gene. (Veilleux et al., 2019)

1.2 The endocannabinoid system

The endocannabinoid system is one of the most important endocrinal regulatory system in the human body. It has a multifaceted role, it takes part in several physiological and pathophysiological processes. It influences the homeostasis, body weight, metabolism, endocrine- and immune system, also it is involved in several processes of the nervous system, regulation of the cardiovascular (CV) system, reproductive functions as a process of fertility and maintaining of pregnancy. Other fields that involve the ECS are the digestive system along with appetite control, body temperature, bone formation and cell cycle. The ECS also influences neurobehavioral and analgesic pathways. (Bányai, Vass, et al., 2023; Bondarenko, 2019; Dörnyei et al., 2023; Kunos et al., 2009; Lowe et al., 2021; Vass et al., 2024)

1.2.1 Contents and morphology

Endocannabinoid receptors are GPCRs that share a 7-TM topology, such as cannabinoid type 1 receptors (CB_1Rs) and cannabinoid type 2 receptors (CB_2Rs). (Huang et al., 2020) Their ligands, such as AEA, 2-AG and endocannabinoid-like compounds, such as N-palmitoylethanolamine (PEA) and N-oleoylethanolamine (OEA), synthesizing enzymes, such as diacylglycerol lipase (DAGL), N-arachidonoyl phosphatidylethanolamine phospholipase D and degrading enzymes, such as monoacylglycerol lipase (MAGL), fatty acid amide hydrolase are all included in ECS and broader concept, the so-called endocannabinoidome (eCBome). (Bányai, Vass, et al., 2023; Pacher et al., 2008; Ueda et al., 2011; Zou & Kumar, 2018) PEA increases the effect of eCBs by competitively inhibiting their hydrolysis or by allosterically modulating their receptor binding, and it has a strong anti-inflammatory effect. However, OEA has an antinociceptive peroxisome proliferator-activated receptor α independent (PPARα) and appetite-suppressor effect through PPARα. (Fezza et al., 2014) The CB_1Rs and CB_2Rs share 44% sequence homology, and have a similar ligand-binding orthosteric pockets, but also have an allosteric binding sites, that might selectively modulate CB receptors' functions. (Huang et al., 2020) There are also some receptors that can interact and are being modulated by the eCBs, for example GPR3, GPR6, GPR12 GPR18, GPR55, GPR19, TRPV1, TRPV2, TRPV3, TRPV4, PPARy receptors. (Aizpurua-Olaizola et al., 2017; Bányai, Vass, et al., 2023; Dörnyei et al., 2023; Guillamat-Prats et al., 2019; Iannotti & Vitale, 2021; Morales

et al., 2017; Morales et al., 2020; Pacher et al., 2005; Pacher et al., 2008; Ramírez-Orozco et al., 2019; Rezende et al., 2023; Veilleux et al., 2019)

1.2.2 The localisation and physiology

ECS has a diverse physiological role. It takes part in synaptic neurotransmission, has cardiovascular effects, regulates metabolism, controls appetite, modulates pain perception, influences on overall well-being, and memory functions. Several metabolic control processes are influenced by the ECS, with CB_1R-dependent signalling increasing appetite and promoting weight gain. The ECS also affects the endocrine system, including modulation of the hypothalamo-pituitary axis and the regulation of gonadotropin-releasing hormone (GnRH) secretion. (Dörnyei et al., 2023; Gyombolai et al., 2012; Huang et al., 2020; Kunos et al., 2009; Pacher et al., 2005, 2006; Schurman et al., 2020; Szekeres et al., 2015; Vass et al., 2024)

The ECS, through CB_1R signalling, has significant effects on CV functions, with a pronounced negative cardiac inotropic and chronotropic, vasodilatory-hypotensive effect and vascular remodeling, as CB_1Rs can be found in the tissues of heart and vessels. (Dörnyei et al., 2023; Miklós et al., 2021; Pacher et al., 2005; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015) This means, that activation of CB_1Rs leads to a decrease in cardiac output and peripheral resistance, which in turn lowers blood pressure. It is shown, that cannabinoids can reduce blood pressure and elevate heart rate through CB_1R agonism. (Rorabaugh et al., 2023) Through GPCRs, endocannabinoid release has been observed, and the coactivation of CB_1Rs reduced the effects of vasoconstriction. (Gyombolai et al., 2012; Karpińska et al., 2018; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015; Szekeres et al., 2012) We had shown earlier that the vasodilatory effect of CB_1R agonist WIN 55,212-2, which effect could not be observed in CB_1R-KO mice, or with the inhibition of CB_1Rs. (Bányai, Vass, et al., 2023; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015) ECS signalling was shown to affect the development of atherosclerosis (AS) and plaque stability via multiple mechanisms such as vascular inflammation, cholesterol metabolism and leukocyte recruitment. (Guillamat-Prats et al., 2019) Furthermore, an elevated endocannabinoid level can be detected in atherosclerosis. Activating CB_1Rs usually leads

to further AS plaque formation, in contrast, CB_2R activation has a protective role against AS. (Rorabaugh et al., 2023)

The ECS was firstly detected in the central nervous system. (Devane et al., 1988) Due to its location, it plays a significant role in learning, memorizing, emotional conditioning and movement learning, regulation of motor planning and motor coordination. It has a crucial role in decreasing pain peripherally and centrally, increasing appetite and food intake, decreasing nausea and vomiting, increased sense of pleasure, pursuit of rewards and in anxiolysis, as well as protection against overstimulation and neurodegeneration. (Gyombolai, 2015; Herkenham et al., 1991; Howlett et al., 2002; Kano et al., 2009; Pacher et al., 2006) CB_1Rs are present in cells of adipocytes and liver. Overall, an anabolic dominance appears in the adipose tissue and liver by activating CB_1Rs: the fat content and size of adipocytes increase, also fatty acid synthesis increases and β-oxidation decreases in the hepatocytes. Parallel with the appetite increasing effect and the upregulation of food intake, CB_1Rs create a systemic anabolic dominance, which can result in gaining weight and become obese. (Gyombolai, 2015; Kunos et al., 2009; Osei-Hyiaman et al., 2005; Roche et al., 2006) CB_1Rs can be also found in the eyes, bone marrow, spermiums, gastrointestinal tract, skin, lungs, pancreas, immune system and kidneys (Järvinen et al., 2002; Lewis & Maccarrone, 2009; Novack, 2016; Pacher et al., 2006; Rezende et al., 2023; Veilleux et al., 2019), thus has an importance in regulation in various physiological processes. (Gyombolai, 2015)

CB_2Rs are present mainly in the immune system (spleen, tonsils, thymus) and in the cells (monocytes, macrophages, T and B lymphocytes, natural killer cells, and neutrophils), also in the peripheral tissues, such as in the bones and bone marrow, skin, liver, central nervous system (microglia) and pancreas. (Rezende et al., 2023; Veilleux et al., 2019) Their functions are modulation of immune cells and contribution to the analgesic and/or antinociceptive effects of cannabinoids. (Li et al., 2020; Veilleux et al., 2019)

1.2.3 Synthesis and degrading of endocannabinoids

Synthesis and degrading endocannabinoids play an important role in the regulation of eCSs and in its tissue levels. 2-AG is formed from membrane phospholipids, which process is catalyzed DAGL through hydrolisation from inositol phospholipids, and its degradation is due to MAGL, into arachidonic acid and glycerol. (Dörnyei et al., 2023;

Ueda et al., 2011) AEA is also produced from cell membrane phospholipid precursor N-arachidonoyl phosphatidylethanolamine (NAPE) through hydrolysis by a phospholipase D. (Liu et al., 2008; Pacher et al., 2008) There are other pathways to create AEA such as sequential deacylation of NAPE, or C-mediated hydrolysis of NAPE and dephosphorilation. (Liu et al., 2008) Degradation of AEA is catalyzed by FAAH. (Dörnyei et al., 2023; Pacher et al., 2008; Ueda et al., 2011; Vass et al., 2024) Other minor degrading pathways are also present *in vivo* of both ligands. (Blankman et al., 2007)

1.2.4 The cannabinoid type 1 receptor and signalling

In the ECS, endocannabinoids mainly bind to two receptors, CB_1Rs and CB_2Rs, but there are some other receptors that eCBs can modulate. (Aizpurua-Olaizola et al., 2017; Bányai, Vass, et al., 2023; Dörnyei et al., 2023; Guillamat-Prats et al., 2019; Iannotti & Vitale, 2021; Morales et al., 2017; Morales et al., 2020; Pacher et al., 2008; Ramírez-Orozco et al., 2019; Rezende et al., 2023; Veilleux et al., 2019) Both CB_1R and CB_2R belong to the family of GPCRs that share a 7-TM topology. (Howlett et al., 2010; Huang et al., 2020) CB_1R was crystallized in 2016 for better understanding. This enables new routes for drug development lately. (Hua et al., 2016; Huang et al., 2020; Shao et al., 2016)

Most of signallings of CB_1Rs are through G-proteins. Activation of $G_{i/o}$-proteins are the main pathway of CB_1R signalling, blocking adenylyl cyclase, thus lowering the cAMP levels of the cell, which inhibits protein kinase-A enzyme and voltage-gated Ca^{2+} channels, suppressing the release of neurotransmitters. (Leo & Abood, 2021; Vandevoorde & Lambert, 2007) Among this pathway CB_1R can modulate metabolism, gene expression, cell growth and differentiation, apoptosis and neurotransmission. Altough, through G_s protein, CB_1Rs elevate cAMP levels and create an opposite effect. The CB_1Rs can couple also to G_s, $G_{q/11}$, and $G_{12/13}$ to less extent. Through $G_{i/o}$-protein βγ-subunit CB_1Rs can block voltage gated Ca^{2+} channels and activate K^+ channels. (Leo & Abood, 2021)

CB_1R is capable of recruiting β-arrestin 1 and 2, in order to induce receptor desensitization-creating tolerance (Bedi et al., 2010; D'Souza et al., 2008), internalization and downregulation, that can underline cannabis dependence. In experimental animals they described cannabinoid-induced tetrad through β-arrestins: analgesia, hypothermia,

hypolocomotion and catalepsy. (Gyombolai et al., 2013; Leo & Abood, 2021; Metna-Laurent et al., 2017)

1.2.5. CB_1R knockout animals

The examination of the functional significance of a receptor is by genetically bred animals that exists without that specific receptor, called knockout animals. However, modern medicine can almost perfectly mimic the missing receptor and alter its function by administering specifically engineered or produced inhibitors, thus several effects of missing function can be reproduced in case of the presence of the receptor. We used CB_1R knockout animals in our experiments (see also subchapter 1.4.). (Bányai, Vass, et al., 2023; Vass et al., 2024)

1.2.6 The endocannabinoid system in pathophysiological conditions

The eCBome is involved in several pathophysiological conditions, such as metabolic syndrome, atherosclerosis, inflammatory bowel diseases, colorectal cancer and cardiovascular diseases. (Di Marzo & Silvestri, 2019; Grill et al., 2019; Morris et al., 2021; Piscitelli & Silvestri, 2019) There might be a connection between the gut microbiome and the endocannabinoidome. (Di Marzo & Silvestri, 2019; Rezende et al., 2023) The extensive distribution of ECS components throughout the body, coupled with the roles of ECS signalling pathways in various physiological and pathological processes, presents significant potential for developing new therapeutic drugs. These could include cannabinergic, cannabimimetic, and cannabinoid-based medications that modulate the ECS either genetically or pharmacologically. Such modulation could involve inhibiting metabolic pathways or targeting ECS receptors through agonism or antagonism, offering new treatment options for a range of diseases. *Cannabis sativa* or medical cannabis is one of the main tool to treat different diseases through the ECS. (Lowe et al., 2021)

Cannabinoids can be a promising novel, alternative therapeutic agents through CB_1Rs and CB_2Rs agonism in panic disorders, social anxiety disorder, generalized anxiety disorder, post traumatic stress disorder, and obsessive-compulsive disorder. (Blessing et al., 2015; Lowe et al., 2021) Also agonists can be effective against acut, chronic and inflammatory pain, and could reduce postoperative hypersensitivity. Nabiximols (Sativex®), a synthetic cannabinoid oromucosal spray is being used to treat cancer-related pain, sclerosis multiplex-related pain and spasmolysis as cannabinoids have also

synergic effects with opioids and non-steroid anti-inflammatory drugs. Cannabinoids exhibit anti-inflammatory and immunosuppressive effects and might help patients suffering from Alzheimer's disease, Parkinson's disease, Huntington's, Batten disease, fatal familial insomnia, schizophrenia and stroke. (Choi et al., 2019; Fernández-Ruiz et al., 2015; Lowe et al., 2021)

Related to cardiac diseases, cannabinoids fights against arrhythmias, heart ischaemia and myocardial infarction, atherosclerosis and stroke. (Dörnyei et al., 2023; Lowe et al., 2021) After cardiac ischaemia, endocannabinoids may be upregulated. Activation of CB_1Rs can lead to cardiac inflammation triggered by the ischemia, and activation of CB_2Rs may promote cardiac healing and limit neutrophil infiltration, that mitigates macrophage polarization and lymphocyte clusters in the pericardial adipose tissue. This means that blocking of CB_1Rs and activation of CB_2Rs in such ischaemic cardiac events may have a protective role for the heart that may promote rehabilitation of the patient. (Puhl, 2020)

Activation of CB_1Rs may lead to impaired lipid metabolism and diabetes, but it was also shown that medications influencing the ECS might have a potential role in treating diabetes mellitus (DM). (Lowe et al., 2021; Puhl, 2020) By blocking of CB_1Rs reductions in trygliceride, plasma glucose, LDL, leptin and insulin levels can be achieved. (Puhl, 2020)

Δ9-THC has shown positive effects on brain oxygenation and enhanced blood flow to the prefrontal cortex, potentially offering benefits in treating frontal lobe strokes. In addition, CBD's antispastic properties may be advantageous for patients experiencing post-stroke spasticity. (Lowe et al., 2021)

Cannabinoids might be capable of treating symptoms of cancer- and of AIDS. Various phyto-, endo-, and synthetic cannabinoids show anticancer properties, their line of actions has a wide range, for example inducing apoptosis, autophagy and cell-cycle arrest, inhibiting DNA synthesis and blocking various signalling pathways, such as phosphoinositide 3-kinase, protein kinase B, mammalian target of rapamycin, adenosine monophosphate, activated protein kinase and epidermal growth factor receptor. In addition, eCBs inhibit cancer cell migration, cancer metastasis, angiogenesis, vascularisation, adhesion, as well as tumor growth and invasion. (Hinz & Ramer, 2022; Lowe et al., 2021; Ramer & Hinz, 2017) In epilepsy, last line of treatment can be medical

cannabis that can reduce the frequency of seizure episodes with the parallel use of CBD. These treatment resistant forms of epilepsies might be treated by phytocannabinoids or synthetic versions of them. (Lattanzi et al., 2018; Lowe et al., 2021)

CB_1R and CB_2R might provide neuroprotection through protection from processes that damage the blood brain barrier such as inflammation, cytotoxicity, cell death and oxidative stress. (Lowe et al., 2021; Vendel & de Lange, 2014)

Activating eCBRs can treat eating disorders such as anorexia nervosa, through orexigenic pathways. (Dörnyei et al., 2023) On the other hand, blocking of specific eCBRs selectively might help loose body weight in obesity. (Costiniuk et al., 2008; Dörnyei et al., 2023; Lowe et al., 2021)

A CB_1R signalling has a regulatory role which was observed in healthy and in pathological condititons. Firstly, in healthy animals, CB_1R-inhibition did not significantly alter hemodynamic characteristics and blood pressure, whereas in pathological conditions for example in hypertension (HT), the absence or inhibition of CB_1Rs had a pronounced effect and significance, such as having a blood pressure (BP) lowering effect in hypertensive rats. (Bátkai et al., 2004; Gyombolai, 2015; Pacher et al., 2005; Randall et al., 2002; Vass et al., 2024)

In conclusion, by modulating the endocannabinoid system offers a potential to treat several diseases, which is not wildly used yet. Further experiments are needed to better understand and develop new targeted drug treatments. This area has high potential and is a promising field of medicine in developing new pharmaceutical agents.

1.3 Atherosclerosis: epidemiology and risk factors

Atherosclerosis is a slowly progressing, multifocal, chronic, immune-inflammatory disease, that involves the intima of large and medium-sized arteries. AS includes accummulation of lipids, inflammatory cells, and smooth muscle cells in the arterial wall, later atheroma and characteristic fibrous plaques appear. Thus in later stages ruptures of plaques and consequently death might occur, but the plaque development can be asymptomatic in early stages postponing the diagnose of AS. Elevated inflammatory markers registered during AS for example C-reactive protein with high low-density lipoprotein (LDL) levels. Environmental, genetic risk factors and other diseases are

involved in developing AS, such as dyslipidemia, hypertension, diabetes mellitus, obesity, inactive lifestyle, inappropriate diet, smoking and alcohol consumption. (Luca et al., 2023) Studies have shown, that AS development may already start before birth, in the womb. (Burlutskaya et al., 2021; Luca et al., 2023)

1.3.1. Atherosclerosis in aortas and other arteries

Atherosclerosis is the main pathological pathway leading to cardiovascular diseases (CVDs), such as AMI and stroke, causing 16.7 million death annually worldwide, 31% of global death, 50% of which had AS. (Fan & Watanabe, 2022; Luca et al., 2023) Due to growth of the aged population CVDs are growing in number globally. (Nedkoff et al., 2023; Vass et al., 2024)

Hypercholesterolemia (HC) predisposes cardiovascular diseases, e.g. atherosclerosis and hypertension, being also the leading cardiovascular risk factors (CVRF). Hypercholesterolemia and AS also alter endothelial function. (Akhmedov et al., 2021; Dörnyei et al., 2023; Jiang et al., 2022; Nedkoff et al., 2023; Singh et al., 2002; Vass et al., 2024; Zhou et al., 2022) While developing CVDs, elevated plasma LDL level and other CVRFs might increase the prevelance of AS that may cause chronic arterial inflammation and a slow lipid build-up in the walls of larger vessels during the progression of AS. This process increases the chance of developing CV events later in life, such as stroke, acute myocardial infarction, stable and instable angina pectoris, or any other chronic CV disease. (Centa et al., 2019; Devesa et al., 2023; Vass et al., 2024) LDL receptor signalling plays a major role in development of AS (Mineo, 2020), and by developing LDL receptor-knockout mouse model, this pathophysiological process can be examined properly. (Vass et al., 2024)

1.3.2 Pathogenesis of hypercholesterolemia and atherosclerosis

Hypercholesterolemia is a worldwide pathophysiological disorder, but its diagnosis can be delayed due to lack of symptoms. It is the leading CVRF and a precursor for cardiovascular diseases, stroke, AMI or peripheral vascular diseases. (Ibrahim et al., 2024; Vass et al., 2024) Hypercholesterolemia by definition occurs if there is an elevated plasma LDL level over 190 mg/dL (4.913 mmol/L) without any other CVRF, or if LDL>160 mg/dL (4.138 mmol/L) with one major CVRF, or also if LDL>130 mg/dL (3.362 mmol/L) with two, or more major CVRFs. (Ibrahim et al., 2024) The risk factors

include if males are more than 45 years, or females are more than 55 years of age, if they have low high density lipoprotein (HDL) levels (HDL<40 mg/dL = 1.0344 mmol/L in males, HDL<1.5 mg/dL = 1.4223 mmol/L in females), if they have also early onset of high LDL levels, have a positive family history of premature atherosclerotic cardiovascular disease (male younger than 55 and female younger than 65 years of age), also if they smoke, have DM or hypertension disease. (Ibrahim et al., 2024) Underlying cause of the hypercholesterolemia can be autosomally inherited genetic mutations, for instance in the LDL receptor gene, which is the most common reason in the development of familial hypercholesterolemia (FH), but also other gene mutations may be involved such as in apolipoprotein B gene, or in pro-protein convertase subtilisin/kexin type 9 (PCSK9) gene. (Ibrahim et al., 2024; Nohara et al., 2021) Unhealthy lifestyle can also cause hypercholesterolemia, which involve sedentary lifestyle and increased intake of animal fat. There are also some secondary causes to hypercholesterolemia, such as in case of hypothyroidism, nephrosis syndrome, cholestasis, during pregnancy and also induced by certain drugs. (Ibrahim et al., 2024)

Low-density lipoprotein receptor-related protein LRP1, LRP5, LRP6, VLDLR, and apolipoprotein E receptor 2 (ApoER2) had been identified as functionally and structurally related to develop AS. During the plaque development, initially fatty streaks appears in the arteries and the process lasts until the buildup of fibrosus plaque and as its consequences, due to the rupture of the lipid rich vulnerable plaques and also thrombosis development, myocardial infarction and stroke can develop. (Luca et al., 2023) LDLR plays a major role in the atherogenesis through the receptor-mediated endocytosis of LDL particles and regulation of cholesterol homeostasis. (Mineo, 2020) Monocytes differentiate to macrophages, and lipids accumulate in them creating foam cells, which keep up the inflammation, that can lead to instable plaque formation. (Jebari-Benslaiman et al., 2022; Luca et al., 2023) In addition, suppression of fibrinolysis promotes proinflammatory mechanisms and atherogenesis. Other cell types such as T-helper 1, 2 and 17 and also cytokines and their receptors are involved. (Lee et al., 2020; Luca et al., 2023; Zernecke & Weber, 2014) The vascular endothelium and smooth muscle cells are also involved in the pathogenesis of AS. The endothelium has a regulatory role in vascular tone, remodeling, inflammation and thrombosis. (Luca et al., 2023) Oxidized LDL, hypercholesterolemia, tobacco use, type 2 diabetes mellitus, or arterial hypertension

promotes endothelial dysfunction and AS. (Willerson & Kereiakes, 2003) Hypercholesterolemia impairs the endothelial nitric oxide (NO) and endothelial nitric oxide synthase (eNOS) responses. (Bányai, Vass, et al., 2023; Luca et al., 2023; Vass et al., 2024) Atherosclerotic lesion formation is also enhanced by a dysfunction of the vascular endothelium thus impairing the production of some endothelial cell (EC) factors. (Choi et al., 2014)

Furthermore, vascular smooth muscle cells migrate to the intima layer and generate collagen, which process appeares in the development of atherosclerotic plaques. Also, the extracellular matrix also aggrevates vascular inflammation. (Lu & Daugherty, 2015)

1.3.3 LDL receptor family and LDL receptors in atherosclerosis

Along with other receptors of the LDL receptor family, structurally and functionally linked to a wild range of biological processes, low density lipoprotein receptor (LDLR) has been proven to play a crucial role in maintaining cholesterol homeostasis and in atherogenesis, through endocytosis of LDL particles, which process is receptor mediated. (Mineo, 2020) Its gene mutations cause elevated serum LDL cholesterol level and coronary atherosclerosis in patients with familial hypercholesterolaemia. (Goldstein & Brown, 2015; Nohara et al., 2021) The LDL receptor family are expressed in multiple types of vascular cells and consist of several transmembrane receptors with common structural features as a large extracellular domain with ligand-binding motifs, a single transmembrane domain, and a cytoplasmic tail with multiple adaptor-binding sites. The LDL receptor family consist of LDLR, VLDLR, LRP1, LRP5/6 and LRP8 (aslo known as ApoER2). In terms of atherogenesis, LRP1 in vascular smooth muscle cells (VSMC) and macrophages demonstrates an effective protective role against atherosclerosis, as well as LRP5 and LRP6 exert antiatherogenic actions. However, ApoER2 and VLDLR have both anti- and pro-atherosclerotic actions. (Mineo, 2020) There are also scavenger receptors such as cluster of differentiation 36, scavenger receptor class B type I, and lectin-like oxidized low-density lipoprotein receptor-1. They can interact with the circulating lipoproteins and modulate vascular inflammation, lipid accumulation, also plaque formation. (Acton et al., 1996; Mehta et al., 2007; Mineo, 2020; Park, 2014; Yamada et al., 1998) Autosomally inherited genetic mutations in the LDL receptor gene lead to FH. (Ibrahim et al., 2024)

1.3.4 Animal models of hypercholesterolemia and atherosclerosis

In order to examine AS, genetically altered experimental animal models are widely accepted in order to create AS prone, hypercholesterolemic animals. (Baltieri et al., 2018; Emini Veseli et al., 2017; Maganto-Garcia et al., 2012; Vass et al., 2024) These animal models are reliable, specific, cost efficient, easy to handle and breed, resemble human anatomy and pathophysiology. Results from animal models can be extrapolated to human medicine, so they have high scientific value. (Emini Veseli et al., 2017) The mouse is the predominant animal model used in hypercholesterolemic-AS research, followed by rabbits, pigs, and other primate species. (Emini Veseli et al., 2017) Each animal model has its limitations and advantages, for instance primate species have very similar plaque formation as compared to humans, both micro- and macroscopically, but these animals are expensive, highly regulated officially and need to be trained. As mentioned, mouse models are the appropriate choice to examine hypercholesterolemia and atherosclerosis, due to their attributions of fast reproductive cycle, low cost of breeding and they are easy to be modified genetically. Wild types of C57BL/6 mouse strain are naturally resistant to AS while kept on control diet (CD), thus they are widely used as controls. (Maganto-Garcia et al., 2012; Vass et al., 2024) AS-prone animal models include Apo-E knockout mouse strain, LDL receptor knockout (LDLR-KO, LDLR−/−) mice, or Apo-E-LDLR double KO mice. (Emini Veseli et al., 2017; Vass et al., 2024) Other mouse models are ApoE*3Leiden, Transgenic ApoB, ApoB100 x LDLR−/−, ApoBEC-1−/− x LDLR−/−, LDLR−/− x ABCG1tg, LDLR−/− x Tbet−/−, ApoE−/− x Rag−/−, PCSK9-AAV, ApoE−/− $Fbn1^{(c1039g)}$. These mouse models kept on high fat diet (HFD) develop serious hypercholesterolemia and AS at a different extent. LDLR−/− mice have an elevated plasma cholesterol level of 200-300 mg/dL kept on control diet, which goes up to 1000 mg/dL while kept on HFD, without change in HDL levels. Among all of the genetically modified mice mentioned here, LDLR−/− strain models human-like familial hypercholesterolemia the best, in which the extent of disease correlates well with the degree of hypercholesterolemia. (Emini Veseli et al., 2017; Maganto-Garcia et al., 2012; Vass et al., 2024)

1.3.4.1 LDL-KO mice and high-fat diet

LDLR knockout mice develop significantly higher plasma levels of cholesterol than LDLR wild-type ones, where most of the cholesterol is transported in HDL form. (Emini

Veseli et al., 2017) In one part of our experiments we used LDLR-KO mouse strain, due to its desired properties of developing LDL-dominant hypercholesterolemia, which is characteristic for humans' familial hypercholesterolemia. (Nohara et al., 2021) LDL represents one of the most prominent risk factors of atherosclerosis as being more atherogenic over VLDL. (Emini Veseli et al., 2017; Getz & Reardon, 2016; Langbein et al., 2015; Maganto-Garcia et al., 2012)

Mice kept on high-fat diet (HFD), develop plaques and AS in a higher extent. (Emini Veseli et al., 2017; Getz & Reardon, 2016; Vass et al., 2024) LDLR-KO mice kept on long term HFD develop plasma cholesterol levels of around 800-1000 mg/dL. (Centa et al., 2019; Emini Veseli et al., 2017; Liu et al., 2022; Maganto-Garcia et al., 2012; Vuorio et al., 2020)

1.4 Endocannabinoid system and atherosclerosis, CB_1R-LDLR-double knockout animal model

During previous experiments, we found that the CB_1R agonist WIN 55,212-2 induced vasodilation, which couldn't be seen in CB_1R-KO mice. (Bányai, Vass, et al., 2023; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015) The effects of CB_1R-KO genotype in female mice (life-long effects), however, were enhanced vasodilatory abilities mediated by enhanced endothelial nitric oxide (eNO) production and by altered endogenous prostaglandin (PG) release. (Bányai, Vass, et al., 2023; Vass et al., 2024) ECBs and CB_1R-signalling were also found to be important in structural remodeling of the vascular wall. (Bányai, Vass, et al., 2023) ECS influencing vascular inflammation, cholesterol metabolism and leukocyte recruitment affected the formation of AS and plaque stability. ECS signalling was also shown to affect the development of AS. (Guillamat-Prats et al., 2019; Vass et al., 2024) We planned to investigate the role of ECS in atherosclerotic vascular wall remodeling by establishing a double LDLR-KO and CB_1R-KO mouse model (See also 3.1.). Keeping these animals on HFD made us capable to investigate the effect of the existence of the CB_1R on structure and function of atherosclerotic remodeling of the aortic wall.

1.5 Female hormonal system, estrogens and its functions

The female reproductive and hormonal system mainly aims to maintain the existance of a species by reproducing, but its hormonal system has a large variety of other roles in

physiological and patophysiological processes, such as development of the body and maintaining homeostasis. In most mammalian animals the reproduction is according to the changes of seasons to give birth in the most optimal months, however in primates the reproduction has been separated, and become independent of the seasonal changes. The female hormonal system is centered around the hypothalamo–pituitary–ovarian (HPO) axis, controlling the production and release of sexual steroid hormones and related metabolites through negative and positive feedback mechanisms. Among other metabolites, these hormones include gonadotropin releasing hormone, luteinizing hormone (LH), follicule stimulating hormone, progesteron (P4), estrone (E1), 17β-estradiol (E2) and estriol (E3), inhibin A and B. (Fonyó, 2014)

In the human body, there are 4 types of estrogen: estrone, 17β-estradiol, estriol, and estetrol (E4). These steroid hormones are lipid-soluble and through estrogen receptor α (ERα), estrogen receptor β (ERβ) and a G protein-coupled membrane receptor (mER or GPER1) they exert their functions. ERα is expressed in the reproductive tissues, bones, white adipose tissue, kidneys, liver and breasts. However, ERβ is expressed in the male reproductive organs, the central nervous system (CNS), cardiovascular system, lungs, immune system, colon and kidneys. GPER1 is expressed in the skeletal muscle, neurons, vascular endothelium, various immune cells and target effector organs. (Chen et al., 2022; Jia et al., 2015; Olde & Leeb-Lundberg, 2009)

E1 is predominant during menopause, E2 has the classical effects, such as developing secondary female sex characteristics and regulating the menstrual cycle, levels of E3 and E4 are elevated during pregnancy. (Chen et al., 2022)

In addition, estrogens play crucial roles in regulating the cardiovascular system, blood pressure, also take part in influencing liver, pancreas, bone, brain, and the immune system. Interestingly estrogens take part in regulating spermatogenesis and male fertility as well. (Chen et al., 2022; Faltas et al., 2020; O'Donnell et al., 2001; Shaha, 2008)

There are some similarities and differences between the human and mouse female reproductive systems. In mice, the ovaries contain numerous follicules in different stages of development, and during the estrus cycle, usually more follicules release oocytas. Mice have a bicornuat uterus. Mice are polyestrus beings, thus can have multiple estrus cycles through the year, while one estrus cycle in female mice is around 4-5 days, regulated by

the HPO axis. After fertilisation, gestation lasts till 19-21 days, and one litter usually contains an average of 6-12 offsprings. (Caligioni, 2009; Quesenberry & Donnelly, 2020)

In terms of vascular functions of estrogens, there are significant gender differences in contractility and endothelial modulation. For example, after ovariectomy the contractility of coronary arteries decreased, while estrogen replacement therapy augmented NO-mediated dilation. (Bányai, Vass, et al., 2023; Matrai et al., 2007; Mericli et al., 2004) Endothelium dependent vasodilations might be strengthened in female vessels by estrogens, also in menopause as hormone replacement therapy, thus inducing vascular protection. (Acs et al., 2000; Bányai, Vass, et al., 2023; Chen et al., 2022; Matrai et al., 2016; Mericli et al., 2004)

1.6 Interplay between the endocannabinoid system and estrogens

Considering the vascular effects of the two estrogen receptors (ERs) both subtypes α and β exert important NO dependent vasorelaxation effects of estrogens. (Darblade et al., 2002) Estrogens exercise rapid, nongenomic, direct effects through ERs creating NO dependent arterial vasodilation. Long-term, genomic effects are changes in gene and protein expressions as creating vasodilatory enzymes, promoting vascular endothelial cell proliferation and decreasing vascular smooth muscle cell proliferation. Indirect effects are elevating serum trigliceride level, HDL level parallel to a decrease of serum LDL level. Other effects of estrogens are to ameliorate vascular injuries, inducing antioxidant effects, lowering LDL oxidation, also affecting coagulation and fibrinolysis (Aryan et al., 2020; Bányai, Vass, et al., 2023; Iorga et al., 2018; Mendelsohn, 2002; Paterni et al., 2014; Savva & Korach-André, 2020)

According to earlier studies, there is an involvement of the ECS in the control processes of the female reproductive system. The precise homeostatic balance and tissue concentration of the endocannabinoids, by their production and degradation, and a well regulated CBR activity are necessary in order to achieve an optimal funtion of the HPO axis and the reproductive tract. (Bányai, Vass, et al., 2023; Bari et al., 2011; Brents, 2016) Among the users of phyto- or synthetic cannabinoids, disturbances of the reproductive endocrine system occur, causing infertility and cycle abnormalities, due to the property of THC that blocks the release of GnRH of the hypothalamus, consecutively impairs the LH production. (Alvarez, 2015; Bányai, Vass, et al., 2023; Brents, 2016; Fonseca &

Rebelo, 2022; Park et al., 2004; Wang et al., 2006) An irregular menstrual cycle, difficulties to harvest enough oocytes during *in vitro* fertilisation (IVF) and complications during pregnancy, such as premature deliveries and abortions have been reported among marijuana user women. (Bányai, Vass, et al., 2023; Wang et al., 2006) The ECS even might control the organisation of the endometrial cycle and also the ovarian follicle maturation (Bányai, Vass, et al., 2023; El-Talatini et al., 2009), as receptors of the ECS and FAAH degrading enzyme are represented in the female reproductive tract, however, their functions are not totally understood. (Bányai, Vass, et al., 2023; Taylor et al., 2010)

2. Objectives

2.1 Objective I.

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 determined plasma estrogen and metabolite levels in order to reveal the possible influences of the ECS on the function of the hypothalamo–pituitary–ovarian axis.

2.1.1 Hypotheses to objective I.

Taking in consideration the literature, firstly we hypothesised, that there might be a connection between the endocannabinoid system and the female hormonal system in terms of vascular functions, remodeling and that by knocking out CB_1Rs may enhance vascular function.

Our second hypothesis was that knocking out CB_1Rs possibly alters female hormonal levels of estrogens and their metabolites.

2.2 Objective II.

In the second part we aimed to reveal the potential role of CB_1Rs and CB_1R signalling 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. Since LDLR-KO animals fed with HFD develop atherosclerosis and hypercholesterolemia, with the double KO model we could investigate the impact of the role of CB_1R in the functional and structural atherosclerotic remodeling of the aortic wall.

2.2.1 Hypotheses to objective II.

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.

Furthermore 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

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. These two groups were used for estrogen level determination and myography in order to examine the impact of ECS and CB_1 receptor activation on vascular functions and the consequences of the missing receptor genotype. Changes in vascular contractility were also examined. These investigations were to examine the potential interplay between the ECS and estrogens. (Bányai, Vass, et al., 2023)

In the second project, male animals (n=62) were used in order to examine the potential role and mechanism of CB_1Rs in atherosclerotic vascular wall remodeling. We also investigated the effect of the existence of the CB_1R on the remodeling of the vascular wall in atherosclerotic-prone LDL-KO mice kept on HFD. These animals were divided into eight groups according to their genetics and their diet as indicated in Table 1. (Vass et al., 2024)

Table 1. Animals by genotype and diet in the second project. *Abbreviations: CD, control diet; HFD, high-fat diet; 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 receptor knockout.* (Vass et al., 2024)

group number	genotype	diet	n
1.	CB_1R+/+; LDLR+/+	CD	9
2.	CB_1R–/–; LDLR+/+	CD	6
3.	CB_1R+/+; LDLR–/–	CD	7
4.	CB_1R–/–; LDLR–/–	CD	7
5.	CB_1R+/+; LDLR+/+	HFD	10
6.	CB_1R–/–; LDLR+/+	HFD	10
7.	CB_1R+/+; LDLR–/–	HFD	6
8.	CB_1R–/–; LDLR–/–	HFD	7

Each group of animals were kept on different, designated diet for them, either kept on Janvier standard chow (Bányai, Vass, et al., 2023), or on high-fat diet and its control diet (see also subchapter 3.1.2.). (Vass et al., 2024)

3.1.1 Genetics of animals

For the first project, female homozygous CB_1R-knockout mice (CB_1R-KO, CB_1R−/−, n = 25) and wild-type counterpart mice (CB_1R+/+; n = 35) were used. (Bányai, Vass, et al., 2023; Zimmer et al., 1999) For the second project, homozygous CB_1R-KO (CB_1R−/−) and WT (CB_1R+/+) mice (C57BL/6J^{Cnr1tm1zim}) were bred in advance. This mouse strain was created with back-crossing of chimeric and heterozygous animals, continued with interbreeding of heterozygous animals as described by Zimmer. (Zimmer et al., 1999) Presence of CB_1Rs in the aorta were tested before. (Szekeres et al., 2015) Mice were bred at the Institute of Experimental Medicine (HUN-REN Research Centre, Budapest). Atherosclerosis-prone LDLR-KO (LDLR−/−) mice were acquired from Jackson Laboratory (B6.$^{129S7\text{-}Ldlrtm1Her/J}$, Jackson Laboratory, Bar Harbor, ME, USA). We have created homozygous double knockout mice by genetically crossing and breeding at the

animal house of Semmelweis University Basic Medical Science Center. (Vass et al., 2024)

3.1.2. Diet of mice during experiments

Mice were either fed with standard Janvier standard laboratory chow from Ssniff Spezialdiäten GmbH (Souris-Elevage ES8189-S096, Soest, Germany, www.ssniff.com) provided by the animal house of Semmelweis University Basic Medical Science Center and Institute of Experimental Medicine, or with special high-fat diet and its control diet in the form of pellets. HFD and control alimentations were also obtained from Ssniff Spezialdiäten GmbH and were given to the animals continuously. Mice, based on their genotype were grouped, and were subjected to a designated diet for them. Control diet contained decreased crude fat (5.1 %), decreased sugar level (11.0 %), gross energy (18.3 MJ/kg), metabolizable energy (15.7 MJ/kg) and it did not contain any cholesterol (0 %), while HFD (Western-type diet) contained elevated crude fat (21.1 %), higher sugar level (34.3 %), gross energy (21.8 MJ/kg), metabolizable energy (19.1 MJ/kg) and cholesterol (0.21 %). However, the female animals (of the 1st project) were kept on 6 months of Janvier standard chow, the male animals (of the 2nd project) were transferred from the age of 1 month onto either HFD or CD for 5 months. Termination age of them was uniformly 6 months. In the LDLR-KO mouse strain kept on HFD showed hypercholesterolemia with pathophysiological vascular remodeling and atheroclerotic plaques could be detected in their aorta (Supplementary Figure 1). (Bányai, Vass, et al., 2023; Baumer et al., 2019; Vass et al., 2024)

3.2 Ethical approvals for experiments on animals

Our experiments were in line with the Guide for the Care and Use of Laboratory Animals (NIH 8th Edn 2011). Institutional and National guidelines for animal care and breeding 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). (Bányai, Vass, et al., 2023; Vass et al., 2024)

3.3 Chemicals

Adrenergic alpha receptor agonist phenylephrine (Phe), an NO-dependent vasodilator acetylcholine (Ach), nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine (LNA) as well as cyclooxygenase (COX) inhibitor indomethacin (INDO), angiotensin II (Ang II),

an angiotensin type 1 (AT_1) receptor agonist, estradiol and standards for estrogen level determination, estron-(-,3,4-13C3) solution, 17β-estradiol-D5, WIN 55,212-2, a CB_1 receptor synthetic agonist and all other salts and chemicals were purchased from Merck KGaA (Darmstadt, Germany). SQ 29,548, a TP receptor inhibitor, was purchased from Cayman Chemical (Ann Arbor, MI, USA). Stock solutions of solvents for INDO were prepared in dimethyl sulfoxide (DMSO) and subsequently diluted in Krebs solution on the day of the experiment. Similar dilution of DMSO was used as "vehicle". LNA was diluted in Krebs solution with ultrasound dispersion. SQ 29,548 was diluted in ethanol as stock solution as suggested by the manufacturer. From the stock solutions further dilutions were made with Krebs. On the day of the experiment, Krebs and high-potassium Krebs solutions were prepared (see in subchapter 3.4). For a list and the sources of immunohistological reagents and their dilutions, see the corresponding sub-chapters. (Bányai, Vass, et al., 2023; Vass et al., 2024)

3.4 Myography

We performed myography in both parts of experiments. Animals were anaesthetized with intraperitonally administered pentobarbital-sodium (Euthasol), first 35 mg/kg, and after BP measurements an additional dose of 20 mg/kg was applied, to reach 55 mg/kg. Depth of anaesthesia was checked by the absence of pain reflexes. The circulatory system was perfused with Krebs in order to remove blood, then aortas were dissected. (Bányai, Vass, et al., 2023; Vass et al., 2024)

Abdominal aortic rings were subjected to wire myography. The isolated segments were put into cold Krebs solution containing: 119 NaCl, 4.7 KCl, 2.5 $CaCl_2 \times {}_2H_2O$, 1.17 $MgSO_4 \times 7H_2O$, 20 $NaHCO_3$, 1.18 KH_2PO_4, 0.027 EDTA, 10.5 glucose (in millimolar). The 3-4 mm long abdominal aortic rings were placed 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, bubbled with carbogen gas (95% O_2 + 5% CO_2), keeping the pH at 7.4. In

line with our protocols (Szekeres et al., 2015), abdominal aortic segments were pre-stretched to 10 mN and were equilibrated for 30 min. After equilibration, reference contraction was performed with high potassium level Krebs solution (K^+ 124 mmol/L). In order to test vascular smooth muscle and endothelium functions, concentration-response curves to vasoconstrictor Ang II (1–100 nmol/L) and Phe (1 nmol/L–10 μmol/L) and also to the vasodilator Ach (1 nmol/L–1 μmol/L) after preconstriction with Phe were obtained (in the 2nd experimental series we obtained Ach dose-responses with Phe 10 μmol/L precontraction). Selective inhibitors were applied to test the mechanism of endothelial function. NOS inhibition was done 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 (10 nmol/L–10 μmol/L) was measured without and with the presence of inhibitors in parallel segments after submaximal preconstriction with Phe (0.5 μmol/L). Vasoconstriction responses were normalized to KCl contraction, vasodilation responses were calculated compared with the precontraction state. (Bányai, Vass, et al., 2023; Horváth et al., 2005; Szekeres et al., 2015) Presence and the effect of CB_1Rs were tested in CB_1R-KO ($CB_1R^{-/-}$) and WT ($CB_1R^{+/+}$) groups with WIN 55,212-2, a synthetic CB_1R agonist (10 μmol/L). WIN 55,212-2-induced relaxation was also compared to "vehicle", in which relaxation effect couldn't be registered. Effects of inhibitors were also calculated as changes in contraction force and as attenuation in relaxation (differences from control, Figure 3A-F) and also as relative (%) changes from control (difference from control/control level x100, Figure 3G). (Bányai, Vass, et al., 2023; Vass et al., 2024) See also in Supplementary Figure 2.

3.5 Blood serum estrogen and metabolites level determination

Free estradiol, conjugated estradiol (cE2), free and conjugated estrone (fE1, cE1), 2-hydroxyestrone (2OH-E1) and free 4-hydroxyestrone (f4OH-E1) levels were determined from blood plasma samples by liquid chromatography-tandem mass spectrometry. Levels of fE1 and cE1, 2OH-E1 and conjugated 4-hydroxyestrone were under threshold levels. (Bányai, Vass, et al., 2023)

3.6 Body weight 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). (Vass et al., 2024)

3.7 Blood pressure measurements

External tail cuff method was used in order to measure the blood pressure 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). (Vass et al., 2024)

3.8 Determination of cholesterol levels

Determination of cholesterol levels was done without long-term fasting to reveal the concentration levels of total serum cholesterol of mice. (Vass et al., 2024) EnzyChrom™ AF Cholesterol Assay Kit (BioAssay Systems, California, US) was used precisely according to the manufacturer's guideline to determine plasma cholesterol levels of the mice. Briefly, cholesterol standards with known concentrations were prepared. Blood plasma samples were subsequently diluted by one hundred times using Assay Buffer (1:100 = Plasma:Assay Buffer). Either 50 µL of cholesterol standard or plasma sample were aliquoted into the corresponding wells of a 96-well plate in duplicate. Equal volumes (50 µL) of the reaction mix, including 55 µL assay buffer, 1 µL enzyme mix, and 1 µL dye reagent were added to both the cholesterol standards and the plasma samples afterwards. Optical density (OD) determination was carried out at 570 nm after 30 minutes of incubation at room temperature. (Vass et al., 2024)

3.9 Immunohystological eNOS staining

To reveal eNOS density we performed immunohystological staining for it. 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 % H_2O_2. 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 labelling 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).

3.10 Statistical analysis

Statistical analysis was performed with two-way ANOVA and Bonferroni post-hoc test for the analysis of comparisons between the groups and one-way ANOVA and Kruskal-Wallis test were used to make comparisons at each concentration levels during myography. If the data didn't meet the normality criterion or the criterion of equality of variances, pairwise comparisons were made using one-way ANOVA, with non-parametric Kruskal-Wallis (Dunn) or Mann-Whitney tests, accepting the test offered by SigmaStat software in case of normality violation. Repeated measures ANOVA could not be used because the measurements were performed independently on separate animals on different days and only could be used in case of the same aortic rings, while aquiring dose response curves, but these results did not differ from non-repeated measures in their merits. Unpaired t-test was applied for comparison between some groups (Figure 1E. and 4.), furthermore curve fitting method was carried out (Supplementary Table 1.). Cholesterol levels were analyzed with one-way ANOVA-Holm-Sidak and two-way ANOVA-Bonferroni post-hoc tests. Pairwise comparison with the absence and presence of CB_1 receptors was tested with one-way ANOVA. During the determination of the levels of estrogen and its metabolites unpaired t-tests were used for the analysis of comparisons between CB_1R wild type and knockout groups. Statistics of body- and heart weight values were made with one-way ANOVA with Bonferroni tests, while blood pressure statistics were made with one-way ANOVA with Holm Sidak post-hoc test. Immunohistochemical results were tested with one-way ANOVA, Tukey and Holm Sidak

post-hoc tests. Values were expressed as mean ± standard error of mean (mean ± SEM) and $p<0.05$ was considered significant. Analysis was carried out by the SigmaStat software 3.5s (Systat Software Inc., San Jose, CA, US) and also with GraphPad PRISM 9.5.0. (San Diego, CA, US). Graphs were made by the latter software.

4. Results

4.1 Results of the impact of CB_1R on the vascular function and on estrogen status in female mice

In the first project we examined the potential interplay between estrogens and the ECS on the vascular functions.

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

4.1.1.1 Effects of presence of CB_1 receptors on vascular contraction and relaxation responses in female mice

In the first project, abdominal aortic rings of female CB_1R knockout and WT were used to obtain concentration-response curves in response to alpha adrenerg receptor agonist phenylephrine (Phe), in concentrations of 10^{-9}–10^{-5} mol/L) and AT1 receptor agonist angiotensin II (Ang II, 10^{-9}–10^{-7} mol/L). As expected, both vasoconstrictors (Ang II and Phe) induced dose-dependent vasoconstrictions, and didn't show any difference between the CB_1R-WT and -KO groups in any dosage (Figure 1A-B). However, 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, Figure 1C-D). Interestingly, in other dosages there were no significant differences indicating the dose-dependent effects of the vasodilators. In order to test CB_1R function we obtained relaxation response with CB_1R agonist. 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). A significant difference between CB_1R-KO and -WT animals could be obtained ($p=0.033$, in unpaired t-test, Figure 1E). (Bányai, Vass, et al., 2023)

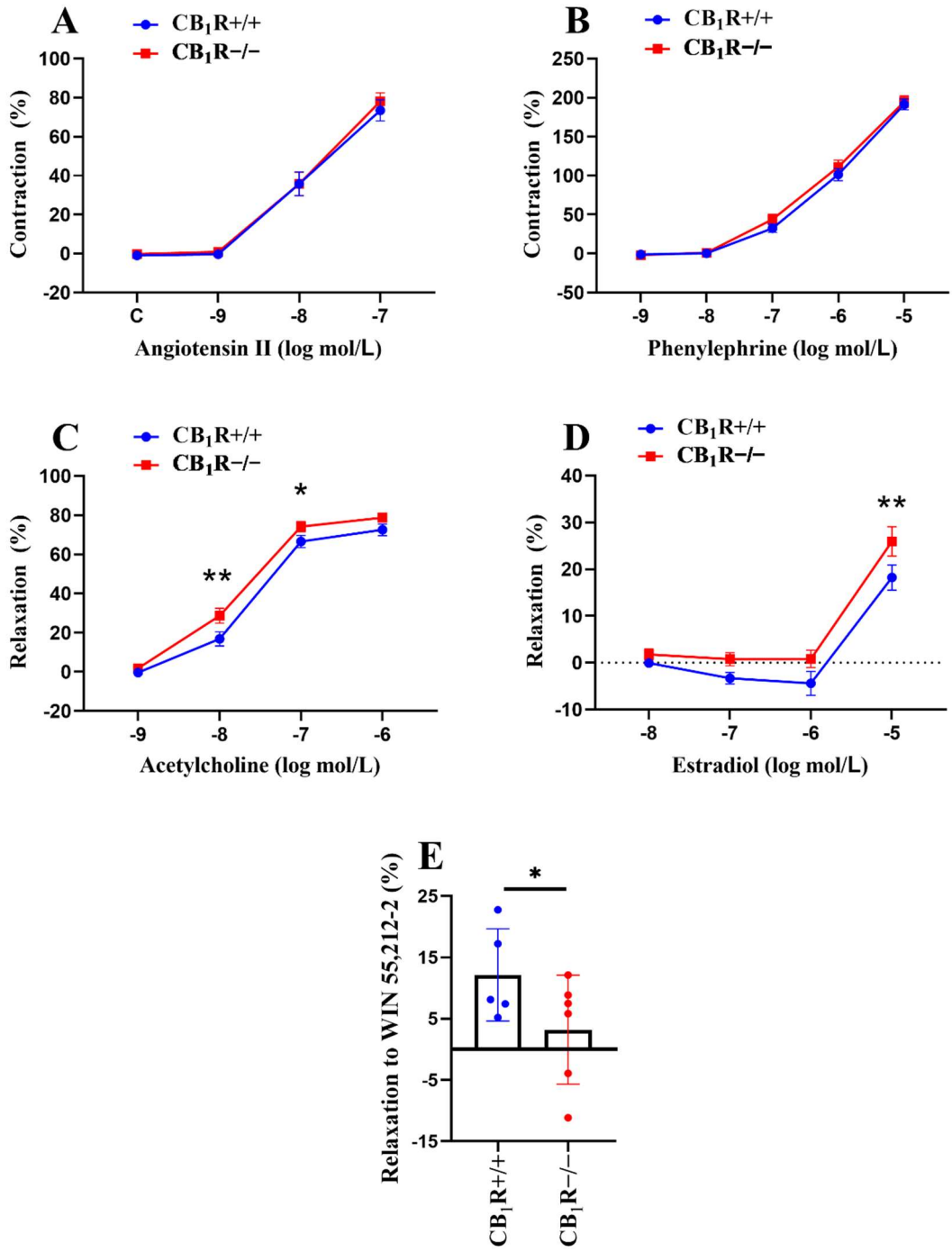

Figure 1. Vascular contraction and relaxation effects in aortic segments of wild-type ($CB_1R+/+$) and CB_1R knockout ($CB_1R-/-$) female mice. **Panel (A).** Dose-dependent contraction curves to angiotensin II in wild-type (n=14–25 aortic ring segments from 9 animals) and in CB_1R knockout (18–26 aortic ring segments from 9 animals) female mice. **Panel (B).** Dose-dependent contraction curves to phenylephrine in $CB_1R+/+$ (n=28 segments from 9 animals) and in $CB_1R-/-$ (n=27 segments from 9 animals) female mice. **Panel (C).** Dose-dependent relaxation curves to acetylcholine in $CB_1R+/+$ (n=26 segments from 9 animals) and in $CB_1R-/-$ (n=27 segments from

8 animals) female mice. **Panel (D).** Dose-dependent relaxation curves to estradiol in $CB_1R+/+$ (n=8–10 segments from 8 animals) and $CB_1R-/-$ (n=7–10 segments from 10 animals) female mice. **Panel (E).** Relaxation response of aortic segments to the CB_1R agonist WIN 55,212-2 (10 μM) in $CB_1R+/+$ (n=5) and $CB_1R-/-$ (n=6) female mice. Mean ± SEM values are shown, while dots showing individual data points, $p < 0.05$ values were considered significant. Contraction data were adjusted relative to KCl contraction and relaxation data were calculated as percent values of precontraction level. *: $p<0.05$ and **: $p<0.01$ between wild-type ($CB_1R+/+$) and CB_1R knockout ($CB_1R-/-$) groups. *Abbreviations: CB_1R: cannabinoid type 1 receptor; $CB_1R+/+$: cannabinoid type 1 receptor wild type; $CB_1R-/-$: cannabinoid type 1 receptor knockout. (Bányai, Vass, et al., 2023)*

4.1.1.2. Effects of specific inhibitors on contraction and relaxation vascular responses in WT ($CB_1R+/+$) and in CB_1R-KO ($CB_1R-/-$) female mice

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 (Figure 2A-F). Baseline vascular tone was not modified by these specific inhibitors, nor by their vehicle (vehicle: by −0.01 ± 0.26 mN, n=9, INDO: by 0 ± 0.25 mN, n=10, LNA: by 0.15 ± 0.37 mN, n=10). Direct comparison between $CB_1R+/+$ and $CB_1R-/-$ genotypes for contractions and relaxations achieved are in Figure 3A-G. (Bányai, Vass, et al., 2023)

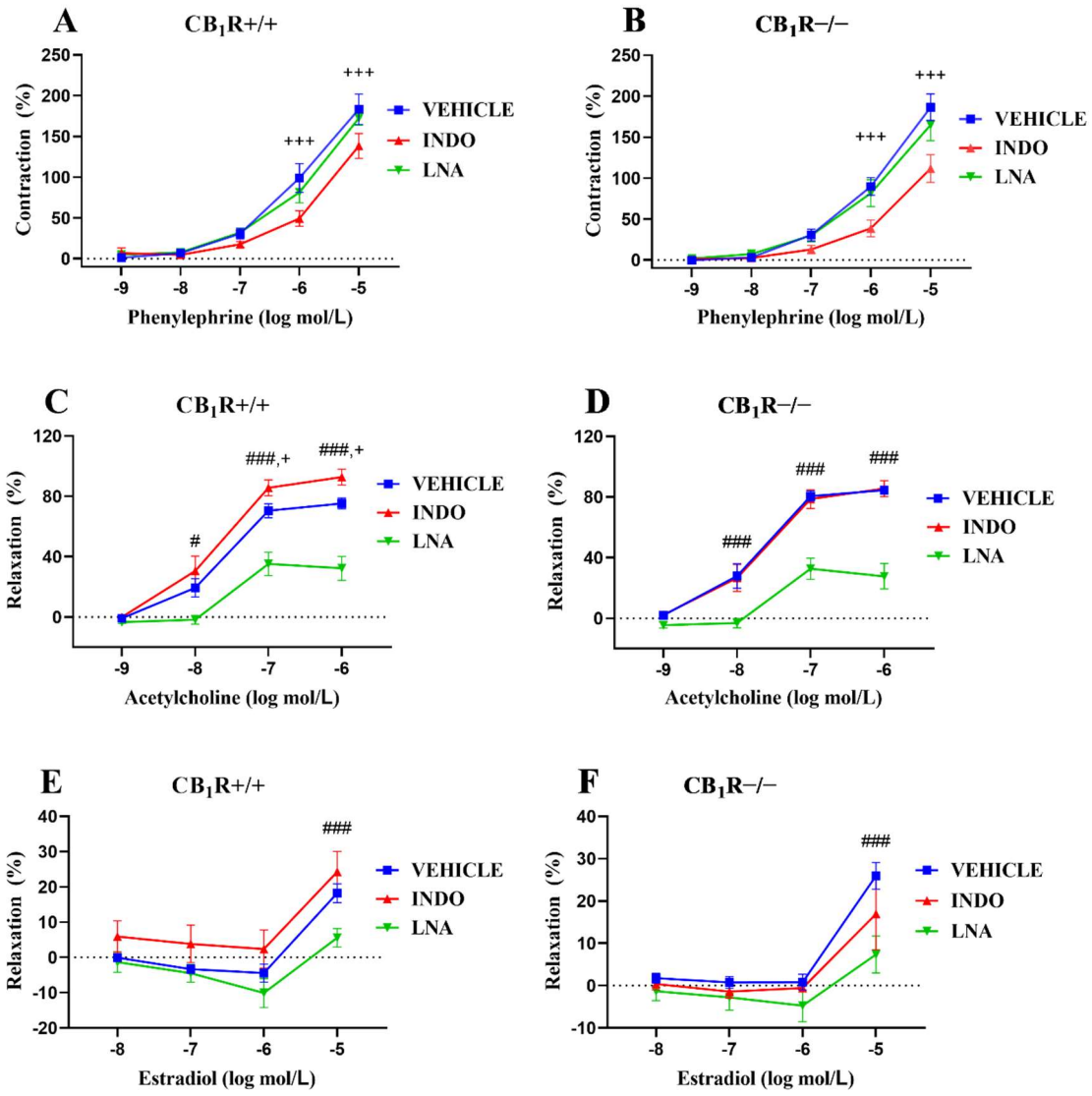

Figure 2. Effects of specific inhibitor of nitric oxide synthase Nω-nitro-L-arginine and cyclooxigenase inhibitor indomethacin on contraction–relaxation vascular responses in abdominal aortas of wild-type ($CB_1R+/+$) and CB_1R knockout ($CB_1R-/-$) female mice. **Panel (A).** Effects of specific inhibitors on phenylephrine-induced vasoconstriction in CB_1R-WT female mice (n=7–9, abdominal aortic segments=7–9). **Panel (B).** Effects of specific inhibitors on phenylephrine-induced vasoconstriction in CB_1R-KO female mice (n=8, abdominal aortic segments=8). **Panel (C).** Effects of specific inhibitors on acetylcholine-induced vasodilation in CB_1R-WT female mice (n=8–9, abdominal aortic segments=8–9). **Panel (D).** Effects of specific inhibitors on acetylcholine-induced vasodilation in CB_1R-KO female mice (n=8–9, abdominal aortic segments=8–9). **Panel (E).** Effects of specific inhibitors on estradiol-induced vascular responses in CB_1R-WT female mice (n=10, abdominal aortic segments=7–10). **Panel (F).** Effects

of specific inhibitors on estradiol-induced vascular responses in CB_1R-KO female mice (n=10, abdominal aortic segments=7–10). Results are presented as mean ± SEM values, $p < 0.05$ were considered significant. #: $p<0.05$, ###: $p < 0.001$ between vehicle and LNA-treated segments, +: $p<0.05$, +++: $p<0.001$ between vehicle and INDO-treated segments (two-way ANOVA with a Bonferroni post-hoc test). Contraction data were normalized to KCl-induced contraction, relaxation data were espressed as percentage of the precontraction level. *Abbreviations: INDO: indomethacin, LNA: Nω-nitro-L-arginine, CB_1R: cannabinoid type 1 receptor, CB_1+/+: CB_1R wild-type, CB1−/−: CB_1R knockout mice. (Bányai, Vass, et al., 2023)*

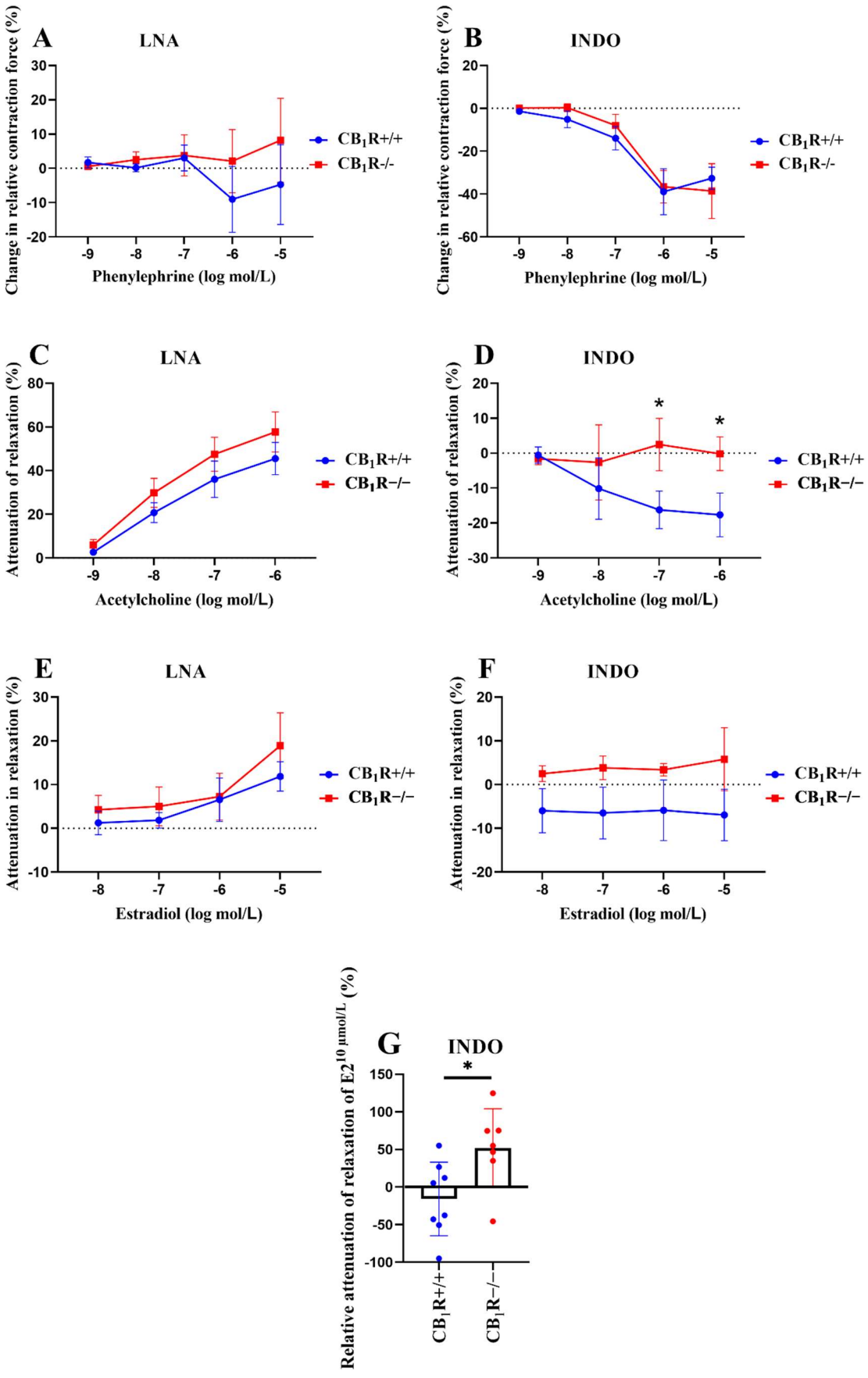
A
LNA
Change in relative contraction force (%)
Phenylephrine (log mol/L)
CB1R+/+
CB1R-/-
B
INDO
Change in relative contraction force (%)
Phenylephrine (log mol/L)
CB1R+/+
CB1R-/-
C
LNA
Attenuation of relaxation (%)
Acetylcholine (log mol/L)
CB1R+/+
CB1R−/−
D
INDO
Attenuation of relaxation (%)
Acetylcholine (log mol/L)
CB1R+/+
CB1R−/−
E
LNA
Attenuation in relaxation (%)
Estradiol (log mol/L)
CB1R+/+
CB1R−/−
F
INDO
Attenuation in relaxation (%)
Estradiol (log mol/L)
CB1R+/+
CB1R−/−
G
INDO
Relative attenuation of relaxation of E2 10 µmol/L (%)
CB1R+/+
CB1R−/−

Figure 3. Comparison of the effects of specific inhibitor of nitric oxide synthase Nω-nitro-L-arginine and inhibitor of cyclooxygenase indomethacin on contraction-relaxation vascular responses in abdominal aortas of WT (CB_1R+/+) and CB_1R-KO (CB_1R−/−) female mice. **Panel (A).** Effects of inhibitor LNA on Phe-induced vasoconstriction in CB_1R-WT (n=6, abdominal aortic segments=6) and CB_1RKO (n=6, abdominal aortic segments=6) female mice. **Panel (B).** Effects of inhibitor INDO on Phe-induced vasoconstriction in CB_1R-WT (n=5, segments=5) and CB_1R-KO (n=6, abdominal aortic segments=6) female mice. **Panel (C).** Effects of inhibitor LNA on Ach-induced vasorelaxation in CB_1R-WT (n=8, abdominal aortic segments=8) and CB_1R-KO (n=8, abdominal aortic segments=8) female mice. **Panel (D).** Effects of inhibitor INDO on Ach-induced vasodilation in CB_1R-WT (n=8, abdominal aortic segments=8) and CB_1R-KO (n=8, abdominal aortic segments=8) female mice. **Panel (E).** Effects of inhibitor LNA on E2-induced vascular responses in CB_1R-WT (n=9) and CB_1R-KO (n=8) female mice. **Panel (F).** Effects of inhibitor INDO on E2-induced vascular responses in CB_1R-WT (n=9) and CB_1R-KO (n=9) female mice. Differences in contraction or relaxation values are plotted. **Panel (G).** Normalized (normalization: {datas from control curves – datas from dose-response curves with the inhibitor} ÷ datas from control curves ×100) effects of INDO on E2 relaxation responses (at 10 μmol/L). Values are plotted as percentage differences from control relaxation. Mean ± SEM values are presented, while dots present individual data points, $p<0.05$ values were considered significant. *: $p< 0.05$ between CB_1R-WT and CB_1R-KO groups. *Abbreviations: Ach: acetylcholine, Phe: phenylephrine, E2: estradiol, INDO: indomethacin, LNA: Nω-nitro-L-arginine, CB_1R: cannabinoid type 1 receptor, WT: wild-type, KO: knockout, CB_1R+/+: cannabinoid type 1 receptor wild-type mice, CB_1R−/−: cannabinoid type 1 receptor knockout mice. (Bányai, Vass, et al., 2023)*

LNA did not significantly influence the contraction dose–response to Phe in either genetic group (Figure 2A-B and Figure 3A). However, 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, Figure 2A) and in the CB_1R-KO (at 10^{-6} to 10^{-5} mol/L, $p<0.001$, interaction: $p<0.001$, 2-way ANOVA, Figure 2B) groups, while no significant differences were shown at 10^{-9}-10^{-7} mol/L. This contraction–attenuation was similar in both of the groups (Figure 3B). (Bányai, Vass, et al., 2023)

Naturally, in CB_1R+/+ mice the concentration-dependent Ach-induced vasodilation was significantly attenuated by the NOS inhibitor LNA ($p<0.05$ at 10^{-8} mol/L, $p<0.001$ at doses of 10^{-7}–10^{-6} mol/L, interaction: $p<0.001$, not significant at 10^{-9} mol/L 2-way

ANOVA, Figure 2C). Ach-induced relaxation was significantly enhanced by the COX inhibitor INDO in CB_1R+/+ female mice ($p<0.05$ at 10^{-7} mol/L and $p=0.026$ at 10^{-6} mol/L, non-significantly at lower doses, Figure 2C). Similarly to CB_1R+/+ mice, in CB_1R−/− mice, Ach-induced relaxation was also significantly attenuated by LNA ($p<0.001$ at doses of 10^{-8}–10^{-6} mol/L, interaction: $p<0.001$, not significant at 10^{-9} mol/L,), but the effect of INDO could not be registered (Figure 2D). Direct comparison of the two strains revealed no significant difference in LNA effect (Figure 3C), while the effect of INDO on Ach relaxation was statistically different ($p = 0.026$ with two-way ANOVA, Bonferroni post hoc test, $p<0.05$ at 10^{-7} and 10^{-6} mol/L Ach, not significant at lower doses, Figure 3D). (Bányai, Vass, et al., 2023)

In CB_1R-WT and also in CB_1R-KO mice, NOS inhibitor LNA significantly reduced E2-relaxation ($p<0.001$ at 10^{-5} mol/L) in both groups, but not significantly at lower doses (Figure 2E-F), while in CB_1R-WT INDO slightly elevated, and in CB_1R-KO animals INDO slightly reduced E2 vasodilatory effect, but this effect did not reach statistical significance (Figure 2E-F). Slight differences in E2-induced relaxation in the two genetic groups with INDO (Figure 3F) were statistically significant if normalized to the original relaxation effect of E2 (Figure 3G). (Bányai, Vass, et al., 2023)

4.1.2 Estrogen metabolite levels

We were unable to determine levels of free- and conjugated estrone, 2-hydroxyestrone and conjugated 4-hydroxyestrone, because they did not reach threshold levels in either of the samples. 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, Figure 4). (Bányai, Vass, et al., 2023)

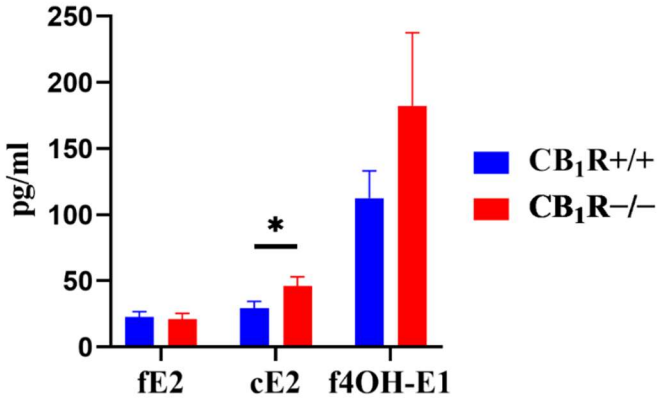

Figure 4. Free estradiol, conjugated estradiol and free 4-hydroxyestrone levels (pg/mL) in CB_1R-WT and CB_1R-KO female mice. Mean ± SEM values are presented, $p<0.05$ values were considered significant. *: $p<0.05$ between CB_1R-WT (n = 14–24) and CB_1R-KO (n = 11–17) animals. Sampling was not adjusted to the estrus cycle, mean values reflect means during estrus cycle. *Abbreviations: fE2: free estradiol, cE2: conjugated estradiol, f4OH-E1: free 4-hydroxyestrone, CB_1R: cannabinoid type 1 receptor, WT: wild-type, KO: knockout, $CB_1R+/+$: cannabinoid type 1 receptor wild-type mice, $CB_1R-/-$: cannabinoid type 1 receptor knockout mice. (Bányai, Vass, et al., 2023)*

4.2 Results of the functional and remodeling effect of CB_1R in double CB_1R-LDLR knockout atherosclerotic male mouse model

In the second part we aimed to reveal the potential role of CB_1Rs and CB_1R signalling mechanisms in the functional vascular remodeling of an atherosclerosis-prone mouse model based on the absence of LDL receptors.

4.2.1 Results of myography in double knockout atherosclerotic male mouse model

4.2.1.1 Endothelium dependent vasodilation of abdominal aortic segments

Acetylcholine-induced dose-dependent vasorelaxation in all groups was tested (Figure 5A-D). Statistical analysis with two-way ANOVA showed a significant difference between CD and HFD groups (p=0.026). $CB_1R+/+$, LDLR+/+ CD and $CB_1R+/+$, LDLR–/–, CD groups showed the greatest Ach-induced endothel-dependent vasodilation, which relaxation was significantly attenuated in HFD groups with the same genotype (p=0.008 between the $CB_1R+/+$, LDLR+/+, CD and $CB_1R+/+$, LDLR–/–, HFD groups). There

weren't any significant differences between CB_1R+/+, LDLR–/–, HFD and CD groups (p=0.064, Figure 5A). On the other hand, animals kept on HFD and CD, when the CB_1R was missing, showed statistical difference: CB_1R–/–, LDLR+/+, CD group showing the most relaxation (p=0.041, Figure 5B) (Vass et al 2024). Endothelium dependent Ach relaxed the vessels the most in LDLR+/+, CD groups, compared to LDLR–/–, CD groups, with a significant difference (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, Figure 5C). In case of HFD groups no significant statistical difference could be obtained between LDLR+/+ vs. LDLR–/– in high-fat diet fed groups in Ach-induced vasodilation, in addition, there was no statistical difference in case of CB_1Rs either. CB_1R+/+, HFD groups had the least relaxation for endothelium dependent relaxant Ach (10^{-8} mol/L). A significant improvement could be registered in vasodilation between CB_1R–/–, LDLR+/+, HFD group and CB_1R+/+, LDLR–/– HFD group (p= 0.043, Figure 5D). At the dosage of 10^{-8} (but not in other doses) HFD groups with the genotype of CB_1R+/+ and LDLR–/– showed significantly weaker Ach-induced vasodilation compared to CB_1R–/–, LDLR+/+, CD (p=0.015, one-way ANOVA, Bonferroni post-hoc test). (Vass et al., 2024)

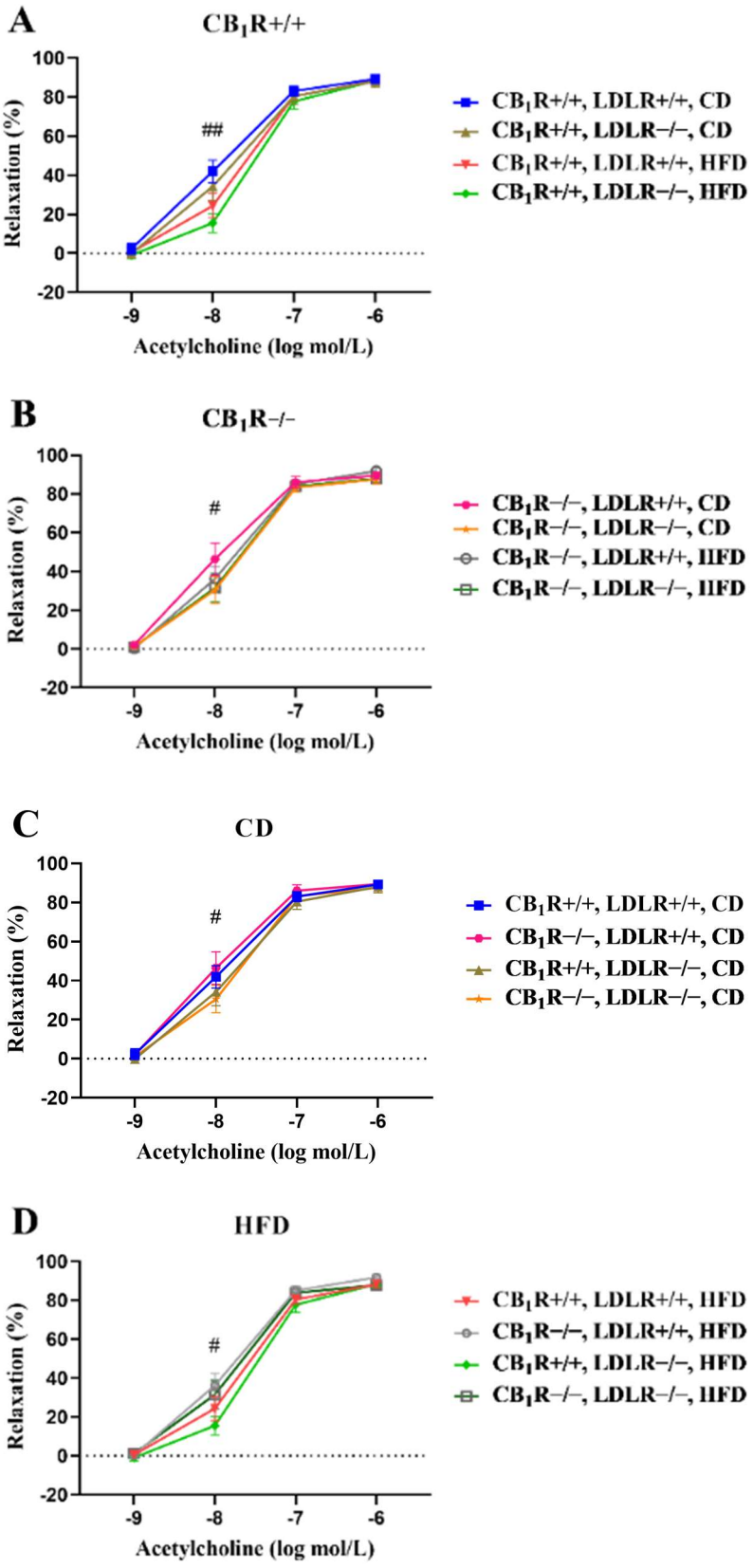

Figure 5. Acetylcholine-induced endothelium dependent vasorelaxation in aortic segments of CB_1R and LDLR wild-type and knockout mice kept on control and high-fat diet. **Panel (A).** Dose-

response vasodilation curves to Ach in CB_1R wild-type groups with different LDLR genotype and diets (##, p=0.008 between CB_1R+/+, LDLR+/+ CD and CB_1R+/+, LDLR−/−, HFD, n=5-10). **Panel (B).** Dose-response vasodilation curves to Ach in CB_1R knockout groups with different LDLR genotype and diets (#, p=0.041 between CB_1R−/−, LDLR+/+, CD and CB_1R−/−, LDLR−/−, CD, n=6-9). **Panel (C).** Dose-response vasodilation curves to Ach in control diet groups with different LDLR and CB_1R genotypes (#, p=0.047 between CB_1R−/−, LDLR+/+, CD and CB_1R−/−, LDLR−/−, CD, n=5-7). **Panel (D).** Dose-response vasodilation curves to Ach in HFD groups with different LDLR and CB_1R genotypes (#, p=0.043 between CB_1R−/−, LDLR+/+, HFD and CB_1R+/+, LDLR−/−, HFD, n=5-10)). Mean ± SEM values. Vasodilatory data were calculated compared to precontraction level with Phe as percent levels. *Abbreviations: CD, control diet; HFD, high-fat diet; CB_1R+/+, endocannabinoid type 1 receptor wild-type; CB_1R−/−, endocannabinoid type 1 knockout; LDLR+/+, low density lipoprotein receptor wild-type; LDLR−/−, low density lipoprotein receptor knockout. (Vass et al., 2024)*

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 (Supplementary Table 1). (Vass et al., 2024)

4.2.1.2 Effects of specific inhibitors on acetylcholine-induced vasodilatory responses

ENOS inhibition with LNA significantly attenuated the Ach-induced vasodilation in all groups in concentrations of 10^{-8}–10^{-6} mol/L (except in CB_1R+/+, LDLR–/–, HFD group in the dosage of 10^{-8} mol/L and 10^{-9} mol/L, Figure 6A-H). COX-inhibition with INDO slightly modulated vasodilation, which was significantly attenuated at 10^{-8} mol/L in the groups of CB_1R+/+, LDLR–/–, CD and CB_1R+/+, LDLR+/+, HFD also in CB_1R–/–, LDLR+/+, HFD groups (Figure 6E-F and H). (Vass et al., 2024)

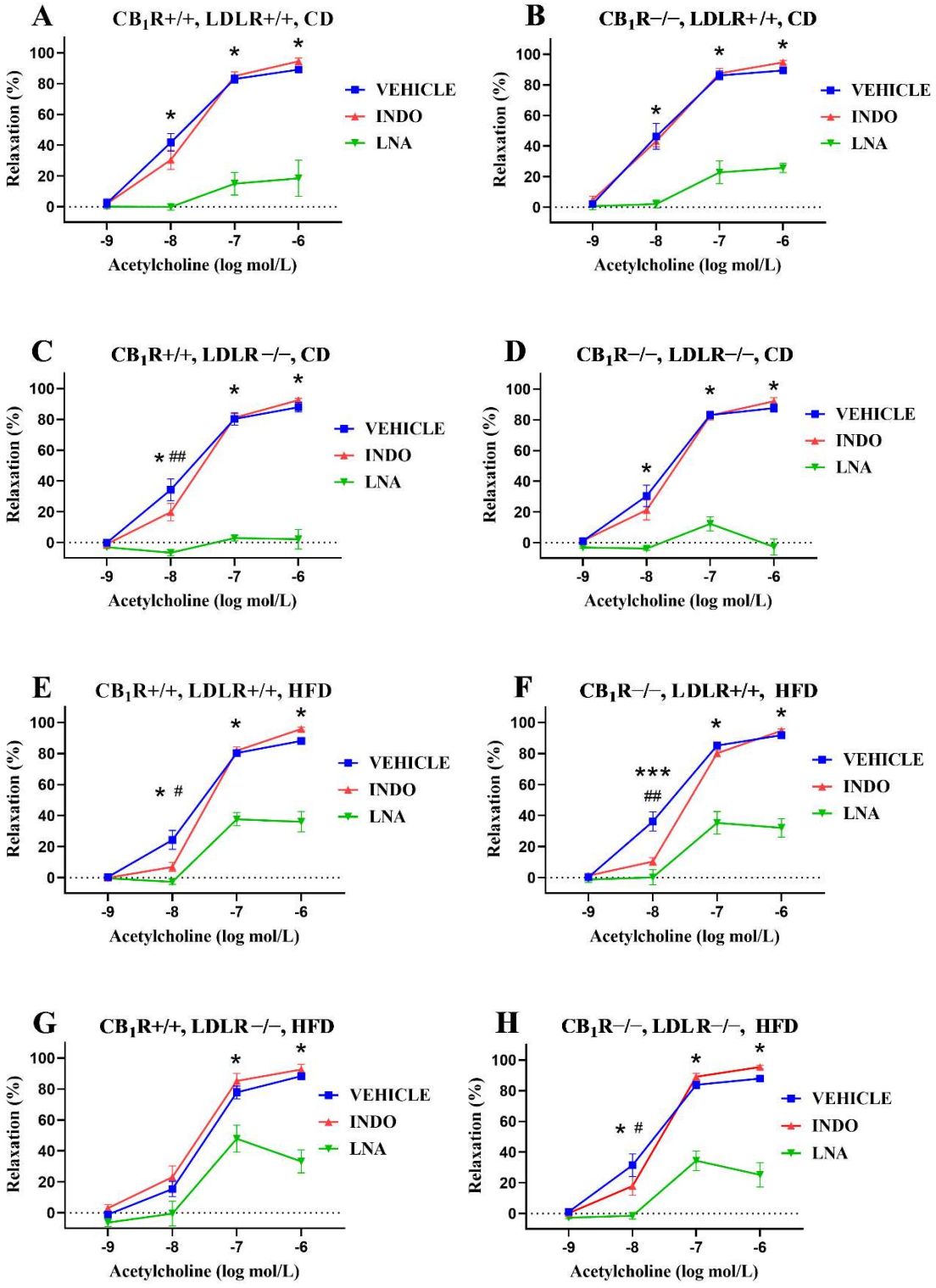

Figure 6. Effects of specific inhibitors of endothelial nitric oxide synthase Nω-nitro-L-arginine and of cyclooxigenase indomethacin on vasodilatory responses in aortas of CB_1R and LDLR wild-type and -knockout mice kept on control diet and high-fat diet. **Panel (A).** Effects of specific inhibitors on vasodilation in CB_1R+/+, LDLR+/+, control diet group (n =5-6). **Panel (B).** Effects

of specific inhibitors on vasodilation in $CB_1R-/-$, LDLR+/+, control diet group (n=6). **Panel (C).** Effects of specific inhibitors on vasodilation in $CB_1R+/+$, LDLR−/−, control diet group (n=7). **Panel (D).** Effects of specific inhibitors on vasodilation in $CB_1R-/-$, LDLR−/−, control diet group (n=6) **Panel (E).** Effects of specific inhibitors on vasodilation in $CB_1R+/+$, LDLR+/+, high-fat diet mice (n=9-10). **Panel (F).** Effects of specific inhibitors on vasodilation in $CB_1R-/-$, LDLR+/+, high-fat diet mice (n=9). **Panel (G).** Effects of specific inhibitors on vasodilation in $CB_1R+/+$, LDLR−/−, high-fat diet mice (n=5). **Panel (H).** Effects of specific inhibitors on vasodilation in $CB_1R-/-$, LDLR−/−, high-fat diet mice (n=7). Data are shown as mean ± SEM values. Significance level was determined at $p<0.05$. *, $p<0.05$; ***, $p<0.001$ between vehicle and LNA-treated segments; #, $p<0.05$; ##, $p<0.01$ between vehicle and INDO-treated segments. *Abbreviations, CD, control diet; HFD, high-fat diet; INDO, indomethacin; LNA, Nω-nitro-L-arginine; $CB_1R+/+$, cannabinoid type 1 receptor wild-type; $CB_1R-/-$, cannabinoid type 1 receptor knockout; LDLR+/+, LDL receptor wild-type mice; LDLR−/−, LDL receptor knockout mice. Relaxation data were calculated as percent values of precontraction level. (Vass et al., 2024)*

4.2.1.3 Comparison of the effects of NOS inhibitor LNA on acetylcholine induced vasodilatory responses

Vasodilation induced by Ach was attenuated by NOS inhibition in all groups (Figure 6A-H), which effect was elevated in all CD groups compared to HFD groups. Pairwise comparisons showed statistical significance between $CB_1R+/+$, LDLR +/+ and $CB_1R+/+$, LDLR−/−, also in $CB_1R-/-$, LDLR−/− genotypes (Figure 7A-D). 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, Figure 7C-D) compared to $CB_1R+/+$ groups (Figure 7A-B). (Vass et al., 2024)

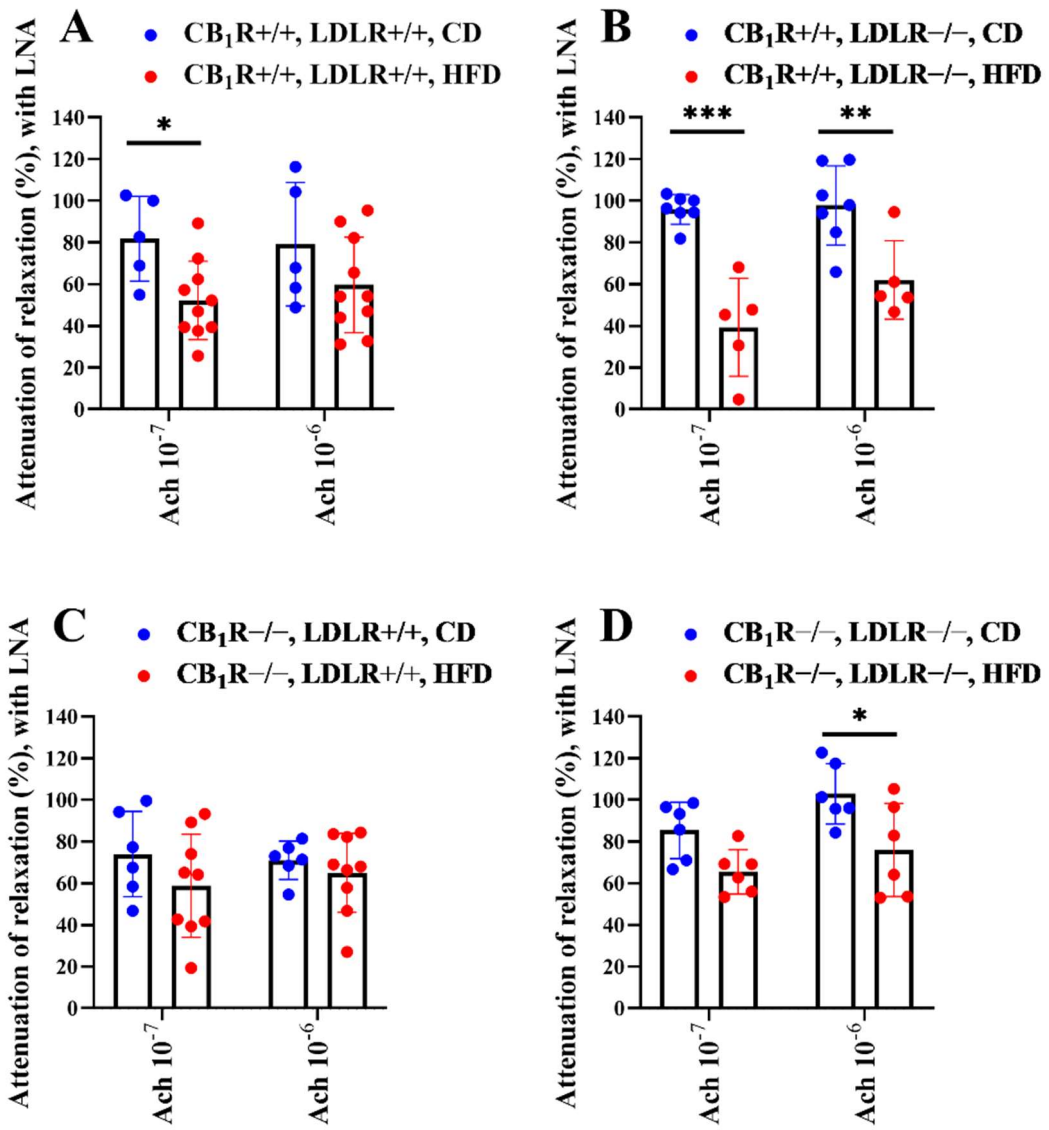

Figure 7. Effect of eNOS inhibitor Nω-nitro-L-arginine on Ach-induced relaxation in aortas of CB_1R- and LDLR-WT and -KO mice kept on CD and HFD, normalized to control values. Attenuation of Ach-induced relaxation with LNA is shown in percent values. **Panel (A).** Attenuation of Ach-induced vasodilation with LNA in groups of CB_1R+/+, LDLR+/+, CD and HFD (n=5-10). **Panel (B).** Attenuation of Ach-induced vasodilation with LNA in groups of CB_1R+/+, LDLR−/−, CD and HFD (n=5-7). **Panel (C).** Attenuation of Ach-induced vasodilation with LNA in groups of CB_1R−/−, LDLR+/+, CD and HFD (n=6-9). **Panel (D).** Attenuation of Ach-induced vasodilation with LNA in groups of CB_1R−/−, LDLR−/−, CD and HFD (n=6-7). $p<0.05$ values were considered significant. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ between CD and HFD groups in the same genotype. Mean ± SEM values are shown, while dots represent individual data points. *Abbreviations, CD: control diet; HFD, high-fat diet; KO, knockout; WT, wild-type; Ach, acetylcholine; LNA, Nω-nitro-L-arginine; CB_1R+/+, cannabinoid type 1 receptor wild type;*

$CB_1R-/-$, cannabinoid type 1 receptor knockout; LDLR+/+, LDL receptor wild-type mice; LDLR−/−, LDL receptor knockout mice. Relaxation data were calculated as percent values of precontraction level. (Vass et al., 2024)

4.2.2 Body- and heart weight values

HFD effectively and significantly increased body weight of mice (Figure 8A), 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. $CB_1R-/-$, Figure 8A). The heart weight of the mice slightly increased while kept on HFD, but this elevation didn't reach statistical significance, just between $CB_1R+/+$, LDLR+/+, HFD group compared to $CB_1R-/-$, LDLR+/+, CD group ($p=0.007$, Figure 8B). Between other groups there was no significant elevation in heart weight, however, slight elevation still can be seen due to HFD (Figure 8B). (Vass et al., 2024)

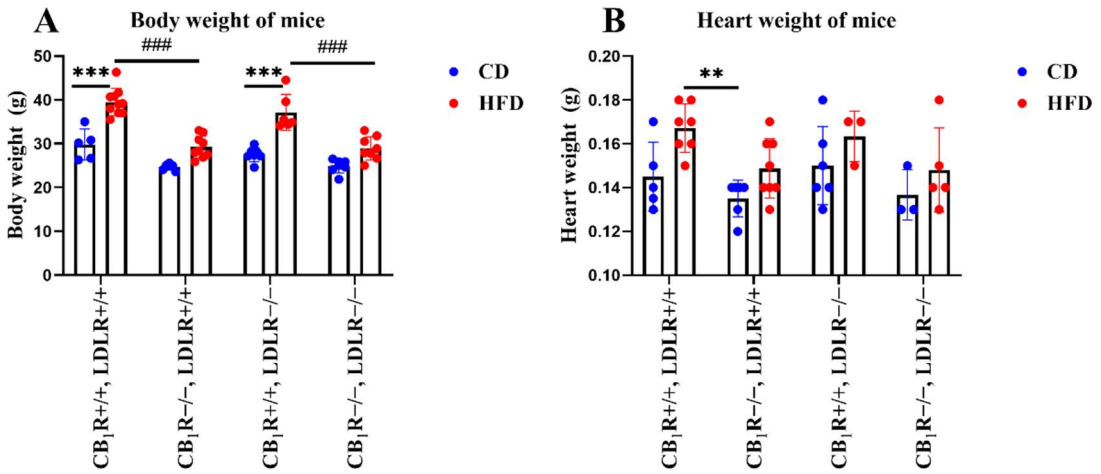

Figure 8. Values of body- and heart weights of CB_1R- and LDLR-WT and -KO mice kept on CD and HFD. **Panel (A).** Body weight of CD and HFD, CB_1R- and LDLR-WT and -KO mice (***, $p<0.001$; ###, $p<0.001$; n=5-10). **Panel (B).** Heart weight of CD and HFD, CB_1R- and LDLR-WT and -KO mice (**, $p<0.007$; n=3-8). Mean ± SEM values are indicated with dots showing individual data points. *Abbreviations: CD, control diet; HFD, high-fat diet; WT, wild-type; KO, knockout; $CB_1R+/+$, CB_1R wild-type; $CB_1R-/-$, CB_1R knockout mice; LDLR+/+, low density lipoprotein receptor wild-type; LDLR−/−, low density lipoprotein receptor knockout mice. (Vass et al., 2024)*

4.2.3 Blood pressure measurements

Significantly elevated systolic and diastolic BP values were registered during pentobarbital-sodium (Euthasol) anaesthesia in $CB_1R+/+$, LDLR−/−, HFD mice compared to $CB_1R+/+$, LDLR+/+, HFD mice ($p<0.001$, Figure 9A-B), 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. There was no statistical difference between CD and HFD groups in LDLR−/− and $CB_1R+/+$ genotypes in terms of systolic and diastolic BP values (Figure 9A-B). (Vass et al., 2024)

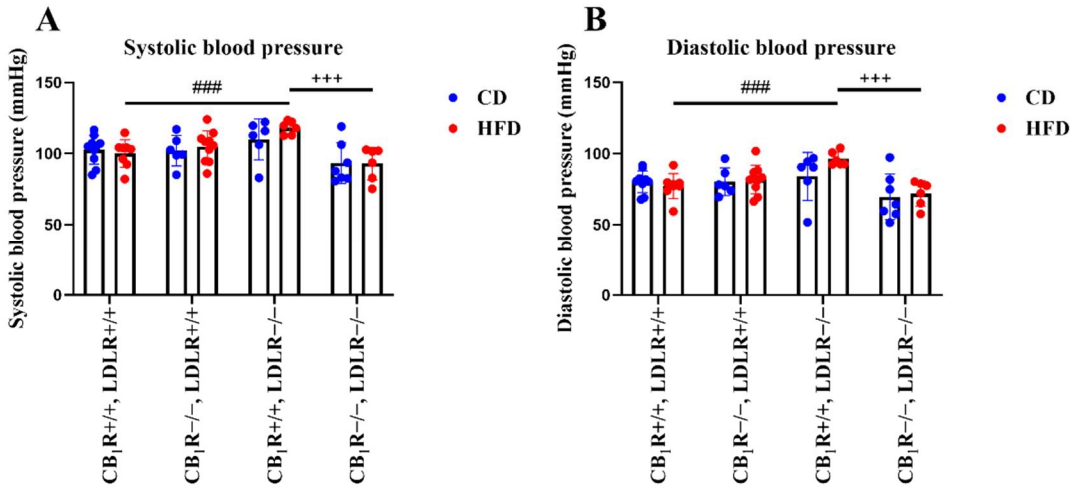

Figure 9. Systolic and diastolic blood pressure values of CB_1R- and LDLR-WT and -KO mice kept on CD and HFD. **Panel (A).** Systolic BP of CB_1R- and LDLR-WT and -KO mice kept on CD and HFD (n=5-10). **Panel (B).** Diastolic BP of CB_1R- and LDLR-WT and -KO mice kept on CD and HFD (n=5-10). +++, $p<0.001$, between $CB_1R+/+$ and $CB_1R-/-$; ###, $p<0.001$ between LDLR+/+ and LDLR−/−, n=5-10). Mean ± SEM values are indicated with dots showing individual data points. *Abbreviations: CD, control diet; HFD, high-fat diet; BP, blood pressure; WT, wild-type; KO, knockout; $CB_1R+/+$, endocannabinoid type 1 receptor wild-type; $CB_1R-/-$, endocannabinoid type 1 knockout mice; LDLR+/+, low density lipoprotein receptor wild-type; LDLR−/−, low density lipoprotein receptor knockout. (Vass et al., 2024)*

4.2.4 Cholesterol level measurements

We examined the impact and efficiancy of HFD on plasma total cholesterol levels in our double KO animal model. We found that plasma total cholesterol levels did not differ significantly in the LDLR+/+ groups, however, there was non-significant elevation in

LDLR+/+ animals HFD compared to CD fed animals (Figure 10). In LDLR knockout groups the HFD significantly elevated the plasma cholesterol levels pushing it into patophysiological 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). In addition, 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 (p=0.013 between $CB_1R+/+$, LDLR+/+, CD and $CB_1R+/+$, LDLR−/−, CD, and p=0.002 between $CB_1R-/-$, LDLR+/+, CD and $CB_1R-/-$, LDLR−/−, CD. Presence of CB_1Rs did not change the plasma cholesterol levels significantly (Figure 10). (Vass et al., 2024)

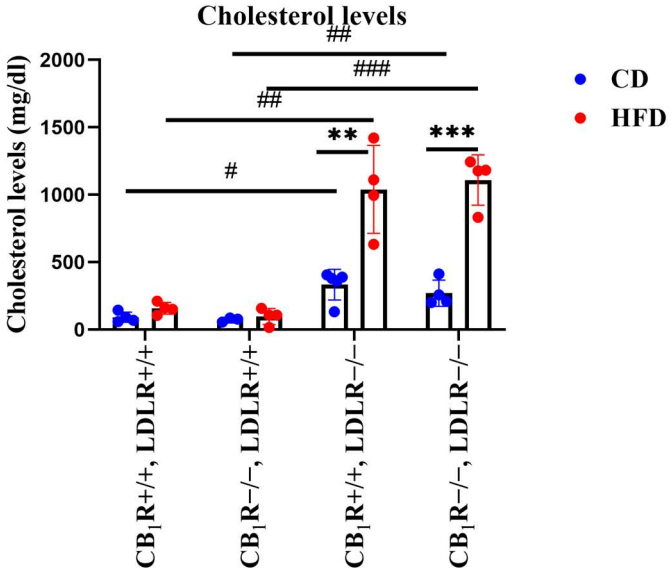

Figure 10. Plasma total cholesterol levels of CB_1R- and LDLR-WT and -KO kept on CD and HFD, **, p=0.006 and ***, p<0.001 between CD and HFD groups. #, p=0.013; ##, p=0.002; ###, p<0.001 between LDLR+/+ and LDLR−/−; n=3-5). Mean ± SEM values are indicated with dots showing individual data points. *Abbreviations: CD, control diet; HFD, high-fat diet; WT, wild-type; KO, knockout; CB_1R, cannabinoid type 1 receptor; $CB_1R+/+$, cannabinoid type 1 receptor wild-type; $CB_1R-/-$, cannabinoid type 1 knockout; LDLR, low density lipoprotein receptor; LDLR+/+, low density lipoprotein receptor wild-type; LDLR−/−, low density lipoprotein receptor knockout. (Vass et al., 2024)*

4.2.5 Immunohistochemistry results of endothelial NOS

In order to assess the effect of diet and CB_1Rs, eNOS expression was measured in LDLR+/+ groups from aortas taken from the upper abdominal level, between the diaphragm and the renal arteries. Endothelial nitric oxide synthase didn't change in CB_1R+/+, HFD group, which 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 (Figure 11 A-B, p=0.016). (Vass et al., 2024)

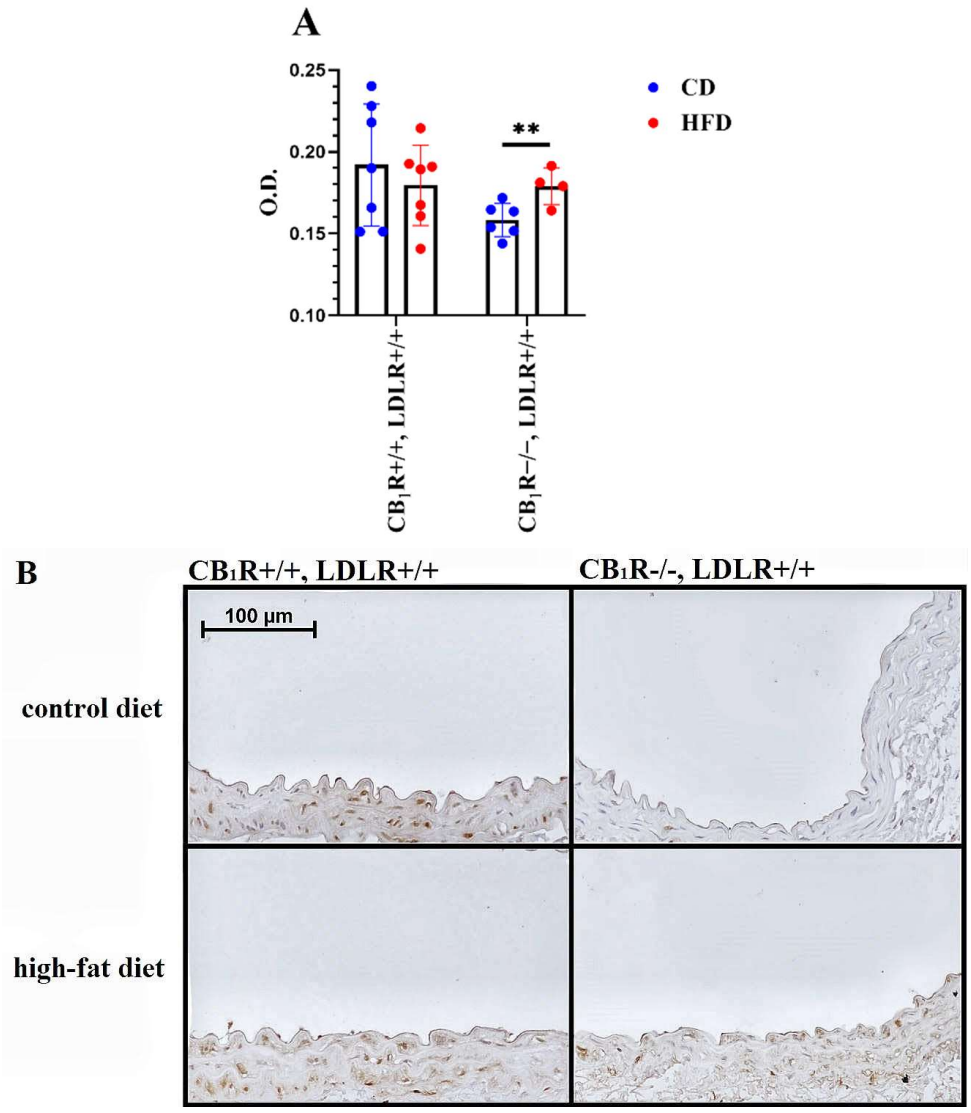

Figure 11. Expression of eNOS in abdominal aortas of LDLR-WT, CB_1R-WT and -KO mice kept on CD and HFD. **Panel (A).** Optical density (O.D.) levels of eNOS expression. n=4-7, **, p=0.016 between CD and HFD groups in the same genotype. Mean ± SEM values are indicated with dots showing individual data points. **Panel (B).** Representative photos indicating eNOS

expression chosen form 4-7 slides of each group. *Abbreviations, CD, control diet; HFD, high-fat diet; WT, wild-type; KO, knockout; O.D., optical density; CB_1R+/+, cannabinoid type 1 receptor wild-type; CB_1R−/−, cannabinoid type 1 receptor knockout; LDLR+/+, LDL receptor wild-type; LDLR−/−, LDL receptor knockout. (Vass et al., 2024)*

5. Discussion

5.1 Main findings

5.1.1 Main findings of the impact of CB_1R on the vascular function and on estrogen status in female mice

In the first part of our experiments we have compared the vascular functions by myography on the aortic segments of CB_1R wild-type ($CB_1R+/+$) and CB_1R-KO ($CB_1R-/-$) female mice, and we also have examined estrogen levels by determinig plasma estrogen and metabolite levels in order to reveal the possible influences of the ECS on the function of the HPO axis.

There were no significant differences among the contraction responses between CB_1R-WT and -KO groups, but the KO animals had significantly greater vasodilatory reactions to Ach and estradiol indicating a functional involvement of eCBs in these relaxation mechanisms. In both CB_1R-WT and -KO animals Ach-induced relaxation was inhibited by NOS inhibition with LNA, however, Ach relaxation response was enchanced by COX inhibition with INDO in the $CB_1R+/+$ mice, which effect could not be seen in the $CB_1R-/-$ animals. Thromboxane-prostanoid receptor (TP-R) inhibition with SQ 29,548 showed similar results. (Bányai, Vass, et al., 2023) The uppermentioned results indicate that Ach-induced vasodilation is influenced by the NO pathway in both CB_1R-WT and -KO groups and that constrictor prostanoids contribute to the vascular effects in $CB_1R+/+$ but not in $CB_1R-/-$ mice. In addition, cE2 levels were significantly higher and an elevated, non-significant tendency for f4OH-E1 levels was also observed in $CB_1R-/-$ compared to $CB_1R+/+$ mice, indicating that CB_1Rs have an impact of influencing serum estrogen levels. NOS inhibition with LNA significantly reduced estradiol-induced relaxation in both groups. While estradiol-induced vasodilation was sligthly augmented in $CB_1R+/+$ genotypes with the COX inhibitor, INDO, still an opposite effect, a slightly depressed vasodilation, could be registered in $CB_1R-/-$ animals, these results didn't reach statistical significance. Comparison of the normalized values showed significant differences between CB_1R-WT and -KO mice, estradiol-induced relaxation of CB_1R-KO animals was more reduced. This observation proves that both endocannabinoids and endogenous prostanoids contribute to estradiol-induced vasodilation. (Bányai, Vass, et al., 2023) We also examined the functional responses of CB_1 receptors. We obtained

significant vasodilation in $CB_1R+/+$ mice for CB_1 receptor agonist WIN 55,212-2 compared to $CB_1R-/-$ mice, where no vasodilation could be observed. Vasorelaxative effects are attributed to the products of eNOS and COX-2 enzymes described above. (Bányai, Vass, et al., 2023)

5.1.2 Main findings of the functional and remodeling effect of CB_1R in double CB_1R-LDLR knockout atherosclerotic male mouse model

In the second part of the study we aimed to reveal the potential role of CB_1Rs and CB_1R signalling mechanisms in the functional vascular remodeling of an atherosclerosis-prone mouse model based on the absence of LDL receptors.

The main findings demonstrate, that the absence of CB_1R counterbalances the functional deterioration of the vascular function in HFD fed mice. (Vass et al., 2024) The increased body weights and the highly-elevated total cholesterol levels in $LDLR-/-$ mice demonstrate the effectivity of the applied high-fat diet. The elevation of total cholesterol levels in LDLR-KO, HFD animals exceeded 1000 mg/dL (>25.86 mmol/L), which indicates a severe hypercholesterolemia with a high probability of inducing the formation of AS. Presence of CB_1Rs did not change significantly the elevation of total cholesterol levels in $LDL-/-$, HFD animals. As expected and described before (Dörnyei et al., 2023), $CB_1R-/-$ mice showed significantly lower body weight values compared to their $CB_1R+/+$ counterpart and this difference was maintained in the present study during their high-fat diet. However, due to HFD, the systolic and diastolic BP values increased in LDLR-KO, $CB_1R+/+$ groups, compared to $LDLR+/+$, $CB_1R+/+$ mice, which was decreased in $CB_1R-/-$ animals. HFD deteriorated the functional remodeling of the aorta as the Ach-induced endothelium dependent relaxation was reduced compared to the CD groups, which was more pronounced in the CB_1R-WT groups. Ach-induced endothelium dependent vasodilation was the most pronounced in the $CB_1R+/+$, $LDLR+/+$ and $CB_1R+/+$, $LDLR-/-$, CD groups, which was decreased by HFD in the same genotype. Within $CB_1R-/-$ groups, the $CB_1R-/-$, $LDLR+/+$, CD group showed the highest Ach-induced vasodilation, which was ameliorated in HFD groups at low concentrations. Curve fitting method showed that EC50 was decreased in the absence of CB_1Rs in LDLR-WT, HFD mice indicating an improvement in Ach-induced vasodilation compared to the corresponding CB_1R-WT mice (Supplementary Table 1.). The importance of CB_1Rs in

vascular damage is also demonstrated by the inhibitor studies. NO-dependent relaxation was attenuated by HFD, but it was partially better in CB_1R-KO groups in between HFD and CD. These findings are underlined by the immunohystochemistry results on the endothelial nitric oxide synthase showing a higher optical density in $CB_1R{-}/{-}$, HFD mice. COX inhibitor INDO modestly modulated Ach-induced vasodilation, which was significantly reduced at lower concentrations in $CB_1R+/+$, LDLR–/–, CD and LDLR+/+, HFD animals. Endogenous prostanoid production is also seems to be rearranged by the diet. (Vass et al., 2024)

5.2 Discussion of the impact of CB_1R on the vascular function and on estrogen status in female mice

5.2.1 Vascular effects of cannabinoids and endocannabinoid signalling

In the first project it has been shown that vascular tone might be altered by cannabinoid receptor-mediated signalling pathways both in physiological and pathophysiological conditions. (Bányai, Répás, et al., 2023; Bátkai et al., 2004; Karpińska et al., 2018; Pacher et al., 2005; Szekeres et al., 2015) Vascular endothelial factors, smooth muscle cells and perivascular neurons are the targets of cannabinoid agonists. (Bányai, Vass, et al., 2023; Dannert et al., 2007; Hillard, 2000; Randall et al., 2002; Wagner et al., 2001) It has been proved that synthetic as well as phyto- and endocannabinoids (e.g.: AEA, THC, WIN55,212-2) induce vasodilation in the coronaries, aorta or in cerebral arteries through different pathways. (Bányai, Vass, et al., 2023; Dannert et al., 2007; Hillard, 2000; O'Sullivan et al., 2007; Randall et al., 2002; Szekeres et al., 2015; Szekeres et al., 2012; Wagner et al., 2001) Vasodilatory effects of eCBs may differ depending on the type of arteries, a more pronounced vasorelaxation effect can be registered on resistance arteries than on the aorta. (Bányai, Vass, et al., 2023; Dannert et al., 2007; Szekeres et al., 2015; Szekeres et al., 2012; White & Hiley, 1998)

Via CB_1R- $G_{i/o}$ protein-coupled signalling eCBs exert acute vasodilatory and hypotensive effects. (Bányai, Vass, et al., 2023; Dannert et al., 2007; Járai et al., 2000; Pacher et al., 2005; Szekeres et al., 2015; Turu & Hunyady, 2010) ECB release was observed during the activation of calcium mobilizing GPCRs, thus regulating vasoconstriction through negative feedback mechanism. (Bányai, Vass, et al., 2023; Gyombolai et al., 2012; Karpińska et al., 2018) These effects have been registered in different vascular beds such as in pulmonary-, cerebral- and coronary arteries, gracilis arteriole or in the aorta.

(Bányai, Vass, et al., 2023; Karpińska et al., 2018; Rademacher et al., 2005; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015; Szekeres et al., 2012) These results suggested a continuous vasodilatory tone of eCBs in the arteries. In previous experiments, Ang II-induced vasoconstriction was higher when CB_1Rs or DAGL were inhibited, while vasoconstrictive mechanisms were attenuated during the blocking of MAGL, suggesting that locally produced 2-AG activates vascular CB_1Rs. (Bányai, Vass, et al., 2023; Szekeres et al., 2015) In addition, in hypertensive rats, the inhibition of AEA degrading enzyme FAAH by URB597 augmented AEA level and improved vascular endothelial vasodilatory functions in small mesenteric arteries. (Bányai, Vass, et al., 2023; Baranowska-Kuczko et al., 2021)

In this first project, we used genetically modified animals (CB_1R knockout) in order to reveal acute, chronic or life-long vascular effects of CB_1Rs. We were able to identify several vasomotor functions of the ECS in controlling vasodilation and vasoconstriction. We have found augmented vasodilatory effects in CB_1R-KO female mice. Applying the specific eNOS inhibitor LNA and COX inhibitor INDO, we showed that nitric oxide-dependent vasodilations to acetylcholine and estradiol have been enhanced in CB_1R knockout mice. Our results also indicate that constrictor prostanoids are present in the aorta of CB_1R wild-type mice, while this mechanism is missing in CB_1R knockout mice. Thus, we can conclude, that in the absence of CB_1Rs, the role in regulating vasomotor functions by vasoconstrictor prostanoids disappears, meanwhile nitric oxide-dependent vasodilatory pathway would be augmented. Previous studies also indicate that vasoconstrictor prostanoids are released together with the vasorelaxant NO, however their impact was decreased in trained animals. (Bányai, Vass, et al., 2023; Szekeres, Nádasy, Dörnyei, et al., 2018; Szekeres et al., 2004; Szekeres, Nádasy, Soltész-Katona, et al., 2018)

Although Stanley found that CBD enchanced the phosporylation of eNOS increasing its enzymatic activity, in this project, the main vasodilatory mechanism was the NO pathway, that was supported by Bányai et al. with eNOS stainings. (Bányai, Vass, et al., 2023; Stanley et al., 2015) In the experiments of Stanley et al., synthetic CB_1 receptor antagonists, such as AM251 and LY320135 inhibited CBD-induced vasorelaxation. This difference can be due to a prolonged endocannabinoid activity via CB_1Rs that might decrease the expression of eNOS, as the existing protein molecules are activated by

cannabinoid agonists and endothelial vasorelaxants such as acetylcholine and estrogen (Stanley et al., 2015) and also CBD can serve as an antagonist in the presence of more powerful stimulators of CB_1Rs. Several other functions of CBD have been shown, for example it can also activate PPARγ and transient receptor potential cation channel vanilloid-1channels and can also stimulate activation of the serotonin receptor. (Bányai, Vass, et al., 2023; Morales et al., 2017)

According to our experiments we conclude that preferable vascular parameters and functional alterations occur in the absence of CB_1Rs. The uppermentioned results indicate that the pharmacologically selective blocking of CB_1Rs or their pathways might propose beneficial novel therapy in several cardiovascular diseases. (Bányai, Vass, et al., 2023)

In addition to the functional changes, Bányai et al. made immunohystological stainings of eNOS, COX-2. The eNOS protein density was elevated in the aortic wall of CB_1R-KO mice compared to their WT counterparts, while endothelial COX-2 decreased in the CB_1R knockout group, further supporting results. (Bányai, Vass, et al., 2023)

5.2.2 Estrogen-induced relaxation, plasma levels

During determination of estrogen levels in the plasma of $CB_1R^{-/-}$ female mice, a significantly higher level of cE2 was found compared to $CB_1R^{+/+}$. In case of fE2 and f4OH-E1, there was no significant difference between the two genotypes. These findings indicate that a reduced activity of the ECS results in an enhanced estrogen conjugation as well as a partially increased metabolism. Regarding the circulating estrogens and their metabolites, only a small portion can be found in a free form, most of them circulate in a conjugated form. Thus our results suggest that in case of female CB_1R knockout mice, the production of estrogens is significantly elevated. This effect is likely to be attributed to the increased activity of the catalyzing enzyme CYP1B1, which can also be activated in numerous pathogenic processes such as tumorigenesis. (Kovács et al., 2017) All these results imply an interplay between the estrogen and the endocannabinoid system. (Bányai, Vass, et al., 2023) Related to our findings, Bányai et al. also carried out ER-α and ER-β immunohystological stainings on the same sample of mice in both the endothelial and the media layers, and found that there were no significant differences between CB_1R-KO and -WT regarding ER-α, but ER-β was reduced in CB_1R-KO compared to WT. (Bányai, Vass, et al., 2023)

It has been shown that E2-induced vasodilation was observed as the result of a complex acute nongenomic effects mediated by endothelial NO. (Chakrabarti et al., 2014; Huang et al., 1997; Kakucs et al., 2001; Kakucs et al., 1998) Vasorelaxant effects contributed to E2 may have a cardioprotective effect in fertile women before menopause. (Kakucs et al., 1998) Our results indicate that the E2-induced vasodilation is enhanced in CB_1R knockout mice. The significance of the endothelium-dependent NO-mediated pathway was also indicated in both CB_1R wild-type and knockout strains by a depressed vasorelaxation during the inhibition of the eNOS, also in accordance with other studies. (Chakrabarti et al., 2014; Kakucs et al., 2001) According to our results (Figure 2C-D) we assume, that the balance of endogenous prostanoids might change and vasodilator prostanoids predominate rather than constrictor prostanoids in the vessels of CB_1R knockout female mice compared to wild-type, resulting in an augmented vasodilatory response in female $CB_1R^{-/-}$ genotype. (Bányai, Vass, et al., 2023; Pabbidi et al., 2018; Zhou et al., 2021) In addition, eCB AEA has been shown to augment the eNOS pathway by potentiating the effect of estradiol. (Bányai, Vass, et al., 2023; Szabó et al., 2020)

5.2.3 Gender differences in vascular responses of estrogens and cannabinoids

In order to explore gender differences in vascular responses of estrogens and cannabinoids, several animal and clinical studies have been performed. Expression and functions of eNOS/NO, endothelium-derived hyperpolarizing factor, ROS, prostacyclin (PGI_2), thromboxane A_2 (TXA_2) are altered due to E2 through ER-α in vascular cells, and thus E2 may change thee prostanoid balance. A thromboxane analogue triggered a higher constrictor effect in male coronaries than in females. (Pabbidi et al., 2018; Varbiro et al., 2006) In this experiment we have obtained an augmented relaxation response in the aortas of CB_1R-KO female mice suggesting that the balance of these processes shifts towards vasodilation. (Pabbidi et al., 2018) Usually NO-mediated vasodilation is augmented, while contraction effects are reduced in experiments of isolated vessels in female mice. (Mericli et al., 2004) There are marked gender-determined differences regarding cannabinoid sensitivity on the expression of different CB receptors and their effects on estradiol and progesterone. AEA-induced vasodilation was enhanced by E2 in isolated mesenteric arteries, which effect was the most prominent in hypertensive rats. (Ho, 2013) This study confirmed an involvement of nitric oxide and prostanoid mechanisms of E2-induced vascular effects. (Bányai, Vass, et al., 2023)

5.3 Discussion of the functional and remodeling effect of CB_1R in double CB_1R-LDLR knockout atherosclerotic male mouse model

5.3.1 Vascular alterations in hypercholesterolemic LDLR-KO mice

The main risk factor in developing cardiovascular diseases is hypercholesterolemia and consecutive atherosclerosis. (Zhou et al., 2022) In our study body weights of mice have significantly increased in case of CB_1R+/+ genotype fed with high-fat diet, however, we couldn't observe significant elevation of the heart weights. Previous experts showed, that high-fat diet fed LDLR−/− mice develop hypercholesterolemia, later become atherosclerotic, developing sclerotic plaques in the aorta. (Baltieri et al., 2018; Bjørnholm et al., 2021; Emini Veseli et al., 2017; Liu et al., 2022; Maganto-Garcia et al., 2012; Vass et al., 2024) HFD is needed in order to develop atherosclerotic lesions in LDL-receptor-deficient animal models. (Getz & Reardon, 2016) In our newly established CB_1R, LDLR double-KO mouse model we also detected an elevated plasma total cholesterol level in case of LDLR-KO mice, that was further elevated high above the pathophysiological level after a 5 month of HFD. (Vass et al., 2024)

In HFD hypercholesterolemic mice Ach-induced vasodilation was attenuated at low concentrations, and NOS inhibitor LNA significantly reduced Ach-induced vasodilation showing and underline the crucial role of the nitrogen oxide pathway. NOS inhibition was less effective in Ach-induced vasodilation in HFD mice compared to CD. This effect also demonstrates the role of alteration in the NO pathway in HFD. These results are partly in accordance with our immunohystochemical eNOS staining results. A possible change of endogenous prostaglandin production caused by the diet is behind the changes in INDO sensitivity. (Vass et al., 2024)

Functional deteriorating changes of the vessels are shown in LDLR−/−, HFD mice even on abdominal aorta sections, where the AS plaques were less visible. Aorta plaques were developed mostly on the aortic arch (Supplementary Figure 1). The development of atherosclerosis, calcificated and necrotic plaque formation through inflammation, smooth muscle cell proliferation and endothelial dysfunction has its risk factors, such as dyslipidemias with high cholesterol levels esepecially of LDL and VLDL. (Bennett et al., 2016; Bjørnholm et al., 2021; Dörnyei et al., 2023; Grootaert et al., 2018; Mahdinia et al., 2023; Novikova et al., 2018; Vass et al., 2024)

Endothelial dysfunction provoked by hypercholesterolemia is driven by cholesterol buildup and inflammatory reactions with changes blood flow patterns in the vessel walls during degenerative remodeling. Endothelial dysfunction and remodeling of the vessels exhibit both functional and morphological changes. (Akhmedov et al., 2021; Beamish et al., 2010; Bernardi et al., 2018; Godo & Shimokawa, 2017; Jiang et al., 2022; Vass et al., 2024; Wang et al., 2022) We have shown the functional endothelial dysfunction of the aorta in HFD mice that is marked by an impaired NO-dependent vasodilatory response. Reduced availability of endothelial nitric oxide predicts the development of AS, but it is reversible in the early stages. (Mudau et al., 2012) The impaired vasodilatory function of endothelial NO might be postponed by compensatory mechanisms such as adipose tissue buildup around the vessels. (Baltieri et al., 2018) Inflammatory biomarkers show a close relation and predictory factor to plaque formation, progression and vulnerability, such as C-reactive protein (CRP), tumor necrosis factor (TNF)α, interleukins 6, 17A, 18, 21, MCP-1, also with CD68- lipid positive areas and macrophage accumulation. (Vass et al., 2024; Wang et al., 2022)

Based on the myography results we have proven a functional vascular remodeling in LDLR-KO, high-fat diet fed mice including an attenuated endothelium-dependent vasorelaxation effect due to impaired NO-production. In addition, an elevated hemodynamic resistance of the circulation is indicated by the elevated body weight and BP values during HFD. (Vass et al., 2024)

5.3.2 Vascular effects of CB_1 receptors and endocannabinoid signalling, CB_1R knockout mice

It is well established, that the endocannbinoid system has a role in maintaining several physiological regulatory processes, including the cardiovascular system eliciting negative inotropic, hypotensive and vasorelaxant, thus BP lowering effect. (Dörnyei et al., 2023; Pacher et al., 2005) Previous experiments demonstrated that vascular tone is modulated by CB_1R signalling, which effect induces vasorelaxation through endothelium-dependent vasodilation. CB_1 receptors are detected on the endothelial cells, smooth muscle cells and perivascular neurons as well. (Hillard, 2000; Randall et al., 2004; Szekeres et al., 2015; Vass et al., 2024)

Vascular functions are significantly modulated by endothelial factors such as NO mediating vasodilation as well as also by vasodilator or vasoconstrictor prostanoids. (Eckenstaler et al., 2022; Koller et al., 1998; Miklós et al., 2021; Szekeres et al., 2004; Zhou et al., 2021) Prostanoids, COX metabolites with different vasoactive mechanisms and vasodilatory NO are produced and released together by endothelial cells in order to maintain normal vascular tone, however effects of the vasoconstrictor PGs were altered in exercise-trained animals to achieve a more pronounced vasodilation. (Szekeres, Nádasy, Dörnyei, et al., 2018; Szekeres et al., 2004; Zhou et al., 2021) Endothelial vasodilatory effects are more pronounced in more muscular resistance arteries. Endocannabinoids exert a vasodilatory effect, which can also be released upon the signalling of vasoconstrictive agents or similar effects can be elicited by CB_1R agonists. (Bányai, Vass, et al., 2023; Bátkai et al., 2004; Dannert et al., 2007; Hillard, 2000; O'Sullivan et al., 2007; Pacher et al., 2005; Randall et al., 2004; Szekeres et al., 2015; Szekeres et al., 2012; Wagner et al., 2001) ECBs bind to CB_1Rs resulting in $G_{i/o}$ protein-coupled signalling, creating a smooth muscle cell hyperpolarization and consecutive vasorelaxation. (Dannert et al., 2007; Járai et al., 2000; Pacher et al., 2005; Szekeres et al., 2015; Turu & Hunyady, 2010) Anandamide, a CB_1R agonist eCB elicits long-lasting hypotension and bradycardia through the CB_1R signalling pathway (Járai et al., 2000), however a continuous vasodilatory tone exists through negative feedback of eCB release during calcium generating GPCR agonists, indicating that vessel walls have a continuous vasodilatory tone. (Dörnyei et al., 2023; Gyombolai et al., 2012; Karpińska et al., 2018; Szekeres, Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015; Vass et al., 2024)

In the first project we also showed that CB_1R knockout female mice had an augmented NO-dependent vasodilation to Ach and estradiol. We found that CB_1R knockout mice had an augmented vasodilatory effect because of the augmented NO-dependent vasorelaxation, and by an attenuated effect of vasoconstrictor PGs, which observation was supported by the results of eNOS and COX immunohystochemistry by Bányai et al. (Bányai, Vass, et al., 2023)

Our present results show a significant role of CB_1R-dependent mechanisms, endothelial factors such as NO and PGs in vascular remodeling of the hypercholesterolemic AS-prone LDLR-CB_1R-double knockout mouse model. (Vass et al., 2024)

5.3.3 Role of CB_1 receptors in hypercholesterolemia-induced vascular alterations in double CB_1R-LDLR knockout mice

A connection between the endocannabinoid system and the development of atherosclerosis had been described before (Guillamat-Prats et al., 2019), now, in this second project we aimed to examine the impact of the ECS on vascular remodeling in double LDLR-CB_1R knockout atherosclerotic mouse model induced by hypercholesterolemia. In this model, we indicated that presence of CB_1Rs significantly influences the functional vascular remodeling. We have found that systolic and diastolic BP values are increased in LDLR−/−, CB_1R+/+ mice with HFD, compared to LDLR−/−, CB_1R−/− HFD group. Myography results indicate that a depressed Ach-induced vasorelaxation with NO-dependency characterizes HFD groups compared to CD animals, while CB_1R-KO partly counterbalanced the deteriorating effects of Ach-induced vasodilation and NO availability. These effects were reduced in hypercholesterolemic, high-fat fed, LDLR−/− mice. The improvement in NO bioavailability is supported by the elevated eNOS expression in CB_1R knockout, HFD mice compared to CD determined by immunohystochemistry. We propose that the absence of CB_1Rs can restore impaired NO production and elevate NO levels in HFD-treated animals. These results indicate that the absence of CB_1Rs can postpone or revise the deteriorating functional and structural impacts of a serious hypercholesterolemic state in LDLR-KO mice. (Vass et al., 2024)

It had been previously described that the ECS has a regulatory role in food intake, appetite and energy metabolism, while the inhibition of ECS signalling mediated by CB_1Rs can decrease food uptake and induce the loss of weight. (Cinar et al., 2020; Dörnyei et al., 2023; Jamshidi & Taylor, 2001; Kunos et al., 2009; Mastinu et al., 2018; Vass et al., 2024) In this second project we have registered lower body weight values in CB_1R−/− mice, which was elevated due to HFD, but not significantly in CB_1R knockout groups. Langbein et al. demonstrated, that alone a voluntary exercise was not enough to improve vascular functions, however they also indicated that a lower body weight and white adipose tissue mass in LDLR−/− mice should be achieved. (Langbein et al., 2015) Our findings indicate that the improvement in vascular function observed in LDLR-KO mice is not in connection with lower plasma total cholesterol levels, but may be due to direct functional effects on the vessel walls. (Vass et al., 2024)

5.3.4 Role of endocannabinoid system and CB_1 receptors in cardiovascular pathologies, vascular remodeling and possible therapeutic effects

Among others, the endocannabinoid system influences BP, vascular tone, cardiac functions, vascular inflammation and angiogenesis, since the components of the ECS are present in numerous organs and tissues having a wide-range of physiological roles. (Bondarenko, 2019; Malinowska et al., 2012) Overactivation and increased expression of CB_1Rs can cause excess lipid uptake, dyslipidemias, hypercholesterolemia, which can lead to obesity and -by the elevated number and severity of the cardiac risk factors- to cardiovascular diseases. (Bányai, Vass, et al., 2023; Dörnyei et al., 2023) AEA upregulated CB_1Rs can induce pro-apoptotic pathways by increasing intracellular Ca^{2+} or ROS. (Rabino et al., 2021) Endocannabinoid system might exert beneficial or deteriorating mechanisms depending on the tissue locations, the conditions (physiological or pathophysiological), and the stimulation or inhibition of it. By the overactivation of cardiovascular CB_1Rs the ECS might have a protective role regarding AS, inflammation, CV diseases, or hypertension, lowering BP and HR in some cases. (Bondarenko, 2019; Mukhopadhyay et al., 2008; Pacher et al., 2005, 2006; Pacher et al., 2008; Pacher & Steffens, 2009) Besides this mechanism, the BP-lowering effect of eCBs can even be harmful in cases of pathological hypotension. (Bondarenko, 2019) However, the heterogenous effects of cannabinoids in the CV function can also be observed by triphasic BP alterations. (Malinowska et al., 2012) During human studies, CBR agonist phytocannabinoid THC had a dose-dependent effect on BP, while it lowered the BP in higher (600mg) dosage, an elevation was observed in smaller (30 mg) dosage. In animal studies of rats, synthetic CB_1R agonist WIN55,212-2 can also elevate BP in normotensive conditions, however, it decreased BP in certain patophysiological conditions such as hypertension. (Bányai, Vass, et al., 2023; Ho & Gardiner, 2009; Vass et al., 2024) Low dose agonism of the ECS activates the sympathetic nervous system causing tachycardia, while high dosage has opposite-parasympathetic effects: low BP and bradycardia. Activation of CB_1Rs can cause catecholamin elevation, thus stress-cardiomyopathy, and also have various inotrop effects. In different types of HT tonic ECS activation was observed as a consequence of increased CB_1R expression in cardiovascular endothelium (Rabino et al., 2021) Selective CB_2R agonist showed dose-dependent effect on cardiac contractility in rabbits. According to these observations, the use of selective CB_1R or

CB_2R agonists or antagonists may offer therapeutic potential in treating cardiovascular diseases such as myocardial infarction, heart failure, atherosclerosis, hypertension and cardiometabolic disorders, because ECS has a pre-homeostatic tone and a Janus-face, dose-dependent effect. (Bátkai et al., 2004; Dörnyei et al., 2023; Kunos et al., 2009; Martín Giménez et al., 2018; Pacher et al., 2005, 2006; Pacher & Steffens, 2009; Rabino et al., 2021; Vass et al., 2024) Strategies to treat these diseases may involve the mechanism of selectively targeting cannabinoid receptors expressed in specific tissues also outside the central nervous system, also of targeting upregulated cannabinoid receptors, and even selectively targeting cannabinoid CB_2Rs. (Pertwee, 2012) In order to reduce undesirable side-effects of cannabinoids, experts are developing more selective drugs which process shows promising results already. (Sierra et al., 2018) Drugs acting on CB_1Rs, CB_2Rs, TRPV1s and PPARs were effective against CVDs such as hypertension, atherosclerosis and myocardial infarction in patophysiological animal models. CBR agonists are also used for their antiemesis properties, for stimulation of appetite, also treating neuropathic pain and symptoms of multiple sclerosis. (Fulmer & Thewke, 2018; Jamshidi & Taylor, 2001; Kunos et al., 2009; Pertwee, 2012) CB_1R antagonists (taranabant, rimonabant) downregulate appetite and food intake, thus have been used to treat obesity and associated metabolic dysregulation in clinical studies, but they have been withdrawn due to their side effects. (Cinar et al., 2020; Dörnyei et al., 2023; Kipnes et al., 2010; Kunos et al., 2009; Vass et al., 2024) Genistein is a new candidate of treating AS as a CB_1R antagonist (Wei et al., 2022), potentially having anti-inflammatory and anticancer effects, too. (Sharifi-Rad et al., 2021) Also, a synthetic CB_1R antagonist AM6545 has been turned into the focus, due to it's property of not penetrating the blood-brain-barrier. (Paszkiewicz et al., 2020) It has turned into a recent focus to develop second and third generation agents of selective CB_1R inhibitors, which have a potential to treat related diseases, but with the minimalisation of the side effects. (Cinar et al., 2020; Dörnyei et al., 2023; Fulmer & Thewke, 2018) Up to date a huge variety of synthetic CB_1R antagonists has been developed, among them O2050, AM251, SR141715 (rimonabant). Previously we have shown their effects to augment GPCR agonist-induced vasoconstriction by calcium signalling mechanisms via DAGL-mediated release of eCBs. These results show the role of signalling-induced release of eCBs regulating CB_1R-mediated vasodilatory effects. (Gyombolai et al., 2012; Szekeres,

Nádasy, Soltész-Katona, et al., 2018; Szekeres et al., 2015; Szekeres et al., 2012) The vasorelaxant effect of the synthetic CB_1R agonists disappeared during blockade of CB_1Rs. (Szekeres, Nádasy, Soltész-Katona, et al., 2018) Related to AS, a previos study demonstrated the reduction of sclerotic plaques of the aorta in LDLR-knockout mouse model treated with a selective CB_1R antagonist rimonabant. (Dol-Gleizes et al., 2009) In addition, Tiyerili et al. described that blocking of CB_1Rs did not change the formation of atherosclerotic plaques in the aorta, however it did improve the endothelium dependent vasorelaxant processes and decreased oxidative stress levels. Furthermore, they demonstrated that in cultured vascular smooth muscle cells treated by rimonabant the production of reactive oxigen species and NADPH oxydase activity were decreased, which might be mediated by Ang II. (Tiyerili et al., 2010) Steffens demonstrated that not just the modulation of CB_1R signalling pathways, but also the activation of CB_2Rs might reduce the degree of atherosclerosis. (Steffens & Mach, 2006) A low dose of the phytocannabinoid nonselective CBR agonist THC has improved the progression of AS plaque formation in mice through immunomodulatory effects. (Steffens et al., 2005)

In the first project we demonstrated that the ECS-signalling can influence vascular remodeling in female mice. Structural vascular changes were found by lowered intima–media ratio, lower COX-2 and higher eNOS expressions in the CB_1R-KO group compared to WT mice in accordance with our functional vacular results of the altered contractile/relaxation responses. (Bányai, Vass, et al., 2023; Vass et al., 2024)

In the second project our findings are in accordance with our previuos results, as we found an altered functional remodeling in CB_1R knockout mice, resulting in higher NO availability and eNOS expression and lower blood pressure in HFD cases. We also found that the slightly higher heart weights with the elevated cholesterol levels, developed in HFD LDLR-KO mice, weren't modified by the presence or absence of CB_1Rs. (Vass et al., 2024)

According to our results we suggest that inhibiting the CB_1Rs selectively may have beneficial effects in hypertensive and dyslipidaemic conditions such as atherosclerosis by lowering blood pressure and delaying or compensating the deterioration of the vascular functions.

6. Conclusions

6.1 Conclusions of the impact of CB_1R on the vascular function and on estrogen status in female mice

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 (Figure 12A-B). In CB_1R knockout mice the vasodilatory responses are lack of the dominance of vasoconstrictor prostanoids, which are present in WT (Figure 12A-B). Bányai and Vass et al. showed structural changes in accordance with the functional results (Bányai, Vass, et al., 2023). 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 blocking CB_1Rs selectively, may enhance favorable vasodilatory effects and vascular remodeling. (Bányai, Vass, et al., 2023)

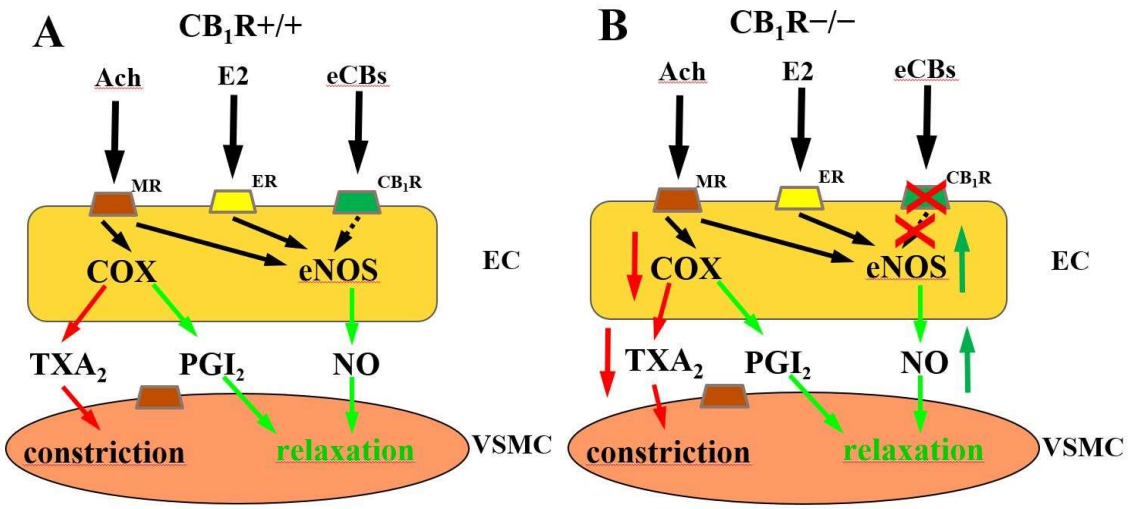

Figure 12. Schematic representation of the functional changes in the aortic wall of CB_1R-WT and CB_1R-KO female mice. **Panel (A).** Functional changes of the aortic wall in CB_1R-WT female mice. **Panel (B).** Functional changes of the aortic wall in CB_1R-KO female mice compared to WT. Knocking out the CB_1Rs induces elevation of eNOS expression and NO availability, parallel 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. (Bányai, 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.

6.2 Conclusions of the investigation of the functional and remodeling effect of CB_1R in double CB_1R-LDLR knockout atherosclerotic male mouse model

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 (Figure 13A-C). According to our results, LDLR knockout mice fed with HFD develop functional vascular deterioration which manifests in a depressed NO-dependent vasodilation (Figure 13B). 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 (Figure 13C). 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. (Vass et al., 2024)

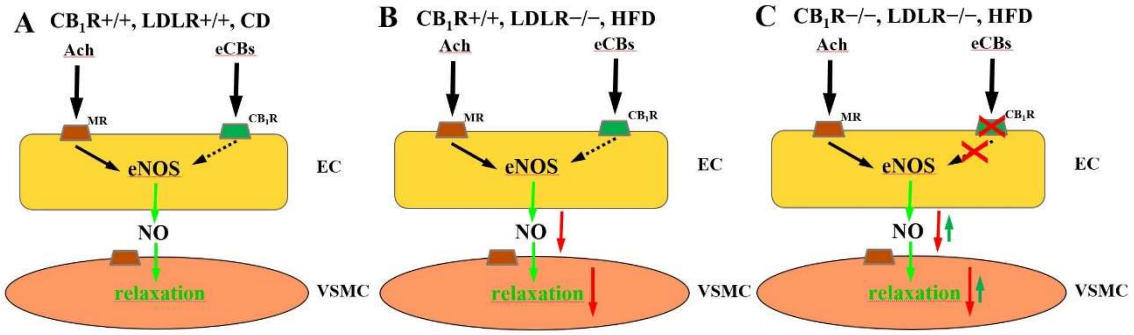

Figure 13. Schematic representation of the functional changes in the aortic wall of CB_1R-LDLR double knockout high-fat diet male mice compared to CB_1R-LDLR-WT, control diet male mice.

Panel (A). Schematic representation of the vasodilatory function of the aortic wall in $CB_1R+/+$, LDLR+/+, CD mice. **Panel (B).** Schematic representation of the vasodilatory function of the aortic wall in $CB_1R+/+$, LDLR−/−, HFD, atherosclerosis-prone, hypercholesterolemic mice, compared to $CB_1R+/+$, LDLR+/+, CD mice. **Panel (C).** Schematic representation of the vasodilatory function of the aortic wall in CB_1R-LDLR double knockout, HFD-fed mice, compared to $CB_1R+/+$, LDLR−/−, HFD 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. (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.*

7. Summary

In the first project we aimed to understand the impact of the ECS and CB_1 receptor activation on vascular functions and to test the impact of the missing receptor function. Also to reveal any potential interplay between the ECS and the female estrogen status on the vascular remodeling using CB_1R wild-type and knockout female mice. We found that the levels of conjugated E2 were elevated in CB_1R knockout mice, compared to their wild-type counterparts. In CB_1R knockout mice, an enhanced vasodilation to Ach and E2 was found, which was attenuated by NOS inhibition. Inhibition of cyclooxygenase decreased phenylephrine-induced vasoconstriction, while it increased acetylcholine-vasodilation in WT mice. This effect wasn't observed in the CB_1R-KO group. Effects of indomethacin on E2-relaxation in CB_1R-KO mice became opposite to those observed in WT. CB_1R knockout female mice represented by better vasodilation responses to be realized by augmented nitric oxide pathway and by a decreased effect of constrictor prostanoids. (Bányai, Vass, et al., 2023)

Related to the second part of the experiments, we examined the potential role and mechanism of CB_1Rs in the hypercholesterolemia-induced remodeling of vascular wall by using our novel developed double-knockout, LDLR-KO and CB_1R-KO AS-prone mouse model kept on high-fat or control diets for 5 months. We investigated the impact of the presence of CB_1R on the hypercholesterolemia-induced functional and structural remodeling of the aortic wall. High-fat diet elevated the cholesterol levels in the LDLR-KO mice into serious patophysiological ranges, which were significantly higher than in the LDLR wild-type mice. Cholesterol levels were not influenced by CB_1Rs. Ach-induced relaxation was depressed to HFD, which was moderated by the absence of CB_1Rs. The BP values were elevated in LDLR knockout animals compared to their WT counterparts, which was significantly higher in HFD groups ($p<0.05$), however, this elevation to HFD was attenuated in CB_1R knockout mice. A depressed eNOS expression was found in the HFD, WT mice compared to CD group, which was enhanced in CB_1R-KO groups. Our findings suggest that deletion of the CB_1R gene significantly attenuates vascular damage in hypercholesterolemic mice. (Vass et al., 2024) We can conclude that in some cases newly designed selective CB_1R inhibitors might have pharmatherapeutic benefits in the future in the treatment of hypercholesterolemia and AS.

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9. Bibliography of the candidate's publications

Publications related to the dissertation:

Journal articles:

1. 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 CB_1 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**

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Publications not related to the dissertation

Journal articles:

1. 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**

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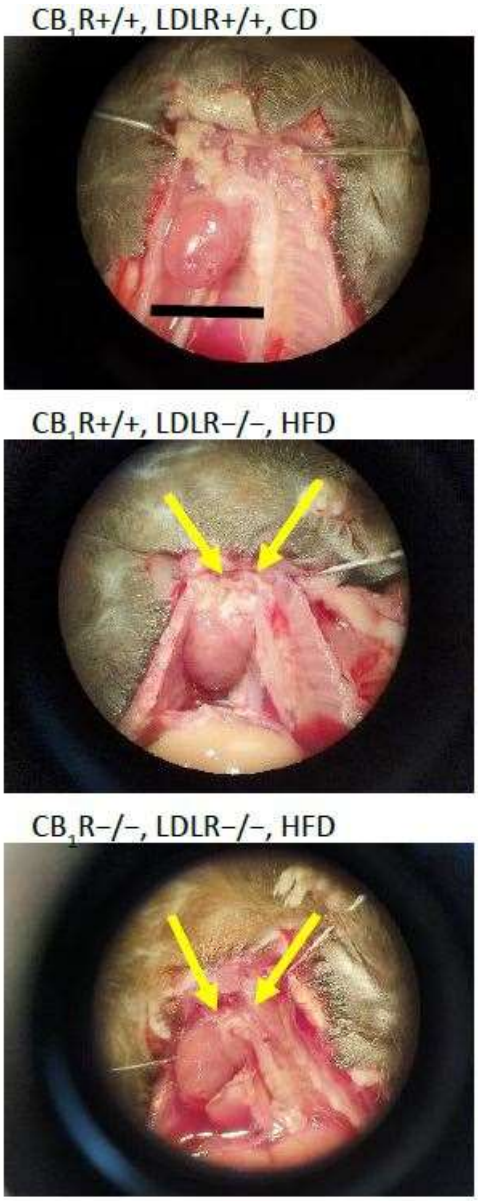
12. Supplementum

Supplementary Figure 1. Microscopic photos of the chest of mice during preparation to visualize the thoracic aorta and the aortic arch



Supplementary Figure 1. Microscopic photos of the chest of mice during preparation to visualize the thoracic aorta and the aortic arch. Panel (A): $CB_1R+/+$, LDLR+/+ kept on CD didn't develop plaques in the arch of aorta. Panel (B-C): $CB_1R+/+$, LDLR−/− and $CB_1R−/−$, LDLR−/− mice kept on HFD has developed sclerotic plaques in the aortic arch shown by arrows. Scale bar shows 1 cm. *Abbreviations: CD: control diet, HFD: high-fat diet, $CB_1R+/+$: cannabinoid type 1 receptor wild-type, $CB_1R−/−$: cannabinoid type 1 receptor knockout, LDLR+/+: LDL receptor wild-type mice, LDLR−/−: LDL receptor knockout mice (Vass et al., 2024).*

Supplementary Figure 2. Hungarian protocol of myography.

Miográf mérések jegyzőkönyve

Project 1: nőstény, CB_1R-KO egerek

Dátum: .....

Állat1: .....súly: .....

Állat2: .....súly: .....

Altatás: .....vérvétel: ……..

Perfundálás: .....

Preparáció kezdete: .....

Erek kiszedése: .....

Erek felszerelése miográfra: ..... Értípus: a. abdominalis

30 min ekvilibráció kezdete 10mN feszülés elérésekor: .....

Mérés kezdete: .....

Miográf mérés protokollja 8 párhuzamos csatornán (ld. Módszerek):

a. abdominalis 1 állatból: 1-4 csatorna, 2. állatból: 5-8 csatorna

Teszt reakciók: Phe (fenilefrin) 10^{-6} M, majd Ach (acetilkolin) 10^{-6} M

mosás 5x

Krebs oldat lecserélése magas K^+ tartalmú (KCl) oldatra 3 min (referencia kontrakció)

mosás 5x, 15 perc

Ang II (angiotenzin II) 10^{-9}-10^{-7} M

mosás 5x

Phe 10^{-9}-10^{-5} M

Ach 10^{-9}-10^{-6} M

mosás 5x

gátlószerek: Vehiculum, Indo (Indometacin) 10^{-5} M, LNA (Nitro-L-Arginin) $5x10^{-5}$ M párhuzamos csatornákra, 15 perc

Phe 10^{-9}-10^{-5} M

Ach 10^{-9}-10^{-6} M

mosás 5x

gátlószerek: Vehiculum, Indo 10^{-5} M, LNA $5x10^{-5}$ M párhuzamos, azonos csatornákra ismételten adva, 10 perc

Phe $5x10^{-7}$ M

1-3, 5-7 csatornára estradiol 10^{-8}-10^{-5} M, 4,8 csatornára WIN55212 10^{-6} M

mosás 5x

Miográf mérések jegyzőkönyve

project 2, him, CB_1-LDL rec. dupla KO egerek

Dátum: .....

Állat1: .....súly: ..... genotípus, ujjkód: …….. etetés: high fat diet/kontroll diéta

Állat2: .....súly: ..... genotípus, ujjkód: …….. etetés: high fat diet/kontroll diéta

Altatás1: ..... Altatás2: .....

Vérnyomásmérés: igen/nem. Vérvétel: igen/nem

Perfundálás1: .....Perfundálás2: .....

Preparáció kezdete: .....

Szövetek kiszedése szövettanra: igen/nem (ld. feldolgozás jk.)

Erek kiszedése: .....

Erek felszerelése miográfra: .....Értipus: a. abdominalis

30 min ekvilibráció kezdete 10mN feszülés elérésekor: .....

Mérés kezdete :.....

Miográf mérés protokollja 8 párhuzamos csatornán (ld. Módszerek):

abdominalis 1 állatból: 1-4 csatorna, 2. állatból: 5-8 csatorna

Teszt reakciók: Phe (fenilefrin) 10^{-6} M, majd Ach (acetilkolin) 10^{-6} M

mosás 5x

Krebs oldat lecserélése magas K^+ tartalmú (KCl) oldatra 3 min (referencia kontrakció)

mosás 5x, 15 perc

Phe 10^{-5} M

Ach 10^{-9}-10^{-5} M

mosás 5x

gátlószerek: Vehiculum, Indo (Indometacin) 10^{-5} M, LNA (Nitro-L-Arginin) $5x10^{-5}$ M párhuzamos csatornákra, 15 perc

Phe 10^{-5} M

Ach 10^{-9}-10^{-6} M

mosás 5x

Supplementary Table 1. Emax and EC50 values of endothelium dependent Ach-induced vasodilation. *Abbreviations: CB_1R: cannabinoid type 1 receptor, LDLR: low density lipoprotein receptor, −/−: knockout, +/+, wild type, CD: control diet, HFD: high fat diet, N: number of animals per group, Emax%: effective maximum response (%), EC50: effective concentration 50, SEM: standard error of the mean (Vass et al., 2024).*

genotype	diet	n	Emax%	SEM (Emax%)	EC50 nmol/L	SEM(EC50)	Statistics of EC50
CB_1R+/+, LDLR+/+	CD	5	91.5659	4.7777	10.6	2.65	p=0.029 vs. CB_1R+/+, LDLR−/−, HFD
CB_1R+/+, LDLR+/+	HFD	10	87.4531	4.645	16.9	4	
CB_1R−/−, LDLR+/+	CD	6	89.5487	5.0845	9.2	2.35	p=0.012 vs. CB_1R+/+, LDLR−/−, HFD

$CB_1R^{-/-}$, LDLR+/+	HFD	8	91.2513	3.8027	14.5	2.4	p=0.04 vs. $CB_1R^{+/+}$, $LDLR^{-/-}$, HFD
$CB_1R^{+/+}$, $LDLR^{-/-}$	CD	7	88.6067	4.8683	13.4	2.93	
$CB_1R^{+/+}$, $LDLR^{-/-}$	HFD	5	88.7236	3.6328	26.4	5.3	p=0.043 vs $CB_1R^{+/+}$, $LDLR^{-/-}$, CD
$CB_1R^{-/-}$, $LDLR^{-/-}$	CD	6	87.7655	4.1828	15	3.1	
$CB_1R^{-/-}$, $LDLR^{-/-}$	HFD	7	88.0499	4.331	14.5	3.02	p<0.05 vs $CB_1R^{+/+}$, $LDLR^{-/-}$, HFD